UNIVERSITY OF SOUTHAMPTON

An investigation into the adequacy of the default kinetic uncertainty (safety) factors used in the risk assessment of food additives.

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ABSTRACT

Faculty of Science

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An investigation into the adequacy of the default kinetic uncertainty (safety) factors used in the risk assessment of food additives

by Sara C. Tullberg

In vivo pharmacokinetic studies were conducted in healthy adult human volunteers and the appropriate test species to determine the adequacy of the default uncertainty factors for inter-species differences and human variability in toxicokinetics. Toxicokinetic parameters from studies in humans were used to estimate the proportion of the population that would be adequately protected by the default factor of 3.16 fold. These data were compared with the results of similar studies in animals (rats or mice) to determine the adequacy of the toxocokinetic default factor of 4.0-fold for inter-species differences.

Four food additives were chosen as test chemicals, butylated hydroxytoluene (BHT), curcumin, propyl gallate (PG) and thiabendazole (TBZ). Separate studies were conducted in which human volunteers received the food additive at the acceptable daily intake (ADI) or 10 x ADI, and studies in which the animal was dosed at the no-observed adverse effect level (NOAEL) or 0.1 x NOAEL. Plasma samples from these subjects were analysed for the additive and used to construct concentration-time curves; where appropriate a mathematical model was used to extrapolate parameters to infinity.

The results of the studies show that the default factor of 3.16-fold for human variability is adequate for greater than 92% of the population for all test chemicals based on all PK parameters derived from the ADI studies. The inter-species default of 4.0-fold was adequate for all 4 test chemicals (comparing ADI and NOAEL data after correction for dose) based on oral clearance, and for PG and TBZ only based on Cmax. Interestingly, there was evidence of saturation kinetics in both humans and animals with TBZ. Oral clearance was ~9-fold and ~13-fold lower at the higher dose for humans and animals respectively.

In conclusion these results support the current regulatory framework and the usual default uncertainty factor. However the data suggest that the dose used in the generation of pharmacokinetic data for deriving chemical-specific adjustment factors, for substitution into the framework, will need to be carefully considered. The dose-response will need to be carefully characterised for the species of interest, and the doses administered should reflect the likely human exposure, so that the correct adjustment factor and therefore a more accurate ADI can be derived.

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Definitions and abbreviations used

AA	arachidonic acid
AhR	aryl hydrocarcon receptor
AOM	azomethane
AUC	area under the concentration time curve
ADI	acceptable daily intake
ARfD	acute reference dose
B(a)P	benzo (a) pyrene
BBDR	biologically-based-dose response
BBN	N-butyl-N-(4-hydroxybutyl) nitrosamine
BDMC	bisdemethoxycurcumin
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
BHT-QM	quinone methide of butylated hydroxytoluene
BMD	benchmark dose
ß-NF	beta-napthaflavone
BNPP	bis-p-nitrophenylphosphate
BSO	buthionine sulphoxamide
CA	chromosomal abberation
CDNB	chloro-2,4-dinitrobenzene
CHA	cyclohexylamine
CI	confidence interval
CL	clearance
CL/F	oral clearance (clearance/bioavailability)
CLm	metabolic clearance
CLO	clofibrate
Cmax	maximal concentration
COV	coeffeicent of variation
COX	cycloxygenase (enzyme)
CpU	concentration os substrate in the plasma unbound (to plasma proteins)
CSAF	chemical specific adjustment factor
Ct	tissue concentration
CYP450	cytochrome P450 enzyme
DHEA	dehydroepiandosterone
DM	di-ethyl maleate
DMAPPH	1-[4-(dimethylamino)phenyl]-6-phenyl hexatriene

DMBA	di-methylbenz(a)anthracene
DMC	demethoxycurcumin
DMH	1,2-dimethyl hydrazine
DMPK	drug metabolism pharmacokinetic (studies)
ECD	electrochemical detection
ECG	electrocardiogram
ECETOC	European centre for ecotoxicology and toxicology of chemicals
ED_{10}	dose giving 10% of the maximal response
EG	ethyl gallate
EHMI	estimated high monthly intake
EM	extensive metaboliser
ENNG	N-ethyl-N'nitro-N-guanidine
EPA	environmental protection agency
EQ	ethoxyquin
EROD	ethoxyresorufin-O-deethylase
EsD	esterase D
EU	European union
FAL	feruroylaldehyde
FDA	food and drug administration
FID	flame ionisation detection
FMK	feruloylmethylketone
FQPA	food and quality protection act
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
GFR	glomerular filtration rate
GOT	glutamate oxaloacetate
GPT	glutamate pyruvate transanimase
GRAS	generally recognised as safe
GST	reduced glutathione
HETE	hydroxyeicosatetranoic acid
HHC	hexahydrocurcumin
HPLC	high pressure liquid chromatography
ILSI	international life sciences institute
I.P.	intra-peritoneal
IPCS	international programme for chemical safety
I.V.	intravenous
JECFA	Joint FAO/WHO Expert Committee on Food Additives

LD_{50}	dose at which 50% of animals under test die
LOAEL	lowest observed adverse effect level
LOD	limit of detection
LOX	lipoxygenase (enzyme)
3-MC	3-methyl cholanthrene
MeIQx	2-amino-3,8-dimethylimidazo (4,5-f) quinoxaline
MMNG	N-methyl-N-'nitro-N-nitroguanidine
MOS	margin of safety
MPBZ	1-methyl-2-phenylbenzimidazole
MRL	maximum residue limit
MROD	methyoxyresorufin-O-deethylase
N-acetoxy-AFF	N-acetoxy-2-acetylaminofluorene
NCI	national cancer institute
NOAEL	no-observed adverse effect level
NAEL	no-adverse effect level
OG	octyl gallate
PAH	polyaromatic hydrocarbon(s)
PB	phenobarbitone
PBI	protein-bound iodine
PBPK	physiology-based-pharmacokinetic modelling
PCB	polychlorinated planar biphenyls
PCDD	polychlorinated dibenzo-p-dioxins
PCDF	polychlorinated dibenzofurans
PG	propyl gallate
PhIP	2-amino-1-methyl—6-phenylimidazo (4,5-b) pyridine
PK	pharmacokinetic
PM	poor metaboliser
PPM	parts per million
PTMI	permitted tolerable monthly intake
PYR	pyrazole
RA	risk assessment
RfD	reference dose
RIF	riampicine
SA	sodium-L-ascorbate
SAR	structure activity relationship
SCE	sister-chromatid exchange
SCF	scientific committee on food

SD	standard deviation
SGOT	serum glutamate oxaloacetate transanimase
T½	half-life
TBHQ	ter-butylhydroxyquinone
TBZ	thiabendazole
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TD	toxicodynamic
THC	tetrahydrocurcumin
TK	toxicokinetic
Tmax	time at which maximal concentration occurs
TPA	12-O-tetradecanoylphorbol-13-acetate
TTC	threshold of toxicological concern
TXB_2	thromboxane (type B_2)
UGT	UDP-glucuronysyltransferase
Vmax	maximal velocity
WHO	World Health Organisation

Chapter 1: Introduction

1. Introduction to the Risk Assessment of Food Additives

1.1 Food and chemicals

Foodstuffs are chemically complex materials, comprising many different types of molecules (proteins, carbohydrates, sugar, fats, vitamins and elements.) In addition to the natural constituents of food, chemicals may be added intentionally (food additives) or unintentionally (contaminants) to foodstuffs during the manufacturing process.

All substances (both natural and synthetic chemicals) are toxic at some dose and it is important to note that synthetic chemicals are not intrinsically more toxic than natural chemicals. It is therefore important to determine the toxicological profile of chemicals present in food, via a process called risk assessment, so that they can be regulated in the diet at levels that present minimal risk to human health (risk management).

Acting to minimise risk to the consumer can raise interesting conflicts of information for risk assessors. For example, in the case of vitamins there can often be a very narrow margin of safety between toxic doses, extrapolated from animal experiments, and the dose calculated to be necessary to maintain human health (Hathcock, 1989). Conversely food additives benefit the producer and the consumer by increasing shelf life, and the range of products available to the customer. However they too are toxic at specific doses and a careful risk-assessment analysis is therefore required in the case of natural and synthetic chemicals.

The general subject of this thesis is concerned with an investigation of regulatory framework used in the risk assessment of food additives. This process normally uses data from chronic animal experiments to derive estimates of safe intake levels for food additives in humans. The rest of this introduction is devoted to introducing and explaining the basis of risk assessment. The specific hypothesis of the thesis will then be introduced and discussed in relation to the current risk assessment paradigm.

1.2 What is the definition of a food additive?

Food additives are a diverse group of chemicals ranging from simple single low molecular weight molecules to complex proteins. Screening of potential food additives in structure-activity relationship databases can flag potentially toxic compounds, but aside from this, the chemical properties of food additives, whilst practically relevant will not necessarily alter the risk assessment process that they are subject to. Food additives are recognised because of the technological function they perform, and have been defined by the international Joint FAO/WHO Expert Committee on Food Additives (JECFA) as

"non-nutritive substances added intentionally to food, generally in small quantities, to improve its appearance, flavour, texture or storage properties" (WHO, 1987).

The JECFA also evaluates substances unintentionally introduced into human food (contaminants), for example polychlorinated biphenyls and dioxins, pesticides, nitrates and heterocyclic amines, polycyclic hydrocarbons and phthalates, heavy metals and fungal toxins (Peshin et al., 2002). The outcome of the risk assessment process for contaminants is different to that of food additives (see later), and so food contaminants will not be discussed specifically in the remainder of the introduction.

1.3 Who performs the risk assessment of food additives?

In recognition of the need to facilitate international trading of food, many of the organisations involved in the risk assessment of food additives are international bodies e.g. the JECFA operating within the auspices of the World Health Organisation. However risk assessment, and more often risk management decisions are also made by local governmental agencies, e.g. the Food and Drugs Administration (FDA, US), the Environmental Protection Agency (EPA, US) and the Scientific Committee for Food (SCF, Europe) or European Food Safety Authority (EFSA, Europe).

Established in 1961, the Codex Alimentarius is an international body, which harmonises the international approach to the risk assessment of food additives (Codex Alimentarius website). Its mandate is to guide and promote the establishment and elaboration of definitions and requirements for food, to assist in their harmonisation, and in doing so, to facilitate international trade. The Codex Committee on Food Additives and Contaminants (CCFAC) identifies food additives and contaminants that should receive priority evaluation and refers them to the JECFA for assessment before incorporating them into Codex standards (Codex Alimentarius website).

The JECFA was established in 1955, upon recommendation by the Joint FAO/WHO Expert Committee on Nutrition (Codex Alimentarius website). It has performed over 1,400 original evaluations of chemicals used as food additives (WHO website). The JECFA also performs evaluations on food contaminants and recommends maximum residue limits (MRLs) for veterinary drugs in animal tissues for human consumption.

Evaluation of a prospective food additive by the JECFA normally involves review of independently conducted scientific studies that have identified potential adverse effects of an animal species fed with the proposed compound. The result is an estimation of the safe exposure limits for humans (incorporating a margin of safety), known as the acceptable daily intake (ADI). This has been defined by the JECFA as "an estimate of the amount of a food additive, expressed in a bodyweight basis that can be ingested daily

over a lifetime without appreciable health risk" (WHO, 1987). The concept of the ADI was "born" in the 1960's during the early meetings of the newly formed JECFA. Truhart (1991) was the originator of the ADI, a concept that has undergone substantial development and refinement in the subsequent years (Vetorazzi, 1987 and Truhart, 1991).

The term "ADI" has been adopted by the JMPR (the Joint FAO/WHO Meeting on Pesticide Residues), the FDA (Food and Drugs Administration, U.S.A) and is known as the reference dose (RfD) by the EPA (Environmental Protection Agency, U.S.A) (Barnes & Dourson, 1988).

1.4 The risk assessment of food additives: a multi-step process

In order to be approved for use in food, the toxicological profile of a novel food additive must be determined. A preliminary analysis of available information indicates the extent of testing required (Renwick, 1999a). If necessary, a battery of rigorous toxicological experiments will be conducted, generating data on which to base a risk assessment. For food additives that are already approved, such evaluations may be performed in light of new experimental data. According to the International Programme for Chemical Safety (IPCS) the risk assessment process can be thought of comprising 5 distinct stages of evaluation and assessment (WHO, 1999) which are then translated into risk management decisions. These five stages are as follows:

1. Hazard Identification: this determines whether or not a chemical represents a possible risk to human health, and under what circumstances the risk occurs.
2. Hazard Characterisation (dose-response assessment): this correlates the dose of the chemical with the incidence of adverse health effects.
3. Exposure Assessment: this is designed to estimate the nature and extent of contact with the chemical in a variety of scenarios.
4. Risk Characterisation: this integrates hazard characterisation and exposure assessment and provides risk managers with the essential scientific evidence and rationale about the risk that they need for decision-making.
5. Risk Management: various organisations (non-regulatory, regulatory, advisory, and technological) evaluate the risk information in light of the exposed population, and consider if the risk needs elimination or reduction.

A variety of methods are used by risk assessors to determine the safe exposure limits of chemicals including food additives. These are briefly outlined in table 1.1. SAR (structure activity relationship) and TTC (threshold of toxicological concern) methods have mostly been used in the evaluation of packaging migrants and flavours, where exposure is expected to be very low. The threshold approach relies on the

use of uncertainty factors (see subsequent sections) and is refined through the use of chemical specific uncertainty factors (CSAFs). The remaining methods (categorical regression, probabilistic modelling and physiologically-based pharmacokinetic modelling) are generally considered to be supportive methods to interpretation of the dose-response data. These three methods importantly allow the special consideration of sensitive sub-populations, when the appropriate data exist. The threshold approach and the chemical specific adjustment factor (CSAF) approach are the main foci of this thesis, and so attention will be devoted to these methods. Where relevant, reference to the other methods will be made.

Method	Strengths	Weaknesses	Applicability	Acceptability
Probabilistic RA[1]	Uncertainties associated with all aspects of the quantitative methods of the RA process can be taken into account Appropriate chemical specific information can be incorporated to reduce uncertainty Provides effect estimates at actual exposure levels.	Requires the use of default distributions in most cases.	Less widely applicable for current studies than threshold methods	Is not currently accepted in the EU[2] Probabilistic approaches to exposure assessment are accepted
Categorical regression	Takes all studies into account and not only the most sensitive one Allows the prediction of a severity effect category at a particular dose (e.g. above the ADI)	Requires toxicological judgement for the categorisation The interpretation of fitted model (different endpoints, observer variation etc) is difficult	Limited because it needs special methodology	Is not accepted in the EU Is used in some case studies (e.g. by EPA) in conjunction with other methods.
PBPK[3]	Is able to model the time course of the amount of the active compound at the target site Is possible for any species and for different exposure and lifetime conditions Allows extrapolation from animal to human without having to have human exposure data Allows target organ dose-response relationships to be used for low-dose extrapolation	Is a data intensive method Does not address dynamics	Limited because it depends on the availability of appropriate data for the specific chemical	OSHA[4] uses and relies on PBPK models for some chemicals Is accepted by EPA Is accepted by JECFA for contaminants Is used in EU countries to some extent for purposes other than RA

[1] RA – risk assessment

[2] EU – European Union

[3] Physiologically-based pharmacokinetic modelling (PBPK)

[4] US Occupational Safety and Health Administration (OSHA)

Table 1.1 Summary of some of the characteristics of the main methods used for the risk assessment of chemicals, including food additives. Adapted from Edler et al., 2002.

1.5 The threshold approach to risk assessment

All chemicals are toxic at a certain dose. Toxic effects arise when the effect of the chemical exceeds the body's natural capacity to contain the change caused by the chemical. This "natural capacity" is fulfilled by a number of mechanisms, for example saturable enzyme processes, active and diffusional transport and renal secretion. Physiological or biochemical processes are maintained at equilibrium, generally via negative feedback mechanisms to ensure survival and optimise performance by a species, a process known as homeostasis (Ganong, 1995). When the chemical level results in parameters fluctuating or being maintained outside the normal homeostatic range the body system can either: a) adapt to the change and show no/few signs of overt toxicity or b) fail to adapt adequately and show signs of adverse effects, i.e. toxicity. The point at which adaptation becomes toxicity is known as the threshold dose.

Three categories of threshold have been defined (Slob, 1999):

Biological threshold:	the dose below which the organism does not suffer from any adverse effects
Experimental:	the dose below which no effects are observed
Mathematical:	the dose below which the response is zero, and above it is non-zero

In hazard characterisation, toxicologists consider the dose at which the food additive produces an adverse (toxic) effect in the test species, which is a combination of biological and experimental thresholds. In order to be directly applicable to the exposed population these studies would have to be conducted in humans, but this would not be ethically acceptable, because it would be necessary to induce adverse effects in the subjects without them deriving any benefit. Instead groups of animals are given the food additive at incremental doses to find the dose at which toxicity occurs.

The adverse effects produced by chemicals have traditionally been divided into two fundamental groups, those having a threshold in the dose-effect relationship, and those for which there is no threshold. A chemical that only begins to have an adverse effect at exposure above a particular concentration shows a threshold effect, whilst a chemical for which there is judged to be a risk at any level (e.g. a mutagen or genotoxic carcinogen) has a non-threshold effect. The risk assessment approaches used for these two classes of compounds are quite distinct from each other. In the case of non-threshold compounds (i.e. genotoxic carcinogens) there is a finite risk of an adverse effect with any dose. According the Delaney Clause, a US government law, compounds with genotoxic or mutagenic non-threshold effects will be rejected as candidate food additives (Somogyi & Appel, 1999). The 95th percentile confidence limit on the dose-response curve is used as the basis for the risk assessment, with the assumption that all such chemicals display linearity at low doses (see figure 1.1(a)) (Price & Stickney, 1997).

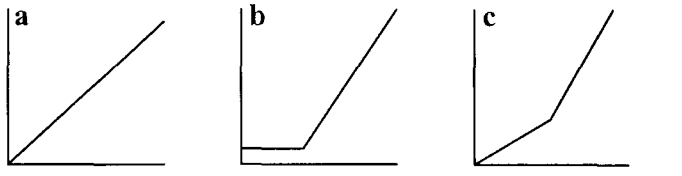


Figure 1.1 Dose-response curves for genotoxic and non-genotoxic chemicals.

In the case of non-genotoxic carcinogens with an estimated threshold (see figure 1.1 (b)), the presence of a threshold negates the use of the dose-response curve. Non-linearity is fundamental to the setting of the NOAEL (no-observed adverse effect level) value, and is assumed to occur in the case of non-genotoxic compounds. However some situations have been proposed where this may not be the case: (Crawford & Wilson, 1996). For example, in the case where the dose-response is for an effect with a high background incidence, and that the chemical under consideration acts by the same mechanism that causes the background incidence. A confounding consideration also raised by Crawford and Wilson is that in the case of genotoxic, non-threshold chemicals, linearity may occur at low doses, but other factors could modify the slope of the dose-response. For example at increasing doses, saturation of kinetics could alter the slope (see figure 1.1 (c)). Another point to consider is that within small ranges on the dose-response, for example, the critical point between non-toxicity and toxicity, the response is likely to be linear.

1.6 Risk assessments based on animal data

It has been recognised by the JECFA that risk assessment databases for food additives will differ widely, as "no single pattern of test could cover adequately but not wastefully the testing of substances so diverse in structure and function as food additives" (WHO, 1987). Internationally agreed protocols have the advantage of minimising differences in requirements that may otherwise impede international trade. Generally speaking a range of toxicological tests are used to ascertain the toxicity of a compound (see table 1.2).

Toxicological Test	Detail
Genetic toxicity	A variety of short-term tests in bacterial and mammalian systems
Acute oral toxicity	Single dose study
Short-term toxicity	Repeated dose study <14 days
Subchronic toxicity	Repeated daily doses for 14-28 days
Long-term or chronic toxicity and carcinogenicity	Repeated daily doses for 1-2 years
Reproductive toxicity	Repeated daily doses prior to, during and after gestation
Immunotoxicity	Investigate effects on structure and function of cells/tissue of immune system. (Incorporated in short-term and subchronic studies.)
Neurotoxicity	Investigate effects on structure and function of the nervous system. (Includes behavioural studies.)

Table 1.2 Standard toxicological studies used for the hazard identification of food chemicals.

The conventional LD_{50} (the dose at which 50% of the test population die) value is of little use in risk assessment of food additives, except in the context of acute accidental exposure (for example in the manufacturing process). Commonly sub-chronic studies (approximately 10% of the animal species lifetime) are used to determine the nature of toxic effect, the organs affected and an estimate of the dose-response relationship. Chronic studies (representing a substantial proportion of the lifetime) in the most sensitive species are then used to observe any cumulative effects and to determine the NOAEL. An adequate database would also include information on reproductive toxicity including the following: parental fertility; teratotoxicity and examination of viability and development of offspring (Walker, 1998).

1.7 When is an effect adverse?

The definition of an adverse effect is the subject of ongoing debate. Deciding whether or not an observed effect from an animal study is adverse, is a matter of expertise and judgement (Iling, 1999). One such effect is a decrease in bodyweight gain, which can be classed as an adverse event in the absence of any other manifestations of toxicity. It is recognised that this may have a nutritional cause (e.g. decreased palatability of the food due to large amounts of the chemical under test) rather than a true toxicological effect (WHO, 1987). Whilst this may be used as the adverse effect in terms of setting the NOAEL for a study, careful reflection may be necessary in the interpretation of the effect of exceeding the ADI derived from such a threshold effect.

Many criteria are used to decide if an observed effect is truly adverse, for example:

- Whether morphological changes have occurred
- Whether the effect is an adaptive response

- Whether the effect is long lasting or transient
- Whether the effect is reversible
- Whether the effect is a precursor event or a secondary event to other changes
- Whether the effect is a direct consequence of the treatment regime

1.8 The NOAEL as a surrogate for the threshold

The aim of animal dose-response studies is to determine the dose where adverse effects do not occur, i.e. to define the precise threshold of toxicity for the given food additive in the most sensitive species (determined in sub-chronic experiments). This experimental study is known as the pivotal study (Benford, 2000). However, this may not be feasible for a number of reasons. Firstly a large number of dosing groups would be necessary, requiring a large number of animals, which may raise ethical problems/considerations. Secondly, and perhaps more importantly, the sensitivity of measurement techniques is generally inadequate to observe the true no-effect level.

Instead, a selection of doses is used (estimated from sub-chronic studies to include the threshold). The intake level at which no effects are observed is called the NOAEL and this is a surrogate for the true threshold. Due to the differences between doses selected for study, the NOAEL can be either close to or considerably below the threshold, (see figure 1.2). In some cases, the NOAEL may be greater than the true threshold because of limited sensitivity of the assay. This can result in the over-estimation of the safe exposure levels for humans (Benford, 2000). It is important to realise that the NOAEL is not an inherent property of the compound, but an experimentally observed value. The design of toxicological studies in animals results in a homogeneous response, with a low background variation and is optimised for hazard identification.

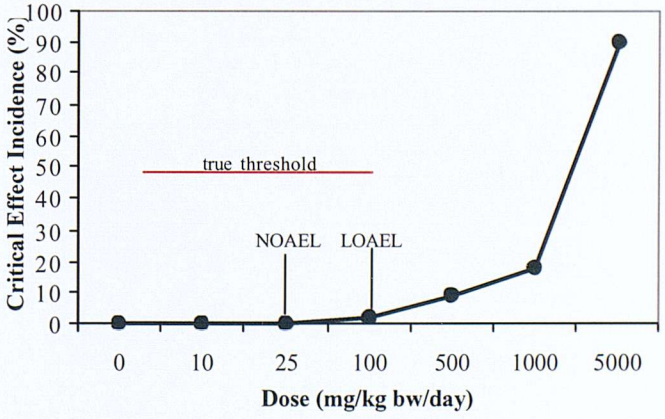


Figure 1.2 A hypothetical dose-response curve, highlighting the NOAEL and LOAEL

1.8.1 Dose selection – critical to the success of NOAEL studies

Dose selection is critical to the success of a study designed to elucidate the NOAEL. The study must perform two essential, almost contradictory functions. In order to fulfil the hazard identification requirement doses at which toxicity is observed must be used, so that the toxic effect can be defined adequately. Conversely, in order to facilitate the risk assessment process there must be a treatment group that has no observed toxicity (Renwick, 1999a). On occasions where the study has not identified a NOAEL, or where the data are inadequate, a LOAEL (lowest observed adverse effect level) (see figure 1.2) may be used as the basis for risk assessment. In some cases an uncertainty factor is used to allow for the potential difference incurred between the two values.

1.8.2 Criticisms of using the NOAEL as the basis for risk assessment

The current methodology of selecting the NOAEL relies on identifying the highest administered concentration that does not cause a statistically significant or biologically significant adverse effect compared to controls. This can be problematic in the case where a NOAEL is not determined in the study (see dose selection discussion above). Several variables have been identified as affecting the precision of the NOAEL, i.e. the extent to which the threshold is under or over-estimated (see table 1.3). (Crump, 1984; Lu et al., 1991; Leisenring et al., 1992; Renwick & Walker, 1993, Speijers, 1999).

Parameter	**Comment**
Duration of study	Duration of toxicity test should be 90% of the species lifespan.
Dose ranges used in study	Margins between doses can lead to imprecision in the estimation of the NOAEL.
Identity and purity of compound under study	Compound purity will affect the level of confidence that can be placed in the outcome of the study.
Parameters studied	The most sensitive and relevant endpoints should be measured
Test species and strain	The most closely related species to humans in terms of sensitivity should be used. Strain-specific differences need to be considered.
Gut microflora of animals	Gut microflora are metabolically active; species differences in activity/resident populations may be required.
Dietary composition	Marginal/deficient diets can result in lower NOAEL values.
Statistics performed	The number of animals/group will depend of the sensitivity of the endpoint and analytical technique

Table 1.3 Experimental parameters affecting the precision of the NOAEL.

Several of these parameters are thought to contribute significantly to the precision of the NOAEL. Firstly, the sensitivity of measurement of the toxicological end-point will affect the lowest detectable response. Group size can also affect the NOAEL and it has been estimated that an increase in group size

from n=10^4 to 10^6 may decrease the NOAEL value estimation by 24-78% (Lu, 1985). However the effect of group size is not considered to be as important for the precision of the NOAEL, as the increment between doses. Different NOAEL values for the same compound may occur when different dosing increments are used (due to study design). It has been calculated that in older studies the NOAEL may be a factor of 5-10 fold less than the marginally effective dose (LOAEL) and that the NOAEL may underestimate the threshold by almost 500% (Renwick & Walker, 1993). The precision of the NOAEL also depends on the slope of the dose-response curve. The steeper the dose-response curve, the more precise the NOAEL estimate and the more secure the ADI estimate. In cases where a chronic study does not identify a NOAEL, the dose-response slope could be used to extrapolate to an estimate of the threshold (Crump, 1984, Slob, 1999), such as the benchmark dose (BMD) (see figure 1.3.).

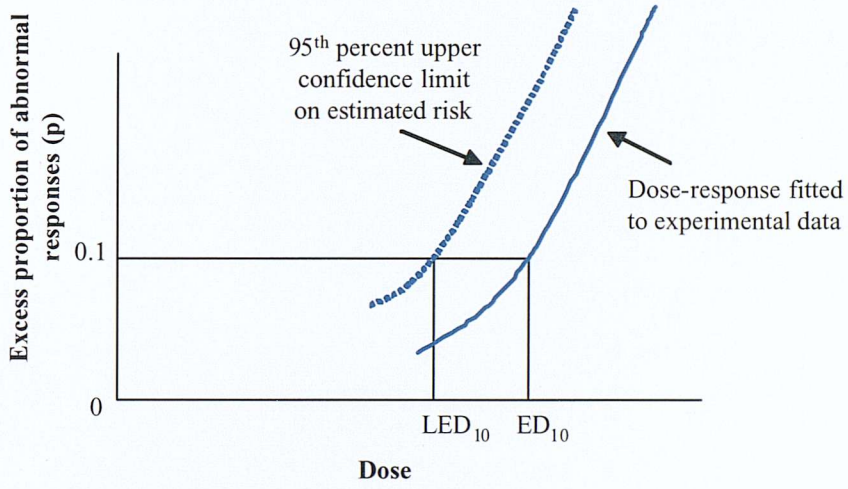


Figure 1.3 The benchmark dose. The BMD is calculated from the upper 95th per cent confidence limit for the dose-response curve at the point between the no-effect level and the just detectable level. The lower confidence limit on the dose giving a 10% response, ED_{10} (LED_{10}) is the BMD. Uncertainty factors would then be used to extrapolate from the BMD a safe exposure level in humans. (Adapted from Iling, 1999.)

The use of the BMD to determine the threshold rather than using the NOAEL has been proposed (Crump, 1984). This approach is thought to confer several advantages:

- The BMD will reflect the dose-response relationship to a greater degree than will the NOAEL.
- The BMD is less dependent on sample size and study design.
- It is not necessary to define a NOAEL in order to determine an ADI.
- The BMD corresponds to defined risks in the experimental range.
- The use of ungrouped data removes any bias encountered through the use of arithmetic or geometric means.

Curve-fitting to the experimentally derived data has also been proposed as an improvement to the current method used to identify the NOAEL. Slob, 1999 proposed that the dose-response should be considered as a continuous function, rather than being used just for the determination of the threshold. In addition, the use of the lower 95th percentile dose in the BMD method "rewards" data from good studies that demonstrate well-defined dose-effect relationships. Kavlock et al., (1996) examined the impact of outcomes of developmental studies on the accuracy and precision of the BMD. Crucial to the determination of an accurate BMD, was the occurrence of 2 responding groups, where one group was dosed at a level close to the ED_{05}. The worst case-scenario was where the only group in which adverse effects were observed was a single group dosed at a level far greater than the ED_{05}. This study demonstrates that just like the estimation of the NOAEL, the successful determination of a BMD is a function of the quality and suitability of the experimental data used.

The BMD approach is less widely accepted that the threshold approach to risk assessment. It is not widely accepted in the EU, however, it is becoming increasingly used by the EPA and Health Canada, and is accepted by WHO (Edler et al., 2002).

In conclusion, the only way to improve the accuracy of the NOAEL, and therefore of the ADI, is to attempt to derive the true NOAEL. As discussed above this is desirable, but not workable. Instead attempts should be made to improve study design, and statistical power leading to a greater likelihood in deriving a NOAEL value close to the true NAEL.

1.9 Using human data to derive a NOAEL

Three sources of human data have been described; studies of intoxication, epidemiological studies and controlled studies (Speijers, 1999). However these data are generally not adequate as the basis of assessment. Data from intoxication studies lack accurate exposure data, and since not all cases are hospitalised and the toxic effects are not consistently recorded. Generally these case studies relate to acute or sub-acute exposure. Epidemiological studies vary considerably in their design (Renwick, 1999a), therefore their interpretation can be difficult. The imprecision of exposure estimates and the lack of diversity in exposure also limit their use in the hazard identification process. Human volunteer studies are most comparable to the standard animal toxicity design. The major limiting factors of these experiments are that the exposure levels and duration, and the age and numbers of people involved will, understandably be ethically constrained.

1.10 Deriving the ADI from the NOAEL/BMD

A series of uncertainty factors are applied to a NOAEL or BMD to derive the ADI. In the case where an additive is without observed adverse effects, it is assigned as "ADI-not specified", and the amount added to food is limited by good manufacturing practice (WHO, 1987). (Approximately 80% of food additives are categorised by an ADI not specified (Groten et al., 2000).

The estimate for the threshold (NOAEL, LOAEL or BMD) is divided by an uncertainty factor (UF) to derive the ADI. (See figure 1.4.) A composite uncertainty factor (also known as a safety factor) is used to ensure an adequate margin of safety between the two values. The uncertainty factor is comprised of several separate factors, designed to allow for extrapolation between the species, the uncertainty in the lack of chemical-specific data for risk assessment and inherent uncertainty in the derivation of the estimate of the NAEL.

$$\frac{\text{Threshold estimate}}{\text{UF}} = \text{ADI}$$

Figure 1.4 The derivation of the ADI from the threshold estimate using the uncertainty factor. Usually an uncertainty factor of 100-fold is applied to the threshold estimate from an animal study to calculate the ADI.

1.11 The 100-fold uncertainty factor

Lehman and Fitzhugh (Lehman & Fitzhugh, 1954) introduced the concept of using a 100-fold default uncertainty factor for cross-species extrapolation to derive safe exposure levels for humans. They reasoned that a 100-fold factor was necessary to account for the following:

- That animals are more toxicologically resistant than man
- That variation in susceptibility exists across species
- That variation exists between different strains of the same species
- That animal experiments are under greater control that conditions in the human environment
- That a particular food additive will be one of many xenobiotic chemicals present in food stuffs, thereby increasing "metabolic strain"

The 100-fold default has been used for the past 60 years, but the theory has been subject to much criticism, primarily due to its arbitrary nature (Calabrese, 1985; Hattis et al., 1987, Vermiere et al., 1999),

but its adequacy has also been supported in reviews (Bigwood, 1973, Lu, 1979 and Vetorrazi, 1987). More recently the theory has been expanded and/or deconstructed and several agencies and academic professionals have defined numerous factors for specific areas of uncertainty. Together with different interpretations of databases, this has resulted in different ADI or RfD values being estimated by different agencies (Dourson & Lu, 1995). Table 1.4 compares uncertainty factors used by different regulatory agencies.

1.12 Refinements/modifications to the 100-fold uncertainty factor

1.12.1 The Health Council of the Netherlands Approach

Whilst this approach is not commonly used in the Netherlands, it is an example of an approach that separates uncertainty factors based on whether they are measured on non-measurable/not measured. A 100-fold factor is employed; but a distinction is made between extrapolation factors based on measurable differences, (for examples extrapolation factors required to compensate for differences in body size between the test species and humans) and safety factors used to compensate for unmeasured uncertainty (for example experimental errors, species specific differences and differences in susceptibility (within and between species).

1.12.2 The EPA, reference dose (RfD) approach.

This method builds from the ADI approach, and shares many of the same uncertainty factors. A series of 10-fold factors are used to account for:

- human variability
- inter-species variability
- use of data from sub-chronic rather than chronic studies
- use of a LOAEL rather than a NOAEL value in the determination of the RfD
- the adequacy of the database (a judgemental decision)

An overall modifying factor ranging from 1-10 can also be applied; for example if a large number of animals were used per dose group, the modifying factor might be 1.

Assessment factors	HC	EPA (RfD)	Calabrese and Gilbert	IPCS	LLN	ECETOC
Inter-individual non-occupational -toxicokinetics -toxicodynamics occupational	10 -	1-10 -	4-10 -	+ 0-3.16 0-3.16 -	+ 1-10 [a] -	10 2
Inter-species -toxicokinetics -toxicodynamics oral route inhalation route	ND [d]	1-10	10	+ 0-2.5 0.4.0	+ 1-10 [a]	+ 4 for oral exposure 1 for inhalation
Duration of exposure Subacute/chronic Subchronic/chronic Other aspects	10	1-10	10	-	+ 1-10[a] 1-10[a]	+ 3 2-3
LOAEL to NAEL	-	1-10	[f]	-	2-10	2-3
Route to route	-	-	[f]	-	-	ND
Type of critical effect	-	-	[f]	-	-	-
Dose-response curve	-	-	[f]	-	-	-
Database adequacy	-	1-10	[f]	1-10	+	1:high, 2:medium,ND:low
Non-scientific factor	-	-	[f]	-	1[a]-10	+
Modifying factor	-	>0-10 [e]	[f]	1-10	+	+
Motivation for choice of factors	-	-	[f]	+/-	+/-	+/-
Overall factor	Other	Multiple	[f]	Multiple	Multiple	Multiple

\+ method accounts for - method does not account for

a default factor

b calculated adjustment factor depending allowing for the differences in metabolic size

c recommended by the FDA

d scaling on bodyweight or caloric demands

e non specific factor to cover unusual uncertainties/dose adjustments not covered by the standard factors

f Calabrese and Gilbert do not describe a full method for the derivation of a safe intake for humans

ND No default value, based on a case by case basis via expert judgement

Table 1.4 A summary of default safety/uncertainty factors used for the derivation of safe intake values for chemicals present in food (adapted from Vermiere et al., 1999).

(HC, Health Council of the Netherlands, EPA, Environmental Protection Agency, IPCS, International Programme for Chemical Safety, LLN, Lewis/Lynch/Nikiforov, ECETOC, European Centre for Ecotoxicology and Toxicology of Chemicals)

1.12.3 The Calabrese and Gilbert method

This method was derived from the EPA approach. Critical to the application of separate uncertainty factors for inter-individual variability and inter-species differences is the assumption that these uncertainty actors are independent of each other. Calabrese and Gilbert challenged this assumption (Calabrese & Gilbert, 1993) and suggested modifications to the defaults used based on their analysis of the situation. (See table 1.5).

Extrapolation Step	
Animal to human	10
Interindividual	
less-than lifetime animal study	5
animal study with normal experimental life time	4
occupational epidemiological study	10
environmental epidemiological study (normal lifespan)	5
LOAEL instead of NOAEL	10
Less-than-lifetime	10

Table 1.5 Modifications to the currently used uncertainty factors, based on the independence and inter-independence of uncertainty factors as proposed by Calabrese & Gilbert (1993).

1.12.4 Method of the IPCS

Again this approach is based on the 100-fold uncertainty factor. By subdividing the factors for inter-individual and inter-species differences to allow for kinetic and dynamic differences a more scientific approach was accommodated, allowing substitution of data when available (Renwick, 1991 & 1993, WHO, 1994). The IPCS also allowed an extra uncertainty factor of 10-fold to allow for particularly toxic effects, e.g. teratogenicity, and a factor to account for the adequacy of the database is used to consider aspects other than the pivotal study. Following a minor amendment, this approach was adopted by the IPCS (WHO, 1994).

1.12.5 Approach described by Lewis/Lynch/Nikiforov (LLN)

This new approach completely re-visited how experimental data could be adjusted to derive safe intake levels for humans. Table 1.6 below summarises the factors they considered necessary, and the defaults suggested in the absence of actual data-derived values.

AF[a]	Description	Range of values	Most likely value	Default value
S	Scaling factor to account for known quantitative differences between species and between experimental conditions and those likely to be encountered by humans	>0	NS[b]	1
I	Inter-individual variability	1-10	1-3[c]	10
R	Inter-species variability	>0-10	NS	10
Q_1	Degree of certainty that the critical effect observed in laboratory animals is relevant to humans	0.1-1		
Q_2	Subchronic to chronic extrapolation	1-10	1-3	10
Q_3	LOAEL to NOAEL extrapolation	NS	2	10
U	Accounts for residual uncertainty in estimates of S, I and R	1-10	NS	10
C	A non-scientific, judgmental "safety" factor	1-10	=3	1

[a] AF = adjustment factor

[b] NS, not stated by authors

[c] Most likely value based on study of high quality

Table 1.6 Adjustment factors, as proposed by Lewis, Lynch and Nikiforov, 1990

A typical composite uncertainty factor of about 250 is calculated using this approach. The main advantage of this method is the discrimination it places between scientific judgement and value judgements. However, the difficulty arises in that in most cases it would not be possible to define the factors adequately. There is also considerable scope for overlapping of the factors, for example between R and S, leading to the possibility of unreasonably high uncertainty factors. The need for the factor Q_1 could be considered unnecessary since the relevance of the critical effect should be considered at the time of NOAEL selection.

1.12.6 The ECETOC approach (European Centre for Ecotoxicology and Toxicology of Chemicals)

The ECETOC approach results in the derivation of the predicted no-adverse effect level (PNAEL). There are three distinct stages in this process:

1. Application of an adjustment factor to the NOAEL/LOAEL of the critical effect in the pivotal study.
2. Application of an uncertainty factor to take account of the level of scientific uncertainty involved
3. Application of a non-scientific safety factor taking into account political and socio-economic

considerations.

The default values used in the absence of a data-informed decision are shown in table 1.7. This method, discriminates between a best estimate for a value and the uncertainty associated with that factor. However, no advice is given as to how to account for this. It also includes advice for setting occupational and non-occupational PNAEL values; however it can be argued that the derivation of separate uncertainty factors (and therefore exposure limits) should be made after the initial derivation of the ADI as this is really a risk management and not a risk assessment consideration.

Element	Factor	Additional Information
Short term repeated/sub-chronic/chronic extrapolation:		
- short-term to subchronic	3	
- subchronic to chronic	2-3	
LOAEL to NOAEL extrapolation	3	Nature of toxicity may justify the use of another factor
Route to route extrapolation	-	Case-by case basis, based on body weight, minute volume and % absorption
Inter-species extrapolation		
-oral route	4	Based on caloric demands
-inhalation route	1	
Inter-individual extrapolation		
-General population	3	
-occupational population	2	

Table 1.7 The recommended default factors for use in the derivation of human PNAELs from human or animal NOAEL/LOAEL values. Adapted from Vermiere et al., 1999.

1.13 Summary and conclusions of the different approaches.

The methods described all share common assumptions, judgements of the critical effect, and the choice of assessment factors or margins of safety. A review of toxicological data results in a composite assessment factor being applied to the NOAEL or LOAEL. The only exception to this is the Margin of Safety Approach (MoS) where the magnitude by which the NOAEL or LOAEL exceeds the estimated exposure is considered in light of any uncertainties involved. Many of the methods reviewed here attempt to refine the use of uncertainty factors by discriminating between single adjustments, separate best estimates and uncertainty. This has the advantage of clarity in approach, demonstrating rational choice and the need to substantiate the uncertainty factor. However, the newly proposed factors are still arbitrary to a greater or lesser extent, and are not supported or validated in the literature, including the widely used 10 x 10 (IPCS) approach. This method does however have a long history of use, although it is not strongly supported in the literature.

Several uncertainty factors are used commonly by a range of regulatory agencies for example inter-species and human variability factors, and these are discussed in the context of the IPCS framework subsequently in this introduction. Some of the other uncertainty factors for example database adequacy factors, and severity of critical effect factors have been the reviewed in the literature and are briefly discussed here.

1.14 A criticism of the default uncertainty factor approach: conservatism

A common criticism of risk assessment processes is one of over-conservatism. It is generally recognised that the use of a composite UF based on multiplication of different factors may result in a very conservative UF and therefore an ADI possibly orders of magnitude above the true NOAEL in humans (which cannot of course be determined). An even greater potential source of conservatism is the precautionary principle. This originated in the early 1980's and basically involves taking protective action even in the absence of scientific evidence to prove a causal link between exposure and effects. Subsequently the United Nations Protection Governing Council adopted a similar proposal. It has been commented upon in the literature (Chapman et al., 1998), where it is exonerated as a method of dealing with cumulative effects, but criticised for marginalising scientific input. I highlight the precautionary principle here, not because of its relevance to the risk assessment of food additives, but to highlight the general acceptance of conservatism in the broader (environmental and industrial risk assessment processes) risk assessment environment.

The focus of this thesis is on evaluating certain aspects of the IPCS approach to risk assessment. Further consideration of the development of this approach, the sources of variability it attempts to discriminate, will now be discussed.

1.15 Further consideration of the IPCS approach.

As outlined in table 1.4, the IPCS framework has uncertainty factors for the following areas of potential difference and uncertainty:

- Inter-species differences (between the test species and humans)
- Human variability
- A factor for the overall adequacy of the database on which to base the risk assessment
- The nature of toxicity (classed as "modifying factor" in table 1.4)

1.16 Nature of Toxicity

Historically (Lewis et al., 1990), additional factors of up to 5000-fold have been used to extrapolate animal dose-response data demonstrating severe toxicity such as.:

- Non-genotoxic carcinogenicity
- Teratogenicity
- Steep dose-response of toxic effect

A factor of 1-10 has been used (Renwick, 1991, WHO, 1994) when the critical effect in the pivotal study is a severe and irreversible phenomenon. The aim of such a factor would be to move the level of human exposure away from the animal dose-response curve. The use of extra uncertainty factors to allow for the nature of toxicity has been identified as a major source of inconsistency between regulatory bodies (Renwick, 1995).

Re-analysis of the databases for BHA (Würtzen, 1993), and erythrosine (Poulsen, 1993) have resulted in the consideration of the use of factors greater than 1 for nature of toxicity where two critical effects were under scrutiny. In both cases, however the critical effect in the chosen pivotal study did not warrant additional weighting factors for existing databases including the cases of BHA and erythrosine.

1.17 Adequacy of Database

1.17.1 Adequacy of the Pivotal study

According to the IPCS (WHO, 1994) a factor designed to evaluate the adequacy of the pivotal study could be applied to reflect the ability of the data to define the NOAEL. The main areas of inadequacy in the pivotal studies were identified as:

- A lack of sensitivity of the analytical method
- Low numbers of animals in the NOAEL dose groups
- An inadequate study duration

A factor lower than the default value of 1 would not be considered unless a study produced exceptional quality or very sensitive data. Instead it was envisaged that dose-effect data from such a study could be used to calculate a slope from which the no-effect level could be determined. An additional factor of 3 or 10 is used when a NOAEL is not defined in a study, to weight the NOAEL produced from mathematical modelling or to extrapolate from the LOAEL. Reports in the literature support a 10-fold factor for LOAEL to NOAEL extrapolation (Kadry et al., 1995, Pieters et al., 1998).

When the NOAEL used is from a sub-chronic study a default factor of 10 is used to extrapolate to an estimated chronic NOAEL. The conservatism of this default has been investigated by Nessel et al., (1995), when matched 90-day and 2-year rodent toxicological studies were compared for inhalation and oral routes. The mean and median values for NOAEL ($_{\text{sub-chronic}}$)/NOAEL ($_{\text{chronic}}$) for 23 oral studies were 2.4 and 2.0 respectively. For the nine-inhalation studies these values were 4.5 and 4.0-fold respectively. Only 1 oral or inhalation study gave a value of 10, and 22/23 oral studies had ratios of 5 of less. The authors concluded that the default of 10 is over-conservative in the majority of cases. Conversely, an analysis by Kramer et al., (1996) where ratios of NOAEL ($_{\text{sub-chronic}}$)/NOAEL ($_{\text{chronic}}$) were generated for 71 compounds resulted in a conversion factor (the conversion factor is defined as the upper 95$^{\text{th}}$ confidence limit of the 95$^{\text{th}}$ percentile for the relevant ratio distribution), of 87 i.e. a UF of 87 would be required as default. The database here was less discriminating; NOAEL data was included irrespective of animal species. This may have resulted in the larger default being calculated.

1.17.2 Adequacy of the overall database

A factor of 1-10 has been proposed to reflect inadequacies in the database used to define the NOAEL (other than the pivotal study). A value of 1 assumes adequacy (assessed against national/international guidelines). A value of >10 should not be applied as this would reflect a database too inadequate to use for a risk assessment.

As presented in table 1.4, the 100-fold factor proposed by Lehman and Fitzhugh (1951) was deemed to consist of two 10-fold factors (see figure 1.5). One to account for the differences between animal species and humans, and one to account for the differences between individual members of the human population (WHO, 1987).

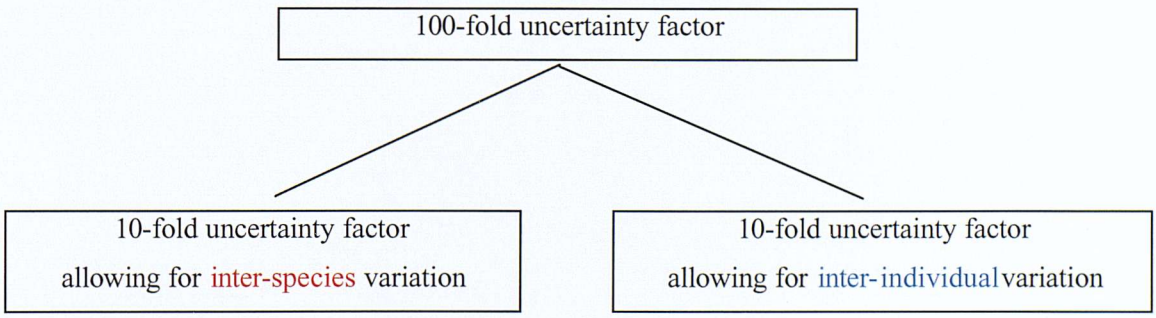


Figure 1.5 Sub-division of the 100-fold factor as used by the IPCS (WHO, 1994).

1.18 Subdivision of the 10-fold factors (the original analyses)

The notion of sub-dividing the 10-fold factors assigned to allow for inter-species and inter-individual differences was first presented by Renwick during a workshop: "The ADI Concept – a Tool for Ensuring Safety" organised by ILSI Europe in 1989. A preliminary analysis (Renwick, 1991) of the adequacy of the two 10-fold factors was carried out where the two 10-fold factors (for inter-species and inter-individual differences) were divided into weighted factors to account for the two major causes of variability:

Variability caused by differences in toxicokinetics, i.e. the differences in the fate of a chemical in the body, including its absorption, distribution, metabolism and elimination (Benford, 2000).
Variability caused by differences in toxicodynamics, i.e. the differences in the effects of the chemical within the body, leading to a toxic response with possible repair and regeneration (Benford, 2000).

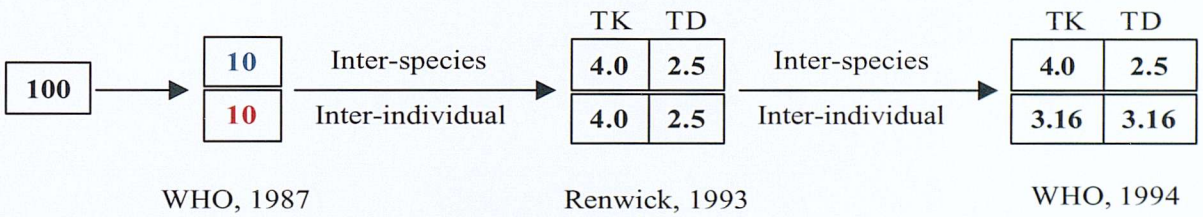
The outcomes of this analysis were several-fold:

- That the observed variability in toxicokinetics (for both inter-species and inter-individual considerations) was greater than the observed variability in toxicodynamics.
- That under differing circumstances the necessary composite uncertainty factor (default of 100) might range from 1.2-10,000-fold, reflecting the best case and worse case scenarios of variability.
- For the vast majority of cases a composite uncertainty factor of at least 10 would be necessary when human toxicological data are available and a factor of 100-fold would be necessary when animal toxicological data are available.

The conclusion of the analysis was that the usual 100-fold default factor was likely to be adequate in the vast majority of cases. In the discussion of this paper, a recommendation was made to revise the regulatory framework so that data pertinent to the specific risk assessment could be used in the risk assessment and the derivation of the ADI. The authors proposed that such a revision would be to reduce uncertainty in the risk assessment of food additives and to define more precise ADI values (Beck et al., 1993; Renwick, 1993). During the ILSI "Workshop on the scientific evaluation of the safety factor" (Kroes et al., 1993) (based on a review of data in the literature) Renwick (1993) went on to propose default factors for differences due to kinetics and dynamics. Few data were available in support of any of the proposed kinetic and dynamic default factors, and most of this data was from drug-response studies rather than studies conducted with food additives or contaminants. The initial observation from the data analysed (Renwick, 1993) pointed to the need to allocate the entire 10-fold factor for general inter-species differences to toxicokinetic variability. It was considered however, that allocating the 10-fold factor to toxicokinetic differences only would be inappropriate because there may be species differences in the

mechanism of action, with humans more susceptible/sensitive than the test animal species. The data presented suggested the need for a greater factor for kinetic variability than dynamic variability. Based on haematological effects, the inter-species difference in toxicodynamics was approximately 3.0-fold, however greater differences were reported for organ-targeted toxicity, and so the factors were spilt, assigning more of the original 10-fold factors to differences in kinetics and less to differences due to dynamics. The human variability default was spilt in the same way, resulting in new default factors of 4.0-fold ($10^{0.6}$) to account for inter-species differences and human variability in toxicokinetics and of 2.5-fold ($10^{0.4}$) to account for inter-species and differences and human variability in toxicodynamics.

On consideration of the so-called "Renwick approach" by the WHO Task Group on Guidance Values for Human Exposure Limits (WHO, 1994), the subdivision of the inter-species factor, resulting in a 4.0-fold ($10^{0.6}$) default factor for toxicokinetic differences, and a 2.5-fold factor ($10^{0.4}$) for toxicodynamic differences was upheld. The division of the 10-fold factor for human variability ($10^{0.5}$) was amended, resulting in equal allocation of two 3.16-fold factors (see figure 1.6).



TK: toxicodynamics

TD: toxicokinetics

Figure 1.6 Subdivision of the 10-fold factors to allow chemical specific data to be introduced (WHO, 1994)

In addition to the two 10-fold factors used to allow for inter-species and inter-individual differences and variability, several other uncertainty factors may be used. The composite UF is then a product of numerous factors (table 1.8). The aim of this approach was to introduce some flexibility into the default-factor based approach used up until this point in the risk assessment of food additives. By sub-dividing the inter-species and human variability default factors of 10-fold into factors specific for kinetics and dynamics, the use of chemical specific data, were it available is now possible. The framework of this scheme will be considered, taking each identified source of variability, and discussing the sources of variability and the support and/or criticism of the defaults in the literature.

Factor	Range of values (default)
Inter-species variability	
- toxicokinetics	0-4.0 (4.0)
- toxicodynamics	0-2.5 (2.5)
Inter-individual variability	
- toxicokinetics	0-3.16
- toxicodynamics	0-3.16
Nature of Toxicity	Up to 10
Adequacy of Database	Variable (1-100; 3, 5, 10 preferred)
Adequacy of Pivotal Study	Variable (3, 5, 10 preferred for LOAEL to NOAEL)

Table 1.8 The uncertainty factors used in the IPCS approach. (WHO, 1994)

1.18.1 Identification of factors contributing to inter-species differences

The 10-fold factor allowing for inter-species differences is designed to account for the extrapolation of a no-observed adverse effect level in a group of animals to a no-observed effect level in the average human. These differences can be divided into differences due to metabolic size and all other remaining species-specific differences.

Support for the 10-fold inter-species default factor is documented in the literature (Dourson & Stara, 1983). This analysis and subsequent discussion (Renwick, 1991; Dourson et al., 1996), including probabilistic modelling (Baird et al., 1996, Price & Stickney, 1997, Swartout et al., 1998 and Slob et al., 1998) indicate that the 10-fold factor for inter-species differences is appropriate.

Different techniques are used to compensate for differences in kinetics in order to provide dose-equivalence. This process is known as allometric scaling. Some differences between species are solely due to the physical size and metabolic capability of the animal (e.g. different hepatic and renal blood flows). Two main methods have been used for allometric scaling. Scaling by bodyweight is used by the JECFA, and most international agencies in evaluations, but there has been debate that surface area would be a better basis for scaling (Calabrese, 1992). Calabrese et al., (1992) also discussed the notion that the scaling factor should be independent of the uncertainty factor used for inter-species variation, and should be considered separately. However the convenience of bodyweight scaling has prevailed.

It is assumed that there may be marked inter-species differences in susceptibility with humans more susceptible than animals (Calabrese et al., 1992). Fundamental differences between species will be related to physiological processes e.g.:

- Heart rate and blood flow to the major organs, including the kidney and liver.
- Differential expression of receptors or metabolic enzymes.
- Different basal metabolic rates, and rates of detoxification.

The variability within the data will also be different for animal versus human data. The test-species data are derived from a more homogenous population. This phenomenon is not currently recognised as a source of uncertainty in the risk assessment process. There is a possibility therefore that outliers in the human population may not be adequately covered based on extrapolation from animal-derived data. However, the hypothesis that humans are more variable in their response to chemicals is inherent is the use of a 10-fold factor to allow for inter-individual differences.

Certain factors have been proposed for particular species to allow extrapolation to humans (Vermiere et al., 1999), and the possibility of a specific default based on the test species used in the pivotal study has been investigated (Walton et al., 2001a).

1.18.2 Identification of factors contributing to inter-individual variability

The 10-fold factor allowing for inter-individual variability is designed to account for the extrapolation of the no-observed adverse effect in the average human to a no-observed effect level in sensitive subgroups of humans (see figure 1.7).

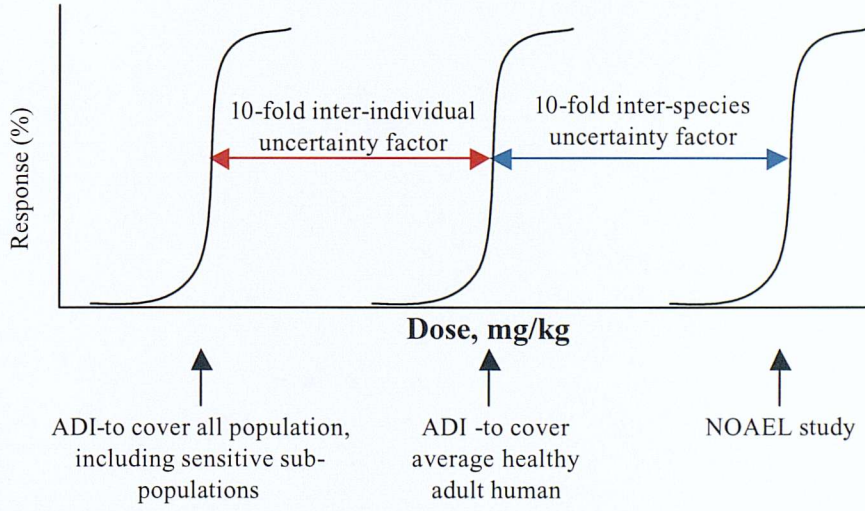


Figure 1.7 The dose-response from the pivotal study in animals is extrapolated to the ADI, using the two 10-fold factors.

The 10-fold factor is also assumed to be sufficient to protect individuals in particularly susceptible subgroups. Differences in susceptibility are due to many factors (Dybing & Soderlund, 1999), affecting both kinetics and dynamics and may be due to differences in:

- Sex (including the effects of sex hormones and general physiological differences)
- Age (possible decreased metabolic capability in neonates and the elderly and increased metabolic capability in children)

- Ethnicity (genetics, cultural differences, diet and other factors contributing to metabolic clearance)
- Xenobiotic metabolism capability and polymorphisms
- Disease state (e.g. hepatic, renal or cardiac impairment affecting clearance of xenobiotics)
- Nutritional status (differing levels of metabolising enzyme induction and cofactors, and anti-oxidant deficiency)
- Other sources of variability, such as glucose-6-phosphate dehydrogenase deficiency

Several analyses have been undertaken, investigating the extent of human variability in the population (Dourson & Stara 1983; Calabrese, 1985; Hattis et al 1987; Hattis & Silver, 1994, Hattis, 1996, Renwick and Lazarus 1998, Burin & Saunders, 1999). These analyses concluded that in the majority of cases the 10-fold factor is adequate and that 80-95% (Calabrese, 1985) or more (Renwick & Lazarus, 1998) of the population are estimated to be covered. Scenarios were identified where the 10-fold factor may not be adequate, e.g. in some pathways of xenobiotic metabolism (Renwick & Lazarus, 1998), and for highly lipophillic compounds. However the data analyses used to make these predictions have some weaknesses:

In some cases it is not entirely clear what part of variability is under investigation, i.e. the pharmacokinetic and pharmacodynamic components are not clearly separated (Hattis et. al 1987).
All the human data analyses have used pharmaceutical data to estimate variability; there are no data available on the variability in susceptibility to food additives. This is a relevant criticism as the variability in response; i.e. receptor-drug interactions vs. a toxic effect are likely to be very different.
Recorded responses are all single measurements, and therefore the measured variability would include experimental variability and intra-individual variability.

1.19 Support for the subdivision of the 10-fold inter-species and human variability default factors.

Following the initial subdivision of the inter-individual 10-fold factor by Renwick (Renwick, 1993), databases of relevant kinetic and dynamic data were compiled using published data from therapeutic drugs (Renwick & Lazarus, 1998). In the absence of specific data for food additives in humans, these data represent the most extensive data available for human pharmacokinetics and pharmacodynamics. The mean coefficients of variation (CoVs) were calculated, and subsequent analyses supported approximately equal sub-division of the 10-fold default factor for inter-individual variability resulting in two equal 3.16-fold factors (see figure 1.6). Using data for nine pharmaceuticals, Suh et al., (1999) calculated that most factors for kinetics and dynamics were less than the default values. Where data for a particular drug included patients with renal or hepatic impairment or the presence of a genetic

polymorphism the kinetic default factor of 3.16 was exceeded. After the inclusion of the chemical-specific data the composite uncertainty factors were less than 100.

Very few data were available to allow adequate assessment of the "new" dynamic default factors, to allow for inter-species or human variability differences (Renwick, 1993), and so most data were derived from drug-response data. Mathematical modelling has been used to generate plasma concentration values to allow comparison of concentrations giving the same response in different subjects. Further validation of the toxicodynamic default factors (for both inter-species and human variability) is required, and depends to some extent on the development of suitable biomarkers of toxic effects. Biomarkers have been defined as "physical sign or laboratory measurement that occurs in association with a (toxicological) effect" (Lesko & Atkinson, 2001). In particular surrogate endpoints for toxicological effects in humans would be required (as it would not be ethically acceptable to induce the toxicological effect itself). Surrogate endpoints are biological measures that are distal changes in the causal chain of events leading to toxicity. These markers could be developed in experimental animals, to ensure that the sensitivity, specificity and probability of false positive or false negative effects are acceptable.

1.20 Ensuring adequate protection for the human population

In terms of inter-individual variability and the application of uncertainty factors, the fundamental consideration, particularly in terms of risk management, is to decide what proportion of the population to cover. Two methods of adequately covering sub-groups of the population have been proposed. The first method (Renwick & Lazarus, 1998) suggests that a consistent percentage of the population, (comparing mean parameter estimates and CoVs), such as the 95^{th}, 97.5^{th} or 99^{th} percentile of the population should be covered. Using this method allowance could be made for varying incidences of sub-groups within the population, and differences such as genetic polymorphism could be taken into account. The other approach (Silverman et al., 1999) suggested an adjustment factor calculated on the basis of a comparison of the 50^{th} percentile of the general population with the 95^{th} percentile of the subgroup. This approach would not accommodate different incidences of sensitive sub-groups for different risk assessments/compounds. This approach may be suitable in considering sub-groups such the elderly, infants and children for which the demography is known and would apply to all risk assessments. However it would not be such a successful approach for sub-groups such as poor or extensive metabolisers, resulting from genetic polymorphism where information regarding the incidence of the sub-group in the population is unknown. In terms of risk management, the ideal would be to cover a set percentage of the population (including subgroups) using chemical-specific data. However it is unlikely that chemical specific data on toxicokinetics or toxicodynamics would be available for the vast majority of compounds.

The theoretical human dose-response for a food additive is assumed to be a unimodal log-normal distribution i.e. a bell-shaped curve, where the 95th percentile of the population lies within 2 standard deviations of the geometric mean (see figure 1.8). However in some cases a bimodal population distribution occurs caused by extreme outliers in sensitive subgroup. Using the approach of Renwick and Lazarus (1998) the factor required to adequately protect the whole population is calculated as a ratio between the 50th percentile of the normal population and the 95th percentile of the sensitive sub-group.

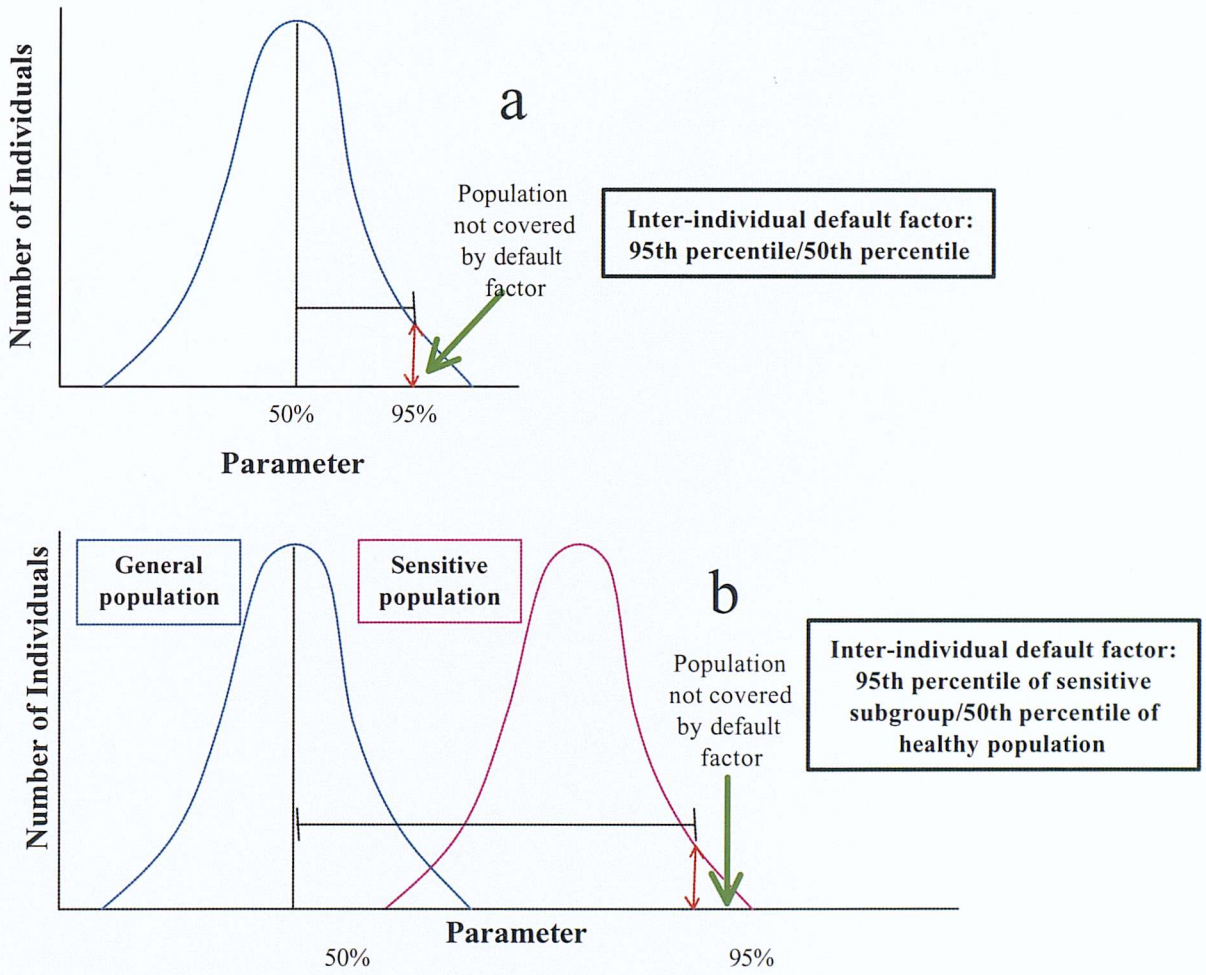


Figure 1.8 Unimodal and bimodal distributions of population effects.

1.21 Does the framework adequately protect sensitive subgroups?

1.21.1 Infants and children

The adequate protection of infants and children using the framework has been the subject of much discussion. Throughout the 1990's there were several reviews of age-related differences in susceptibility. (The ILSI conference in the USA, 1990, Renwick et al., 1991 the US National Research Council in 1993, the passing of the Food Quality Protection Act (FQPA) in 1996 and the ILSI working group in 1996/7)

(Olin, 1998). In 1996 the FQPA proposed the use of an additional 10-fold factor to allow for adequate protection of infants and children (Environmental Protection Agency, 1999). In Europe the issue was discussed at an ILSI workshop (Olin, 1998) where the suitability of the ADI to children and infants was discussed, in relation to susceptibility and developmental study protocols.
Two major arguments exist for the notion that food additives may pose a greater risk to children than to adults (Larsen & Pascal, 1998):

- Infants and children are more susceptible to some types of chemical insults (both in their ability to eliminate potentially toxic compounds, and via enhanced organ toxicity.
- Infants and children have a higher food intake compared to adults on a per kg bw basis.

The conclusion of the multitude of data presented was that infants and children are/should be adequately covered using the conventional risk assessment framework. The ADI is designed to cover the entire population (WHO, 1987), and covers infants and children, based on the following assumptions:

- It is assumed that the toxicokinetic and toxicodynamic parameters in the developing animal mirror those in humans (Østergaard & Knudsen., 1998).
- If animal studies indicate that the foetus/immature animal is the most sensitive group, then the results of this study will be used for determination of the NOAEL and therefore the ADI
- Children generally have higher clearance per kg body weight and would have a lower corresponding body burden than adults when they receive the same dose expressed in mg/kg bw/day (as used to define the ADI) (Larsen & Pascal, 1998)

The major criticism of the battery of toxicological tests performed was that the chronic studies commence when rats are 6 weeks of age. As a result the very young equivalent to human infants less than 12 weeks of age are not reflected in the toxicological database (Larsen & Pascal., 1998; Renwick, 1998; Østergaard & Knudsen., 1998). Consequently most food additives are not permitted in infant formulas/foods (although infants could also be exposed through breast milk).

A substantial review of biological systems revealed age-related differences in most body-systems (liver, lung, kidney, brain and blood) (Østergaard & Knudsen., 1998). The most important were the age-related differences in renal clearance (increased in infants and children) and xenobiotic metabolising enzyme activity (generally increased – but see below) (Østergaard & Knudsen., 1998). Examination of a database of therapeutic drugs to determine differences in toxicokinetic parameters highlighted the increased clearance and elimination capacity of infants and children in comparison to adults (Renwick, 1998). Of course, this may be beneficial (for metabolically deactivated compounds) or may be detrimental, in cases where compounds are metabolically activated. In the case of the latter, special consideration may be

required for infants and children. Increased clearance and elimination in infants and children may go someway to compensating for perceived increased toxicodynamic susceptibility. The ideal situation would be to correct the 10-fold human variability factor by an experimentally derived neonate: adult ratio. Age-related differences in toxicodynamics could not be evaluated, due to a lack of data, and the ethical issues involved with conducting the necessary studies in infants and children. Where kinetic data do exist there are often no relevant comparable animal data, e.g. where *in vivo* human data exist, but neonatal animal data do not. There is a need to address some "data gaps" before any further analysis/refinement of the risk assessment process regarding infants and children can progress (Renwick et al., 1991 and Renwick et al., 2000).

1.21.2 Ethnic groups

It is important to consider what is meant by the term "inter-ethnic variability", as there are two main possible sources of variation that may affect susceptibility to a food additive. Genotypic differences could affect dynamic or kinetic parameters, and cultural differences could affect exposure, e.g. through the diet (Smith, 1996). From a risk characterisation perspective, any potential differences in these factors would need to be assessed against the values for the population upon which the risk assessment is based, most often Caucasian or Japanese population groups.

An analysis of the inter-ethnic differences in xenobiotic metabolism (Renwick, 1996) reviewed data for type I and type II metabolic pathways, comparing a variety of races. Consideration was given to the following points:

Differences in the range of values observed for metabolism/elimination of probe substrates between ethnic populations. (Measurements in one ethnic group may not necessarily apply to other ethnic groups.) Differences in the incidence of poor metabolisers (PMs) in the population. (Poor metabolisers would be at increased risk from an equal exposure if the parent compound were the active entity.)

Major ethnic differences have been observed in cytochrome P450 mediated metabolism and in type II metabolic systems such as glucuronidation, reflecting in pharmacokinetic differences (Pacifci & Pelkonen, 2001). Pharmacodynamics would be expected to vary as well, particularly in parameters such as receptor expression. Certainly differences in the incidence of PMs in the population of consideration should not prove a problem providing that this incidence is compared and used in the risk assessment. The overall conclusion based on reasonable kinetic data, but poor dynamic data was that inter-ethnic differences are not anticipated to require an additional factor related to pharmacokinetics or pharmacodynamics (Renwick, 1996). There are few data that suggest that risk assessments made on the basis of data from one ethnic group would not be applicable to another.

1.21.3 Genetically polymorphic groups

A genetically polymorphic population occurs when two or more forms of a gene species that are subject to simple inheritance exist in the population (Laurence, 1998). In the case of risk assessment these would impact the adequacy of the defaults if they occurred in genes affecting kinetics, e.g. enzymes or plasma proteins or in genes affecting dynamics, e.g. receptor isoforms. Many polymorphic enzymes involved in detoxification mechanisms are described in the literature including polymorphisms of CYP2D6, CYP2C19, alcohol and aldehyde dehydrogenases, N-acetyl transferase and glutathione-S-transferase enzymes (Lee, 1991 and Ketterer, 1996).

The impact of a polymorphism would be dependent on the route of metabolism of the chemical under assessment. Potentially the inter-individual toxicokinetic default may be inadequate for poor metabolisers if the substrate for the enzyme is the active chemical species (Dorne et al., 2002). This is of concern to risk assessors, as these individuals are not clearly recognisable, and specific advice cannot be given to them.

1.22 How can the CSAF (chemical specific adjustment factor) framework be used?

The new IPCS approach (see figure 1.6), in particular the sub-division of the 10-fold factor represented an important departure from traditional risk assessment frameworks. Firstly default 10-fold factors can be replaced by toxicologically appropriate sub-factors. Secondly, the new default factors allow scientifically valid and relevant data to contribute to the derivation of a modified uncertainty factor (chemical-specific adjustment factors) and therefore a more appropriate ADI. The use of CSAFs would result in the risk assessment process being less uncertain and the resulting ADIs being more appropriate.

Aside from the primary aim of making the provision for experimentally derived data to be used in risk assessment, the model proposed by the IPCS allows for greater transparency in the setting of ADI values. It also reduces the need to allocate temporary ADIs, as the necessary uncertainty can be built into the composite uncertainty factor.

1.23 What data are necessary to replace the default factors? Case studies of chemicals where CSAFs have been used.

In order to replace the inter-species default factor, data would be required to compare the mean parameter values between humans and the animal test species. The magnitude of any difference would be used to calculate the CSAF. To replace the inter-species default, an analysis of the population distribution of relevant data would be required. Having decided what proportion of the population to cover, the CSAF

could be calculated. The types of data required in order to derive CSAFs have been defined (IPCS website). *In vivo* toxicokinetic studies or *in vivo* measurements following environmental or occupational exposure, or *in vitro* measurement of the elimination processes incorporated into a physiologically-based pharmacokinetic (PBPK) model would constitute the type of kinetic data necessary.

The main advantage of using *in vivo* human data is that all *in vivo* processes are included in the estimate, including the role of extra-hepatic tissues. PBPK modelling can be used to estimate the internal dose based on animal data followed by inter-species scaling, and has been used in low-dose extrapolation (non-threshold approach), but it could also be used to replace the kinetic inter-species default factor.

In theory data from any step in the mode of action from the interaction of a toxicant with its molecular target up to the final toxic response could be used to replace the toxicodynamic default. Potential sources of toxicodynamic data include *in vivo* or *in vitro* studies studying the final toxic effect, or an intermediate in the chain of events, in which variability due to pharmacokinetics has been excluded. (Certain problems may arise when using surrogate endpoints.) Biologically based dose-response models (BBDRs) could be used to replace both the toxicokinetic or toxicodynamic aspects. (*In vitro* analysis of sub-group susceptibility could be conducted using BBDRs).

The potential of *in vitro* data to be used in the calculation of a CSAF has been investigated (Walton et al., 1999). Examination of 65 JECFA evaluations, revealed that 18 evaluations had associated *in vitro* data that were not used at the time of evaluation. Nine of the original eighteen *in vitro* studies were related to the pivotal study used to calculate the ADI. If *in vitro* studies are carefully designed, for example to provide mechanistic, toxicokinetic or toxicodynamic data, providing that they are relevant to the pivotal study, they could and should be used to reduce uncertainty in the setting of an ADI value.

The following sections describe assessments for specific chemicals and **are the only examples to date** where CSAFs based on experimental data have been used to replace the default uncertainty factor.

1.23.1 Boron

A review of the database for boron (Dourson et al., 1998) resulted in the inter-individual 10-fold factor being replaced with a 6-fold (composite) factor. The renal clearance of boron in female rats was about 4 times greater than in humans, indicating that the inter-species toxicokinetic default was appropriate in the absence of reliable data. Based on the critical effect of foetal weight, the clearance of boron in pregnant rats was found to be lower compared to non-pregnant rats. This effect is due to the physiological effect of increased glomerular filtration rates (GFR) in pregnancy. It was assumed that the 4fold difference between non-pregnant rats and humans would also apply during early pregnancy. Data do not exist

describing the clearance of boron in pregnant humans, however the inter-individual toxicokinetic default of 3.16 was replaced with a factor of 1.8, reflecting the potential human variability in GFR between pregnant and non-pregnant humans (see figure 1.9 below).

Mean GFR in pregnancy (human)	144
Mean GFR - two standard deviations	144 – (2 x 32) = 80
Ratio of :	
Mean GFR/Mean GFR – 2 standard deviations	144/80 = 1.8

Figure 1.9 Calculation of the CSAF for pharmacokinetic inter-individual differences.

No additional data were available to replace the default factor for inter-species differences, although all that would be required to complete the database would be a pharmacokinetic study in the rat.

1.23.2 Cyclamate

The risk assessment of cyclamate (E952) is based on the toxic metabolite, cyclohexylamine (CHA). The conversion of cyclamate to cyclohexylamine is effected by intestinal bacteria and is subject to wide human variability. Most people form very small amounts of CHA. The SCF evaluated cyclamate, cyclohexylamine and dicyclohexylamine in 1985 (cited in SCF, 2000) and allocated a temporary ADI of 0-11 mg/kg bw/day expressed as cyclamic acid for cyclamate and its sodium and calcium salts. The ADI was based on the NOAEL of 100 mg/kg bw/day from a study, where the critical effect was testicular toxicity. The ADI for cyclamate was calculating assuming absorption of~63% of cyclamate and a conversion rate of ~30%, giving an overall conversion rate of 18.9%. The ADI was temporary due to uncertainties regarding the relevance of testicular toxicity in rats to man.

Subsequent evaluations in 1988, 1991 and 1995 reviewed new data on the metabolism of cyclamate to cyclohexylamine in humans, and the results of primate studies (in *Cynomolgus* monkeys) showing that sensitivity to CHA was similar to that of the rat. Human studies had revealed substantial variability in the population in the proportion of cyclamate metabolised to cyclohexylamine. Requests for further data were made, to include the following:

- To establish the proportion of low converters that can convert to high converters with sustained exposure to cyclamate.
- For high converters, to establish the range in conversion and the persistence of conversion at this rate.

- If possible, to conduct in vitro studies to establish the relative sensitivity of the rat, monkey and human testes to cyclohexylamine
- To receive updates on three epidemiological intake studies.

The most recent evaluation of cyclamate (SCF, 2000) reviewed the outcome of these studies. Three intake studies in Spain revealed that only 0.16% of the population (0.9% of consumers) were estimated to consume >11mg/kg bw/day. Intake data from other countries, (Brazil, Germany and the Netherlands) indicated that high consumers were not exceeding the ADI of 11 mg/kg bw/day.

The outcome from metabolic studies in humans was that none of the previously identified non-converters (n=31) became high converters. Considerable variability was observed in the data from the 13 high converters studies. In 6 subjects, the average conversion rate exceeded 18.9% by up to 2.5-fold.

A review of available data in relation to the potential use of *in vitro* testicular systems to determine species sensitivity revealed that such studies were not feasible due to the lack of validated models.

An epidemiology study investigating the fertility of cyclamate workers was inconclusive regarding the possible toxicological effects of occupational exposure to cyclamate or CHA. This was in part due to the lack of statistical analysis because of confounding factors, such as alcohol intake and smoking. Subjects attending an infertility clinic were compared with control subjects in a case: control study (n=405 and 379 respectively). Semen and urine samples were taken and a food frequency questionnaire was compiled (using the years 1994-1996). No significant difference in intake was found between cases and controls or in urinary excretion of either cyclamate or CHA.

Taking into account all the new data, the committee concluded that the questions regarding human variability in conversion had been satisfactorily answered, and that the conversion rate used previously (18.9%) was no longer relevant. The committee concluded that due to the large inter-individual differences in conversion rate, the maximal conversion rate would be 85%. This was deemed most appropriate for calculating the ADI. Since conversion rate was now being taken into account a reduced uncertainty factor should be applied to allow for human variability. A full ADI for cyclamate was established, using the original NOAEL of 100 mg/kg bw/day, taking into account the difference in molecular weight of cyclamate and cyclohexylamine. The conversion rate of cyclamate to cyclohexylamine was used (85%) and an overall safety factor of 32-fold was applied to the NOAEL resulting in an ADI of 0-7 mg/kg bw/day expressed as cyclamic acid for cyclamate and its sodium and calcium salts (refer to figure 1.10 below).

Maximum overall conversion rate of cyclamate to cyclohexylamine and absorption of the latter is 85%

NOAEL for cyclohexylamine is 100 mg/kg bw

Allowance for difference in molecular weights between cyclamic acid and cyclohexylamine:

$$\frac{\text{MW cyclamate}}{\text{MW cyclohexylamine}} \quad 1.81 \text{ rounded up to } 2$$

Safety factor : 10 for inter-species extrapolation
3.2 for inter-individual variations in toxicodynamics
1 for inter-individual variations in toxicokinetics

$$\text{ADI} = \frac{\text{NOAEL for CHA x MW ratio}}{\text{Safety factor x conversion}} = \frac{100 \times 2 \times 100}{32 \times 85} = 7.35, \text{ rounded down to 7 mg/kg bw}$$

Figure 1.10 Derivation of an ADI for cyclamate, using chemical-specific adjustment factors (from SCF, 2000)

1.23.3 Dioxins

Dioxins are potent toxins that share a common mechanism of toxicity, and include compounds such as polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated planar biphenyls (PCBs). Biodegradation of dioxins is slow, and they are widely distributed in the food chain; humans are therefore under continuous exposure to dioxins. The risk assessment of dioxins as a whole is based upon 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) which has been subject to intensive study. TCDD is one of the most potent dioxins, and has one of the longest half-lives. It is slowly eliminated by humans, resulting in bioaccumulation, and the steady state-body burden only occurs at 30-40 years of age. The most recent risk assessment for dioxins was made in 2002 by the JECFA (WHO, 2002). Whilst dioxins are contaminants of food rather than additive chemicals, the fundamental risk assessment process is identical, and it is one of the few chemical risk assessments where toxicokinetic default factors have been replaced with uncertainty factors derived from chemical-specific data.

Previous risk assessments of dioxins were based on carcinogenicity. More recently, a new critical effect of developmental toxicity has been observed in the male offspring of female rats exposed to TCDD. A NOAEL of 12.5 ng/kg bw was observed in a study (Ohsako et al., 2001 cited in WHO, 2002) where pregnant rats were given a single oral dose of TCDD on day 15 of gestation. The critical effects were reduced ventral prostate weight and decreased anogenital distance in male offspring. In another study a LOAEL of 25 ng/kg bw was observed in a study (Faqi et al., 1998, cited in WHO 2002) where dams were treated subcutaneously before mating and throughout mating, pregnancy and lactation. The reported

critical effects were decreased sperm production and altered sexual behaviour in male offspring.

The maternal body burden following long-term dosing was calculated, using an estimate of bioavailability (60%) and a linear model predicting maternal body burden after repeated dosing (Hurst et al., 2000, cited in WHO, 2002). Additionally, the background body burden in rats was found to be 3-12 ng/kg bw, depending on age. The JECFA correspondingly added 3 ng/kg bw to the body burden estimate. In conclusion, these toxicokinetic considerations resulted in a NOAEL of 16 or 22 ng/kg bw (based on linear or power model respectively) and a LOAEL of 28 or 42 ng/kg bw (based on linear or power model respectively). As discussed previously, a more chronic intake limit would be preferable, and so the committee derived extrapolated high monthly intakes (EHMIs) taking into account the difference in half-life between rats and humans. Based on the NOAELs these were (237 pg/kg and 330 pg/kg respectively) and on the LOAELs (423 pg/kg and 630 pg/kg respectively).

The composite uncertainty factors (one based on the NOAEL and the other on the LOAEL) were estimated as follows. Because body-burden scaling has been used in the derivation of the EHMI no inter-species toxicokinetic-scaling factor is required. Due to limited data relating to variability in human kinetics, the default of 3.2 (3.16) was used. The dynamic data indicate that humans are probably less sensitive than rats for some effects, but the Committee could not ignore the possibility that humans could be at least as sensitive as the most sensitive rat and so the combined inter-species and human toxicodynamic variability uncertainty factors were set to 1. In total the composite safety factor was 3.2, based on the NOAEL. This resulted in PTMIs of 74 pg/kg bw/month and 103 pg/kg bw/month (based on the linear or power model derivation of body burden). In the case of the LOAEL data an extra factor of 3-fold was added to the composite uncertainty factor, giving a value of 9.6 which resulted in PTMIs of 44 pg/kg bw and 66 pg/kg bw (based on linear or power model derivation of body burden). The estimated PTMIs ranged from 44-103 pg/kg bw. The Committee decided to choose the midpoint in the range of 70 pg/kg bw as the PTMI for dioxins. Dioxins are of major public health concern and the subdivision of the usual 10-fold uncertainty factors was essential in deriving a logical PTMI.

1.24 The aims of this research project: evaluating the adequacy of the default kinetic uncertainty factors as proposed by the IPCS.

As previously discussed, the default toxicokinetic factors for inter-species differences and human variability was proposed using pharmaceutical data. The previous section has highlighted some chemicals (food additives and contaminants) for which there is sufficient information to replace the default factors for toxicokinetics and toxicodynamics. These chemicals are the exception rather than the rule; very few databases exist where relevant and adequate chemical-specific data can be used to replace default uncertainty factors. The majority of data pertaining to humans is from intoxication or

epidemiological studies, where duration and magnitude of exposure are generally not recorded adequately. Where data of the necessary quality exists, it would normally apply to the replacement of the inter-species default for variability in toxicokinetics.

Clearly the ideal situation for the risk assessment of a novel food additive, would be a full complement of human *in vivo* data, adequately quantifying the variability in toxicokinetics and toxicodynamics across the entire population. This would remove most of the inherent uncertainty present in default frameworks. For obvious reasons, (both economical and ethical) this is not achievable. However, it is desirable that in some way the constantly evolving risk assessment frameworks are tested.

The testing of the adequacy of the inter-species and human variability toxicokinetic default factors (4.0 and 3.16-fold respectively) using *in vivo* data is the subject of this thesis. There were three hypotheses under investigation:

1. That the inter-species default factor of 4.0-fold is adequate
2. That the human variability default factor of 3.16-fold is adequate
3. That saliva can be used as a biomaker of exposure to food additives

In order to test these hypotheses, four probe substrates were chosen for investigation. These compounds were food additives for which there was a numerical NOAEL and ADI. They were all of low molecular weight and were metabolised via different pathways. The substrates chosen were:

- Butylated hydroxytoluene
- Curcumin
- Propyl gallate
- Thiabendazole

Background information regarding the metabolism and toxicity of these compounds is given in subsequent chapters.

In order to address the three hypotheses, a series of experiments were conducted in animals and humans. The animal experiments were conducted by post-doctoral research fellows (Dr Kim Walton and Dr Warren Keene), whilst all the human experiments were conducted by myself (Sara Tullberg). I collaborated with data analyses of the animal studies, and conducted all the WinNonlin™ analyses (see chapter 2, materials and methods). The fundamental design of the experiments was identical. Both species received a single oral bolus dose at the no-effect level, the NOAEL for animals and the ADI for humans. Human subjects received the dose as a capsule whilst the animals received a gavage dose. Post-

dose blood samples were used to quantify the concentrations of food additive. In the human studies, saliva and urine samples were also taken to evaluate the use of saliva as a biomarker, and to examine the excretion of parent drug in the urine. The precise detail of time points for each substrate is given in the compound specific chapters.

The desired outcome of the human and animal studies was to construct a concentration time profile from the individual quantified concentration of food additive at each time point from which pharmacokinetic parameters could be calculated. Figure 1.11 shows a typical concentration-time profile obtained after oral dosing.

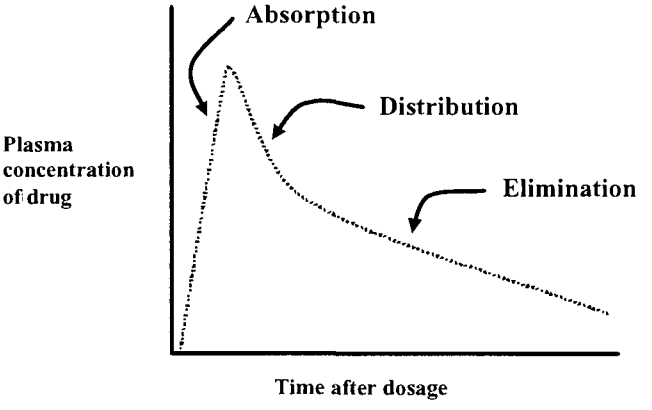


Figure 1.11 A typical concentration-time profile for a substrate following oral dosing.

From the concentration time-profile it is possible to derive and calculate some pharmacokinetic parameters that can be used to compare concentration time profiles between species and between individuals in the human population. The maximal concentration reached is called the maximal concentration as is designated Cmax. This measure is most important where considering acute exposure to chemicals, since it represents maximal exposure. The units of measure are amount per volume. In the case of most of the food additives under study this was ng/ml. The area underneath the concentration time profile is known as the AUC (area under the curve) and is a measure of overall exposure, hence it is a better indicator of chronic exposure, as it reflects the rate of elimination of the chemical as well as the absorption and distribution. AUC is reported as AUC observed (AUC_{obs}) and AUC extrapolated (AUC_{inf}) to infinity in this thesis, depending on the whether the trapezoid formula or the mathematical model was used (see chapter 2 for detail of mathematical model). The units of measurement are ng x min/ml. Clearance is a measure of the rate of elimination of the compound. It is measured as volume of blood cleared of the compound per unit of time, in this case ml/min. In this thesis all clearance values are actually CL/F, where CL is clearance and F is bioavailability. Bioavailability is the fraction of the dose absorbed x fraction of dose escaping first pass metabolism. Bioavailability was not estimated, as intravenous-dosing experiments would have been required (dose entering the systemic circulation directly by-passes absorption and first pass metabolism), and the time constraints of the project meant that this

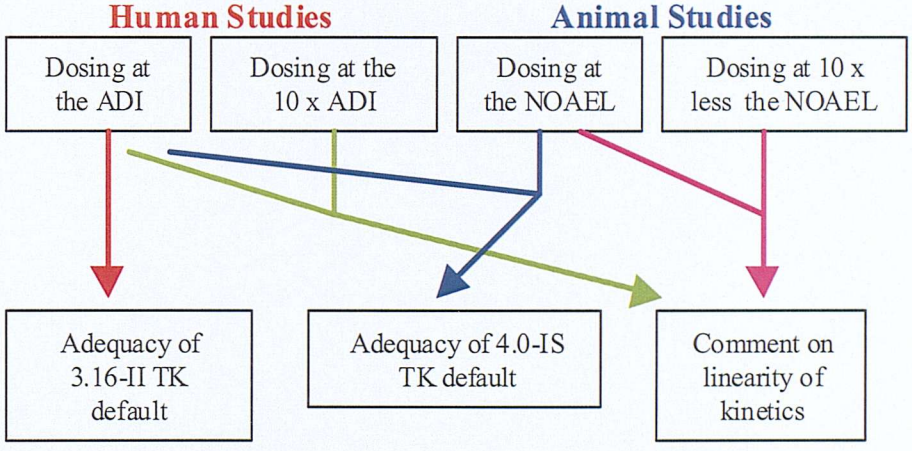
was not possible. In order to estimate bioavailability, the following equation (figure 1.12) would need to be solved:

$$\frac{AUC_{oral}}{AUC_{iv}} = F$$

Figure 1.12 Deriving bioavailability from oral and i.v AUC values

The initial studies in humans at the ADI revealed very low concentrations of food additives post-dose. Experiments where volunteers received the food additives at doses of 10 x ADI were conducted to aid reliable quantification of the food additives, and to produce data upon which kinetic comparisons could be made. Justification for giving a higher dose was based on the estimation of an acute reference dose (ArfD) for each compound (see compound specific chapters). Dose equivalent studies (0.1 x NOAEL) were also conducted to complete the data set so that the linearity of kinetics between the low and high doses in each species could be made.

The final outline of experiments conducted and presented in this thesis is given in figure 1.13. In addition, pharmacokinetic parameters from saliva concentration-time profiles were compared with plasma PK parameters to assess the possible use of saliva as a biomarker of internal exposure.



I.I – inter-individual variability or human variability

I.S – inter-specied variability

Figure 1.13 Experimental plan of the data presented in this thesis.

The following chapter describes the materials and methods used to derive the data sets for the probe substrates. Subsequent chapters are organised in alphabetical order (relative to the substrate) and give background information regarding metabolism and toxicity of the compound, results from the

experiments described above and discussion of the compound specific data. The final discussion provides conclusions regarding the three hypotheses under examination and addresses the possible impact of non-

Chapter 2: Materials and Methods

2. Materials and Methods

This chapter describes how the clinical studies were conducted; the subsequent processing and analysis of the samples, and gives details of all chemicals reagents, and laboratory equipment used. Details of the integration and mathematical processes used to generate the plasma and saliva concentrations of the food additives under study are also given. Additionally, a description and explanation of the mathematical modelling system used is presented.

I conducted all of the studies in humans, and together with the help of the laboratory technician, analysed all the samples. The animal studies were conducted by Drs Walton and Keene, as indicated in the text. I then used Excel to calculate the PK parameters, and where possible, used a mathematical model, WinNonlin™, to fit both the human and animal data.

2.1 Conduct of Clinical Studies

Both sets of studies were subject to ethical approval by the South West Local Research Ethics Committee. Additionally the studies complied with all aspects of the Research Governance Framework in place at Southampton General Hospital, including the data protection act.

2.1.1 Recruitment of Volunteers

Healthy adult volunteers were recruited into the study using a standard advertisement, through poster, email and word of mouth. The inclusion criteria for the study were as follows:

- Subjects must be between 18-65 years of age
- Subjects must be non-smokers
- Subjects must not have taken prescription medication for three weeks prior to the study day
- Subjects must be healthy (no serious illness for three months prior to the study day)

Once informed consent was obtained, volunteers were given food diaries for completion over the two days prior to the study, to include as much detail as possible including brand names.

2.1.2 Preparation of capsules containing the additive

Volunteers were weighed on calibrated weighing scales up to 2 days before the study. The dose was calculated as follows:

Volunteer weight* x ADI ** (or 10 x ADI) = Dose (mg/kg bodyweight)

* Volunteer weight in kg recorded to the nearest 0.5 kg

** ADI for BHT = 0.3 mg/kg

ADI for curcumin = 1.0 mg/kg

ADI for propyl gallate = 1.4 mg/kg

ADI for thiabendazole = 0.1 mg/kg

For all compounds except BHT, the appropriate weight of solid compound powder was weighed directly into an appropriately sized capsule. In the case of BHT, solutions of BHT were made in corn oil, and then the corn oil was weighed into a gelatine capsule. For the ADI studies, a 0.1 mg BHT/mg oil solution was prepared. It was not possible to make a 1mg/mg solution of BHT for the 10 x ADI studies, and a 0.5 mg BHT/mg oil was prepared. Vortexing and sonication were used to dissolve BHT into the corn oil. In the case of a 70kg volunteer, 210 mg of the 0.1mg/mg solution would result in a dose equal to the ADI, whilst 420mg of the 0.5mg/mg solution would result in a dose equal to 10 x ADI.

A size 1 gelatine capsule was placed on weighing scales (previously sterilised with ethanol) and the appropriate weight of the BHT corn oil solution was transferred using a Pasteur pipette. Once sealed, the capsule was stored in an appropriately labelled 6 ml glass blood tube in the refrigerator prior to the study. The details of materials used for all the clinical studies are given below in table 2.1.

Material	**Supplier**
Size 0, 1 or 3 gelatine capsules Colour-cap OP, white (AJA) E9906228	Shinogi Qualicaps, SA Calla de Granja 49 28100, Alcobendas, Madrid, Spain
Intravenous Catheter (Cannula) 18G, 20G JELCO™	Johnson and Johnson Medical, Ethicon SpA, Via del Mare, 56-00040, Pomezia, Italy
B.D-Connecta ™ Plus 3 (for ADI studies)	Becton Dickinson, Infusion Therapy AB, SE-25106, Helsongborg, Sweden
SmartSite® Needle –Free Extension Set	Alaris Medical UK Ltd., Basingstoke, Hants, RG22 4BS, UK
B.D Plastipak 2, 5, 10 ml syringes	Becton Dickinson, S.A, S.Auguston del Guadalix, Madrid, Spain
5ml Sodium Chloride BP POM, 0.9% w/v	B. Braun Melsugen AG, D-34209 Melsungen, Germany

Table 2.1 Materials used in the clinical studies

2.2 Collection of Samples

2.2.1 Cannulation

Volunteers arrived at the Wellcome Trust Clinical Research Facility (WTCRF), at Southampton General Hospital between 08.30 and 09.00 hours. A cannula was inserted (size dependent on volunteer – normally 18 or 20 gauge) and a three-way tap or smart site was attached to the cannula. Saline (5 ml) was flushed through the cannula to ensure patency.

2.2.2 Baseline Sampling

To ensure that saline was not present in the blood, the saline and a sample of about 2 ml blood was removed via the cannula and discarded. A 10 ml blood sample was then taken, using a 10 ml plastic syringe. Blood was transferred from the 10 ml plastic syringe into a pre-labelled heparinised 10 ml blood tube and placed on ice prior to centrifugation. A 5 ml saline flush was then introduced back through the cannula to prevent blood clotting in the cannula.

A baseline saliva sample was taken simultaneous to the plasma sample. The volunteer placed a dental roll in his/her mouth for several minutes. The dental roll was then placed in a 5 ml syringe and the saliva extracted by depressing the syringe plunger. The saliva was collected in a pre-labelled 6 ml blood tube and stored on ice prior to being frozen. A baseline urine sample was taken after the blood and saliva sample. Volunteers were asked to completely empty their bladder into a pre-labelled plastic beaker.

2.2.3 Processing the samples for storage

The 10 ml blood tube was placed in the centrifuge and spun at 4°C, 2,600g (2,800 rpm) for 10 minutes. 1 ml aliquots of plasma were transferred to further pre-labelled glass tubes using a Gilson pipette. Samples were then frozen whilst in the upright position. The total urine volume was measured in a plastic measuring cylinder, and the total volume recorded. A 40 ml aliquot of urine was retained in 2 x 20ml pre-labelled sterilin bottles. Samples were then frozen upright in wire sterilin racks.

2.2.4 Collection of post-dose samples

Samples of blood (10 ml), saliva and urine (total volume) were taken at several time points post dose as described above. The precise sample collection times were determined in the initial studies for each compound, and details of the time courses are given in the relevant result chapters. Typically the early collection times were half hourly, changing to hourly collection after 3 hours. Only BHT had a 24-hour

collection point, because the early studies indicated that BHT remained in the plasma 7-8 hours post-dose. When there were delays in taking a sample (for example, difficulties with the cannula) the actual times at which samples were taken were recorded on the protocol sheet. Samples that could not be collected were also recorded on the protocol sheet.

2.3 Study Food

Volunteers were asked to arrive for the study, having fasted overnight, from 19.00 hours the previous day. Volunteers were also asked to provide a diary of foods they had eaten in the two days prior to the study. After the baseline samples were taken, volunteers were given breakfast consisting of the following:

- 1 x 200 g Muller light virtually fat free yoghurt
- 1 x braeburn apple
- cup of tea or coffee, or water

The actual food eaten by the volunteer was recorded on the protocol sheet.
Immediately after the last mouthful of food the volunteer was asked to swallow the capsule containing the food additive. Water was made available to the volunteers to drink, as they required throughout the day. The volunteer consumed no other food apart from the food detailed in this section.

Lunch was provided for the volunteers after collection of the samples at 180 minutes. This consisted of:

- 1 x 400 g Tesco cheese and tomato bake
- 1 x bowl of Tesco mixed crispy salad
- 1 x KitKat (chocolate covered biscuit)

2.4 Transportation and storage of samples

At the end of the study, samples that had frozen were placed in pre-labelled plastic bags, which were then sealed. Samples that had not completely frozen were retained in the wire racks. An icebox, with lockable lid was used to transport the samples between the clinical facility and the analytical laboratory. Ice blocks were used to keep the samples cool during transportation. Prior to analysis the samples were stored in a –20°C freezer.

2.5 General Laboratory Reagents

All laboratory reagents used in the analysis of the clinical samples are given below in table 2.2

Material	Supplier
Butylated hydroxytoluene, (BHT; 2,[6]-Di-tert-butyl-p-cresol)). Minimum 99.0% [128-37-0] EC No 204-881-4 B-1378 100 g Lot 70K0140	Sigma Chemical Co. PO BOX 1450863178, St Louis, MO, USA
Butylated hydroxyanisole, (BHA; 2,[3]-t-Butylhydroquinone monomethyl ether) Mixed isomers (minimum 90% 3-isomer/9% 2-isomer) [25013-16-5] EC No 246-563-8 B-1253 100 g Lot 19H0261	Sigma-Aldrich Chemie GmbH PO 1120, 89552 Steinheim, Germany
Curcumin, 10 g Cat no. 81025, Lot 11466c 1-[4-(dimethylamino)phenyl]-6-phenyl hexatriene (DMAPPH), 25 g, 39290, Lot 406945/135199	Cayman Chemical Company Fluka Chemie AG CH9471, Buchs, SW, Sigma-Aldrich Chemie GmbH PO 1120, 89552 Steinheim, Germany
DMAPPH, 1-[4-(Dimethylamino) phenyl]-6-phenyl-hexatriene. 25 mg Cat no. 12322. Lot 406945/135199	Fluka Chemie AG CH9471, Buchs, SW, Sigma-Aldrich Chemie GmbH PO 1120, 89552 Steinheim, Germany
Propyl gallate, n-propyl gallate (3,4,5-Trihydroxybenzoic acid n-propyl ester) [121-79-9] EC No. 204-498-2,P-3130, 100 g, Lot 117110526	Sigma Chemical Co. PO BOX 1450863178, St Louis, MO, USA Sigma-Aldrich Chemie GmbH PO 1120, 89552 Steinheim, Germany
Ethyl gallate, Fluka, 48640, 100 g, 424126/1 30302	Fluka Chemie GmbH, CH-9471, Buchs, Sigma Aldrich Chemie GmbH, PO 1120, 89552 Steinheim, Germany
Thiabendazole, (2-[4-Thiazolyl]benzimidazole) Minimum 99% [148-79-8] EC No 205-725-8 T-8904, 100g Lot 119H0697	Sigma-Aldrich Chemie GmbH PO 1120, 89552 Steinheim, Germany
MPBZ, 1-methyl-2-phenylbenzimidazole Catalogue no. S463620, 5g	The Sigma-Aldrich Chemical Company, PO Box 366, Milwaukee,W163201,USA
Ethanol (AnalR grade) Methanol (HiperSolv for HPLC grade) Ammonia Solution sp. gr. ~0.88 (HiperSolv for HPLC grade) Acetic acid (AnalR grade)	BDH Laboratory Supplies, Poole, Dorset, BH15 1TD, England
Double-distilled water (for HPLC mobile phase)	Produced "in-house"
Cyclohexane (Residue analysis grade) Ethyl acetate (Analytical reagent grade) Pasteur pipettes Fisher brand, 150mm Product code FB50521	Fisher Scientific UK Limited Bishop Meadow Road Loughborough Leicestershire, LE11 5RG UK
Mazola, Pure Corn Oil 1 Litre	Bestfoods UK Ltd, Claygate House Esher, Surrey, KT10 9PN, UK

Table 2.2 General laboratory reagents used in the analysis of the clinical samples.

2.6 General Laboratory Equipment

A variety of laboratory equipment and general consumables were used during the analysis of the plasma and saliva samples, and details are given below in tables 2.3 and 2.4.

Equipment	Information
Sonicator	Ultrasonics Ltd., Soniclear, Type 6444
Centrifuges	In the laboratory at the hospital: Beckman Coulter Allegra TM 6R In the laboratory at the University: Heraeus Sepatech, Megafuge 1.0. Heraeus Sepatech GmbH, PO BOX 1220, Am Kalkberg, D-3360, Oseterode, Germany
Shaker	Edmund Bühler, 7400 Tubingen, Laborgeratebau glastechnik, umwelttechnik, KS19 (420 min-1)
Mixer	Denley Spiramix 5
Waterbath	Grant Instruments Ltd., Barrington Cambridge, CB2 5QZ
Protein binding assay equipment (cells and rotating device)	Dianorm Ltd

Table 2.3 General laboratory equipment used in the analysis of plasma and saliva samples.

Consumable	Supplier
GC vials, inserts and caps	Kinesis Ltd, P.O Box 3958, Epping, Essex, CM16 4AW
HPLC Vials, caps, inserts and plugs (Product codes 1NWV, 8-NPWP-003 and 03-MTV, 8-NPWP-003)	Chromacol Ltd, 3 Little Mundals, Welwyn Garden City, Hertfordshire, AL7 1EW, UK
6 ml Blood tube (no anticoagulant) Product code 4C19 10 ml Blood tube (no anticoagulant) Product code 4319	Labco Ltd, Brow Works, Copyground Lane, High Wycombe, Buckinghamshire, HP12 3HE, UK
Luna 5µ C18 100 x 4.6 mm column and C18 guard cartridges	Phenomonex, Queens Ace., Hurdsfield Industrial Estate, Macclesfield, Cheshire

Table 2.4 General laboratory consumables used in the collection/analysis of plasma and saliva samples.

2.7 Analytical Methods

Analytical methods were developed *de novo*, except where indicated. Calculating the relative recoveries of the substrate and the internal standard validated the extraction procedures. Spiked samples were extracted as per protocol, and the AUC value on the resulting GC/HPLC trace was compared with that of

mobile phase spiked to the same concentration. Percentage recoveries are reported for each method below.

2.8 Analysis of samples following oral dosing with butylated hydroxytoluene.

2.8.1 Preparation of Standard Curve

Triplicate analyses of a series of spiked standard plasma samples were run simultaneously with analysis of each batch of clinical samples. The resulting standard curve was used for quantification of BHT in the clinical samples used. The standard concentrations used to derive the standard curve for analysis of the ADI samples were 0, 10, 20, 40, 160, 320 ng/ml BHT, and 0, 10, 50, 250, 500, 1000 ng/ml BHT in the case of the 10 x ADI samples.

Six individual pools of plasma (one for each standard concentration) were spiked with a BHT solution (in 10µl ethanol) to obtain the required ng/ml concentration. The 0 ng/ml standard was prepared by spiking with the equivalent volume of ethanol. After vortexing to ensure mixing, the pools were divided into 250 µl aliquots in pre-labelled HPLC vials and capped prior to storage at –20°C.

2.8.2 Extraction method for standards and samples

Triplicate or duplicate 1 ml aliquots (in glass GC vials) from each time point for a single study were removed from the freezer, and were allowed to defrost on the bench. Using a positive displacement pipette, 10 µl of a 350 µg/ml solution of butylated hydroxyanisole (BHA) in ethanol was added to each standard vial and sample vial. This resulted in a concentration of 3.5µg/ml BHA in each standard and sample.

Samples were briefly vortexed to ensure mixing of the internal standard (BHA) with the plasma. Cyclohexane (125 µl) of was then added to the HPLC vial using a calibrated 200 µl Gilson pipette. Caps were placed on all vials, and they were shaken vertically on a mechanical shaker for one hour. After shaking, the vials were centrifuged at 2,600 x g (4000 rpm) for 10 minutes to separate the solvent and aqueous layers. Approximately 50 µl of the solvent layer was removed and placed in a GC vial insert, the remaining sample and solvent layer were frozen upright.

2.8.3 Determining the source of contamination in the early BHT studies

As reported in Chapter 3, there were much higher than expected baseline concentrations of BHT in the preliminary studies. After ruling out contamination of the samples during processing and analysis, an

experiment was conducted to check whether the blood sampling method used at that time (the Vacutainer system) was the source of contamination. Three volunteers were cannulated in one arm, and had a single 10 ml blood sample taken via the bung and Vacutainer system. In the other arm, two samples were taken, firstly using a standard needle and syringe, and secondly using the Vacutainer system alone. Comparison of the blood from the two Vacutainer samples would determine if the cannula were the source of contamination. The resulting plasma was then analysed using extraction with cyclohexane, and analysed using the GC-MS method described below. The results are shown in figure 3.7.

2.8.4 GC-MS Method

As described in sections 3.6.1 and 3.6.2, the early BHT samples were analysed by GC-MS. Because this method was used for studies to determine the source of contamination of the samples, it is described here. Details of the GC-MS equipment and method are given below in tables 2.5 and 2.6.

Parameter	**Detail**
Autosampler	Fisons Instruments A200S
Gas Chromatography apparatus	Fisons Instruments GC 8000 series 8060, coupled to an MS (see below).
Mass Spectrometer	Fisons Instruments Trio 1000

Table 2.5 Details of the GC-MS equipment

Parameter	**Value**
Column	J&W Scientific DB-5ms 30mx0.25mm, 0.25um
Injection	Splitless, 260°C, 2µl
Temperature ramp	100°C - 200°C at a rate of 20°C /min
Final plateau	200°C for 4 minutes
Gas pressure	He at 50kPa
Source temperature	240°C
Interface temperature	240°C
Source pressure	-4.0
Analyser pressure	-4.0
Filament current	0.2 amps
E Energy	-70 electron volts

Table 2.6 Details of the GC-MS method

Solutions of BHT and BHA in cyclohexane were analysed by GC-MS in order to identify parent ions or parent ion fragments suitable for monitoring. The molecular weight of BHT is 220.34. The spectrum (figure 2.1) showed a dominant peak at 205.07. Single ion monitoring of the 205.07 fragment (EIC) between 7.05 and 8.00 minutes was used to detect BHT.

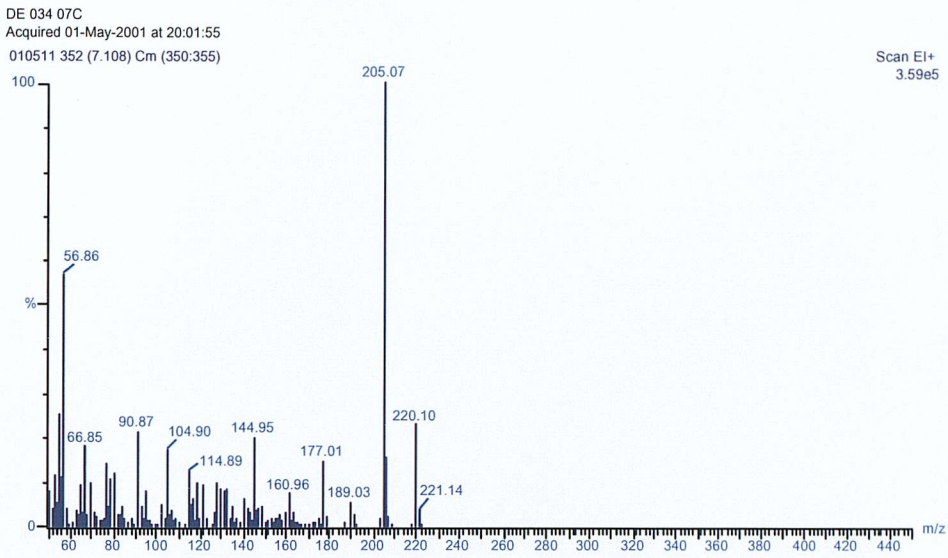


Figure 2.1 Mass spectrum of a 1 µg/ml solution of BHT in cyclohexane

The molecular weight of BHA is 180.24. The spectrum (figure 2.2) showed a dominant peak at 164.96. Single ion monitoring of the 164.96 fragment (EIC) between 6.00 and 7.05 minutes was used to detect BHA.

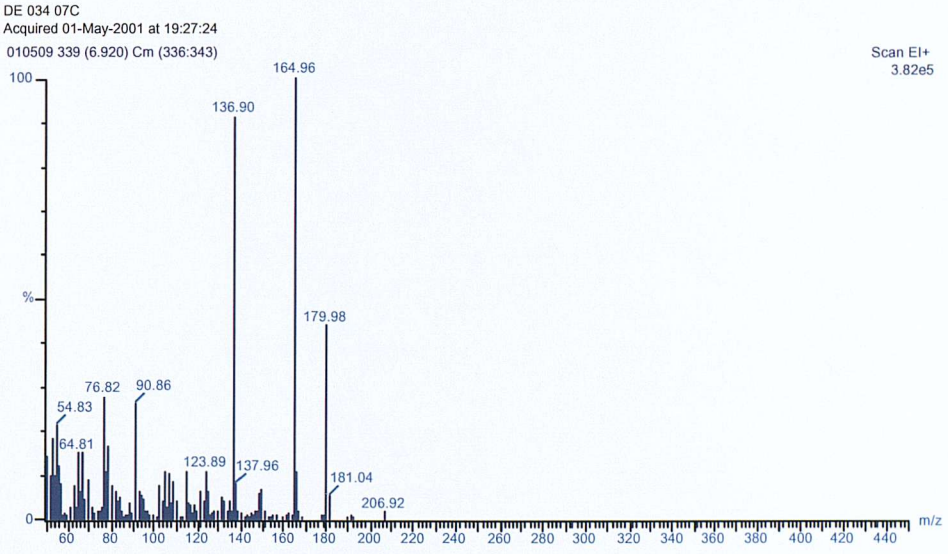


Figure 2.2 Mass spectrum of a 1 µg/ml solution of BHA in cyclohexane

The GC-MS was run with the conditions given below using single ion monitoring at 164.96 (EIC) between 6.0 and 7.05 minutes (BHA) and then at 205.07 (EIC) between 7.05 and 8.00 minutes (BHT). The details of the GC and MS conditions are given in table 2.6.

2.8.5 GC method

Problems were experienced using GC-MS, because the ratio of BHT/BHA for a standard solution was not stable (see Chapter 3, figure 3.8). Due to the unreliability of this method, a GC method was developed for BHT, using BHA as the internal standard, which proved more robust, without any of the problems of GCMS, and with an acceptable limit of quantification/detection. A Varian/Chrompack 9003 GC fitted with an auto-sampler was used for the analysis of all BHT plasma and saliva samples included in the final data set. The details of the GC method are given below in table 2.7.

Parameter	**Measure**
Detection type	Flame ionisation detection
Column type	Chrompack capillary column, CP-Sil 5CB 50m 0.32mm, 0.25µm
Injector and detector temperature	250 °C
Starting temperature	60°C
Temperature ramp	20°C/min up to 180°C then held for 5 minutes

Table 2.7 Details of the GC method employed for the analysis of BHT clinical samples.

A typical trace for BHT and BHA is given below in figure 2.3. Inset is the area of integration, which has been magnified to show the peaks for BHT and BHA. The retention of BHA and BHT was 9.1 and 9.5 minutes respectively.

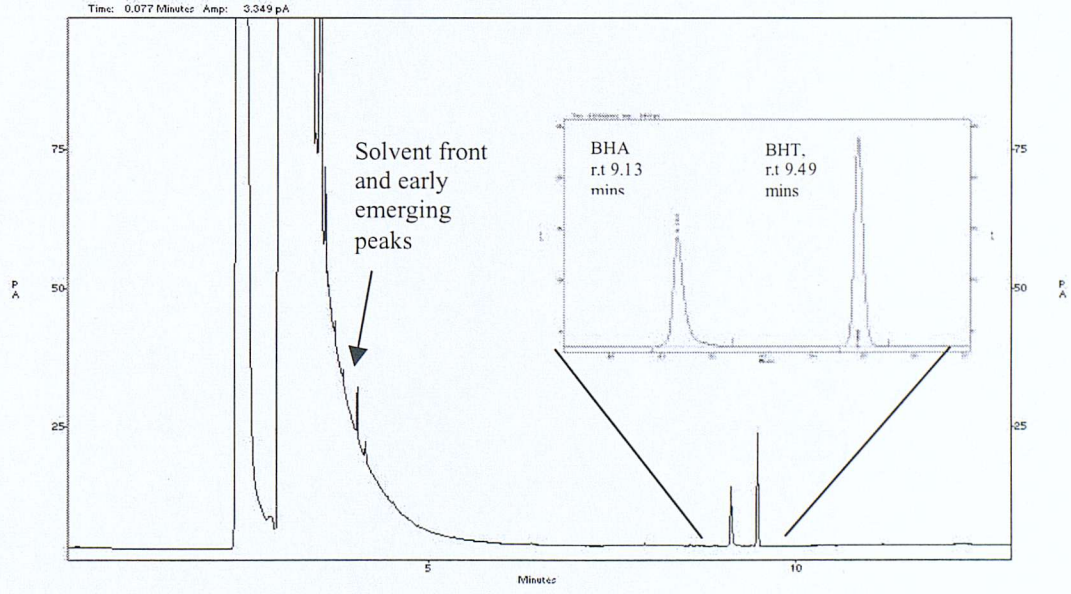


Figure 2.3 A typical GC chromatogram for BHT and BHA.

As discussed earlier in this chapter a triplicate standard curve was analysed simultaneously with the analysis of clinical samples. The slope of the curve for the peak area ratios (BHT/BHA) vs. concentration

of BHT in standards was used to quantify the concentration of BHT present in the clinical samples, based on the ratio in the sample. Figure 2.4 is an example of a standard curve generated from such analyses. The average recovery of BHT and BHA from plasma, saliva and water was ~70% and ~20% respectively. The recovery for BHA was lower than is normally deemed acceptable; however, it was a consistent recovery, resulting in good standard curves on which to quantitate BHT.

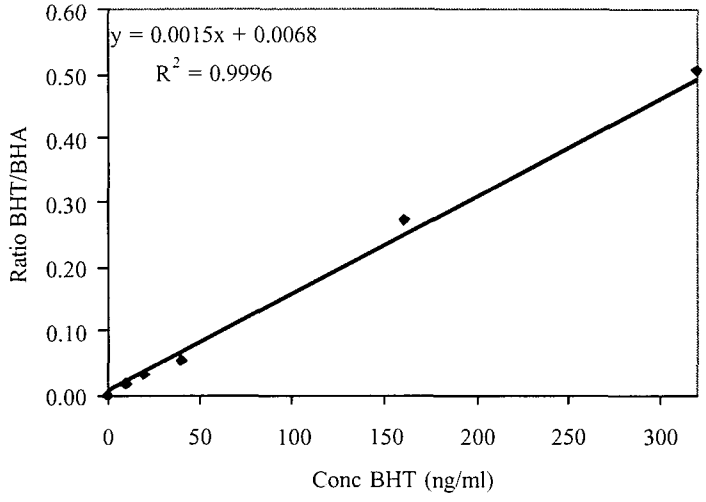


Figure 2.4 An example of a standard curve used for the quantification of BHT in clinical samples.

The limit of quantification for the analysis of clinical samples was 10 ng/ ml BHT (defined as the lowest standard used for the construction of the standard curve) and the limit of detection was ~2 ng/ml (defined as 3 x baseline noise). The average R^2 values from the standard curves used to quantify the clinical samples were 0.9934 (ADI samples), 0.9992 (10 x ADI samples) and 0.9910 (10 x ADI saliva samples). Sample sets with poor standard curve R^2 values were rejected from the overall analysis and a re-analysis was performed. This was judged by the coefficient of variation measures on the standard curves. The average CoV for the lowest standard (10 ng/ml standard) was 11 %, and for the highest standards was 5 % (320 ng/ml standard used in the ADI analyses) and 7% (1000 ng/ml standard used in the 10 x ADI analyses). In the case of the BHT samples, mis-injections were resolved by either 1) re-injection of the same extracted samples, or 2) re-extraction and subsequent injection of another sample. When this occurred either the BHT was quantified with I) the original standard curve or 2) with the standard curve run with the newly extracted samples. These additional data points were then combined with the majority of the data already collected.

2.9 Analysis of samples following oral dosing with curcumin.

2.9.1 Preparation of standard curves

Triplicate analyses of a series of spiked standard plasma samples were run simultaneously with the analysis of the clinical samples. The resulting standard curve was used for quantification of curcumin in the clinical samples. The standard concentrations used to derive the standard curve for the ADI studies were 0, 12.5, 125, 500, 1000, 10000 pg/ml curcumin. The standard concentrations used to derived the standard curves for the 10 x ADI studies were 0, 10, 50, 100, 500, 1000, 5000 pg/ml. Six pools of plasma were prepared (one for each standard concentration). The pools were spiked with a curcumin solution (in 10 µl ethanol) to obtain the required pg/ml concentration (except for the 0 ng/ml standard, where clean ethanol was added). The pools were divided into 1 ml aliquots in pre-labelled 10 ml glass blood tubes, sealed and frozen at –20°C pending analysis.

2.9.2 Extraction method for standard and samples

Triplicate or duplicate 1 ml aliquots (in 10 ml glass blood tubes) from each time point for a single study were removed from the freezer, and were allowed to defrost on the bench. Using a calibrated positive displacement pipette, 10 µl of a 5 µg/ml 1-[4-(Dimethylamino) phenyl]-6-phenyl-hexatriene (DMAPPH) solution in ethanol was added to each standard and sample vial, to give a concentration of 50 ng/ml DMAPPH.

The tubes were briefly vortexed to ensure mixing of the internal standard (DMAPPH) with the plasma. Ethyl acetate (3ml) was then added to the 10 ml glass blood tubes using a burette. Caps were placed on all tubes, and they were shaken vertically on a mechanical shaker for 20 minutes. After shaking, the vials were centrifuged at 5000 rpm for 10 minutes to separate the solvent and aqueous layers. The solvent layer was subsequently removed and placed in a pre-labelled 10 ml glass blood tube. The extraction was repeated with another 3 ml ethyl acetate and the combined solvent layers were evaporated to dryness under nitrogen at 40°C. The standards and samples were then re-dissolved in 130 µl of the HPLC mobile phase, and roller-mixed for 10 minutes prior to centrifugation at 5000rpm. The resulting solution was then placed into a pre-labelled HPLC vial containing an insert. Details of the HPLC equipment and run conditions are given below in tables 2.8 and 2.9.

Parameter	Detail
HPLC Pump	Waters™ 510 Pump
HPLC Autosampler	Waters™ 717 plus autosampler
HPLC Fluorescence detector	Waters™ 2475 multi wavelength fluorescence detector
Waterbath	40°C
HPLC Column	Phenomonex 5μ amino NH_2 aminopropyl 100A° 150 x 46 mm column

Table 2.8 HPLC equipment used for the analysis of plasma samples following oral dosing with curcumin.

Parameter	Detail
Mobile phase	0.05% acetic acid in HPLC grade ethanol
Flow rate	1.5ml/minute
Injection volume	100μl
Column temperature	40°C
Fluorescence excitation wavelength	422nm
Fluorescence emission wavelength	539nm

Table 2.9 Details of the HPLC method used for the quantification of curcumin in plasma samples

Early experiments with curcumin confirmed that curcumin is unstable in aqueous solutions at pH>7. Upon exposure to higher pH values, curcumin undergoes ionisation, accompanied by a colour change (Tønnesen & Karlsen, 1985). The HPLC method was designed in light of the fact that curcumin is vulnerable to hydrolytic degradation reactions. A non-aqueous system was used, and an acidic pH was maintained through the use of acetic acid in the mobile phase. A typical trace for curcumin and DMAPPH is given below in figure 2.5. The retention of curcumin was ~3.7 minutes, and was ~1.6 minutes for DMAPPH.

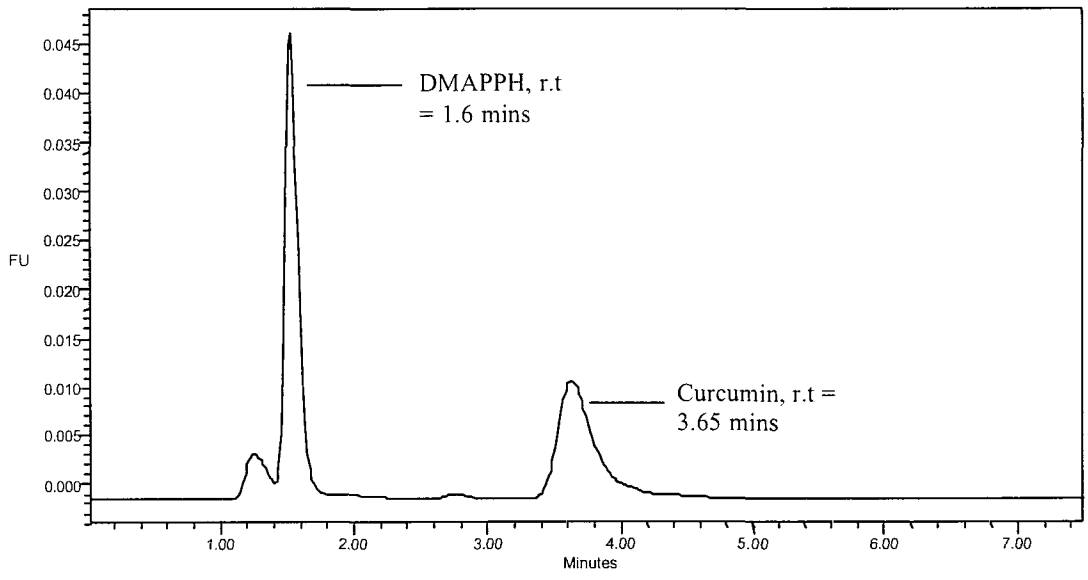


Figure 2.5 A typical chromatogram for a standard plasma containing 5000 pg/ml curcumin.

As discussed earlier in this chapter a triplicate standard curve was analysed simultaneously with the analysis of clinical samples. The slope of the peak area ratios vs. concentration curve was used to quantify the concentration of curcumin present in the clinical samples. Figure 2.6 is an example of a standard curve generated from such analyses. The limit of quantification for the analysis of clinical samples was 31.25 pg/ ml curcumin (defined as the lowest standard used for the construction of the standard curve) and the limit of detection was ~10 pg/ml (defined as 3 x baseline noise). The average recoveries of curcumin and DMAPPH in clinical sample sets analysed were 86% and 92% respectively.

The average R^2 values from the standard curves used to quantify the clinical samples were 0.997 (ADI samples) and 0.999 (10 x ADI samples). Sample sets with poor standard curve R^2 values were rejected from the overall analysis and a re-analysis was performed. This was judged by the coefficient of variation measures on the standard curves. The average CoV for the lowest standard (31.25 pg/ml standard) was 30%, and for the highest standards was 4 % (10 or 12.5 ng/ml) standard used in the ADI analyses and 16% for the lowest standard (10 pg/ml standard) and 3.5% for the highest standard (5 ng/ml) used in the 10 x ADI analyses).

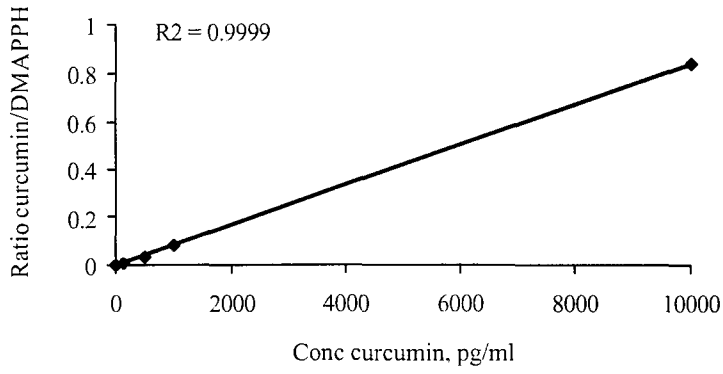


Figure 2.6 An example of a standard curve used for the quantification of curcumin in clinical samples.

2.10 Analysis of samples following oral dosing with propyl gallate.

2.10.1 Preparation of standard curves

Triplicate analyses of a series of spiked standard plasma samples were run simultaneously with the analysis of the clinical samples. The resulting standard curve was used for quantification of propyl gallate in both the ADI and 10 x ADI clinical samples. The standard concentrations used to derive the standard curve were 0, 3.125, 6.25, 12.5, 25 and 50 ng/ml propyl gallate for the ADI analyses and 0, 6.25, 12.5, 50, 100, 250 ng/ml propyl gallate for the 10 x ADI analyses. Six pools of plasma were prepared (one for each standard concentration). The pools were spiked with a propyl gallate solution (in 10µl methanol) to obtain the required ng/ml concentration. (Except for the 0 ng/ml standard, where clean methanol was added). The pools were aliquoted out into 1 ml aliquots in pre-labelled 10 ml glass blood tubes and sealed and frozen until analysis.

2.10.2 Extraction method for standard and samples

Triplicate or duplicate 1 ml aliquots (in 10 ml glass blood tubes) from each time point for a single study were removed from the freezer, and were allowed to defrost on the bench. Using a calibrated positive displacement pipette, 10µl of a 10µg /ml ethyl gallate solution in methanol was added to each standard and sample vial, to give a concentration of 100 ng/ml ethyl gallate.

The tubes were briefly vortexed to ensure mixing of the internal standard (EG) with the plasma. Ethyl acetate (5ml) was then added to the 10 ml glass blood tubes using a burette. Caps were placed on all tubes, and they were shaken vertically on a mechanical shaker for 20 minutes. After shaking, the vials were centrifuged at 5000 rpm for 10 minutes to separate the solvent and aqueous layers. The solvent

layer was subsequently removed, placed in a pre-labelled 10 ml glass blood tube and evaporated to dryness under nitrogen at 40°C. The standards and samples were then re-dissolved in 130 µl of the HPLC mobile phase, and roller mixed for 10 minutes prior to centrifugation at 5000rpm. The resulting solution was then placed into a pre-labelled HPLC vial containing an insert. Details of the HPLC equipment and run conditions are given below in tables 2.10 and 2.11.

Parameter	Detail
HPLC Pump	Waters™ 510 Pump
HPLC Autosampler	Waters™ 717 plus autosampler
HPLC Electrochemical detector	Waters™ M460 electrochemical detector
Waterbath	40 °C
HPLC Column	Phenomonex Luna 5µ C_{18} 100 x 4.6 mm column

Table 2.10 HPLC equipment used for the analysis of plasma samples following oral dosing with propyl gallate

Parameter	Detail
Mobile phase	35% methanol. 1% acetic acid, 64% double-distilled water
Flow rate	1 ml/minute
Injection volume	100µl
Column temperature	40°C
ECD potential	+0.9 V*

Table 2.11 Details of the HPLC method used for the quantification of propyl gallate in plasma samples. *Bianchi et al (1987) used electrochemical detection to quantify propyl gallate in plasma samples.

A typical trace for propyl gallate and ethyl gallate is given below in figure 2.7. The retention of propyl gallate was 3.5 minutes, and was 1.8 minutes for ethyl gallate.

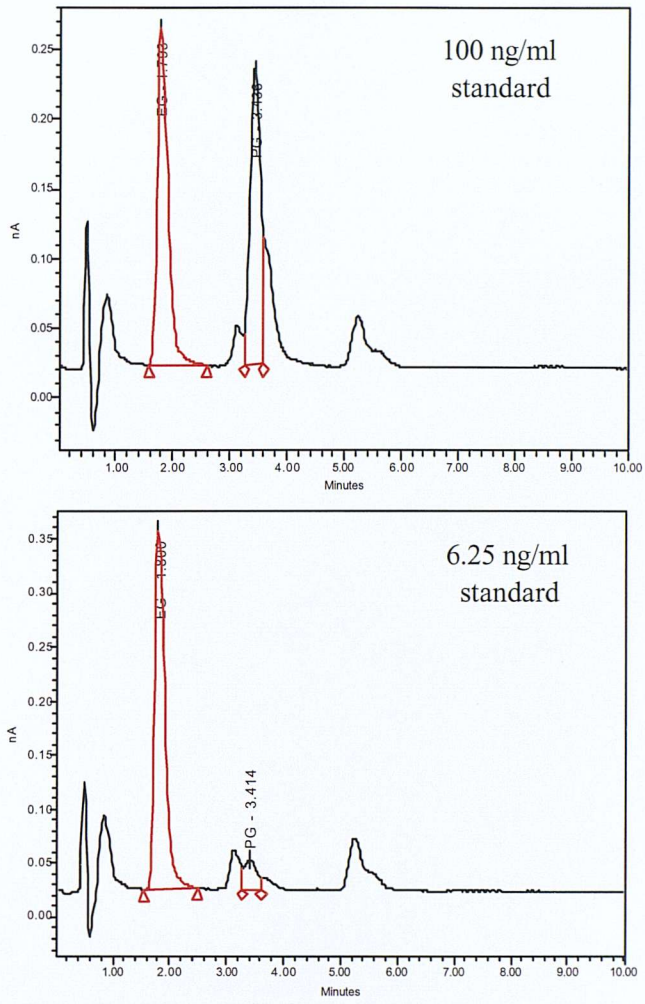


Figure 2.7 A typical chromatogram from the clinical analysis of propyl gallate

As discussed previously a triplicate standard curve was analysed simultaneously with the analysis of clinical samples. The slope of the peak area ratios vs. concentration curve was used to quantify the concentration of propyl gallate present in the clinical samples. Figure 2.8 is an example of a standard curve generated from such analyses. The limit of quantification for the analysis of clinical samples was 6.25 ng/ ml propyl gallate (defined as the lowest standard used for the construction of the standard curve) and the limit of detection was ~4 ng/ml (defined as 3 x baseline noise). The average recoveries of propyl gallate and ethyl gallate from plasma were over 100% (due to noise in the baseline).

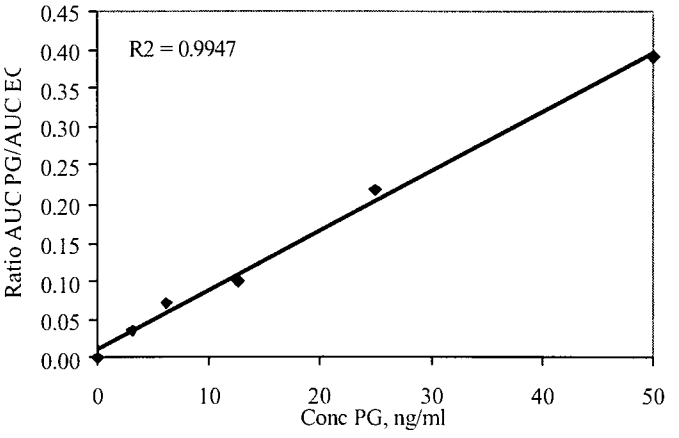


Figure 2.8 An example of a standard curve used for the quantification of propyl gallate in clinical samples.

The average R^2 value from the standard curves used to quantify the clinical samples were 0.994 (ADI samples), 0.9965 (10 x ADI samples) and 0.9848 (10 x ADI saliva samples). Sample sets with poor standard curve R^2 values were rejected from the overall analysis and a re-analysis was performed. This was judged by the coefficient of variation measures on the standard curves. The average CoV for the lowest standards (3.125 or 6.25 ng/ml standard) was 19%, and for the higher standard in the ADI analyses (50 ng/ml) was 11% and 7% respectively. The average CoV for the highest standard in the 10 x ADI analysis (250 ng/ml) was 6%.

2.11 Analysis of samples following oral dosing with thiabendazole.

2.11.1 Preparation of Standard Curves

Triplicate analyses of a series of spiked standard plasma samples were run simultaneously with analysis of the plasma samples. The resulting standard curve was used for quantification of TBZ in the clinical samples. For analysis of the low dose (ADI studies) the standard concentrations used to plot the standard curve were 0, 125, 250, 500, 1000, 2000, 10000 pg/ml thiabendazole. For the high dose studies the standard concentrations were 0, 0.3125, 3.125, 12.5, 50, 125 ng/ml thiabendazole. Due to the very low concentrations present in the samples from the ADI studies, the lowest standard in the standard curve was the limit of detection for the assay (defined as three times baseline noise level) as well as being the minimal quantifiable amount of TBZ in this assay. This point was on the linear part of the standard curve, and allowed quantification of low concentrations of TBZ in the ADI plasma samples.

Six pools of plasma were prepared (one for each standard concentration). The pools were spiked with 10µl of a TBZ solution (in 10 µl methanol) to obtain the required pg or ng/ml concentration. (Except for

the 0 ng/ml standard, where methanol was added.) The pooled standards were pipetted as 1 ml aliquots into pre-labelled 10 ml glass blood tubes and sealed and frozen at –20°C until analysis.

In the case of the saliva samples obtained from the 10 x ADI studies, triplicate analysis of a series of spiked standard water samples was run simultaneously with analysis of the saliva samples. The resulting standard curve was used for quantification of TBZ in the clinical samples. For analysis of the high dose (10 x ADI studies) the standard concentrations used to plot the standard curve were 0, 0.25, 1, 5, 10, 100 ng/ml thiabendazole. Six pools of water were prepared (one for each standard concentration). The pools were spiked with a TBZ solution (in methanol) to obtain the required ng/ml concentration. (Except for the 0 ng/ml standard, where methanol was added.) The pooled standards were pipetted as 0.5 ml aliquots into pre-labelled 5 ml glass blood tubes and sealed.

2.11.2 Extraction method for standard and samples

Triplicate or duplicate 1 ml aliquots from each time point for a single study were removed from freezer and were allowed to defrost on the bench. Using a calibrated positive displacement pipette, 10 µl of a 1 µg/ml 1-methyl, 2-phenyl benzimidazole (MPBZ) solution in methanol was added to each standard and sample vial, to give a concentration of 10 ng/ml MPBZ. (MPBZ was used as an internal standard in the analysis of TBZ in plasma samples by Hardee et al., 1987.)

The tubes were briefly vortexed to ensure mixing of the internal standard (MPBZ) with the plasma. Ethyl acetate (5 ml) was then added to the 10 ml glass blood tubes. Caps were placed on all tubes, then they were shaken vertically on a mechanical shaker for 20 minutes. After shaking, the vials were centrifuged at 5000 rpm for 10 minutes to separate the solvent and aqueous layers. The solvent layer was subsequently removed and placed in a pre-labelled 10 ml glass tube. The solvent layer was then evaporated to dryness under nitrogen at 40°C. The standards and samples were re-dissolved in 130 µl of the HPLC mobile phase, and roller mixed for 10 minutes prior to centrifugation at 5000rpm. The resulting solution was then placed into a pre-labelled HPLC vial containing an insert. Details of the HPLC equipment have been given previously in table 2.10.

The single saliva sample from each time point (~1-3 ml) was removed from the freezer and allowed to defrost on the bench. Aliquots of 0.5 ml of saliva were prepared in labelled 6 ml glass blood tubes, resulting in duplicate or triplicate samples for analysis. Where insufficient saliva was present, the sample was diluted with distilled water to obtain duplicate samples. Using a positive displacement pipette, 10 µl of a 1 µg /ml 1-methyl, 2-phenyl benzimidazole (MPBZ) solution in methanol was added to each standard and sample vial to give a concentration of 40 ng/ml MPBZ (used as an internal standard in the analysis of TBZ) in the sample . The tubes were briefly vortexed to ensure mixing of the internal standard (MPBZ)

with the plasma. Ethyl acetate (2.5 ml) was then added to the 6 ml glass blood tubes. The rest of the extraction procedure employed was the same as for the plasma samples.

Samples were analysed using the same HPLC system as used for the analysis of the curcumin samples (see table 2.10). The HPLC conditions used for the analysis of the thiabendazole samples is given below in table 2.12. At typical HPLC trace using this method is given in figure 2.9.

Parameter	**Condition**
Column	Phenomonex Luna 5μ C_{18} 100 x 4.6 mm column
Mobile phase	40% methanol, 60% double distilled water containing 0.05% ammonia, filtered through a 0.2μM filter and degassed using the sonicator.
Flow rate	1.5ml/minute (isocratic for the ADI plasma analyses; revised to 1.5ml/min for 6 mins, then 2ml/min for 10 x ADI and saliva analyses.)
Injection volume	100μl
Column temperature	40°C
Fluorescence excitation wavelength	300nm*
Fluorescence emission wavelength	350nm*

*Excitation and emission spectra as used by Isshiki et al., 1980 and by Hardee et al., 1987.

Table 2.12 Details of the HPLC method used for the quantification of thiabendazole in plasma and saliva samples.

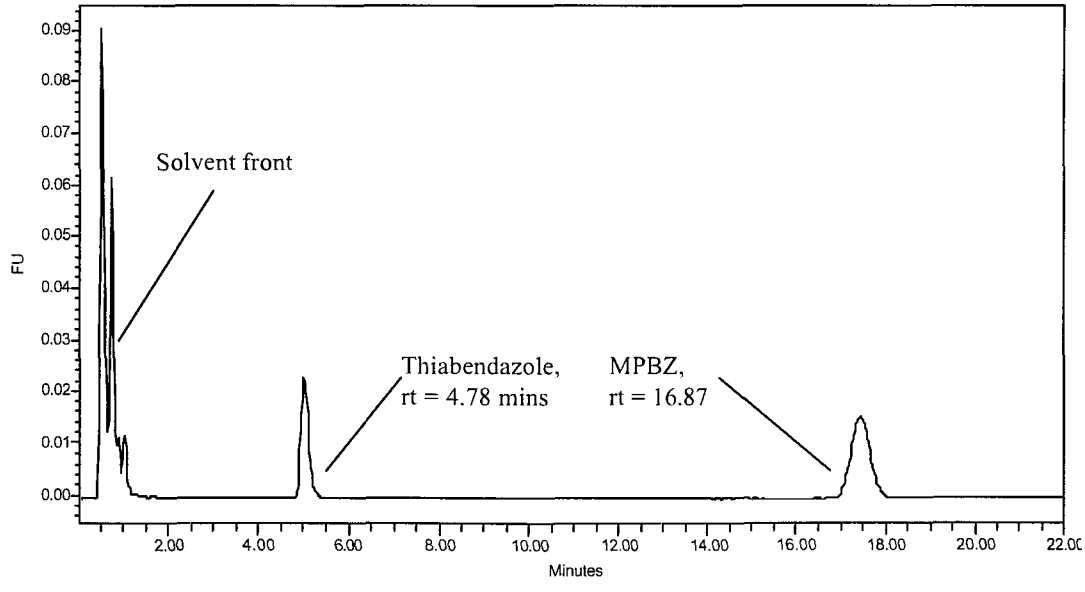


Figure 2.9 A typical chromatogram from the clinical analysis of thiabendazole.

2.11.3 Reliability of the analytical method

As discussed previously a triplicate standard curve was analysed simultaneously with the analysis of clinical samples. The slope of the peak area ratios vs. concentration curve was used to quantify the concentration of TBZ present in the clinical samples. Figure 2.10 is an example of a standard curve generated from such analyses. The limit of quantification for the analysis of clinical samples was 125 pg/ml thiabendazole (defined as the lowest standard used for the construction of the standard curve) and the limit of detection was ~250 pg/ml (defined as 3 x baseline noise). The average recovery of TBZ and MPBZ in clinical sample sets analysed was 90% and 75% respectively. A recovery experiment to verify that TBZ and MPBZ were extracted equally well from saliva and water was conducted prior to using water for the standard curve in the analysis of TBZ saliva samples. The recoveries were found to be 105% (TBZ) and 88% (MPBZ)

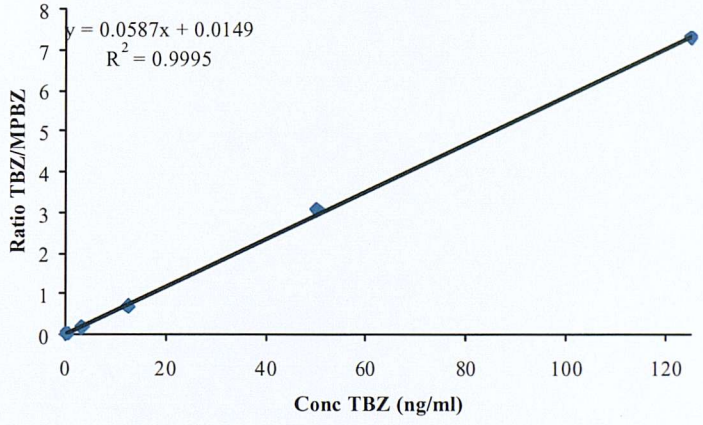


Figure 2.10 An example of a standard curve used for the quantification of thiabendazole in clinical plasma samples.

The average R^2 value from the standard curves used to quantify the clinical samples were 0.9965 (ADI samples), 0.9892 (10 x ADI samples) and 0.9998 (10 x ADI saliva samples). Problems were experienced in obtaining reliable standard curves for the plasma analysis, particularly with the ADI analyses, this was because of interfering peaks in the baseline. These could sometimes make it difficult to integrate the TBZ peak, and the lowest standard used in the ADI analyses was very close to the limit of detection. Sample sets with poor standard curve R^2 values were rejected from the overall analysis and a re-analysis was performed. This was judged by the coefficient of variation measures on the standard curves. The average CoV for the lowest standard (125 pg/ml standard) was 9%, and for the higher standard in the ADI analyses (10 ng/ml) was 8%. The average CoV for the highest standard in the 10 x ADI analysis (125 ng/ml) was 10%. Where re-analyses were performed, and the ratios between the internal standard and TBZ were identical in the rejected and acceptable analyses of the samples, the results were pooled, using the slope value from the reliable standard curve to quantify TBZ.

2.11.4 Recovery of TBZ from the dental roll

Following the analysis of saliva samples from studies where volunteers received TBZ at a dose of 10 x ADI, it became apparent that there was a discrepancy between the predicted saliva concentrations of TBZ based on the protein-binding studies, (described below) and the concentrations of TBZ that were actually detected in the saliva. Because the recovery of TBZ from the saliva was good, it was hypothesised that TBZ was being retained on the dental roll. An experiment was conducted, in which dental rolls were saturated in saliva or saliva spiked with TBZ. The saliva was removed from the dental roll as described earlier (via a syringe) and underwent the extraction method described above. The results of this experiment are reported in chapter 6 (tables 6.16 and 6.17).

2.12 Protein Binding Experiments

Protein-binding experiments were conducted for BHT, PG and TBZ because the extent of protein binding of the substrates would affect the saliva:plasma concentration ratio. The equipment/chemicals used for the protein binding experiments (extra to general laboratory reagents listed in table 2.3) are given below in table 2.13

Material	**Supplier**
Zenlab human albumin 4.5% solution Ph.Eur	Bio Products Laboratory, Dagger Lane, Elstree, Herts., WD6 3BX
Spectra/Por molecularporous membrane tubing (Size 1 6,000-8,00 daltons.	Spectrum Laboratories Inc., 18617 Broadwick St., Rancho Dominguez, CA 90220-6435, USA

Table 2.13 Equipment/chemicals used specifically for the protein-binding experiments.

The membranes used in the experiment were soaked overnight in de-ionised water to remove the preservative, glycerine, prior to be fitted between the compartment of the model and cut to size. The cells were then placed in a holding frame, and equal amounts (1.5 ml) of the Ringer's solution and spiked albumin (spiked with the food additive so that albumin remained in molar excess) were loaded into the pre-labelled compartments using 2ml syringes and 25 gauge needles. After sealing the cells, the frame was placed in an automated carousel in a 37°C waterbath and the cells were rotated overnight (for 16 hours).

Aliquots of 0.5ml were taken from each compartment and placed in pre-labelled 6 ml glass blood tubes. The samples were then extracted and analysed by GC or HPLC using the same method as the plasma and saliva samples. Appropriate control samples (extracted albumin, extracted Ringers', extracted spiked albumin, extracted spiked Ringers') were run with the analysis to allow the calculation of the percentage binding as shown in figure 2.11.

$$\% \text{ protein binding} = \frac{(\text{total-proportion unbound}}{(\text{total}))} \times 100$$

i.e.

$$\% \text{ protein binding} = \frac{(\text{ratio TBZ:MPBZ in albumin - ratio TBZ:MPBZ in Ringers'})}{(\text{ratio TBZ:MPBZ in albumin})} \times 100$$

Figure 2.11 Calculating protein binding of substrates

2.13 Integration of raw data

The output from the GC FID was fed directly into a data management system Maitre Chromatography Data System (CP Maitre v 2.5). The output from the ECD or the fluorescence detector was fed directly into a data management system: Waters Millennium™ system. Each individualvolunteer data was stored in a separate file in a database system. Peaks corresponding to the internal standard and food additive were identified using the retention times of the peaks in the standard samples. The peaks were integrated manually, sample sets were printed, and peak heights for the internal standard and food additive were entered manually into Excel™ spreadsheets. The ratio food additive/internal standard was used to plot a standard curve, and the slope of this curve was used to transform the food additive/internal standard ratio determined in volunteer samples into a concentration of food additive present, in pg or ng/ml. The resulting concentration-time data were subsequently used as inputs in a mathematical modelling system to generate pharmacokinetic parameters.

2.14 Calculation of pharmacokinetic parameters

A range of pharmacokinetic parameters was used to compare inter-individual and inter-species differences. The maximal concentration (Cmax) is an observed value, and is simply the maximal quantity of the food additive observed in a subject in the duration of the study. The time of the maximal concentration is also an observed value (Tmax). The human and animal studies resulted in two distinct types of data:

1. Data in which the food additive was present in the plasma or saliva at concentrations very close to or below the limit of detection.
2. Data in which the food additive was present in the plasma or saliva at concentrations above the limit of quantification.

In adequate data sets, a mathematical model, WinNonlin™ was used to model the concentration-time profile. A non-compartmental, extra-vascular bolus dose model was used. The model uses the observed

data points to extrapolate the terminal phase of the concentration-time curve to infinity, and thus gives predictive values of AUC to infinity, T½ and CL/F (estimated as dose/AUC_{inf}).

In data sets that were too poor to analyse using WinNonlin™, the trapezoid formula was used to calculate AUC, using the observed data points. CL/F values were derived by dividing the dose/AUC_{obs}.

In some cases the time of the final sample collection in humans did not match that of the corresponding animal studies. For example in the TBZ studies, the final human time point was 420 minutes, whilst it was 1440 minutes in the rat studies. In this instance, where the quality of the data allowed, WinNonlin™ was used to model the existing animal data, to generate a value for T½. The T½ was then used to extrapolate the animal data to the final point of the human data, i.e. to match the final time point. The formula used to extrapolate the observed time point to an extrapolated time point using T½ is shown below in table 2.14:

Concentration of substrate present in extrapolated time point =	Concentration of substrate present in last time point x Exp(((0.693 x time difference between last animal data point and last human point)/T½))

Table 2.14 Formula used to extrapolate from observed values

In order to compare pharmacokinetic parameter equally between groups, data analysed using either the AUC with extrapolation or the AUC observed was compared. In other words, extrapolated data was only compared with other extrapolated data, and non-extrapolated data was only compared against other non-extrapolated data. Where both methods were employed to analyse the same data set, an evaluation of the impact of the mathematical extrapolation upon the resulting pharmacokinetic parameters was possible.

Because Cmax and AUC are dose-dependent PK parameters, the following equation was used to correct the PK parameters for dose in order to generate the inter-species PK ratios. The example in table 2.15 shows how the inter-species factor for TBZ (based on the ADI and NOAEL data) was calculated.

(Human value/Human dose) (Animal value/Animal dose):		
Which in the example of Cmax equates to:	(0.57/0.1) (2815/1)	0.020

Table 2.15 Correcting dose-dependent PK parameters for dose, and calculating the inter-species kinetic ratios (using as an example, the TBZ ADI and NOAEL data for Cmax).

2.15 Estimating the proportion of the population covered by the 3.16-fold default factor for human variability in kinetics.

The proportions of the population that would be covered by the 3.16-fold default factor, and conversely the uncertainty factors that would be required to adequately protect the 95th, 97.5th and 99th percentile were calculated using the CoV values for the mean PK value generated in the human studies. The method used has previously been described by Dorne et al., 2001. To summarise, the data was assumed to be log-normally distributed (Renwick & Lazarus 1998, Dorne et al., 2001) and to analyse the variability assuming a log-normal distribution the data was converted from normal data into a geometric mean (GM), geometric standard deviation (GSD) using the equations in figure 2.12.

$$GM = \frac{X}{\sqrt{(1+CV^2_N)}}$$

$$GSD = \exp\{\sqrt{(1+CV^2_N)}\}$$

Figure 2.12 Calculating the geometric mean and geometric standard deviation.

The uncertainty factor associated with the CoV was then calculated using the GSD for the parameter. The uncertainty factor for any percentile can then be calculated using the corresponding Zscores for each percentile, as shown below in figure 2.13. The uncertainty factor required to protect a given percentile can also be calculated (figure 2.14). The Zscore for the 95th, 97.5th or 99th percentiles are 1.64, 1.95 and 2.33 respectively.

$$\text{nth percentile} = (\ln(GSD) \times \text{Zscore})$$

Figure 2.13 Calculating the Zscore for a given percentile

The uncertainty factor for the kinetic parameter was the antilog of the value for that percentile.

$$UF = e^{(\text{nth percentile})}$$

Figure 2.14 Extrapolating the uncertainty factor required to protect a given percentile.

The calculations were performed using the NORMSINV function in ExcelTM.

2.16 Statistical considerations

The scope of the studies presented in this thesis was two-fold. Firstly to determine the deasibility of conducting pharmacokinetic studies in humans using relevant doses of food additives, and secondly to provide some provisional data regarding the scale of the inter-species differences and human variability. The latter were used to evaluate the likely adequacy of the default inter-species and human variability factors currently used in the risk assessment of food additives.

The studies presented are pilot studies and there are not sufficient data (i.e. not enough volunteers of a representative population were studied; n was too small) to make any policy recommedations. A larger group size, would give greater confidence in the mean parameter estimate, in this case the size of uncertainty factor required to protect a given percentage (normally 95%) of the population. Similarly a larger data set would have allowed a greater predictive effect of the data, i.e. there would be smaller confidence intervals for the predicted values of any single parameter estimate.

This thesis allows an assessment of the adequacy of the default kinetic factors based on limited data, and gives an indication of the extent of variability that might be expected when deriving pharmacokinetic parameters in humans.

Chapter 3 : Studies with BHT

3. Studies with butylated hydroxytoluene (BHT)

3.1 BHT as a food additive

Butylated hydroxytoluene (BHT) (E321) (see figure 3.1 below) is a lipid soluble antioxidant commonly found in foods containing fats. It is added to foods to prevent oxygen-induced lipid peroxidation. BHT is a phenolic compound with a sterically hindered hydroxyl group and acts as a chain breaker in free-radical reactions. It was patented in 1947, and in 1959 was given GRAS (generally recognised as safe) status by the FDA, at levels not exceeding 0.5 mg/ kg bw/ day.

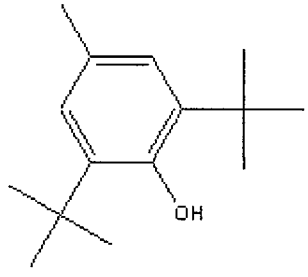


Figure 3.1 Butylated hydroxytoluene

BHT was first evaluated by JECFA in 1961 and allocated an ADI of 0-0.125 mg/kg bw/day (WHO, 1996). In subsequent evaluations it was assigned a group ADI (together with butylated hydroxyanisole and tert-butylhydroquinone) of 0-0.5 mg/kg bw/day and prior to its last assessment in 1995 a temporary single ADI of 0-0.125 mg/kg. Following the most recent assessment BHT (as a single entity) was allocated an ADI of 0.3 mg/kg bw/ day based on a NOAEL of 25 mg/ kg bw/ (WHO, 1996).

The metabolic fate of BHT

3.1.1 Animal studies with BHT

The distribution of BHT has been investigated in the rat and mouse following oral dosing. Very little is retained in the body of either species, 3.8% was in rats 4 days post-treatment, and <1% of the dose in mice 24 hours post-treatment (Daniel & Cage, 1965, cited in Jori, 1983). The highest concentrations of BHT (determined in a radiolabel distribution study) were found in the liver, adipose tissue, kidneys and lungs, with maximal concentrations achieved at 6 hours post-dose (Tye et al, 1965 & Ichikawa et al. 1976 cited in Jori, 1983). The concentrations of BHT in adipose tissue were reduced more rapidly after 3 days due to the induction of microsomal enzymes (Gibert & Goldberg, 1965).

The distribution of BHT within the liver and kidney varies over time. For the first 6 hours following an oral dose, BHT is found in the supernatant fraction, whilst in the next 6 hours, it is found in the

microsomal fraction where it is bound to the microsomes (Nakagawa et al. 1976 and Ikawa et al 1976, cited in Jori, 1983). BHT preferentially binds to hepatic rather than pulmonary microsomes (Takahashi & Hiraga, 1979 cited in Jori, 1983) and its metabolism is increased by pre-treatment with phenobarbital, a microsomal monoxygenase system inducer (Nakagawa et al. 1979, cited in Jori, 1983).

Early investigations demonstrated that BHT is metabolised rapidly in rabbits and rats following intravenous (i.v.) or oral dosing (El-Rashidy & Niazi, 1980). In rats BHT is mainly metabolised by hepatic microsomes (Shaw & Chen, 1972 cited in Jori, 1983). The main route of metabolism involves the oxidation of the methyl group of BHT first to the alcohol, then to the aldehyde and the acid that is then conjugated prior to elimination in the urine (see figure 3.3 for metabolic pathways and structures). Large amounts of unmetabolised and unconjugated BHT are found in the faeces after oral dosing (Daniel et al., 1968). BHT is excreted into the bile where the majority of an oral dose is found within the first 6 hours (Ladomery et al 1976a). Radioactivity-distribution studies demonstrated that BHT does not accumulate in tissues (Ladomery et al 1967b). Excretion of the radiolabel was slow, with only 40-60% of an oral dose (Tye et al, 1965, cited in Witschi et al., 1989) or 69% of an intra-peritoneal (i.p.) dose (Ladomery et al, 1967) found in the urine and faeces after 2-4 days. These findings suggested enterohepatic recirculation of BHT and/or the metabolites. This was confirmed in a study where 95% of the radioactivity of an intravenous dose and 52% of an i.p dose was recovered in the bile after 6 hours. Furthermore, 10% of the total dose could be found in the bile 4 days post-dose (Ladomery et al., 1967). Evidence exists to suggest that neither the BHT-acid nor its glucuronide is responsible for enterohepatic recirculation in the rat (Holder et al., 1970).

Data from studies where BHT was given orally suggest low body burden, however studies determining the distribution of radiolabelled BHT can be interpreted to suggest that BHT may remain in the body for sustained periods of time. It is possible that the metabolites rather than the parent compound are re-circulating. The separate analysis of BHT and the metabolites was not determined in these studies.

3.1.2 Observations in humans with BHT

BHT has been found to accumulate in adipose tissue in humans (Collings & Sharrat 1970 & Mizutani & Che 1976 cited in Jori, 1983). Trace amounts were detected in blood 24 hours after administration and no free metabolites could be detected (Wiebe et al., 1977). Conflicting information exists regarding urinary metabolites. In one study, the main metabolite was identified as the BHT-acid and its glucuronide (Holder et al. 1970) (see figure 3.2). However a more recent study identified the main urinary metabolite as 5-carboxy-7-(-1-carboxy-1methylethyl)-3,3-dimethyl-2-hydroxy-2,3-dihydrobenzofuran (Wiebe et al., 1977). Elimination of this metabolite occurs mostly in the first 6 hours following oral administration and it cannot be detected 72 hours after dosage (Wiebe et al., 1977). However this compound only represents

22% of the administered dose (Wiebe et al., 1977), suggesting other routes of metabolism, and possibly hepatic biliary excretion. To date the occurrence of enterohepatic recirculation in humans has been neither validated nor excluded.

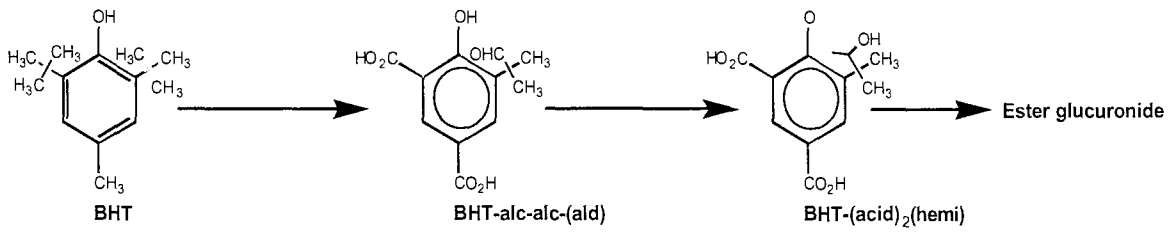


Figure 3.2 Proposed metabolic pathway of BHT for humans. (Adapted from Witschi et al., 1989.)

3.2 The metabolites of BHT and their toxicological significance

3.2.1 Metabolic pathways of BHT

The identified metabolic pathways for BHT are outlined in figures 3.3, 3.4, 3.5 and 3.6. There are three main metabolic pathways:

1. Benzylic hydroxylation, resulting in the formation of the 4hydroxymethyl derivative (BHT-BzOH) (see figure 3.3)
2. Tert-butyl hydroxylation resulting in the formation of BHT-t-BuOH (see figure 3.4)
3. p-electron oxidation, resulting in the formation of non-aromatic products including peroxyquinol and quinone methides (see figure 3.5 and 3.6)

a) Benzylic hydroxylation

BHT is hydroxylated on the 4-methyl group via microsomal CYP450 enzymes. Benzylic hydroxylation (see figure 3.3) has been studied in the liver and lung microsomes of rats and mice (Thompson et al., 1987). Additional metabolic products were observed, including the benzaldehyde, acid and glucuronidated products. Quantitative data show that BHT-BzOH is the principal metabolite in rat lung and liver microsomes, whilst mice produce large amounts of both BHT-BzOH and BHT-t-BuOH in these tissues with similar metabolite formation. Mouse lung was shown to produce more of the quinone metabolite, a more toxic metabolite (see following section on tert-butyl hydroxylation and p-electron oxidation).

b) Tert-butyl hydroxylation

The oxidation of the tert-butyl side chains is catalysed by CYPP450 enzymes. The product (BHT-t-BuOH) is more toxic than BHT, so this represents a bioactivation step. The greater toxicity of BHT-t-BuOH (produced preferentially in mice) compared to BHT can also be explained by the chemical nature of the hydroxylated quinone methide (BHT-OH-QM) (see figure 3.4). BHT-OH-QM is more electrophillic than the quinone methide BHT-QM due to intramolecular hydrogen bonding. This increases the positive charge on the exocyclic methylene group resulting in greater reactivity with nucleophiles.

Investigation of the potential for rat and mice liver and lung microsomes to produce BHT-OH-QM from BHT via BHT-t-OH has been investigated (Thompson et al,. 1987 and Bolton et al., 1990). Both rat and mouse liver microsomes produced similar amounts of the quinone methide, and both species could readily oxidise BHT-t-OH to BHT-OH-QM, but the concentrations of BHT-t-OH generated by rat liver microsomes were extremely low compared to the mouse, suggesting that rats are not capable of tert-butyl hydroxylation of BHT. Rat lung microsomes do not produce BHT-OH-QM and are much less active (~2-fold lower) than mice in oxidising BHT-t-OH to BHTOH-QM.

In vitro studies (using rat and mouse liver and lung microsomes) with BHT (Bolton & Thompson, 1991), in the presence of inducers (phenobarbital, PB) or inhibitors (SKF-525A, ABT or Cedrol) of CYP450s confirmed the major differences between rats and mice in their metabolism of BHT. PB inducible enzymes were proposed as the enzymes responsible for BHT biotransformation. The major PB-inducible form of CYP450 was capable of oxidising BHT to the quinone methide and BHT-t-OH to BHT-OH-QM. PB treatment has an inducing effect only in the liver of rats or mice. The conversion of BHT-t-OH to BHT-OH-QM in rat liver microsomes is low in controls, but is induced 37-fold by PB-treatment. Basal levels of activity are higher in mice, and activity is induced 10-fold by PB-treatment. Basal activity of pulmonary microsomal enzymes was substantially higher in mice compared to rats, but was unaffected by PB-treatment in either species.

A series of cytochrome P450 inhibitors (SKF-525A, piperonyl butoxide and carbon disulphide) and P450 activators (phenobarbital or cedar oil) were administered concomitantly with buthionine sulfoximine (BSO) and BHT (Kawano et. al 1981). BSO is a selective and potent inhibitor of ?-glutamyl cysteine synthetase and reduces glutathione synthesis, thus disabling glutathione conjugation of electrophillic BHT quinone methide metabolites, which can then bind covalently to tissue macromolecules resulting in toxicity (see figure 3.4) (Nakagawa, 1984 cited in Powell & Conolly, 1991 and Powell & Conolly, 1991). Eleven analogues of BHT were also concomitantly administered with BSO to assess their hepatoxicity. Analysis of these results suggests that there are several structural requirements for liver toxicity:

- Phenolic ring
- Benzylic hydrogen atoms at the 4' position and an ortho-alkyl group that moderately hinders the hydroxyl group.

Positive correlations between toxic responses in liver and lung suggest that the structural requirements are the same for both the lung and the liver.

The use of CYP450 inhibitors resulted in decreased liver toxicity whist the use of inducers increased liver toxicity in the mouse. Generally these results complement studies in the mouse lung, suggesting that the cause of toxicity is via metabolic activation of BHT.

The organ-specificity of BHT toxicity in mice could be due to the fact that concentrations of GSH in mouse liver are high compared to lung. Glutathione conjugates of BHT and BHT-t-OH were found in the bile of mice pre-treated with PB prior to intra-peritoneal injection of BHT (Bolton et. al 1990).

Intra-peritoneal administration of 1800mg/kg bw/day BHT alone does not result in liver toxicity (Kawano et al,. 1981). In contrast, GSH-depleted mice show liver damage when treated with 200mg/kg bw/day BHT. This glutathione conjugation of BHT and BHT-t-OH represents a detoxification pathway.

c) p-electron oxidation

In vitro data demonstrate that BHT is oxidised by a CYP450 catalysed reaction forming the peroxyquinol (Shaw and Chen, 1972, cited in Witschi et al., 1989) (see figure 3.5). The peroxyquinol metabolite rapidly undergoes further CYP450-mediated oxidation to produce the corresponding quinol which is partially oxidised in a tert-butyl group to the hydroxyquinol (Thompson et al., 1987, Takahashi et al. 1983 cited in Witschi et al., 1989). During the homolytic cleavage of the O-O bond present in peroxyquinol, the quinone, free radicals and electrophillic species are produced (see figures 3.5 and 3.6). It is therefore not surprising that formation of the peroxyquinol is associated with toxic effects. The peroxyquinol is more potent than BHT-t-BuOH, the quinone methide or BHT-SCH_3 (see figure 3.6) at inducing haemorrhagic death in rats (Yamamoto et al. 1980). It is equipotent with benzyl peroxide as a tumour promoter in mouse skin (Taffe et al., 1987 cited in Witschi et al., 1989). The peroxyquinol is approximately 10-fold more cytotoxic than BHT to isolated hepatocytes (Thompson & Ross, 1988 cited in Witschi et al., 1989).

The quinone (DBQ) and hydroxylated quinone (DBHQ) (see figure 3.5) have been identified as metabolites in rat urine (Yamamoto et al., 1979 cited in Witschi et al., 1989). The glucuronide conjugate of DBHQ has also been found in rat bile (Tajima et al. 1983 cited in Witschi et al., 1989). The quinone

has been identified as a metabolite in rat liver and lung and mouse lung microsomes. It is formed from the peroxyquinone via beta-scission of the corresponding quinoxy radical generated by homolytic cleavage of the O-O bond (Wand & Thompson 1986, cited in Witschi et al., 1989). The quinone can be measured in the urine, after i.p administration of the peroxyquinol to rats, suggesting that the peroxyquinol is the intermediate in the formation of the quinone from BHT (Yamamoto et al. 1979 cited in Witschi et al., 1989). The quinone methide (BHT-QM) can is the major unconjugated metabolite of BHT present in the liver of rats (Takahashi & Hiraga, 1979, cited in Witschi et al., 1989). It is also present in the urine and bile of rats following BHT administration (Tajima, 1981, cited in Witschi et al., 1989). Quinones are known to be highly reactive, as they are potent electrophiles. They react with nucleophiles via the exocyclic methylene carbon. The quinone binds covalently to microsomal proteins by attacking thiol groups; the binding of radioactive BHT (presumably via the quinone methide) occurs more in liver microsomes compared to lung microsomes (Nakagawa et al, 1979 and 1983 cited in Bolton et al. 1990). It is likely that the glutathione conjugate found in rat bile is formed by reaction of glutathione with the quinone methide. The formations of rat urinary BHT-NAC (Daniel et al, 1968) and BHT-SCH_3 (see figure 3.5) (Yamamoto et al., 1979 cited in Witschi et al., 1989) are thought to occur via enzymatic degradation of BHT-SG.

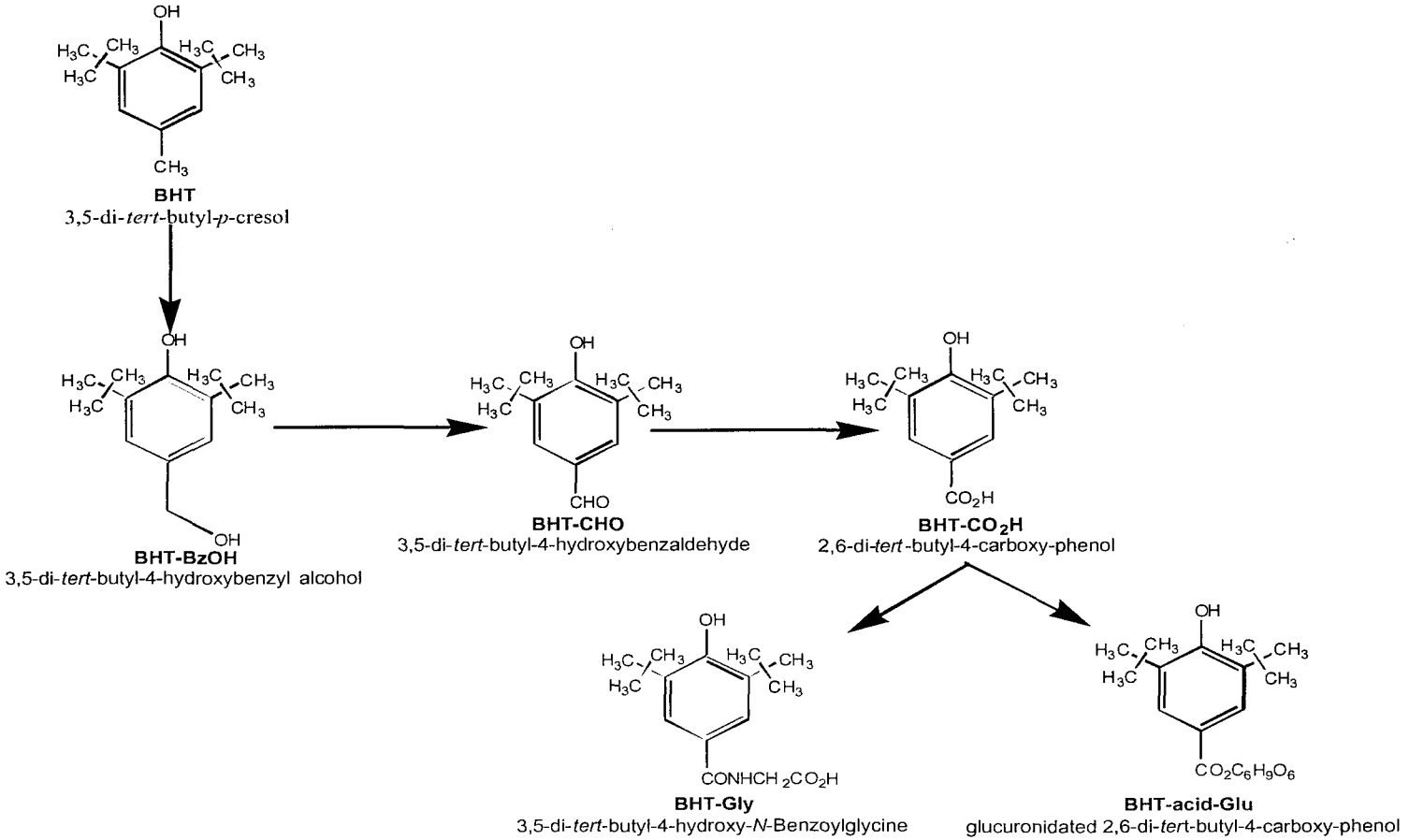
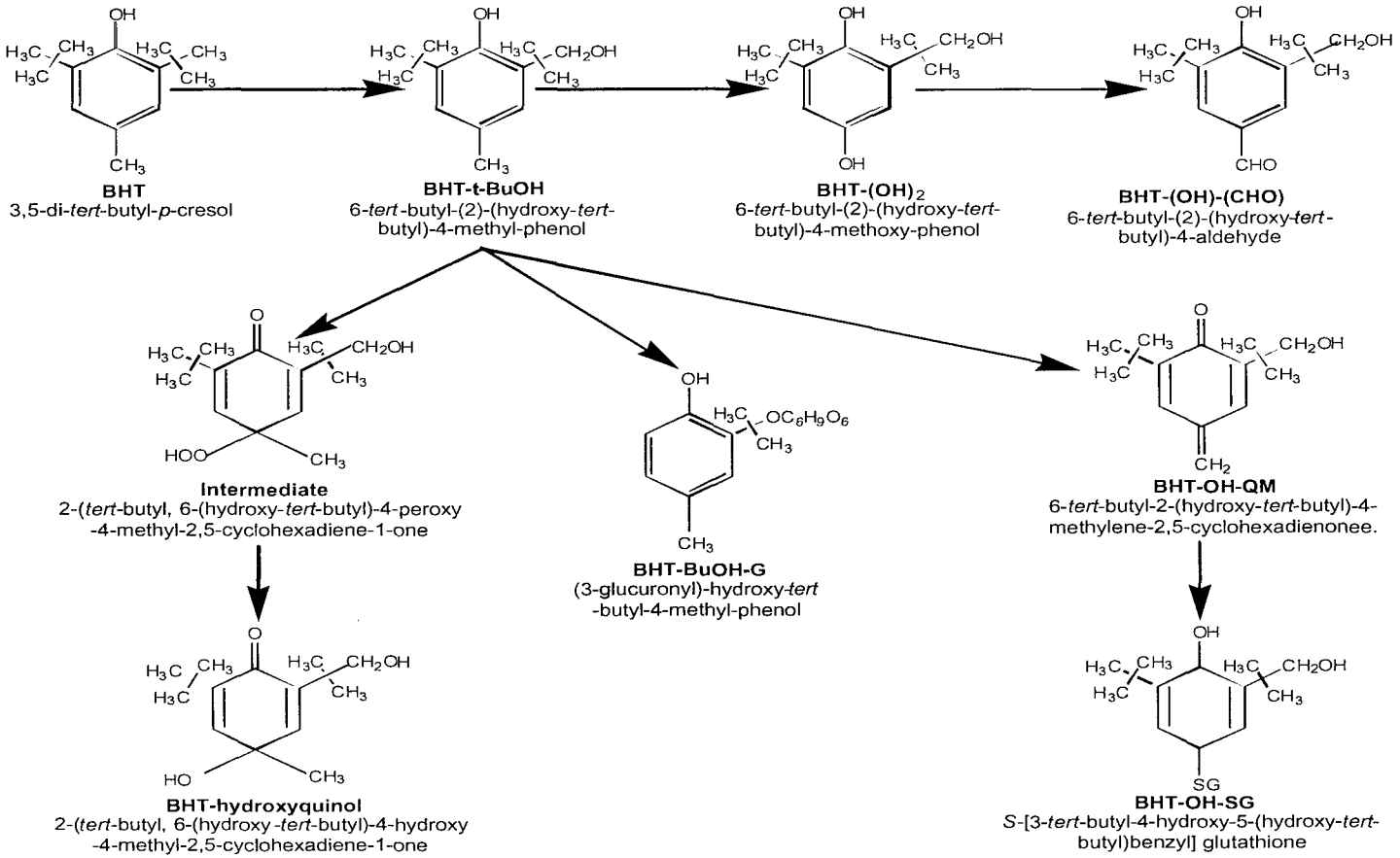


Figure 3.3 Alkyl oxidation of BHT – preferential route of metabolism for rats and rabbits. Adapted from Witschi et al., 1989

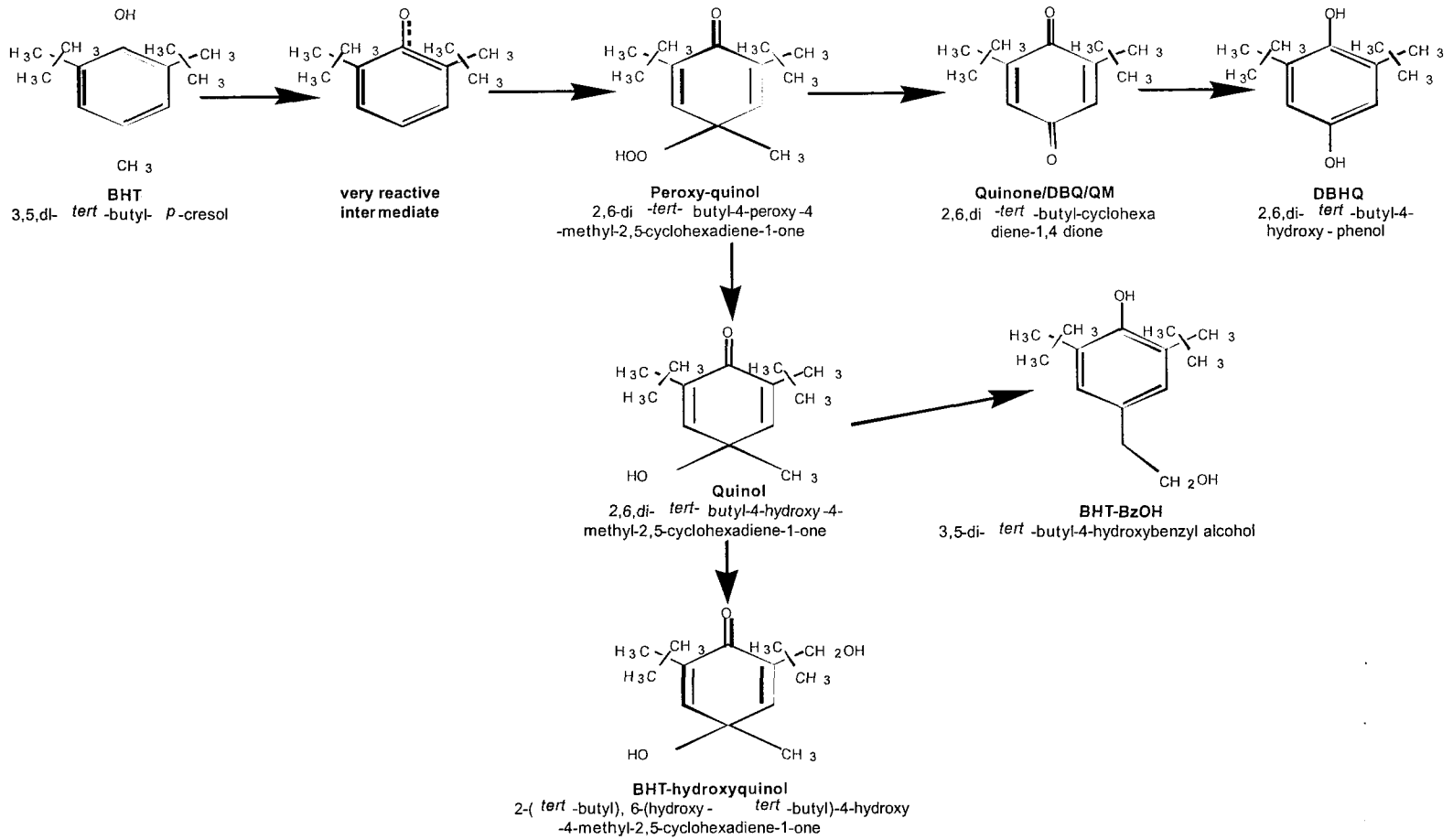
NB there is experimental evidence that hydroxyquionone (and hydroxyquinol) and are produced from BHT-t-BuOH in rabbits.

i



NB BHT-hydroxyquinone is also produced from the intermediate metabolite.

Figure 3.4 Alkyl oxidation of BHT – preferential route of metabolism for mice. Adapted from Witschi et al., 1989; Bolton et al. 1992; Bolton and Thompson 1991



NB Metabolic transformation of hydroxyquinone from quinone has not been demonstrated experimentally

Figure 3.5 p-electron oxidation of BHT forming reactive intermediates and electrophillic metabolites. Adapted from Thompson et. al 1987)

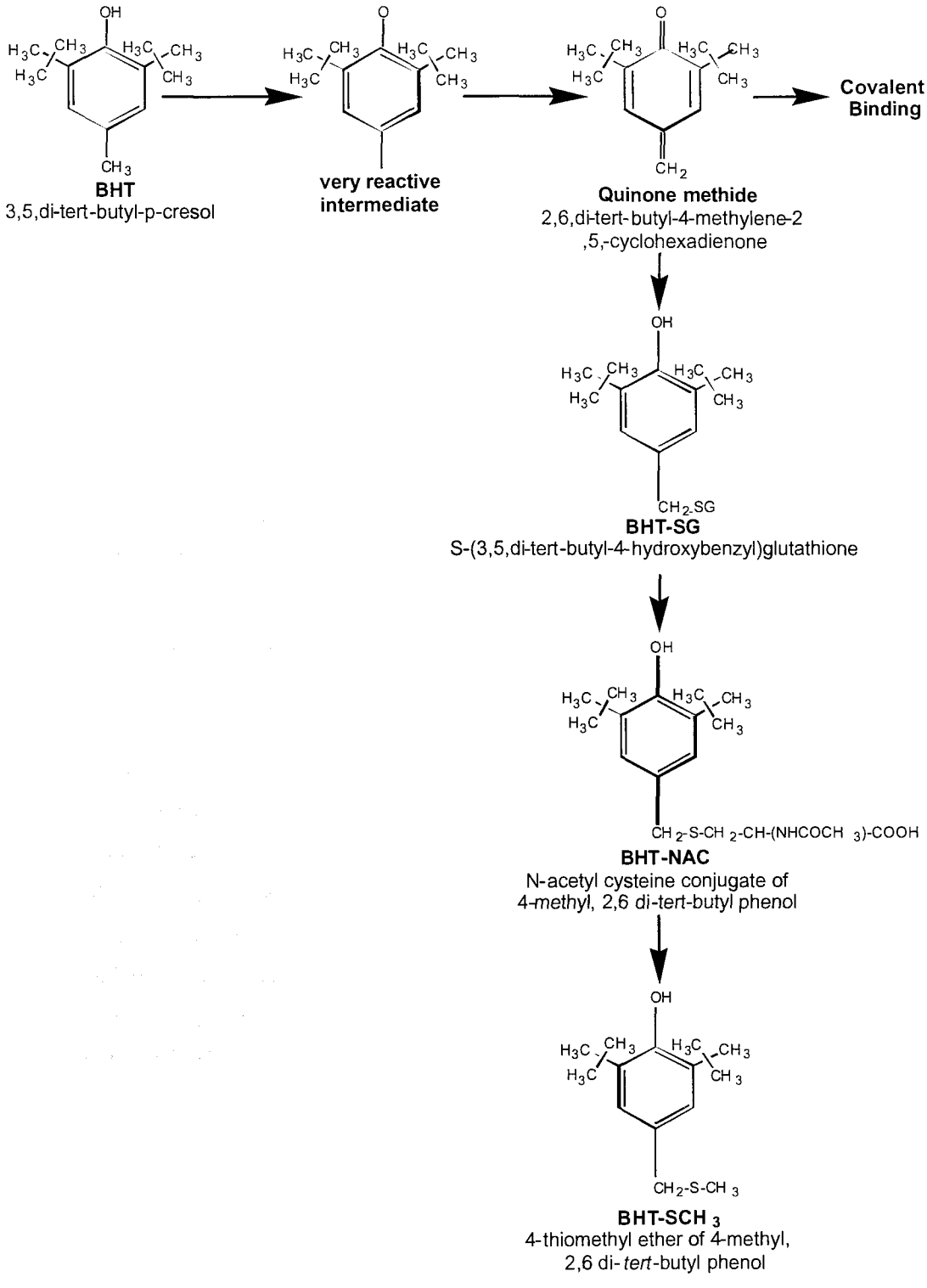


Figure 3.6 p-electron oxidation of BHT forming reactive intermediates and electrophillic metabolites. (Adapted from Witschi et al.,1989)

3.3 Toxicological effects of BHT

3.3.1 Acute and sub-acute toxicity of BHT

The acute toxicity of BHT was investigated in multiple species by Deichmann et al., 1955. The LD_{50} values or approximate lethal doses are in the range of 1-10 g/kg.

3.3.2 Sub-chronic toxicity of BHT

Søndergaard & Olsen (1982) investigated the effect of BHT on the thyroid. Rats fed BHT in the diet (500 and 5000 ppm) for 90 days showed increased iodine uptake, and increased thyroid weights. After 13 days, the half-life of thyroxine was found to increase transiently. The no-observed effect level was 500 ppm, equal to 25 mg/kg bw/day. No clear pathological mechanisms were identified, and the toxicological significance of the finding remains unexplained.

3.3.3 Haemorrhagic effects of BHT.

The haemorrhagic effect of BHT in rats has a threshold of 0.69% in diet (fed *ad libitum* for 40 days), with a dose-dependent increase in prothrombin time (Takahashi & Hiraga, 1978). The results indicated that vitamin K deficiency might be implicated in the haemorrhagic effect of BHT. Subsequent studies (Takahashi et al. 1978b cited in Cottrell et al., 1994, Takahashi &Hiraga, 1979 and Takahashi, 1986 cited in Cottrell et al., 1994) revealed that haemorrhagic effects could be detected at much lower doses (0.017% in diet) and that supplementation of the diet with vitamin K prevented BHT-induced hypothrombinanemia. Takahashi et al, 1986 (cited in Cottrell et al., 1994) demonstrated that the prothrombin and several vitamin K-dependent clotting factors were rapidly decreased after the administration of BHT.

BHT has been shown to require metabolic activation in order to exert its haemorrhagic effects. The BHT quinone methide (refer to figure 3.5) is more active than the parent compound at reducing in the vitamin-K dependent clotting factors. The ?-carboxylation of glutamate residues in blood clotting factors II, VII, IX and X is dependent on vitamin-K epoxide. It is thought that BHT (and the quinone methide) inhibits phylloquinone (vitamin K1) epoxide reductase. Redox cycling of vitamin K is prevented, and the production of functional blood clotting factors is inhibited, preventing adequate clotting (Takahashi, 1988). One criticism of these studies (Takahasi & Hiraga 1978b cited in Cottrell et al., 1994) in which supplementation of the diet with vitamin K prevented BHT-induced hypothrombinanemia was that haemorrhagic effects were also observed in control animals which indicates a possible dietary vitamin-K deficiency.

A further study was therefore conducted to ascertain whether or not BHT could induce haemorrhagic death in rats provided with at least the recommended level of vitamin K (3.0ppm, NRC, 1978 cited in Cottrell et al., 1994). The findings were that a high intake of BHT in the diet (3000 mg/kg/day) was associated with decreased activity of vitamin-K dependent clotting factors (Cottrell et al., 1994). Supplementation of the diet with vitamin K3 simultaneously with BHT administration reversed this effect for all factors except prothrombin.

This effect is a high dose phenomenon with a steep dose-response curve. However the doses causing haemorrhagic effects are far in excess of any likely human exposure. The LOAEL in this study (Cottrell et. al 1994) was 600 mg/kg/day, which is 2000 times greater than the ADI for BHT (0.3 mg/kg bw/ day).

3.3.4 Hepatotoxicity and carcinogenicity studies with BHT

Early studies indicated that BHT caused liver enlargement (hepatomegaly caused by hyperplasia) in rats (Gilbert & Goldberg, 1965, Creaven et al., 1966, Crampton et al., 1977) and bile duct hyperplasia in mice (NCI, 1979 cited in WHO, 1996). Liver hypertrophy, proliferation of hepatic endoplasmic reticulum and modified enzyme activity of liver microsomes was also reported in monkeys given 500 mg/kg bodyweight for 28 days (Allen & Engblom 1972). It was considered that liver hypertrophy and hyperplasia was a physiological adaption rather than an hepatotoxic response to BHT exposure and, together with the induction of enzyme activity in the liver the data suggested that BHT is not hepatotoxic *per se* (Gilbert & Golberg, 1965, Gaunt et al ., 1965, Feuer et al., 1965)

Hirose et al., (1981) did not observe increased tumorigenesis in Wistar rats fed 1% BHT for 104 weeks. Another study (Shirai et al., 1982) failed to demonstrate carcinogenic effects of BHT in B6C3F1 mice. An increased incidence of hepatoadenomas was observed in male BC3F1 mice receiving 2% BHT orally for 104 weeks (Inai et al., 1988). There was a clear dose-response effect, but due to excessive incidences of tumours in control mice (a known problem in the strain under study), the results were deemed inconclusive.

A two generation chronic feeding study (Olsen et al., 1986) showed that BHT was capable of inducing benign and malignant hepatocellular neoplasms in male and female Wistar rats fed up to 500 mg/kg bw for 144 weeks. The dose-response relationship was particularly pronounced in the male rats. It is believed that the abnormal duration of the study meant that late-developing tumours could be detected. Another single generation study, (Williams et al., 1990) conducted for 76 to 110 weeks failed to demonstrate the carcinogenic effect of BHT.

Powell et al., (1986) discussed a hypothesis regarding the mechanism of BHT tumorigenesis. BHT is

thought to induce tumours by a non-genotoxic mechanism, and previous studies have shown biochemical and pathological changes in centrilobular and periportal areas of the liver. Centrilobular damage would be expected, given the lipophillicity of BHT and a correlation has been made between areas of damage and CYP450 activity, again reinforcing the notion that a metabolite of BHT was responsible for toxicity. The fact that there is a lag-time between exposure to BHT and toxicity was explained by Powell et al., who suggest that a cycle of necrosis and proliferation provides favourable conditions for neoplastic transformation.

A further study was designed to characterise the hepatocellular changes and their role in the development of carcinomas (Price et al., 1994, cited in WHO, 1996). The experiment was very similar in experimental design to the Olsen study (Olsen et al., 1986). In a two-generation feeding study doses of 0, 25, 100 or 250 mg/kg were fed to F_1 male Wistar rats. Significant hepatic enzyme induction (of cytochrome P450 enzymes and in particular pentoxyresorufin-O-dealkylase (CYP450 2B1)) and thyroid hyperactivity were observed in rats in the high and mid-dose groups. These effects were not observed in the 25 mg/kg bw/ day group.

3.4 The most recent determination of a NOAEL for BHT

The outcomes of the Price study, (cited in WHO 1996.) (enzyme induction and thyroid hyperactivity) together with the findings of the Olsen study, (Olsen et al .,1986) (tumour induction) were used to derive the NOAEL for BHT of 25 mg/kg bw/day. This was used as the basis for estimation of the ADI.

3.5 Clinical studies with BHT

3.5.1 Clinical procedure

The materials and methods used in the clinical studies with BHT are given in chapter 2 (materials and methods).

3.5.2 Dose selection and safety

As discussed in the introduction, one of the aims of this project was to compare human and animals' kinetic parameters derived from studies where BHT was administered at the no-effect level (the ADI for humans and the NOAEL for animals). These doses are 0.3 mg/kg bw/day and 25 mg/kg bw/day respectively. A secondary aim of the project was to investigate linearity of the kinetic parameters. Doses equivalent to 10 x ADI and 0.1 x NOAEL were administered to humans and animals respectively. In order to gain ethical permission for these studies, the acute reference dose (ArfD) was estimated for BHT.

In cases of acute exposures to hazardous chemical, the ArfD is estimated based on short-term animal toxicology studies. The ArfD is an estimate of a safe acute exposure, and therefore is normally calculated from no-effect level demonstrated in acute animal toxicity testing. Since our human volunteers in the high dose studies were effectively being exposed to the food additives in much larger quantities, a review of all available acute toxicity data to assess the margin of safety between the ARfD in the animal database and the 10 x ADI dose. Table 3.1 lists all the currently available acute toxicity data for BHT.

Based on the short-term toxicity data presented in table 3.1, the NOAEL for short-term effects for BHT would be estimated to be >85 mg/kg/day or >5950 mg/person/day. Comparing this with the intended single dose for this study (210 mg/person) gives a margin of safety of >28.

Study performed	Species	Duration	NOAEL(mg/kg bw/day)	Human intake (mg/day)	Reference
Short-term study (1)	Mouse	10 weeks	2% MTD = 2,000	140,000	Inai et al., 1988
Short-term study (2)	Rat	7 weeks	6,200 mg/kg food (MTD) = 0.62% = 310	434,000	NCI, 1979 (cited in WHO, 1996)
Short-term study (3)	Rat	24 months	0.5%, = 250	17,500	Deichmann et al., 1955
Long-term study (4)	Rat	Lifetime	0.062% = 31	2,170	Hiraga, 1978 (cited in WHO, 1996)
Long-term study	Rat	Two-generation study	25	1,750	Olsen et al., 1986
Long-term study	Rat	Two-generation, two-year study	25	1,750	Price, 1994 (cited in WHO, 1996)
Reproductive toxicity study (5)	Mouse	Two-generation lifetime study	0.1% = 100	7,000	Johnson, 1965
Reproductive toxicity study	Mouse	Three-generation study	<20	<1,400	Tanaka et al., 1993
Reproductive toxicity study	Rat	Three-generation study	1000	70,000	Frawley et al., 1965
Reproductive toxicity studies (6)	Mouse Rat	1- generation and 3- generation studies	0.1% 100 (Mouse) 50 (Rat)	7,000 (based on mouse) 3,500 (based on rat)	Meyer and Hansen, 1980, Brunner et al., 1978; Vorhees et al., 1981 (cited in WHO, 1996)
Hepatotoxicity study (7)	Rat	7 days	25	1,750	Powell et al. 1986
Haemorrhagic effects study	Rat	1-4 weeks	85	5,950	Takahashi and Hiraga, 1979
Haemorrhagic effects study	Rat	3 consecutive doses	380	26,600	Krasavage, 1984

(1) MTD = maximum tolerated dose 2% diet converted to mg/kg bw/day as follows: 2% converted to ppm (= 20,000 ppm), then multiplied by 0.1 = 2,000
(2) 6,200 mg/kg food = 6,200 ppm. 6,200 x 0.05 = 310
(3) NOAEL calculated as follows: 0.5% = 5000 ppm. 5000 x 0.05 = 250
(4) NOAEL calculated as follows: 0.062% = 620 ppm. 620 x 0.05 = 31
(5) NOAEL calculated as follows: 0.1% = 1000 ppm. 1000 x 0.1 = 100
(6) NOAEL for mouse calculated as (4). For rat, 0.1% = 1000 ppm. 1000 x 0.05 = 50 (Studies considered together as part of the Chemical Manufacturers Association comments submitted to JECFA in 1980)
(7) Mild hepatocyte damage was found at the next highest dose. (250 mg/kg bw/day, equivalent to 17,500 mg/day in humans)

Table 3.1 A summary of toxicity studies on BHT.

3.6 Results

3.6.1 Early clinical studies with BHT and method development

Analysis of the initial clinical studies performed with BHT revealed relatively high BHT concentrations present in the baseline (pre-dose) plasma samples. In the analyses of the first 6 volunteers using GC-MS, the average BHT concentration in baseline samples was ~30 ng/ml (see table 3.2). Volunteers had all fasted for over 12 hours by the time baseline sampling was performed and it was anticipated that there would be negligible pre-dose concentrations of BHT. In one subject (BHT-7), the baseline plasma concentration of BHT was higher than any subsequent post-dose measurement of BHT. (The analysis of samples from BHT-2 did were unsuccessful, and no data area available.)

Time,	Contaminated from the vacutainer system						New syringe collection method		
minutes	BHT-1	BHT-3	BHT-4	BHT-5	BHT-6	BHT-7	BHT-8	BHT-9	BHT-10
0	0 (11.4)	0 (20.1)	0 (18.2)	0 (16.1)	0 (25.0)	0 (89.5)	0 (3.0)	0 (4.4)	0 (9.7)
30	0.8	-2.2	6.7	-3.8	7.5	-41.2	0.0	-0.1	4.2
60	2.2	-1.0	-1.2	2.5	-5.7	-50.2	0.0	-0.7	-0.8
90	14.2	16.8	-5.3	4.4	-4.5	-24.1	1.4	-0.8	56.9
120	1.9	-0.7	-1.7	5.8	-5.9	29.2	8.6	-0.6	220.7
150	-4.8	5.4	41.8	0.4	-25.0	-11.6	13.6	1.3	88.8
180	-11.3	86.9	8.2	1.4	42.0	-5.2	11.1	1.2	52.2
240	14.1	48.5	73.3	2.1	3.8	-26.6	7.6	32.0	13.5
300	3.0	1.4	30.9	5.8	7.9	-46.8	7.0	27.4	12.1
360	-6.3	7.3	-1.1	16.0	-3.6	-54.1	5.0	14.0	15.5
420	3.7	-1.8	22.2	1.7	1.6	-58.0		13.5	6.6
480									7.1

(Values in parentheses are the baseline values quantified as BHT that were subtracted from all subsequent measurements.)

Table 3.2 Plasma concentrations of BHT in ng/ml from the initial 9 individuals, who received BHT at the ADI.

Studies with different reagents and equipment (shown in figure 3.7) indicated that the source of BHT contamination was the blood sampling method used. A full description of the materials and methods employed is given in chapter 2 (materials and methods).

Figure 3.7 shows the concentrations of BHT measured in blood samples taken using a variety of methods (needle and syringe, vacutainer system directly and vacutainer system via a cannula). The results shown are for 3 individuals.

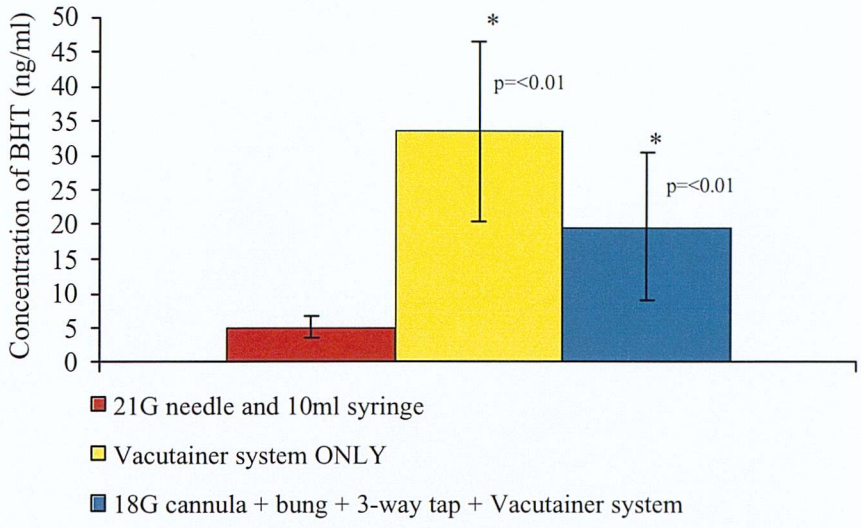


Figure 3.7 Identifying the source of BHT contamination of plasma samples obtained in the initial studies.

Clearly there was a contamination issue arising from the use of the vacutainer system. There were significant differences between the concentrations of BHT detected in plasma following blood sampling using a needle and syringe compared with blood sampling using a vacutainer on its own, or in conjunction with a cannula and associated equipment. Subsequently the collection method was revised (see chapter 2), and further sets of clinical samples (BHT 8, 9, 10) were analysed. Here, the average BHT concentration present in the pre-dose samples was ~6 ng/ml.

3.6.2 Problems with the analysis of BHA (internal standard) using the GC-MS method

Another problem encountered, resulting in the need for frequent re-analysis of the initial clinical samples, was the observation that the area under the curve (AUC) of the integrated BHA peak would decrease during the run. Consequently the ratio of BHT to BHA was not constant and increased on repeated injection of the same solution. Figure .3 8 illustrates this effect.

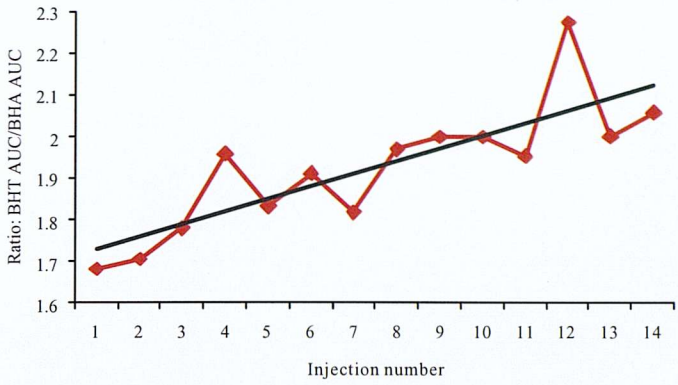


Figure 3.8 Changing ratio of BHT:BHA through a number of injections. (There was approximately 15 minutes between injections – see section 2.8.4)

Following several attempts to rectify the problem, (replacement of the inlet filter, increasing the temperature on the final temperature ramp) it became clear that it was unlikely that optimal performance was going to be achieved from the GC-MS available. Had the GC-MS performed optimally, it should have resulted in a more sensitive assay. Due to the problems already discussed, it was decided to abandon GC-MS and to use GC as the method of quantifying BHT.

3.6.3 Studies in which human volunteers received BHT at the ADI

Due to the analytical problems encountered with the early studies, plasma concentration-time data from the first nine volunteers (as detailed in table 3.3) could not be used. In consequence a further 10 volunteers were given BHT at the ADI at a dose of 0.3 mg/kg, and these data sets were used for calculation of pharmacokinetic parameters (see table 3.4 for details of these volunteers). The mean age of the volunteers was 26 years old, (30 for the males and for the 23 females). The mean weight of the male volunteers was 78 kg and for the female volunteers was 61 kg.

	Age	Weight	Ethnicity
M1	20	79	Caucasian
M2	37	79	South Asian*
M3	18	87	Caucasian
M4	21	80	Caucasian
M5	18	66	Caucasian
Male Mean	**23**	**78**	**N/C**
F1	33	59	South Asian*
F2	24	69	Caucasian
F3	30	48	Caucasian
F4	44	71	Caucasian
F5	18	57	Caucasian
Female Mean	**30**	**61**	**N/C**
Overall Mean	**26**	**69**	**N/C**

*South Asians are volunteers originally from the Indian sub-continent.

N/C – mean not calculated, non –arithmetic values.

Table 3.3 Details of volunteers who received BHT at the ADI.

3.6.3.1.1 Plasma analyses

Table 3.4 shows the concentrations of BHT, expressed as ng/ml detected using GC at each time point for each individual included in the pharmacokinetic comparisons.

The plasma concentrations shown in table 3.4 show that BHT concentrations at many time points was below the lowest concentration in the standard curve (10 ng/ml). However, reliable concentrations of BHT could be detected below 10 ng/ml, and good CoV values (<10%) on the triplicate analyses were obtained in some samples for concentrations as low as 2 ng/ml. Individual concentration-time profiles were obtained, and they are represented graphically in figure 3.9.

Concentration of BHT in plasma samples, ng/ml

Time	Male Data					Female Data				
(minutes)	M1	M2	M3	M4	M5	F1	F2	F3	F4	F5
0	0.0	0.0	0 (1.6)	0.0	0.0	0.0	0 (2.6)	0.0	0.0	0.0
30	1.3	3.4	0.0	0.0	0.9	2.7	0.0	0.0	0.0	0.8
60	0.9	1.3	0.0	0.0	2.0	2.4	0.0	0.0	0.0	2.9
90	**27.3**	9.2	0.2	1.2	**10.3**	**36.1**	0.0	1.4	0.0	3.0
120	**143.2**	**16.9**	4.9	**22.1**	**10.2**	**49.0**	4.7	4.3	**34.2**	6.9
150	**58.7**		**26.5**	**19.1**	8.9		**13.5**	9.4		**40.2**
180	**36.3**	**35.6**	**26.5**	**13.5**	9.3	**38.4**	**29.5**	**45.1**		**64.0**
210		**37.3**	**15.4**	**10.7**	7.1	**34.0**	**31.3**	**46.9**	**126.6**	**54.2**
240	**11.7**	**17.4**	**10.5**	4.8	4.8	**21.4**	**10.8**	**19.9**	**43.9**	**21.2**
300	8.9	9.7	6.1	2.2	3.6	8.2	5.4	8.0	**12.1**	9.5
360	7.7	5.0	5.8	3.2	3.8	8.3	2.6	5.8	**10.7**	6.3
420	7.0	4.1		1.9	2.0	2.5	1.5	4.0	**10.5**	5.8
480	7.0									
1440		0.2	0.8	0.0	2.0	0.8	0.0	0.0	3.0	3.4

NB The **red bold typeface** denotes concentrations that were above the limit of quantification for the method. Values given are following baseline subtraction; baseline values quantified as BHT are shown in parentheses.

Table 3.4 Plasma concentrations of BHT from studies in which volunteers received the ADI

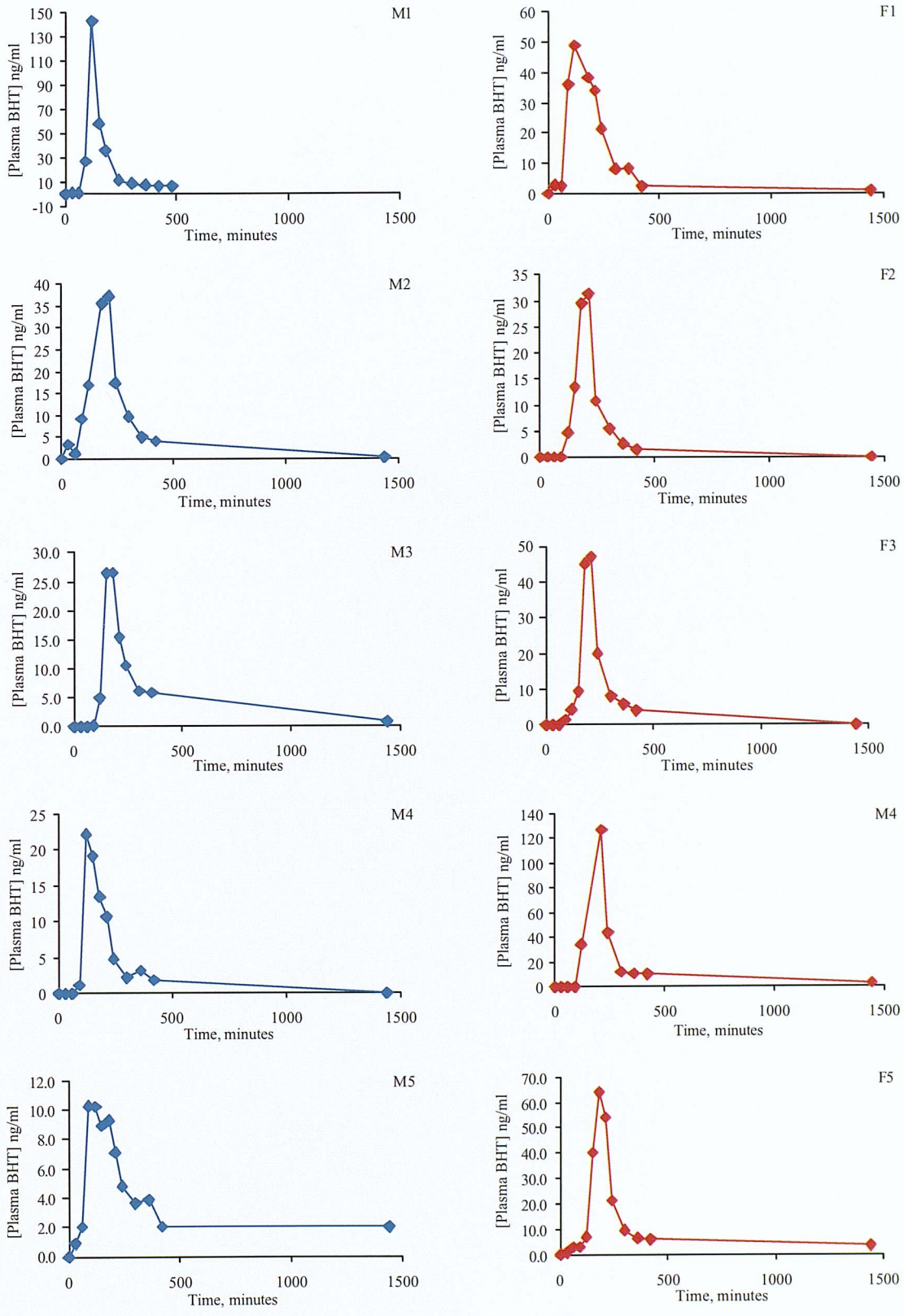


Figure 3.9 Concentration time profiles for plasma BHT in male (blue graphs) and female (red graphs) volunteers after dosing at the ADI.

3.6.4 Pharmacokinetic parameters calculated for BHT using the data from ADI studies

As discussed in the previous section, concentration time curves were observed in the case of most individuals, therefore the kinetic model (WinNonlin™, described in chapter 2), was able to predict terminal phase pharmacokinetic values for AUC to infinity $AUC_{(inf)}$, CL/F to infinity or a value for the terminal half-life. The pharmacokinetic parameters available for comparison comprise observed values; Cmax, Tmax, $AUC_{(obs)}$ (calculated using the trapezoid formula) and extrapolated values $AUC_{(inf)}$, T½ and CL/F (using the observed values extrapolated to infinity). These parameters are shown below in table 3.5. These values are calculated using baseline-subtracted data, as shown in table 3.4.

	Observed values			Parameters extrapolated to infinity			
	Cmax (ng/ml)	Tmax (minutes)	AUC (ng x min/ml)	AUC (ng x min/ml)	T1/2 (minutes)	?z values used to determine T1/2	CL/F (ml/min)
M1	143	120	11276	16117	478	300-480	19
M2	37	210	7863	6212	78	240-420	48
M3	27	180	6790	7224	384	210-360	42
M4	22	120	3547	2869	100	120-420	105
M5	10	90	4243	4712	162	120-360	64
Male Mean	**47.8**	**144**	**6744**	**7427**	**240**	**N/C**	**55**
F1	49	120	9871	9730	48	180-300	31
F2	31	210	4205	3521	65	210-420	85
F3	47	210	7104	5720	118	300-420	52
F4	127	210	17320	19064	456	300-420	16
F5	64	180	11938	16382	1045	360-1440	18
Female Mean	**63.6**	**186**	**10088**	**10883**	**346**	**N/C**	**41**
P value (M vs. F)	0.606	0.174	0.238	0.386	0.621	N/C	0.464
Overall Mean	**56**	**165**	**8416**	**9155**	**293**	**N/C**	**48**
Overall SD	45	47	4288	5911	312	N/C	30
Overall CoV	**80**	**29**	**51**	**65**	**106**	**N/C**	**62**

N/C not calculated. Values in **bold** are the mean data and overall CoV used in later comparisons. P-values compare the male and female data, using unpaired student t-test (2 tails, equal variance)

Table 3.5 Pharmacokinetic parameters for BHT generated using the data from studies in which volunteers received the ADI.

As reflected in the overall CoV values, there was extensive variability in pharmacokinetic parameters amongst individuals, with very high variability in Cmax, and T½. The variability in the T½ values largely arose from the data for subject F5 who showed only a limited decrease in concentration between 480 and 1440 minutes. The group size was not large enough to make any comment regarding differences in kinetic parameters relating to ethnic group. No sex differences in PK parameteres were detected.

(Using a student's t-test no significant sex differences were detected.)

In particular, three individuals, M1, F4 and F5 stand out from the majority of the data, with high T½ and/or Cmax values and low CL/F values. The high variability in Cmax is probably not due to varying rates of absorption, because there is no inverse correlation with Tmax. The large variability in T ½ shown in table 3.5 is probably not a true reflection of T½, because the concentrations of BHT quantified in the last time points were very low, and the replicates showed large CoV values. Therefore, in the majority of cases the 1440 minutes time point was not used in the ?z range for most individuals. The sensitivity of the analytical method prevented the accurate projection of the terminal elimination phase.

3.6.5 Studies where human volunteers received BHT at the 10 x ADI.

3.6.5.1 Plasma analyses

A total of 10 volunteers were given BHT at a dose of 3.0 mg/kg (10 x ADI). From these 10 data sets, 10 were used for comparison of pharmacokinetic parameters (see table 3.6 for details of these volunteers). The mean age of the volunteers was 25 years old, (26 for the males and for the 24 females). The mean weight of the male volunteers was 77 kg and for the female volunteers was 67 kg.

	Age	Weight	Ethnicity
M6	21	72	African
M7	31	68	Caucasian
M8	33	88	Korean
M9	26	67	Caucasian
M10	22	84	South asian
M11	22	84	South asian
Male Mean	**26**	**77**	**N/C**
F6	32	61	Caucasian
F7	19	59	Caucasian
F8	21	67	Caucasian
F9	24	82	Caucasian
Female Mean	**24**	**67**	**N/C**
Overall Mean	**25**	**73**	**N/C**

Table 3.6 Details of volunteers who received BHT at 10 x ADI.

Concentration of BHT in plasma samples, ng/ml

Time	Male Data						Female Data			
(minutes)	M6	M7	M8	M9	M10	M11	F6	F7	F8	F9
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0 (0.79)
30	6.6	0.0	0.9	0.0	0.0	0.0	0.0	0.0	0.0	19.1
60	5.8	20.7	0.0	15.5	69.5	3.2	42.6	0.0	0.0	151.4
90	22.5	95.1	0.0	122.2	62.3	3.0	307.5	6.6	5.4	341.2
120	126.0	158.5	9.5	180.3	64.8	16.7	183.0	13.5	17.9	432.6
150	170.9	278.3	74.5	229.2	55.7	155.2	151.6	221.5	104.3	644.6
180	170.4	222.6	190.6	216.7	92.4	339.7	117.4	558.6	274.6	490.0
210	102.2	144.7	419.1		453.2	433.0	81.8	606.8	579.8	183.4
240	53.0	65.7	379.4	81.0	378.4	237.3	36.7	286.7	300.8	141.0
300	24.5	29.4	242.2	53.5	211.6	95.4	15.5	114.0	60.1	63.5
360	16.9	22.6	130.0	27.7	127.6	83.2	13.9	98.7	48.6	50.2
420	10.6	21.2	93.3		120.9	51.1	12.3	90.0	32.9	47.9
1440	4.0	7.1		6.4	15.2	10.1	6.6	15.0	11.9	9.5

Values are given following baseline subtraction. Baseline values quantified as BHT are shown in parentheses.

Table 3.7 Plasma concentrations of BHT from studies in which volunteers received 10 x ADI.

The plasma concentrations (table 3.7) show that much higher concentrations of BHT were detected in samples from the 10 x ADI studies, compared with the previous ADI studies (table 3.4). Individual concentration-time profiles showing absorption and elimination phases were obtained and these are plotted in figure 3.10.

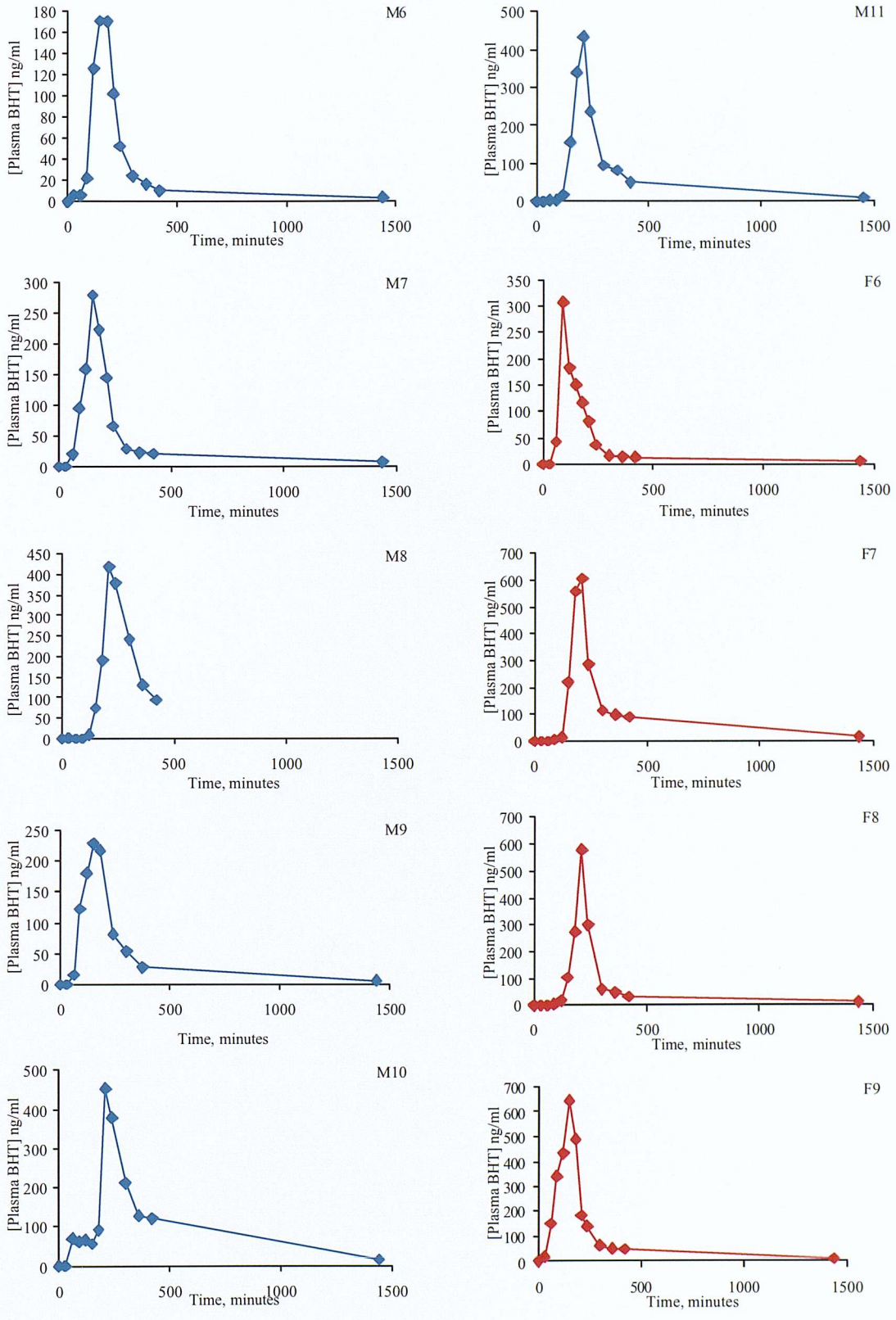


Figure 3.10 Concentration time profiles for BHT in male (blue graphs) and female (red graphs) volunteers after dosing at 10 x the ADI.

3.6.6 Pharmacokinetic parameters for BHT calculated using the data from the 10 x ADI studies

As with the ADI data, concentration time curves were observed in the case of most individuals, and so the kinetic model (WinNonlin™, described in chapter 2), was able to predict terminal phase pharmacokinetic values for AUC to infinity $AUC_{(inf)}$, CL/F to infinity or a value for the terminal half-life. The pharmacokinetic parameters available for comparison comprise observed values; Cmax, Tmax, $AUC_{(obs)}$ (calculated using the trapezoid formula) and extrapolated values $AUC_{(inf)}$, T½ and CL/F (using the observed values extrapolated to infinity). These parameters are shown below in table 3.8. These values are calculated using baseline-subtracted data, as shown in table 3.7.

	Observed values			Parameters extrapolated to infinity			
	Cmax (ng/ml)	Tmax (minutes)	AUC (ng x min/ml)	AUC (ng x min/ml)	T1/2 (minutes)	?z values used to determine T1/2	CL/F (ml/min)
M6	171	150	34541	30819	608	360-1440	87
M7	278	150	55364	48740	646	360-1440	54
M8	419	210	75435	63043	92	300-420	40
M9	229	150	57908	57093	443	300-1440	52
M10	453	210	141897	134335	346	360-1440	21
M11	433	210	88762	82795	389	300-1440	34
Male Mean	**331**	**180**	**75651**	**69471**	**421**	**N/C**	**48**
F6	307	90	50111	39973	1062	360-1440	60
F7	607	210	132693	124087	396	360-1440	23
F8	580	210	83870	73348	613	360-1440	36
F9	645	150	117772	111736	422	360-1440	25
Female Mean	**535**	**165**	**96111**	**87286**	**623**	**N/C**	**36**
Pvalue (M vs.F)	0.044	0.775	0.411	0.460	0.303	N/C	0.334
Overall Mean	**410**	**170**	**83288**	**75908**	**514**	**N/C**	**44**
Overall SD	174	N/C	38775	38090	267	N/C	21
Overall CoV	**42**	N/C	**47**	**50**	**52**	**N/C**	**48**
Mean -M8	**413**	**177**	**83072**	**75365**	**464**	**N/C**	**43**
SD - M8	174	N/C	38692	37825	223	N/C	22
CoV - M8	**42**	N/C	47	**50**	**48**	**N/C**	**51**

N/C not calculated. Values in **bold** are the mean data and overall CoV used in later comparisons. P-values compare the male and female data, using unpaired student t-test (2 tails, equal variance)

Table 3.8 Pharmacokinetic parameters for BHT generated using the data from studies in which volunteers received the ADI.

The Cmax values range from 171-645 ng/ml, and show a statistically significant sex difference. Tmax remains relatively constant, with 90-210 (1½ to 3½ hours). The AUC values showed the greatest inter-subject variability (M10, F7 and F9 have lower values for AUC). There are some outliers in the Cmax data, notably M6, M7 and F6, who have lower Cmax values. The T½ was fairly consistent, with the exception of M8. Subject M8 only had data points until 7 hours, therefore T ½ may have been

underestimated and cannot be compared with the other subjects. The values for CL/F show limited variability, although there are some outliers, M10, F7 and F9 who have a much smaller AUC values resulting in a higher CL/F.

3.6.7 Comparing plasma kinetics for BHT at the two human doses.

Assuming linear kinetics, a 10-fold increase in concentration dependent parameters (Cmax and AUC) would be anticipated in volunteers receiving 10 x ADI. CL/F however, would be expected to remain the same, because it is a concentration-independent parameter. Table 3.9 compares the kinetic parameters calculated from observed values as a ratio (10xADI/ADI).

	Observed values			**Parameters extraolated to infinity**		
	Cmax (ng/ml)	Tmax (minutes)	AUC (ng x min/ml)	AUC (ng x min/ml)	T1/2 (minutes)	CL/F (ml/min)
ADI Mean	**56**	**165**	**8416**	**9155**	**293**	48
ADI SD	45	47	4288	5911	312	30
ADI CoV	80	29	51	65	106	62
10 x ADI Mean	**410**	**170**	**75908**	**83288**	**514**	**44**
10 x ADI SD	174	N/C	38090	38775	267	21
10 s ADI CoV	42	N/C	50	47	52	48
Ratio 10 x ADI/ADI	**7.4**	**1.0**	**9.0**	**9.1**	**1.8**	**0.92**
P-value	<0.001	0.66	<0.001	<0.001	0.087	0.64

N/C not calculated. Values in **bold** are the mean data (except for the ratio calculation). P-values compare the high and low dose data, using unpaired student t-test (2 tails, equal variance)

Table 3.9 Comparison of pharmacokinetic parameters for BHT generated from the two studies conducted in humans.

Taken collectively, the ratios in table 3.9 indicate that the kinetics are linear at the high dose. The Cmax ratio of 7.4 suggests that the amounts of BHT entering the systemic circulation at the higher dose are not 10 times those at the ADI. The Tmax values are similar, as one would expect. The ~10-fold differences in AUC between the low are high dose indicate that, as expected for concentration dependent parameters, a 10-fold increase in dose results in a 10-fold increase in the parameter value. The T½ and CL/F ratio values suggest that the removal or elimination of BHT is unaffected by dose, i.e. saturation of (of elimination) is not occurring at either dose.

3.7 Protein-binding studies with BHT

The analysis of the saliva samples only commenced once the feasibility of detecting BHT in the saliva samples was determined. Prior to analysing the saliva samples taken in the 10 x ADI studies, the extent to

which BHT is protein-bound was determined in protein-binding studies. Due to the low concentrations of BHT observed in the plasma of subjects dosed at the ADI corresponding saliva samples were not analysed.

Protein-binding studies were conducted to determine the level of plasma-protein binding of BHT. Albumin was spiked with BHT, and equilibrium dialysis performed using a protocol described in chapter 2 (material and methods), BHT was highly protein bound (see table 3.11), indicating that the saliva samples would contain only about 5-10% of the concentration in the corresponding plasma samples.

	Ringers'	**Albumin**	**Protein Binding, expressed as a percentage.**
Ratio BHT/BHA	0.3033 ± 0.131	0.0108 ± 0.00456	93

The results are the mean ± SD for 5 replicate measurements.

Table 3.10 Results of an experiment to determine the percentage protein binding of BHT. (See chapter 2, materials and methods for details).

The appropriate controls were analysed simultaneous to the samples; baseline subtraction was not conducted as the replicate concentrations of extracted unspiked albumin were not consistent. (The levels of BHT in unspiked albumin were ~ 1% of the spiked albumin.)

3.7.1.1 Analysis of saliva samples from the BHT 10 x ADI studies

Saliva samples collected at the time of Cmax and a sample either side of the Cmax from the subjects dosed at 10 x ADI were analysed. (In some cases one of the samples adjacent to the Cmax was very similar to the Cmax, and so a fourth sample was analysed.) The maximal concentrations in plasma at the ADI studies ranged between 171-645 ng/ml. Based on 93% protein binding, one would therefore expect maximal concentrations in the saliva of between 12-45 ng/ml. Given the limit of detection of the assay (~2 ng/ml) it would be unlikely that a concentration time-profile for BHT could be followed in the saliva for a significant number of subjects. Saliva samples were analysed (as outlined in Chapter 2) and the concentrations quantifie d in the samples is shown in table 3.11.

Very small amounts of BHT were detected in the saliva. In many cases the levels were close to or below the limit of quantification and the CoV values were high. The Tmax in saliva showed no consistent correlation to the Tmax in plasma. In addition, there was no consistent ratio between plasma

concentration of BHT and salivary concentration of BHT either within an individual's samples, or between the samples from different individuals. The mean overall value for relative concentration of BHT in saliva was ~6% for those samples with measurably concentrations, which supports the outcome of the protein-binding study. However the quality of the saliva data does not allow too much emphasis to be placed on this mean value. In conclusion, salivary analysis of BHT was not a reliable marker for BHT in the plasma at a dose of 10 x ADI.

Volunteer	Time point	Conc. of BHT in Plasma (ng/ml)	Conc. of BHT in Saliva (ng/ml)	CoV (of Saliva)	Relative BHT in saliva (%[saliva]/[plasma])
M6	120	126.0	**6.2**	173	4.9
	150	170.9	0.0	N/C	N/C
	180	170.4	0.0	N/C	N/C
	210	102.2	0.0	N/C	N/C
M7	120	158.5	**7.5**	173	4.7
	150	278.3	**7.7**	173	2.8
	180	222.6	0.0	N/C	N/C
M8	180	190.6	0.0	N/C	N/C
	210	419.1	**3.6**	173	0.9
	240	379.4	**2.0**	173	0.5
M9	120	180.3	**5.4**	141	3.0
	150	229.2	16.8	173	7.3
	180	216.7	24.5	58	11.3
	210		**9.6**	94	N/C
M10	180	92.4	0.0	N/C	N/C
	210	453.2	12.9	87	2.9
	240	378.4	**2.3**	173	0.6
M11	180	339.7	0.0	N/C	N/C
	210	433.0	0.0	N/C	N/C
	240	237.3	0.0	N/C	N/C
F6	60	42.6	18.7	39	43.8
	90	307.5	0.0	N/C	N/C
	120	183.0	**5.6**	173	3.1
F7	180	558.6	0.0	N/C	N/C
	210	606.8	**5.4**	173	0.9
	240	286.7	0.0	N/C	N/C
F8	180	274.6	0.0	N/C	N/C
	210	579.8	**2.5**	173	0.4
	240	300.8	0.0	N/C	N/C
F9	90	341.2	0.0	N/C	N/C
	120	432.6	12.8	51	2.9
	150	644.6	0.0	N/C	N/C
	180	490.0	16.4	9	3.4

The timepoint marcated in **bold** was the Cmax in plasma. Data in **red font** denote concentrations below the LoQ of 10 ng/ml.

Table 3.11 Salivary concentrations of BHT from studies in which volunteers received BHT at 10 x ADI.

3.7.2 Calculating the chemical-specific inter-individual toxicokinetic factors for BHT

The default uncertainty factory, allowing for inter-individual variability in toxicokinetics is 3.16 (WHO, 1999). The coefficient of variation was determined for the three primary kinetic parameters calculated from the human experimental data. Assuming a log-normal distribution the variation observed in the parameters was used to calculate what proportion of the population would be protected by the current default of 3.16-fold. The uncertainty factor that would be required to protect 95, 97.5 and 99% of the population was also calculated. The results, using parameters derived from analyses of plasma (after dosing at the ADI and 10xADI) are shown in table 3.12.

	ADI PK Parameters			**10 x ADI PK Parameters**		
	Cmax (ng/ml)	AUC (ng x min/ml)	CL/F (ml/min)	Cmax (ng/ml)	AUC (ng x min/ml)	CL/F (ml/min)
Mean	56	9155	48	410	75908	44
SD	45	5911	30	174	38090	21
CoV	80	65	62	42	50	48
Uncertainty factors	Cmax	AUC	CL/F	Cmax	AUC	CL/F
~% Covered by 3.16	**94.9**	**97.4**	**97.8**	**99.8**	**99.3**	**99.4**
95th Percentile	**3.18**	2.66	2.56	1.95	2.17	2.13
97.5th Percentile	**3.97**	**3.20**	3.06	2.22	2.52	2.46
99th Percentile	**5.14**	**3.98**	**3.77**	2.58	3.00	2.91

Uncertainty factors in **red font** denote those greater than the default of 3.16-fold.

Table 3.12 Calculating the inter-individual toxicokinetic factors for BHT using plasma kinetic data derived from the ADI and 10 x ADI studies. (AUC and CL/F values are values extrapolated to infinity.)

This summary table shows contrasting results. In the case of the low dose studies, the high variability in Cmax values means that none of the usual risk management default percentage population values (used for exposure considerations) would be adequately protected by the default of 3.16. The results based on AUC are intermediate whilst using CL/F, the acceptable defaults would be protected, except for the 99th percentile. With the high dose data, the low variability found in Cmax, AUC and CL/F values means that, large proportions of the population are adequately protected.

3.7.3 Studies in which rats were dosed with BHT at the NOAEL

Eleven rats, 5 male and 6 female were dosed with 25mg/kg bw BHT, (the NOAEL), in corn oil (2.5%). Dr K. Walton conducted these studies and analysed the samples. Table 3.14 is a summary of the concentrations measured at each time point in each rat.

Concentration of BHT in rat plasma, ng/ml

Time	Male Data					Female Data					
(minutes)	M1	M2	M3	M4	M5	F1	F2	F3	F4	F5	F6
0	0	0	0	0	0	0 (29)	0 (32)	0	0	0	0
30	31	27	50	46	27	26	46	155	97	96	41
60	27	59	56	49	67	98	104	186	144	132	111
120	52	130	208	163	222	178	159	281	172	188	106
240	111	74	140	184	85	136	107	146	145	179	75
360	102	64	99	81	52	146	98	98	143	82	67
480	90	53	75	66	60	92	77	63	126	65	54
600	80	65	79	52	45	94	57	62	103	50	57
720	68	66	74	47	57	61	51	69	80	39	62
1440	31	26	80	21	70	1	25	39	43	18	26

Values are given following baseline subtraction. Baseline values quantified as BHT are shown in parentheses.

Table 3.13 Plasma concentrations of BHT in rats from studies in which rats were dosed with the NOAEL.

BHT was readily detectable in the rat plasma samples, which resulted in clear absorption, distribution and elimination profiles. Figure 3.11 below shows the average concentration-time profile for the male and female rats. Absorption appeared to be slower than for TBZ or PG (see chapters 5 and 6), and elimination was also slower, with detectable concentrations present at 1440 minutes (24 hours) post dose.

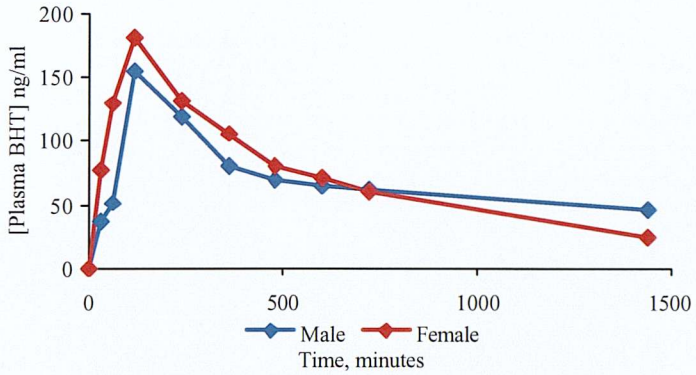


Figure 3.11 Concentration time-profiles generated from mean male and mean female rat data after dosing at the NOAEL. (The results are the average for 5 male and 6 female rats.)

3.7.4 Pharmacokinetic parameters for BHT calculated using the data from the NOAEL studies

Pharmacokinetic parameters were calculated for each individual parameter (see table 3.14) using the same method as for human ADI data, i.e. baseline subtraction, followed by mathematical modelling of the data to obtain pharmacokinetic values extrapolated to infinity.

	Observed values			Parameters extrapolated to infinity			
	Cmax (ng/ml)	Tmax (minutes)	AUC (ng x ml/min)	AUC (ng x ml/min)	T1/2 (minutes)	?z values used to determine T1/2	CL/F (ml/min)
M1	111	240	92531	120820	628	300-1440	207
M2	130	120	83275	105570	593	600-1440	237
M3	208	120	129667	334699	1777	120-1440	75
M4	185	240	91228	109939	626	600-1440	227
M5	222	120	101942	N/C	N/C	N/C*	N/C
Male Mean	**171**	**168**	**99175**	**167757**	**906**	**N/C**	**186**
F1	178	120	103252	103426	127	600-1440	242
F2	159	120	91110	115684	693	600-1440	216
F3	281	120	125594	185435	1072	600-1440	135
F4	172	120	136034	176435	655	120-1440	142
F5	188	120	93389	108730	597	600-1440	230
F6	111	60	81882	110138	741	60-1440	227
Female Mean	**181**	**110**	**105210**	**133308**	**647**	**N/C**	**199**
P value (M vs. F)	0.755	0.074	0.659	0.493	0.378	N/C	0.761
Overall Mean	**177**	**136**	**102719**	**147088**	**751**	**N/C**	**194**
Overall SD	50	54	19053	72306	426	N/C	57
Overall CoV	**29**	**40**	**19**	**49**	**57**	**N/C**	**29**

N/C WinNonlin™ could not extrapolate the slope of the terminal half life from the data. Values in **bold** are the mean data and overall CoV used in later comparisons. P-values compare the male and female data, using unpaired student t-test (2 tails, equal variance)

Table 3.14 Pharmacokinetic parameters for BHT generated from studies in which rats were dosed with the NOAEL.

The Cmax values had a small range, from 111-281 ng/ml, with a Tmax typically of around 2 hours. There were no statistically significant sex differences, although the T-test approached significance for sex differences in Tmax value. The T½ and CL/F values were consistent, with the exception of two noticeable outliers; M3 and F3 which have higher T½ values, and correspondingly lower CL/F values. In these two animals, the very long T½ values resulted in considerably higher AUC extrapolated to infinity and correspondingly lower CL/F.

3.7.5 Studies in which rats were dosed with BHT at 0.1 x less the NOAEL

As previously reported (earlier in this chapter), studies were conducted in human, where volunteers received 10 x ADI. A dose equivalence study was conducted in rats (receiving 0.1 x less the NOAEL), using the same experimental protocol as previously described. Table 3.15 reports the summary of the concentrations measured at each time point in the rats.

Concentration of BHT in rat plasma, ng/ml

Time	Male data				Female data					
(minutes)	M1	M2	M3	M4	F1	F2	F3	F4	F5	F6
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30	0.0	39.2	48.0	0.0	0.0	0.0	0.0	9.6	0.0	0.0
60	14.3	0.0	13.9	0.0	0.0	0.0	0.0	10.5	0.0	0.0
120	0.0	16.2	20.3	0.0	0.0	15.6	20.2	14.8	11.4	0.0
240	0.0	14.4	21.4	0.0	10.6	0.0	13.4	0.0	10.3	0.0
360	39.5	0.0	40.2	27.1	12.7	0.0	11.6	11.8	9.6	0.0
480	0.0	14.3	0.0	0.0	0.0	0.0	0.0	0.0	10.1	0.0
600	10.2	59.6	8.2	0.0	0.0	28.2	0.0	12.3	0.0	0.0
720	17.0	10.1	0.0	0.0	0.0	12.9	0.0	10.9	0.0	0.0
1440	0.0	0.0	0.0	0.0	0.0	0.0	0.0	11.4	0.0	0.0

Table 3.15 Plasma concentrations of BHT from studies in which rats were dosed with 0.1 x the NOAEL.

Concentrations of BHT in the 10x<NOAEL samples were close to the limit of detection. The low concentrations of BHT observed in these samples were lower than the anticipated 10-fold dose-dependent decrease. Based on the data in table 3.15 the concentration-time profiles did not show clear and consistent characteristics, and so are not plotted. The plasma samples from one rat (F6) did not contain BHT at any time point post-dose. This data was not used in subsequent PK calculations, since an AUC of zero would result in an infinite CL/F value which could not be used to derive a mean, SD or CoV for CL/F for the group of animals.

3.7.6 Pharmacokinetic parameters for BHT using data from the 0.1 x NOAEL studies

Pharmacokinetic parameters were calculated for each individual parameter using concentrations after baseline subtraction, followed by calculation of AUC using the trapezoid formula (see table 3.16). CL/F was calculated as dose/AUC. As discussed above, the animal data did not show clear concentration-time profiles, and so the data could not be extrapolated to infinity. The AUC for the human and rat NOAEL data were also calculated using the trapezoid formula and CL/F as dose/AUC, to allow direct comparison of the data from this study with the human and NOAEL PK data (see table 3.18).

	Cmax (ng/ml)	Tmax (minutes)	AUC (ng x ml/min)	CL/F (ml/min)
M1	39	360	13758	182
M2	60	600	17463	143
M3	48	30	12266	204
M4	27	360	3255	768
Male Mean	**44**	**338**	**11686**	**324**
F1	13	360	2794	895
F2	28	600	10193	245
F3	20	120	4818	519
F4	15	120	13658	183
F5	11	120	4635	539
Female Mean	**22**	**276**	**7964**	**451**
P value (M vs. F)	0.01	0.64	0.24	0.46
Overall Mean	**29**	**297**	**9205**	**409**
Overall SD	17	212	5428	282
Overall CoV	**58**	**72**	**59**	**69**

Table 3.16 Pharmacokinetic parameters for BHT generated from studies in which rats were dosed at 0.1 x NOAEL data.

The Cmax values had a small range, from 11-60 ng/ml. Tmax was quite variable, 30 minutes for one rat (M3) and 600 minutes for another (F2). There were no statistically significant sex differences. AUC values, and therefore CL/F values varied (3-4 fold, and 6-7 fold respectively) with some outliers (M4 and F1) having particularly low AUC values, and corresponding high CL/F values.

In order to examine the effect of the smaller dose on the kinetics within the rat, table 3.17 was prepared, showing the ratio between the NOAEL and 0.1 x NOAEL pharmacokinetic parameters. (AUC and CL/F values calculated by the trapezoid rule without extrapolation to infinity.)

	Cmax (ng/ml)	Tmax (minutes)	AUC (ng x ml/min)	CL/F (ml/min)
0.1 x NOAEL	29	297	9205	409
Overall SD	17	212	5428	282
Overall CoV	58	72	59	69
NOAEL Mean	177	136	102719	250
Overall SD	48	54	19053	42
Overall CoV	35	40	19	17
Ratio NOAEL/0.1 x NOAEL	**6**	**N/C**	**11**	**0.61**

Table 3.17 Comparison of PK parameters for BHT generated from the two studies conducted in rats.

This comparison shows that differences in concentration-dependent parameters do not exceed the 10-fold increase in dose (Cmax) or barely exceed the expected increase (AUC). The 2-fold difference in CL/F is not statistically significant, suggesting that saturation is not occurring at the high dose.

3.7.7 Calculating the chemical-specific inter-species toxicokinetic factor for BHT

Human and animal kinetic parameters were compared to generate the chemical-specific inter-species toxicokinetic factor for BHT (see table 3.18). The kinetic parameters (excluding CL/F, which is independent of dose) were corrected for dose using the equation show in chapter 2, materials and methods (table 2.15).

	Observed values		Extrapolated parameters		
	Cmax (ng/ml)	Tmax (minutes)	AUC (ng x ml/min)	T1/2 (minutes)	CL/F (ml/min)
Human ADI Mean	56	165	9155	293	48
Overall SD	45	47	5911	312	30
Overall CoV	80	29	65	106	62
Animal NOAEL Mean	177	136	147088	751	194
Overall SD	50	54	72306	426	57
Overall CoV	29	40	49	57	29
Inter-species factor	**26.27**	**N/C**	**5.19**	**N/C**	**4.04**

Table 3.18 Calculating the interspecies toxicokinetic factor comparing ADI and NOAEL data.

The data in table 3.18 show that based on Cmax or AUC the toxicokinetic default factor of 4.0-fold is not adequate. The human Cmax value is 30-times greater than the rat Cmax after correction for dose, and the human AUC value is 5 times greater that the animal AUC; i.e. humans are exposed to a greater proportion of the (corrected) dose than animals. CL/F is faster in animals than humans, but the species difference

does not exceed the 4.0-fold default. Tmax values are lower in animals than humans, indicating that humans would have an overall greater exposure to BHT than animals.

3.7.8 The magnitude of the inter-species toxicokinetic at different dose levels.

The inter-species toxicokinetic differences were calculated using all possible combinations of data (see tables 3.19 and 3.20). Differences were calculated based on extrapolated data and on the calculated data, except in the cases of dose equivalence, and in the case of CL/F, which is calculated as animal value/human value, without correction for dose.

	Inter-species differences		
	Cmax	AUC	CL/F
ADI/NOAEL	**26.3**	**5.2**	**4.0**
ADI/0.1 x NOAEL	17.8	N/C	N/C
10 x ADI/NOAEL	19.3	4.7	4.4
10 x ADI/0.1 x NOAEL	13.1	N/C	N/C

Table 3.19 Inter-species differences (based on extrapolated PK values).

	Inter-species differences		
	Cmax	AUC	CL/F
ADI/NOAEL	**26.3**	**6.7**	**5.1**
ADI/0.1 x NOAEL	17.8	8.3	8.2
10 x ADI/NOAEL	19.3	6.2	5.0
10 x ADI/0.1 x NOAEL	13.1	7.6	8.2

Table 3.20 Inter-species differences (based on observed PK values).

The alternative ways in which the mean values for each study group were calculated (i.e. extrapolated vs. non-extrapolated) reflect the influence of the terminal phase slope. In the majority of cases, the terminal slope was an accurate reflection of the terminal half-life, especially in the high dose data. Unfortunately, because T½ could not be calculated in the 0.1 x NOAEL studies, not all the comparisons can be made using extrapolated data. The comparisons using observed data however, underestimate AUC, resulting in higher CL/F. Although the differences between the inter-species ratio values are small, from a margin of safety perspective, they would be misleading. Comparison of these species differences with the default uncertainty factor of 4.0-fold (WHO, 1999) shows that in both cases (using observed and extrapolated to infinity parameters) the default of 4.0-fold is exceeded for all parameters based on all dose comparisons. The use of observed or extrapolated values makes very little difference to the inter-species difference. The inter-species differences are greatest based on comparison of Cmax values, and exceed the default of

4.0-fold by a smaller margin based on AUC and CL/F. The margin of difference is greatest when the dose gap is biggest (ADI compared with NOAEL), smallest when based on dose equivalence (10 x ADI compared with 0.1 x NOAEL) and is an intermediate value based on comparison of one of the intermediate doses with the no-effect level dose.

3.8 Discussion

3.8.1 Plasma kinetics from studies dosing human volunteers with BHT at the ADI.

The LoD (limit of detection) of ~2 ng/ml for the BHT assay proved to be adequately sensitive to quantify BHT detected in the ADI plasma samples. However, unless members of the population at large were consuming large bolus doses of BHT it is unlikely that this assay would provide the sensitivity required to monitor dietary BHT. A more sensitive analytical method would have improved the estimation of the terminal elimination slope. Within the available analytical techniques, the method developed represented the best possible limit of detection that could be achieved.

The sensitivity of the BHT assay is probably the cause of the large variability (indicated by high CoV values) seen in T½. The large variability in T½ shown is probably not a true reflection of T ½ , as the concentrations of BHT quantified in the last time points were very low, and subject to large CoV values. This was why, the 1440 minutes time point is not used in the ?z range for most individuals, which meant that the terminal half-life was estimated to varying degrees of accuracy in different volunteers, resulting in an overestimate of the true variability in T½ for these volunteers.

Despite the difficulty in quantifying the concentrations of BHT in very early and late time points, the quality of the data is generally good (reflected in the CoV values of the standard curves and samples with >2 ng/ml). Overall there was greater variability in Cmax, compared to AUC or CL/F (values of 80, 65 and 62% respectively). There was also greater variability in the AUC extrapolated to infinity compared to AUC calculated from observed values, some of which can again be attributed to the confidence in the early and late time points and the accuracy of the T½ used in the extrapolation The high variability in Cmax, with relatively constant Tmax suggests that differences in bioavailability amongst individual volunteers may be contributing to the overall variability in AUC and CL/F. In particular, three individuals, M1, F4 and F5 stand out from the majority of the data, with high Cmax values and low CL/F values. There was no evidence of a difference within the data based on ethnicity. Only two of the volunteers were non-Caucasian (M2 and F1) and the PK parameters for these individuals were not radically different to the range observed in the Caucasian data. Using a Student's t-test no significant sex differences were detected; a sex difference would not be expected based on animal studies reported in the literature (see earlier in this chapter). The age range of the volunteers in this data set is too limited to

make any observations regarding any influence of age.

3.8.2 Plasma kinetics from studies dosing human volunteers with BHT at 10 x the ADI.

Dosing of human volunteers at 10 x ADI resulted in higher plasma concentrations of BHT, so that most time points had quantifiable concentrations of BHT. This allowed more reliable determination of the terminal elimination slope. The use of the pharmacokinetic model resulted in larger mean values for AUC and CL/F since the use of the slope allowed extrapolation to infinity

There are some outliers in the data M10, F7 and F9 all have CL/F values on the low end of the range of values reported. However these lower CL/F values correlate to larger AUC and T½ values, indicating that the source of variation is likely to be the difference in bioavailability between subjects. The subject M8 had a considerably lower CL/F value compared to the rest of the group, which was due to the T½ being projected from a shorter ?z range, i.e. because a 1440-minute sample was not obtained. For this reason table 3.8 also shows that mean PK parameters derived without M8 being included in the data set. This results in lowered variability for T½ and CL/F values for the group.

As with the data derived with a dose at the ADI the age range of subjects in these studies was small (see table 3.6, and so no comment can be made regarding any trend with age. This volunteer group comprised more ethnically diverse subjects (compared to the ADI group) but again there was no obvious effect of ethnicity. However, there was a significant sex difference based on comparison of Cmax, where the female group, consisting entirely of Caucasian volunteers had larger Cmax values than the male group (where only two of the volunteers were Caucasian).

In general the inter-individual variability observed in the PK parameters from this higher dose study was small (relative to the ADI studies), with CoVs of 42%, 50%, 47%, 52% and 48% for Cmax, AUC_{obs}, AUC_{inf}, T½ and CL/F respectively. In particular, the variability in Cmax was much lower. This presumably would be due to more uniform absorption amongst these volunteers. The Tmax values were very similar; suggesting that rate of absorption was not different.

Table 3.9 compares the PK parameters at the ADI and 10 x ADI. Whilst there is some evidence of non-linear kinetics occurring at the higher dose, there is no clear indication of saturation kinetics. Cmax values have increased ~7-fold, but variability in the individual values was decreased at the higher dose. The mean Tmax values have not changed significantly at the higher dose, and AUC has increased ~10-fold as expected given the 10-fold difference in dose. T½ values are ~ doubled in the 10 x ADI group, reflective of the use of a longer ?z range, but the graphs (figure 3 9 and 3.10) show that the trend in shape of the concentration-time curves is the same at the high and the low dose. CL/F was unchanged at the higher dose, although variability was lower, probably due to a more accurate quantification of the data

points.

As previously discussed the enterohepatic circulation of BHT has been identified in rodents, but not in humans (Ladomery et al., 1967a, Holder et al., 1970). The possibility of BHT accumulating in adipose tissue has also been suggested. The data presented here do not support or refute either suggestion, since biliary concentrations of BHT were not determined, and both possibilities would give a prolonged terminal half-life. Figures 3.9 and 3.10 do not show any unexpected increases in BHT plasma concentrations at any time point after the initial peak, which might have indicated the presence of an enterohepatic recirculation.

3.8.3 Salivary concentrations of BHT as a biomarker of internal dose

Given that the estimated plasma protein binding of BHT was 93%, analysis of BHT in the saliva samples from individuals receiving BHT at the ADI dose was not undertaken. The analytical range required to be able to monitor BHT in these saliva samples would be in the very low nanogram/hundreds of picograms range. Given that it is unlikely that BHT would be consumed in bolus doses saliva monitoring of BHT is the general population would require the development of much more sensitive analytical techniques.

BHT plasma protein binding (determined by PPB studies) was found to be approximately 93%. Based on the mean Cmax values (410 ng/ml) observed in the plasma from the 10 x ADI studies one would expect mean Cmax saliva concentrations of BHT to be ~30 ng/ml. However true observed values were ~10 ng/ml. These results were not in agreement with the PPB studies, and suggest a smaller proportion of BHT is protein-bound in the plasma.

It is a criticism of the study that the effect of collection method upon the BHT recovered from the saliva was not investigated prior to the human studies. The standard curves used for the quantification of the saliva samples were prepared in water. The extraction efficiency of BHT from water was investigated and found to be satisfactory (see chapter 2). It is unlikely that the concentration of BHT in the saliva is being underestimated quantitatively, or that extraction from saliva was incomplete. Another possible explanation between the expected and observed concentrations of BHT in the saliva could be that BHT is not stable in the saliva, and therefore we observe smaller concentrations of BHT in the saliva than we would expect. (A stability study was not conducted for BHT in saliva, since a problem with stability was not anticipated.)

3.8.4 Conclusions regarding the development of biomarkers of exposure based on the pharmacokinetic studies in humans.

In the case of BHT it was possible to monitor the plasma concentration profile in healthy adults after they had consumed the ADI as a bolus dose. However, if one wanted to monitor dietary exposure to BHT, particularly via saliva samples from potentially sensitive sub-groups, a considerably more sensitive analytical method would be required.

Unfortunately, without further investigation into why the detected saliva concentrations are less than anticipated, it is difficult to conclude whether or not saliva could be used as a suitable biomarker for exposure to BHT. It is clear from the protein-binding experiment that in order to monitor BHT in saliva, a more sensitive analytical method would be required. The only PK parameter that exhibited statistically significant sex differences was Cmax values in the 10 x ADI group. A sex-defined Cmax might have an impact on risk assessment of BHT, particularly when acute exposure was considered. Interestingly this effect was observed only at the higher dose, suggesting that increasing the numbers of subjects may help elucidate whether or not the effect is real.

3.8.5 The adequacy of the inter-individual toxicokinetic default factor (3.16-fold) for BHT

The default factor of 3.16 adequately protects greater proportions of the population exposed to BHT at 10 x the ADI rather than at the ADI (see table 3.12). This is to be expected, given that the magnitude of variability was greater at the lower dose. Whilst the percentage of the population covered by the 3.16 default was greater at the higher dose, the percentage of the population covered by the 3.16 default based on chronic parameters such as AUC and CL/F is reasonable at the ADI (~97 and 98% respectively). In all cases, the 3.16 default could be expected to protect adequately the population exposed to 10 x ADI, however one would need to consider the remaining margin of safety between an acute and chronic exposure in animal studies and the 10 x ADI dose.

3.8.5.1 Sources of variability in the human BHT data.

Approaching the subject of variability logically, there are 4 main pharmacokinetic processes in which individuals may demonstrate inter-individual differences; absorption of the compound, distribution of the compound, metabolism of the compound and excretion of the compound. Most compounds will cross the intestinal epithelium via passive diffusion, and because of the high perfusion of the gut, this is not likely to become a rate limiting process for lipid soluble compounds. If an active transport mechanism were in place, then saturation of this process could occur, and at increasing doses of BHT, proportionally less BHT would enter the systemic circulation. The data in this chapter do not support this notion, and it can be assumed that passive diffusion is responsible for BHT being absorbed into the systemic circulation. The bioavailability of BHT, i.e. the fraction of the administered dose reaching the systemic circulation, cannot be estimated from the data presented here. A study in which BHT was given as an i.v. bolus dose

would be required to calculate absolute bioavailability, a regimen which although of scientific interest was outside the scope of this study. Similarly the distribution of BHT into the tissues could not be calculated from the oral dosing data generated. The metabolism of BHT was recorded, albeit indirectly, through the disappearance of the compound, and is reflected in the reported parameter, CL/F. The rate of clearance of BHT from the body was due to two factors, firstly excretion of unchanged drug in the urine, and secondly biotransformation to metabolites. There is no published human kinetic data for BHT, giving the fraction of BHT that is metabolised and the proportion that is excreted unchanged in the urine and bile.

The animal data reviewed earlier in this chapter suggest that whilst BHT is metabolised rapidly, a reasonable proportion of BHT is recovered in the urine and faeces as the unchanged parent compound. This suggests that cytochrome P450 activity may not be as important a source of variability as it is for some of the other compounds described in other chapters. Also, biliary excretion of BHT is likely to contribute significantly to the variability in kinetics. Due to the ethical constraints of the human studies, bile samples were not collected from either species. Urine samples were taken as part of the clinical studies with BHT, but priority was given to analysing the plasma and saliva samples given the time constraints. Analysis of these samples would have generated some information regarding the excretion of BHT. The metabolites (both human and animal) are not readily commercially available, and again, the quantification of metabolites was not the primary aim of these studies.

3.8.6 Plasma kinetics in rats which were dosed with BHT at the NOAEL.

The dosing of rats with BHT equivalent to the NOAEL resulted in quantifiable concentrations of BHT being recorded at the majority of time points. Clear concentration–time profiles were evident, (figure 3.11), and showed very similar profiles to those observed in humans. Generally speaking the resulting PK parameters show less variability than that observed in either human data set. This is due to the rat population being more homogenous. No significant sex differences were seen in any PK parameter, which can be supported in the literature (see earlier in this chapter); this also helps validate the lack of sex differences generally seen in the human data sets.

3.8.7 Plasma kinetics in rats which were dosed with BHT at 0.1 x NOAEL.

Dosing rats with BHT equivalent to 0.1 x NOAEL (2.5 mg/kg) resulted in data where BHT was detectable at very few time points. Concentration-time curves were not apparent; consequently WinNonlin™ was unable to model the data. The observed PK parameters showed large variability, 70%, 71% and 69% for Cmax, AUC_{obs} and CL/F respectively. Due to the quality of the data it is very difficult to comment on outliers and possible reasons for deviation from the mean for specific individual data sets.

Table 3.17 compares PK parameters from the low and high dose animal studies. The 10-fold difference in dose has resulted in 7-fold difference in Cmax values, a 12-fold difference in AUC values and a 2-fold difference in CL/F values, indicating the possibility of slight saturation at the higher dose. However considering the quality of the data from the 0.1 x NOAEL studies it is difficult to claim that saturation is occurring at the higher dose in rats.

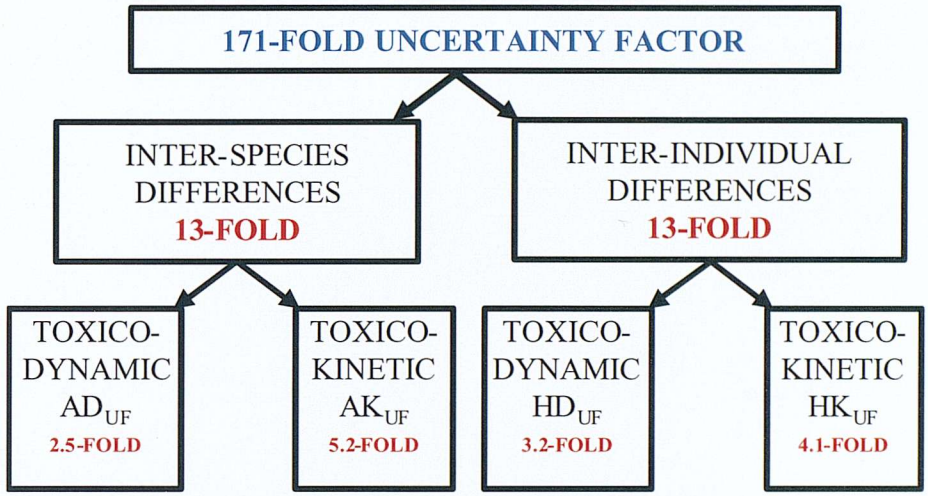
3.8.8 The adequacy of the inter-species toxicokinetic default factor (4.0-fold) for BHT

Table 3.18 shows the inter-species comparison of BHT PK parameters, using the appropriate comparison of the ADI vs. the NOAEL. It is clear that the default of 4.0-fold is exceeded for all PK parameters. However a distinction can be made between the reported factor based on Cmax, and the reported values based on AUC or CL/F. The ADI is related to chronic toxicity and so the factors based on AUC and CL/F are more important, and whilst these exceed the default of 4.0-fold (only marginally in the case of CL/F) the difference is negligible. It is worth considering however, that the differences in AUC and CL/F figures are likely to be conservative estimates of the true values, due to the likely underestimation of the T½ in humans. The factor for Cmax, which far exceeds the default of 4.0-fold is an indicator of acute toxicity, and is of less consequence when considering the appropriateness of the ADI for the population at large, consuming chronic low doses.

Tables 3.19 and 3.20 show all possible combinations of the animal data and human data in order to derive inter-species differences. Clearly the comparison between 10 x ADI and 0.1 x <NOAEL shows the smallest inter-species factor as these are at dose equivalence. This highlights that the fundamental assumption in risk assessment that humans are subject to a greater exposure to a nominal dose is correct in the case of BHT, and that in this case the default factors are not overly conservative. Roughly speaking there was a 10-fold margin between the PK parameter in the rat and the PK parameter in human subjects.

3.8.8.1 Calculating chemical-specific adjustment factors for BHT

The aim of the 1994 IPCS model is to encourage and accommodate the inclusion of chemical-specific data in the derivation of the ADI. Figure 3.12 below shows the derivation of the safety factor that would result if the two toxicokinetic factors (based on AUC) were substituted into the framework. (The factor for inter-individual differences in toxicokinetics is based on covering the 99th percentile of the population, using the CoV for AUC from the ADI data.)



NB – the inter-species factor and the inter-individual factors have been rounded to the nearest whole number

Figure 3.12 Substituting data-derived toxicokinetic values into the IPCS framework, resulting in a chemical specific adjustment factor for BHT.

By using the *in-vivo* data for BHT, the overall composite factor is increased from 100 to a factor of 171old. This would have the impact of decreasing the ADI from 0.3 mg/kg/day to approximately 0.15 mg/kg/day. It is unlikely however, that the data presented in this chapter would be considered sufficiently robust to make such a change (WHO, 2002). This is mostly because of the quality of the ADI PK data and the number of subjects studied. Whilst it is sufficient to estimate the likely human variability and the difference between species, the failure to characterise the terminal slope makes the data sub-optimal, in terms of its predictive values of chronic exposure kinetics.

The human data are likely to be underestimating the variability in PK that would be seen in larger samples of the population. Our subjects represented healthy adults and it is likely that variability will larger in the extended population, reflecting age, ethnicity and range of socio-environmental contributors. Both of these experimental limitations mean that the overall default could not be over-ruled by *in vivo* data at this moment in time. Repeat studies in animals at 0.1 x NOAEL and in humans at the ADI using a more sensitive analytical method would strengthen this database. An extended population study, including sub-groups would be recommended prior to re-evaluation.

3.8.9 Intake estimates for BHT

Whilst the overall uncertainty factor derived in figure 3.12 results in a composite uncertainty factor greater than the default of 100-fold, it is important to consider the impact of this with respect to intake estimate data for BHT. If the population is consistently exposed to BHT at concentrations below the ADI this may compensate for the smaller margin of safety between the NOAEL and the ADI based on kinetics that has been reported in this chapter. In the case of BHT, preliminary intake assessment of BHT and proposed maximum limits of the draft General Standard of Food Additives indicated that intakes of these food additives might approach or exceed the ADI (Codex Alimentarius Commission, 1996). JECFA assessed the potential for BHT intake to exceed the ADI at their 51st meeting (WHO, 2000). Their assessment showed that estimate of intake varied according to the estimate method used. When national maximum permitted levels or European maximum levels were used, estimates of per capita intakes of BHT ranged from 0.003-0.11 mg/kg bw based on poundage data. Additionally, using the household economic surveys or retail sales survey intakes were estimated to be 0.052-0.1 mg/kg bw. Finally, based on model diets intake estimates ranged (except in the case of the USA) from 0.02--0.1 mg/kg bw.

Several other intake estimates have been conducted; the summaries of these findings are presented in table 3.21. In all estimates except one (Kirkpatrick & Lauer, 1986), the estimated daily intake of BHT is less than or very close to the current ADI of 0.3 mg/kg bw/day. However, it is generally accepted that most of the methods used to estimate intake result in over-estimation, rather than under-estimation of true consumption, therefore it is likely that true intake is less than the ADI for most individuals. It has been recognised that children (under 18 years of age, and including infants) may be consuming relatively more BHT per unit of bodyweight, and so may incur a smaller margin of safety compared to adults (Verhagen et al., 1990 and Leclercq et al., 2000).

Estimated intake of BHT	Method	N	Year	Country	Reference
0.351 mg/day	Semi-quantitative food frequency questionnaire	2035	2000	The Netherlands	Botterweck et al., 2000
0.315 mg/ kg/day	Budget method		2000	Italy	Leclercq et al., 2000
0.125 mg/kg/day	Food consumption data in aggregated categories		2000	Italy	Leclercq et al., 2000
0.109 mg/person/day	Deduced daily intake from the mean concentrations and amounts of food consumed.	34,489	2002	Japan	Ishiwata et al., 2002a
0.05-0.10 mg/kg bw/day	Food consumption data (from a household economic survey) Packaged goods market survey	16014 households 300 sources	2001	Brazil	Baunwart & Toledo, 2001
>0.05 mg/kg bw/day	Dietary record survey.	-	1989	The Netherlands	Verhagen et al., 1990
<1 mg/kg bw/day (TMDI) <0.4 mg/kg bw/day on average	Dietary recall data and maximum permitted use levels	-	1986	Canada	Kirkpatrich & Lauer, 1986

Table 3.21 A summary of BHT intake estimates.

The critical effect was not developmental toxicity, and given the general conclusion that adults are not generally exceeding the ADI, it is unlikely that another uncertainty factor would be used specifically to cover infants and children without validated intake data estimating consumption of BHT in this group.

Chapter 4 : Studies with curcumin

4. Studies with curcumin

4.1 Curcumin as a food additive

Tumeric is an essential ingredient of curry and is used extensively in Indian cuisine in curries and mustards. Curcumin (E100), (see figure 4.1) the major yellow pigment derived from the rhizome of *Curcuma longa* (Conney et al., 1991) is used as a colouring agent in foodstuffs such as cake mixes, cheese, pickles and fruit drinks (Schich et al., 1994). It is one of three phenolic compounds known as the curcuminoids that make up tumeric derived from the rhizome.

Curcumin is present in tumeric in varying quantities, depending on the preparation. Curcumin is present at levels of 1-5 % in powdered tumeric, whilst in the oleoresin (organic extract) it is present in much larger quantities (40-85%) (NCI, DCPC, 1996).

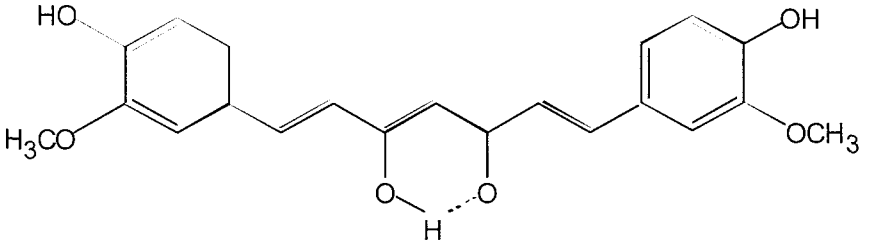


Figure 4.1 Chemical structure of curcumin

Curcumin was first evaluated by JECFA in 1974 when a temporary ADI of 0-0.1 mg/kg bw/day was established based on a NOAEL for tumeric with an assumed average concentration of 3% of curcumin present in the tumeric tested. This ADI was retained until 1995 when a new temporary ADI for curcumin of 0-1 mg/kg bw/day was established, based on a NOAEL of 220 mg/kg bw/day (derived from a carcinogenicity study in mice) to which a 200-fold factor was applied (WHO, 1995). The temporary ADI was extended at a recent meeting of the JECFA until 2003 pending the result of a reproductive study (WHO, 2000).

4.2 The metabolic fate of curcumin

4.2.1 Animal studies with curcumin

Curcumin was poorly absorbed (maximal blood concentration = 20 ng/ml), following gavage dosing of 1-5g/kg bw to rats (Wahlstrom & Blennow, 1978), and 65-85% of the administered dose was excreted in the faeces within three days. The most rapid rate of excretion was in the first 24 hours. A negligible amount of curcumin was detected in the urine. The biliary concentration of curcumin rose to 1 μg/ml

after 30 minutes and remained constant for the duration of the study (72 hours). Following i.v. administration of 1-5g/kg bw curcumin to rats, metabolism was rapid, with plasma concentrations of curcumin returning to zero after only 60 minutes. After 3 hours, 2.1% of curcumin detected in the bile was present as free curcumin, whilst 20% of the administered dose was present as conjugated curcumin. The results of this study suggest that curcumin undergoes extensive biliary excretion as conjugates which are eliminated in the faeces.

The rates and routes of elimination of curcumin following oral ingestion were confirmed by Holder et al., (1978). Following oral administration of [benzene ring-^{3}H] curcumin to rats, 89.4% of the dose was recovered in the faeces, and 6.3% in the urine within 72 hours. After i.p. administration these values were 73% and 11% respectively. As previously reported by Wahlstrom & Blennow (1978) the majority of elimination occurred within 24 hours, with no radioactivity being detected after 72 hours. Biliary excretion was found to be the major route of elimination. Following lower i.v. doses, 85% of the radioactivity was detected in the bile within 6 hours. Elimination of the i.v dose was rapid, with 75% of the radioactivity was detected in the bile within the first hour. Following i.p administration, the rate of excretion was slower, with 80% recovery within 8 hours. Biliary metabolites following the i.p. dose were identified as the glucuronides of tetrahydrocurcumin and hexahydrocurcumin (figure 4.2). Dihydroferulic acid was identified as a minor metabolite. Curcumin was considered to have undergone endogenous reduction, as it would not be in contact with gut microflora in this experiment. It was speculated by the authors that curcumin is likely to be a substrate for the a,ß-unsaturated ketone reductase with dihydroferulic acid produced via oxidative cleavage of the aromatic rings of curcumin metabolites. Alternatively it is possible that alkyl chain cleavage of the metabolites occurs resulting in dihydroferulic acid.

After 400 mg/kg bw oral dose to rats, nearly 40% of the dose was excreted in the faeces after a 5 day period (Ravindrath & Chandrasekhara, 1980). In contrast to previous studies, conjugated metabolites were detected in the urine. Elevated concentrations of the glucuronide and sulphate metabolite of curcumin were detected in the urine for up to 44 days post administration. A follow-on study investigated the tissue distribution of curcumin after gavage administration of 400mg curcumin (equivalent to 2-2.7g/kg bw). Following administration of the dose, 40% was excreted in the faeces over 5 days, suggesting that at least 60% of the dose was absorbed. Conjugates of curcumin were detected in the urine; and sustained levels of the glucuronide and sulphate were detected for up to 7 days post-dose. These results suggest curcumin may have undergone entero-hepatic re-circulation, or may be binding to plasma proteins. Very small amounts of curcumin were detected in the blood, kidney, gut, and liver after administration. However, 90% of the dose was found in the stomach and small intestine after 30 minutes, and the total recovery of curcumin in the caecum and large intestine was ~70% after 60 minutes. These results indicate that curcumin is being absorbed from the caecum and large intestine one hour post-dose;

and that curcumin is probably undergoing biotransformation prior to or during absorption as there was no corresponding increase in blood concentrations at around one hour.

This hypothesis was the subject of further investigation (Ravindrath and Chandrasekhara, 1981) using inverted sacs of jejunal tissue and radioactively labelled curcumin. Curcumin and an unidentified radioactive compound could be detected on the mucosal side, but only the unidentified compound could be detected in the mucosal tissue or in the serosal fluid. The results of this study suggest therefore, that curcumin undergoes biotransformation during its transport across the intestinal mucosa.

A subsequent excretion and tissue distribution study (Ravindrath and Chandrasekhara, 1982) substantiated the earlier findings. Following oral administration of [^{3}H] curcumin at doses of 0.05, 0.4 or 2g/ kg bw/day to rats' urinary excretion was found to be negligible. Most of the label was found in the faeces after 72 hours, but only 35% was present as curcumin, 65% of the label was assumed to be metabolic products of curcumin. At the lower doses, excretion was rapid, 80-90% excreted within 3 days, whilst at the higher dose excretion was found to be slower, 60% of the dose excreted after 12 days, suggesting enterohepatic re-circulation of biliary metabolites.

Piperine is a potent inhibitor of drug metabolism, in particular, hepatic and intestinal glucuronidation, and alters the disposition and bioavailability of a number of drugs, including propranolol and theophylline (Atal et al., 1985, Bano et al., 1991), and it increased the bioavailability of curcumin in rats (Shoba et al., 1998). Rats receiving 20 mg/kg piperine in addition to 2 g/kg curcumin had higher serum concentrations (mean of 1.55 μg/ml) compared to rats receiving curcumin alone (1.0 μg/ml). Tmax was delayed by concomitant administration of piperine, but AUC was not significantly increased (from 2.36-3.64 μg/h/ml), and the elimination half-life was reduced from 1.70 h to 1.05 hr. These data suggest that normal pathways of drug metabolism and glucuronidation are not important in curcumin elimination.

A recent study (Asai and Miyazawa, 2000) identified curcumin, demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC) in the plasma of male rats following gavage administration of 100 mg/kg bw curcumin. Treatment of samples with ß-glucuronidase confirmed that the conjugates of the curcuminoids were the major species present in the plasma post-dose. Interestingly, and in contradiction to earlier findings where curcumin was given i.v or i.p. (Holder et al, 1978 and Pan, 1999) neither THC nor its metabolite was detected post-dose in this study. This suggested that while THC and its glucuronide are important metabolites in the biliary system they may not be a major plasma component following oral dosing in rodents.

Curcumin was administered orally and i.p. to BALB/c female mice (Pan et al., 1999). There was rapid absorption following oral administration; curcumin could be detected in the plasma within 15 minutes,

and elimination was rapid, as curcumin could not be detected after 6 hours. Absorption and elimination were even more rapid after i.p. administration; curcumin was undetectable in the plasma after one hour. The Cmax was higher after i.p. administration compared to oral administration suggesting limited oral bioavailability. The intestine contained the most curcumin, with very small amounts present in the blood and brain one hour after an i.p. dose. Metabolite analysis supported the theory that curcumin and/or its metabolites were substrates for an endogenous reductase system. Curcumin, hexahydrocurumin (HHC), tetrahydrocurcumin (THC) and their corresponding glucuronide conjugates could be detected in plasma via HPLC analysis.

The experiments above highlight the importance of route of administration regarding curcumin metabolism. It would seem that following an oral dose, curcumin is hydrogenated whilst being absorbed from the gut. It is subject to extensive first pass metabolism in the liver and is excreted as conjugates. After i.p. or i.v. administration hydrogenation occurs in the tissue, and curcumin and its reduction products are absorbed into the adipose tissue. These compounds are slowly released and then undergo conjugation prior to excretion.

4.2.2 In vivo studies in humans

As part of the study conducted by Shoba et al., (1998) the effect of piperine (20 mg) on curcumin kinetics following a single oral dose of 2 g curcumin in humans was investigated. In the curcumin-only study serum levels of curcumin were low/undetected at most time points. A Cmax of 6 ng/ml, and an AUC of 4 ng/h/ml was reported. The concomitant administration of piperine (20 mg/kg) resulted in clearly measurable concentrations (180 ng/ml) an increased AUC of 80 ng/h/ml (although the difference between dosing regimens was not significant), an earlier Tmax, and an increased Cmax of curcumin, presumably due to increased absorption and reduced metabolism.

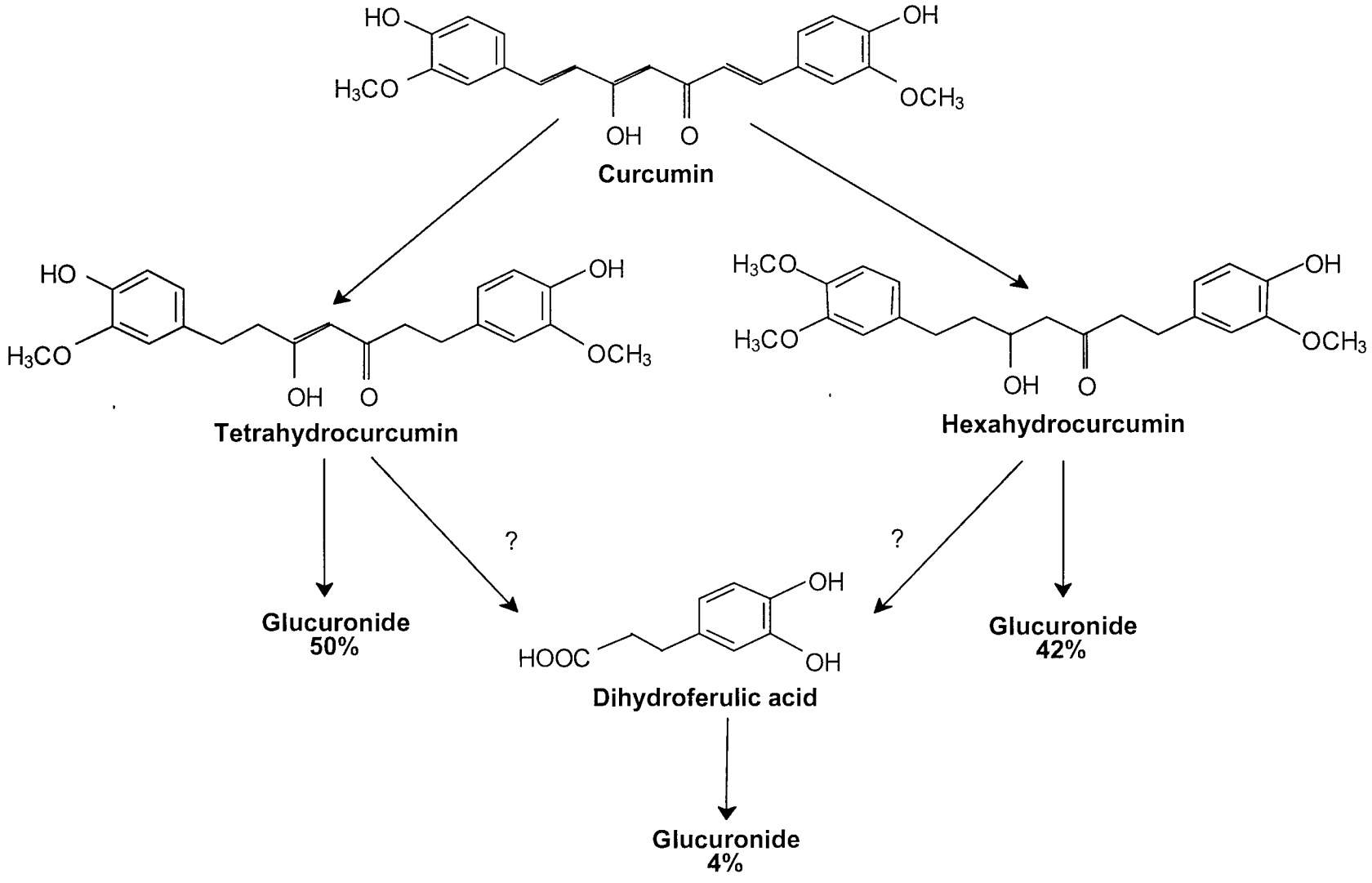


Figure 4.2 Biliary metabolites of curcumin (Holder et al., 1978)

4.3 Xenobiotic enzymes implicated in curcumin biotransformation

Curcumin was found to be a potent inhibitor of CYP1A1 and CYP1B1, a less potent inhibitor of CYP2B1 and CYP1B2 and a weak inhibitor of CYP2E1 using microsomal preparations from the livers of rats treated with phenobarbital (PB), ß-naphthoflavone (ß-NF), or pyrazole (Pyr) (Oetari et al., 1996). In addition, treatment of rats with 1% tumeric in the diet resulted in a significant decrease in B(a)P-induced CYP1A1 and CYP1A2 and PB-induced CYP2B1 in liver, lungs and stomach (Thapliyal & Maru, 2001). Curcumin was also found to be an inhibitor of glutathione-S-transferase (GST) using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate with a 20-fold lower inhibitory effect compared to the effect on CYP1A1/1A2. The selective inhibition of certain CYP450 isoenzymes, and the inhibition of GST may modulate the bioactivation of other chemicals and result in anti-carcinogenic, anti-mutagenic and cytoprotective effects; all of which are reported as effects of curcumin (Commandeur & Vermeulen, 1996).

GSH-curcumin conjugates were detected, and the effects of recombinant human glutathione-S-transferase (GSTP1-1) on the reaction kinetics were explored by Awasthi et al., (2000). Products of curcumin-GSH interaction identified using mass spectroscopy included mono and di-glutathionyl adducts of curcumin as well as cyclic rearrangement products of GSH adducts, feruloylmethylketone (FMK) and feruloylaldehyde (FAL). The presence of GSTP1-1 accelerated the initial rate of GSH-mediated metabolism of curcumin, but was also shown to catalyse the reverse "deglutathione" reaction. GSTP1-1 is the major GSH isoenzyme in the small intestine (Aswasthi et al., 2000 and Pacifici et al., 2001), it would be predicted therefore, that a significant fraction of curcumin could be conjugated with GSH. This process is a reversible one, particularly in conditions of low GSH concentrations for example in the bile. The deconjugation could therefore mediate a carrier function in biliary transport.

4.4 Biochemical effects of curcumin

Rao et al., (1970) reported that the levels of cholesterol in serum and liver in rats fed cholesterol and curcumin were ~1/2-1/3 of those in the control group (i.e. rats fed cholesterol alone). Curcumin increased the fecal excretion of bile acids and cholesterol in both hypercholesteremic and normal rats, suggesting an increase in bile formation, which would explain the reduction in tissue cholesterol upon curcumin feeding. The effective level was estimated to be <0.1% of curcumin in the diet.

Tetrahydrocurcumin caused a dose-dependent inhibition of the oxidation of LDL (low-density lipoprotein) cholesterol in rabbits (Naito et al., 2002). Although THC could not be detected in the serum or the liver, THC was detected in samples treated with ß-glucuronidase and sulphatase, suggesting that THC is present as a conjugate with glucuronic acid or sulphate.

Studies with human serum albumin (HSA) and curcumin revealed two binding sites for curcumin, one of low affinity and one high affinity site (Reddy et al., 1999). The equilibrium constant for the curcumin-HSA interaction was independent of temperature, suggesting hydrophobic interactions. It is possible that curcumin binds in the hydrophobic pockets of HSA, and it has structural similarities with caffeic acid which binds via this site. Due to resemblance to fatty acids, it is also possible that curcumin binds via the fatty acid sites on HSA.

4.5 Pharmacological activity of curcumin

4.5.1 Anticarcinogenicity of curcumin

Curcumin has been shown to inhibit tumour initiation in animals by benzo(a)pyrene, 7,12 dimethylbenz(a)anthracene and dimetylbenz[a]anthracene (DMBA) (Huang et al., 1988, Soudamini and Kuttan, 1989, Huang et al., 1992, Azuine & Bhide, 1992). It also inhibits tumour promotion in animals by phorbol esters and 3-MC (3-methyl cholanthracene) (Soudamini and Kuttan, 1989, Conney et al., 1991, Nagabhushan & Bhide, 1992). Curcumin was also found to be highly cytotoxic to normal human lymphocytes, to human leukaemic cells and to Dalton's lymphoma cells (Kuttan et al., 1985). The effective concentrations were 1-4µg/ml.

As previously discussed, curcumin has been shown to reduce the tumorigenic potential of many compounds such as BaP (benzo(a)pyrene), AOM (azomethane) and ENNG (N-ethyl-N'-nitro-N-nitroguanidine) administered to mice and rats (Huang et al., 1988, 1994, Rao et al., 1993 and 1995). It is postulated that curcumin may be exerting these effects through inhibition of COX or LOX enzymes. Metabolic products of lipoxygenase (LOX), including 8, 12 and 15-HETE (hydroxyeicosatetranoic acid) and products of cycloxygenase (COX), including PGE_2, $PGFa_2$, keto- $PGFa_2$, and TXB_2 are reduced in tumour sites by curcumin (Huang et al., 1988, 1994, Rao et al., 1993 and 1995).

Tetrahydroxycurucmin (THC) was also the only compound (in a study comparing curcumin (C), demethoxycurcumin (DMC), bis-demethoxycurcumin (BDMC) and tetrahydrocurcumin (THC)) not to inhibit TPA (12-O-tetradecanoylphorbol-13-acetate)-induced transformation of JB6 (P+) cells (Huang et al., 1995). In mice pre-treated with DMBA prior to TPA only curcumin and demethoxycurcumin inhibited tumourigenesis in a dose-dependent manner. The results of this study suggest that the double carbon-carbon bond and both methoxy groups are necessary for anti-tumorigenic activity (refer to figure 4.2).

Curcumin has also been shown to be protective against *in vivo* chromosomal damage induced by ?-radiation (Abraham et al., 1993). The mouse bone marrow micronucleus test showed that curcumin (5,

10, 20 mg/kg bw) was able to significantly reduce the frequencies of micronucleated polychromatic erythrocytes. Administration 2 hours prior to or post exposure did not alter the protective effect observed and effects were observed up to 48 hours after exposure to radiation.

In addition to its anti-mutagenic activity *in vitro*, and anti-cancer effect in animals and cell culture, curcumin has also been shown to decrease the levels of B(a)P-induced mutagens in the urine of rats (Polasa et al., 1991). Rats were fed with curcumin for up three months prior to B(a)P exposure via the i.p route, followed by urine collection for 24 hours. Similar results were obtained using ^{32}P-postlabelling analysis, where tumeric (0.2% or 1% tumeric in the diet followed by oral dose of B(a)P) was found to reduce levels of B(a)P-DNA adducts in the liver, lung and forestomach of Swiss mice in comparison to animals fed a control diet prior to B(a)P exposure (Thapliyal et al., 2002).

4.5.2 Curcumin affects arachadonic acid (AA) metabolism and has anti-inflammatory activity.

Several studies have shown that curcumin is an effective anti-inflammatory agent. Using the carrageenan-induced rat paw oedema model, curcumin has been found to be as effective as phenylbutazone (Srimal & Dhawan, 1973, Yegnanarayan et al., 1976, Rao et al., 1982, Mukhopadhyay et al., 1982) and was as effective as phenylbutazone in the treatment of arthritis in humans (Deodar & Srimal, 1980). Sodium curcuminate, 4-hydroxy cinnamoyl methane and tetrahydrocurcumin were more potent inhibitors of inflammation than curcumin itself (Ghatak & Basu, 1972, Rao et al., 1982 and Mukhopadhyay et al., 1982). In addition, curcumin has been found to be as effective as cortisone acetate in reducing inflammatory swelling using the Freud's adjuvant induced arthritis model in rats. Curcumin reduced inflammation in both the early and late response.

Huang et al (1991 and 1995)., showed that curcumin (and metabolites of curcumin) reduced ear oedema in female CD1 mice treated with TPA (12-O-tetradecanoylphorbol-13-acetate) or AA (arachadonic acid). Curcumin inhibited the formation of 5-HETE and 8-HETE (metabolic products of AA) in a dose-dependent way. A later study (Began et al., 1998) demonstrated that curcumin competitively inhibits lipoxygenase-1 (LOX-1).

4.5.3 Antimutagenicity of curcumin

Nagabhusan & Bhide (1986) tested curcumin for mutagenicity in *Salmonella typhimurium* strains with and without metabolic activation. None of these were mutagenic using the Ames test. Chillies and their principal alkaloid capsaicin, were mutagenic in TA98 using S9; however curcumin was able to affect dose-dependent decreases in mutagenicity of chilli extract and capsaicin. In a similar experiment (Nagabhusan & Bhide, 1987), curcumin inhibited the mutagenic action of bidi, cigarette smoke

condensates, tobacco and masheri extracts in the TA98 assay, in the presence of induced S9.
Curcumin was found to be anti-mutagenic in the Ames test using BaP or DMBA as substrates (Nagabhushan and Bhide, 1987). Feeding 1% tumeric in the diet decreased the incidence of chemically-induced stomach papillomas as well as spontaneous mammary tumour incidence in C3H Jax virgin mice.

Tumeric was found to be anti-genotoxic in a dose-dependent fashion, using the SMART (somatic mutation and recombination test) (el Hamss et al., 1999).

4.5.4 Curcumin as an antioxidant

The antioxidant properties of curcumin are predictable upon consideration of its chemical structure, and its activity as an antioxidant is well documented in the literature (Kunchady & Rao, 1990; Schaich et al., 1994; Osawa et al., 1994 and 1995). Curcumin acts as a hydroxyl radical scavenger *in vitro* at high concentrations (>0.6 μM). In the same *in vitro* system, curcumin was found to be a highly efficient superoxide radical scavenger.

Many studies have focused on the structure-activity of curcumin in relation to its antioxidant activity. Sharma (1976) investigated the ability of curcumin, demethylated derivatives (bis-3, 4-dihydroxycinnamoylmethane and caffeic acid) and methylated derivatives (3, 4, 5-trimethoxycinnamic acid) to inhibit lipid peroxidation *in vitro*. The demethylated derivatives were most potent, whilst methylation abolished the activity. It was also concluded that the hydroxyl group in the benzene ring must be in the para-position. Three curcuminoids (curcumin, demethoxycurcumin and bisdemethylcurcumin) were able to inhibit iron stimulated lipid peroxidation in rat brain homogenate and rat liver microsomes (Rao & Rao, 1994). All the curcuminoids were shown to be equally active, and more potent than a-tocopherol which suggested that the methoxy and phenolic groups had little effect on activity. All three species can bind iron, and it was hypothesised that the chelation of iron inhibited iron-catalysed lipid peroxidation. Subsequent investigation of the anti-lipid peroxidation capabilities of the curcuminoids demonstrated that curcumin was the most potent inhibitor of these three curcuminoids (Osawa et al., 1995). However the hydrogenation products of curcumin and demethoxycurcumin were more potent inhibitors than curcumin itself, suggesting that the antioxidative actions of curcumin are mediated via these two species. Subsequent work has shown that the ßdiketone moiety is involved because analogues of curcumin without a phenolic group still displayed redox activity (Tønnesen & Greenhill, 1992)

4.6 Toxicological effects of curcumin

4.6.1 Acute and sub-acute toxicity of curcumin

The LD_{50} of curcumin in the rat and the mouse is >10 g/kg bw/day (Lilja et al., 1983 cited in WHO, 1986).

Tumeric containing ~2.5% curcumin and an alcoholic extract of tumeric (dose not indicated in publication) was fed to male and female Wistar rats, male guinea pigs and male adult monkeys (*Macacus sp.*) for 3 weeks (Shankar et al., 1980). All animals showed normal increases in bodyweight and no mortality was observed in any group. The gross appearance of liver, heart and kidney was normal, with no significant increase in any organ weight. Histological examinations did not reveal any pathological manifestations.

4.6.2 Sub-chronic toxicity of curcumin

Groups of 10 male and 10 female F344/N rats were fed diets containing 0, 1000, 5000, 10000, 25000, or 50000 ppm tumeric oleoresin for 13 weeks (50, 250, 480, 1300, 2600 mg/kg bw to the males and 60, 300, 550, 1450 or 2800 mg/kg to the females (NTP, 1993). All rats survived until the end of the study. The mean body weight of males receiving 50000 ppm was 5% lower than that of the controls. Feed consumption was unchanged. The absolute and relative liver weights of female rats and the relative liver weight of male rats receiving 5000, 10000, 25000 and 50000 ppm were significantly greater than the controls. There were no biologically significant differences in haematology, clinical chemistry or urinalysis parameters. Hyperplasia of the cecum and colon of male and female rats was observed in the high dose group.

Very similar results were obtained in a study in male and female mice, (male doses were the equivalent of 150, 750, 1700, 3850 or 7700 mg/kg and female doses were the equivalent of 200, 1000, 1800, 4700, 9300 mg/kg). Absolute and relative liver weights of male mice that received 5000ppm and male and female mice that received 10000, 25000 and 50000 ppm were significantly greater than those of controls. As before there were no biologically significant changes in haematological, clinical chemical or urinary parameters. There were no chemical-related histopathological lesions

4.6.3 Carcinogenicity studies with curcumin

The majority of these studies were conducted the National Institute of Health (NIH), USA, as part of the National Toxicology Program.

B6C3F_1 mice were fed diets containing 0, 220/320, 1520/1620, 6000/8400 mg (male/female doses) tumeric oleoresin mg/kg diet in a chronic study. Survival rates were not affected by dietary tumeric, but bodyweight were significantly reduced in the two highest dose groups. At 15 months absolute and relative liver weights were elevated in the two highest dose groups, but had returned to normal by the end of the study (103 weeks). A small non-treatment related increase in neoplasms was observed. The NOAEL in this study was 220 mg/kg bw/day based on the dose in male mice associated with reduced bodyweight and increased absolute and relative liver weights (NTP, 1993).

F344/N rats were fed diets providing 0, 80/90, 460/540, 2000/2400 mg tumeric oleoresin/kg bw/day in a chronic study (male/female doses). Survival rate was unaffected, and as with the mouse study, absolute and relative liver weights were significantly elevated in females in the two highest dose groups. Non-neoplastic lesions recorded in the gastro-intestinal tract of the high dose male group included ulcers, hyperplasia and hyperkeratosis of the forestomach, hyperplasia and inflammation of the caecum and colon, and sinus ectasia of the mesenteric lymph node. Similar effects were observed in the high dose female group. Neoplasms were not observed in any of the male dose groups; clitoral gland adenomas and carcinomas were reported in the female high dose group, but were considered unrelated to dose, or hyperplastic observations. The NOAEL in this study was 460mg/kg bw/day based on the dose in male rats (NTP, 1993).

4.6.4 Reproductive studies with curcumin

Bhavanishankar and Murthy (1987) performed a 2-generation multi-litter study in Wistar rats fed either tumeric (2.5% curcumin) 500mg/kg bw/day or 60mg/kg bw/day of an alcoholic extract of tumeric (equivalent to 500 mg/kg bw/day tumeric). The lactation index of the second litter of the F_1 generation was increased significantly compared to controls, however this is unlikely to be biologically significant. Some changes in the litter weights of the F_0 and F_1 generations were reported, however, these were also not considered to biologically significant. No histological abnormalities were reported, and curcumin was considered safe at the doses administered.

4.6.5 Observations in humans receiving curcumin

Generally curcumin has been found to be well-tolerated in studies in which volunteers have been given curcumin (Shoba et al., 1998). In one report contact dermatitis was reported in a mill-worker in India exposed to tumeric (Goh and Ng, 1987).

Eighteen patients with rheumatoid arthritis received either 1200 mg/day curcumin or 300 mg phenylbutazone (Deodar et al., 1980). The anti-rheumatic activity of curcumin was found to be equal to

that of the phenylbutazone and no side effects related to intake of curcumin were reported.

Curcumin (0.5g/day) administered to 10 human volunteers significantly lowered serum lipid peroxides and cholesterol (Soni & Kuttan, 1992). Treatment also increased the amount of plasma HDL-cholesterol and there was a non-significant decrease in serum triglycerides. No significant side effects were reported, although a slight lowering of blood pressure was observed.

4.7 The most recent determination of a NOAEL for curcumin

The outcomes of the NTP study in mice (see earlier in this chapter), (NTP 1993) was used to derive the NOAEL for curcumin of 220 mg/kg bw/day. The JECFA applied a safety factor of 200-fold to this dose (WHO 2001). Overall a temporary ADI of 0-1 mg/kg bw/day was allocated to curcumin, pending the outcomes of a reproductive/multi-generation study.

4.8 Clinical studies with curcumin

4.8.1 Clinical procedure

The materials and methods used in the clinical studies with curcumin are given in chapter 2 (materials and methods).

4.8.2 Dose selection and safety

As discussed in the introduction, one of the aims of this project was to compare human and animals' kinetic parameters derived from studies where curcumin was administered at the no-effect level (the ADI for humans and the NOAEL for animals). These doses are 1.0 mg/kg bw/day and 220 mg/kg bw/day respectively. A secondary aim of the project was to investigate linearity of the kinetic parameters. Doses equivalent to 10 x ADI and 0.1 x NOAEL were administered to humans and animals respectively. In order to gain ethical permission for these studies, the acute reference dose (ArfD) was estimated for curcumin. In cases of acute exposures to hazardous chemical, the ArfD is estimated based on short-term animal toxicology studies. The ArfD is an estimate of a safe acute exposure, and therefore is normally calculated from no-effect level demonstrated in acute animal toxicity testing. Since our human volunteers in the high dose studies were effectively being exposed to the food additives in much larger quantities, a review was made of all available acute toxicity data. This review was used to assess the margin of safety between the ARfD in the animal database and the 10 x ADI dose. There are very few acute or sub-acute toxicity data for curcumin. Based on the toxicity data presented in earlier in this chapter, the NOAEL for short-term effects for BHT would be the same as for chronic effects, i.e. 220 mg/kg bw/day. Comparing

this with the intended single dose for the 10 x ADI studies (700 mg/person) gives a margin of safety of 22-fold.

4.9 Results

4.9.1 Studies in which human volunteers received curcumin at the ADI

A total of 8 volunteers were given curcumin as a single oral dose at the ADI (1.0 mg/kg) (see chapter 2 for protocol and table 4.1 for details of these volunteers). The mean age of the volunteers was 22 years, (24 for the males and for the 20 females). The mean weight of the male volunteers was 77 kg and for the female volunteers was 58 kg.

	Age	Weight	Ethnicity
M12	24	65.8	South Asian*
M13	25	72.7	Caucasian
M14	29	87.5	Caucasian
M15	19	83	Caucasian
Male Mean	**24**	**77**	**N/C**
F10	19	61.4	Caucasian
F11	19	57.1	Caucasian
F12	21	60.2	Caucasian
F13	22	53.7	Caucasian
Female Mean	**20**	**58**	**N/C**
Overall Mean	**22**	**68**	**N/C**

*The South Asian subject was originally from the Indian subcontinent.

Table 4.1 Details of volunteers who received curcumin at the ADI.

4.9.1.1 Plasma analyses

Table 4.2 shows the concentrations of curcumin, expressed as pg/ml detected at each time point for each individual. Values given are following baseline subtraction; baseline values quantified as curcumin are shown in parentheses.

Concentration of curcumin in plasma samples, pg/ml

Time	Male Data				Female Data			
(minutes)	M12	M13	M14	M15	F10	F11	F12	F13
0	0.0	0.0	0 (156.7)	0 (173.5)	0 (7.9)	0 (10.3)	0 (8.2)	0 (20.6)
10		0.0	0.0	23.7				
20		0.0	0.0	0.0				
30	0.0	0.0	0.0		0.0	0.0	0.0	0.0
40		0.0	30.6	0.0				
60	0.0	0.0	0.0	0.0	0.0	0.0	0.7	0.0
90	0.0	0.0	0.0	0.0	0.0	0.0	13.1*	0.0
120	0.0	0.0	0.0	0.0	22.4*	0.0	0.9	0.0
150	0.0	0.0	0.0		0.0	0.4	0.0	0.0
180	0.0	0.0	381.2		0.0	7.1	6.1	0.0
240	0.0	0.0	8.7	0.0	48.8*	0.0	0.0	0.0
300	0.0	0.0	44.1	0.0	0.0	0.0	0.0	0.0
360	0.0	0.0			0.0	0.5	15.3	0.0
420	0.0	0.0			0.0	0.0	18.4	0.0

Values given are following baseline subtraction. Baseline values quantified as curcumin are shown in parentheses.

*Indicates that one of the duplicate samples contained curcumin at a similar concentration to the pre-dose level.

Table 4.2 Plasma concentration of curcumin from studies in which volunteers received curcumin at the ADI.

The plasma concentrations given in table 4.2 show that curcumin concentrations at most time points were below the limit of quantification for the method of analysis employed, despite a limit of detection of ~10-50 pg/ml. The sample at 180 minutes in subject M14 showed a clear curcumin peak with good duplicate analysis. The data also show that in some cases (M15, F11 and F13) an HPLC peak corresponding to curcumin was present in baseline samples at concentrations exceeding those observed post-dose. Due to the fact that curcumin was not detected at concentrations above baseline in the majority of sample sets, concentration-time profiles were obtained and so no further pharmacokinetic analysis was undertaken. Comparison of observed values is of limited use, since in all subjects any estimate of AUC or CL/F would be reliant on a maximum of three data points that were above the limit of detection.

4.9.1.2 The stability of curcumin in human plasma

Following the observation that curcumin could not be detected in plasma samples from volunteers who received the ADI it was hypothesised that curcumin might be unstable in plasma. All samples were analysed within one month of the study taking place, and so a one month stability study was conducted. A pool of human plasma was spiked with curcumin; three aliquots were analysed on the day that the stability study was set-up, the remaining aliquots were stored at –20°C pending analysis. The results of the stability study are shown in figure 4.3.

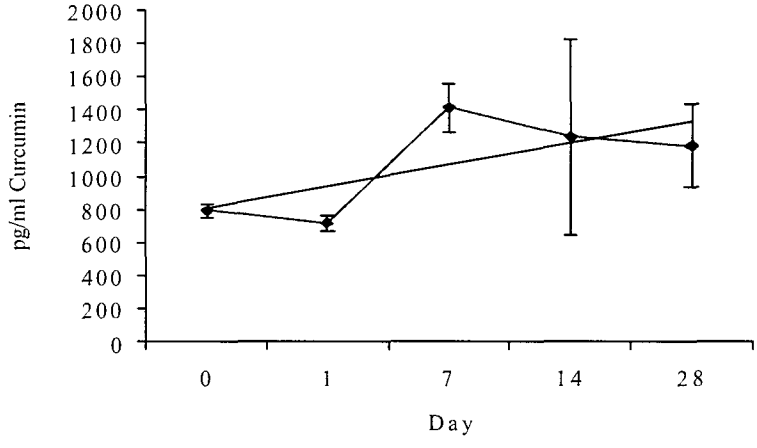


Figure 4.3 Results of the one-month stability test (at –20°C) with curcumin in human plasma

Despite larger standard errors in the later analyses, the data in figure 4.3 show that curcumin was stable for the duration of the stability study. The results from the ADI studies were therefore considered to an accurate reflection of the true situation; that curcumin was not present in the systemic circulation following an oral bolus dose of the ADI in human subjects.

4.9.2 Studies in which human volunteers received curcumin at 10 x ADI.

Because of the very low concentrations of curcumin detected in the plasma of subjects receiving the ADI, a total of 4 volunteers were given curcumin at 10 x ADI (10.0 mg/kg) (see table 4.3 for details of these volunteers). It was hypothesised that the higher dose might result in the detection of curcumin at adequate concentrations to allow the derivation of pharmacokinetic parameters for comparison with animal data. The mean age of the volunteers was 24 years old, (23 for the males and for the 25 females). The mean weight of the male volunteers was 71 kg and for the female volunteers was 60 kg.

	Age	Weight	Ethnicity
M16	23	76.5	Caucasian
M17	23	65.2	Caucasian
Male Mean	**23**	**71**	**N/C**
F14	24	54	Caucasian
F15	25	65.7	Caucasian
Female Mean	**25**	**60**	**N/C**
Overall Mean	**24**	**65**	**N/C**

Table 4.3 Details of volunteers who received curcumin at 10 x ADI.

4.9.2.1 Plasma analyses

Table 4.4 shows the concentrations of curcumin, expressed as pg/ml detected at each time point for each individual. In all four subjects an HPLC peak was present in the pre-dose (baseline) plasma samples with the same retention time as curcumin. Values given are following baseline subtraction; baseline values quantified as curcumin are shown in parentheses.

Concentration of curcumin in plasma samples, pg/ml

Time	Male Data		Female Data	
(minutes)	M16	M17	F14	F15
0	0 (101.8)	0 (87.0)	0 (117.5)	0 (94.4)
20	25.6	24.8	82.4	72.3
40	48.2	28.4	81.0	33.1
60	41.8	35.3	62.4	43.9
90	24.8	14.6	59.5	-
120	24.9	26.3	-	-
150	21.2	21.4	79.9	25.1
180	33.2	30.7	50.7	78.8
240	27.9	10.7	53.2	58.5
300	8.9	7.1	0.0	0.0
360	3.6	12.9	0.0	0.0
420	1.7	0.0	0.0	0.0

Values given are following baseline subtraction. Baseline values quantified as curcumin are shown in parentheses.

- = no sample

0.0 = concentration less than pre-dose concentration

Table 4.4 Plasma concentrations of curcumin from studies in which volunteers received curcumin at 10 x ADI

The plasma concentrations in table 4.4 show that more curcumin was detected in plasma samples from volunteers receiving curcumin at 10 x ADI than from volunteers receiving the ADI. Again there were significant background concentrations (at approximately the limit of quantification, 100 pg/ml) of a compound that was integrated as curcumin. This peak interferred with quantification of low concentrations of curcumin in both the ADI and 10 x ADI studies. Attempts were made to separate the interfering peak from the curcumin peak by altering the chromatographic method by changing the relative amount of acetic acid present in the mobile phase (see chapter 2). These efforts were not successful but it was considered that should curcumin be present in the plasma samples from the ADI or 10 x ADI study in any significant amounts, the interfering peak was not so large that it would preclude quantification. The small concentrations of curcumin present in the samples were the underlying limit to the derivation of pharmacokinetic parameters rather than the interfering peak. Individual time-concentration profiles were obtained, and they are represented graphically in figure 4.4.

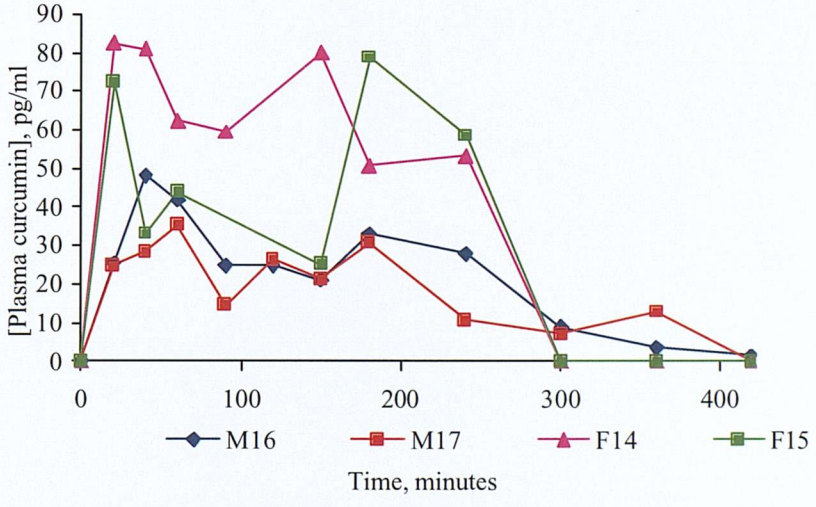


Figure 4.4 Concentration time profiles for curcumin for male and female volunteers after dosing at 10 x the ADI.

The concentration profiles in figure 4 do not show any clear trend regarding human exposure to curcumin following an oral dose, for example there is no constant Tmax. It is possible that the female volunteers have higher Cmax values compared to the male volunteers, and that the elimination appears to be more rapid compared to the male volunteers. No conclusions are possible on such limited data in 2 subjects of each sex only.

These initial results from a total of four individuals were not particularly promising in terms of generating PK parameters on which to perform inter-individual and inter-species analyses. Therefore it was decided to cease with any further studies using curcumin and concentrate on the other substrates.

4.9.3 Pharmacokinetic parameters calculated using the data from the 10 x ADI studies

Whilst concentration time profiles were obtained from the 10 x ADI data, the shape of the profiles (see figure 4.4) meant that the data could not be fitted using the mathematical model WinNonlin™ (as outlined in chapter 2). Instead, observed pharmacokinetic parameters, Cmax, Tmax, AUC (calculated using the trapezoid formula) and CL/F (calculated as dose/AUC) are reported in table 4.5. These values are calculated from the baseline-subtracted data, as shown in table 4.5.

	Cmax (pg/ml)	Tmax (minutes)	AUC (obs) (pg x ml/min)	CL/F (ml/min)
M15	48	40	8618	1160387
M16	35	60	7037	1421130
Male Mean	**42**	**50**	**7827**	**1290758**
F15	82	20	14480	690614
F16	79	180	11013	908041
Female mean	**81**	**100**	**12746**	**799327**
Overall Mean	**61**	**75**	**10287**	**1045043**
Overall SD	23	N/C	3238	315772
Overall CoV	**38**	**N/C**	**31**	**30**

N/C not calculated. Values in **bold** are the mean data and overall CoV used in later comparisons.
NB very little confidence can be placed in these reported values for AUC and CL/F, since the individual profiles (see figure 4.4) do not have clear absorption, distribution, elimination profiles.

Table 4.5 Pharmacokinetic parameters for curcumin generated from studies in which volunteers received curcumin at 10 x ADI.

Due to the limited number of subjects (n=2 for each sex) it is difficult to interpret these pharmacokinetic data. It seems that female volunteers had a larger internal exposure than male volunteers, reflected in larger Cmax, AUC values and lower CL/F values. Further studies would be needed to validate this hypothesis, but since the results of these four studies were not particularly promising, time and effort was instead dedicated to studies with the other substrates. The magnitude of the CL/F values reported in table 4.5 suggest that the bioavailability of curcumin is negligible, so that CL/F is about one order of magnitude greater than cardiac output. This value that can only be explained by massive and almost complete first-pass metabolism giving a very low value of F (bioavailability).

Since pharmacokinetic parameter values were not obtained from the studies in which volunteers received curcumin at the ADI, comparison data from the two different doses was not possible. However, based on Cmax values from the 10 x ADI studies, and assuming linearity, one could expect Cmax values in the 3-8

pg/ml range, which would have been below the limit of detection (~10-50 pg/ml) as well as the limit of quantification (100 pg/ml). The only possible way to determine curcumin in plasma samples following an oral bolus dose equivalent to the ADI would be to use an analytical method that offered a much lower limit of quantification.

4.9.4 Analysis of saliva samples from the curcumin studies

Salivary concentrations were not determined because the plasma concentrations of curcumin were so low. Since the saliva concentration of curcumin would reflect unbound compound and assuming some protein binding of curcumin, it was predicted that the levels of curcumin in saliva would be approximately one to two orders of magnitude (i.e. 0.1-1.0 pg/ml curcumin) below the limit of detection. Since analysis of the saliva samples was not undertaken, the plasma-protein binding efficiency was not determined for curcumin.

4.9.5 Calculating the chemical-specific inter-individual toxicokinetic factors for curcumin

The default uncertainty factory, allowing for inter-individual variability in toxicokinetics is 3.16. The adequacy of the default factor for human variability and the factors necessary to cover different percentages of the population were estimated as described for BHT in chapter 3. The results, using parameters derived from analyses of plasma (after dosing at 10xADI) are shown in table 4.6

	Cmax (pg/ml)	AUC (obs) (pg x ml/min)	CL/F (ml/min)
Overall Mean	**61**	**10287**	**1045043**
Overall SD	23	3238	315772
Overall CoV	**38**	**31**	**30**
95th Percentile	1.83	1.65	1.62
97.5th Percentile	2.1	1.81	1.78
99th Percentile	2.35	2.02	1.98
% Covered by 3.16	**>99.9**	**>99.9**	**>99.9**

Table 4.6 Calculating inter-individual toxicokinetic factors for curcumin using plasma kinetic data generated from the 10 x ADI studies.

It must be emphasised that this analysis is based on very small numbers of subjects, and has been given as an indication only. The results in table 4.6 suggest that based on data where individual received curcumin at a dose equivalent to 10 x ADI, the inter-individual default of 3.16-fold allowing for inter-individual variation is probably adequate in the case of curcumin. In terms of acute exposure, it seems that the

default 3.16-fold factor would be adequate to protect a significant proportion of the population. However it is difficult to place much confidence in this assertion, because the plasma concentration time profiles were not regular in their shape (i.e. there was no clearly defined absorption, distribution or elimination) the AUC and CL/F values may not be truly representative of the true chronic exposure situations.

4.9.6 Studies in which mice were dosed with curcumin at the NOAEL

Groups of 5 B6C3F1 mice (male and female) were used per time-point and received curcumin (220 mg/kg bw) by gavage as a suspension in carboxymethylcellulose. Dr K. Walton conducted these studies and sample analyses. Table 4.7 is a summary of the concentrations measured at each time point in each rat.

Curcumin was detected in plasma of mice given curcumin as a bolus dose at the NOAEL (220 mg/kg bw). Unlike the human data, curcumin was quantified at the majority of time points, at levels above the limit of quantification. Interestingly, a significant sex difference is observed between 90 and 420 minutes, with female mice having higher concentrations of curcumin present. This seems to be echoed in the human data from the 10 x ADI studies (table 4.5) where females have higher Cmax and AUC values, and smaller CL/F values. Concentration time profiles were obtained from the NOAEL data (see figure 4.5).

Mean curcumin concentration in plasma of mice, ng/ml

Time (minutes)	Male (n=5)	SD	Female (n=5)	SD	P value (M vs. F)
0	N/C	N/C	N/C	N/C	N/C
15	0.99	0.57	2.53	2.14	0.2
30	1.29	0.79	5.82	8.73	0.3
45*	1.14	1.04	4.54	5.25	0.2
60	1.05	1.28	5.93	5.79	0.1
90	0.46	0.22	2.67	1.13	0.003
120	0.41	0.38	1.66	1.14	0.05
240	0.34	0.51	1.69	0.89	0.02
420	0.46	0.53	2.10	1.49	0.05
660*	0.01	0.03	0.02	0.04	0.8
1440*	0.02	0.05	0.11	0.11	0.1

*These samples were taken in a study subsequent to the initial study which originally had a final time point at 420 minutes.

Table 4.7 Plasma concentrations of curcumin from studies in which mice were dosed with the NOAEL.

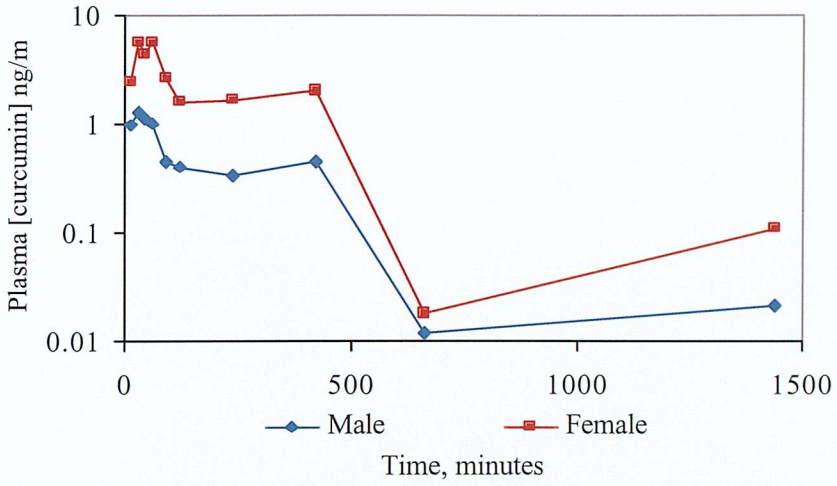


Figure 4.5 Concentration time profiles for curcumin generated from mean male and mean female mouse data after dosing at the NOAL (220 mg/kg bw).

Figure 4.5 shows that curcumin is rapidly absorbed and that there are two distinct phases of elimination. The concentration of curcumin in both sexes decreased rapidly between 60 and 420 minutes. However after the initial rapid clearance of curcumin, presumably dependent on the action of CYP 450 enzymes, there is evidence, at the 420 minute timepoint that re-circulation of curcumin, possibly via entero-hepatic circulation of curcumin in the bile may be occurring. (The later increase in curcumin concentration, at 1440 minutes is assumed to be due to coprophagy.)

Initially the studies were only extended to 420 minutes. However, because of the upwards trend of curcumin between 240 and 420 minutes (table 4.7), a further 30 mice (5/time point for three time points, 45, 660 and 1440 minutes, and both sexes) were dosed with curcumin (equivalent to the NOAEL) in a separate study. It is presumed that the resulting "dip" in plasma concentrations from female mice at 45 minutes, is the result of some experimental inconsistency, possibly due to different litters of mice, or the different dosing solution.

4.9.7 Pharmacokinetic parameters calculated using the data from the NOAEL studies

Whilst concentration time profiles were obtained from the NOAEL, the shape of the profiles (see figure 4.5) meant that the data could not be fitted using the mathematical model WinNonlin™ (as outlined in chapter 2). Instead, as with the human 10 x ADI data, observed pharmacokinetic parameters, Cmax, Tmax, AUC (calculated using the trapezoid formula) and CL/F (calculated as dose/AUC) are reported in table 4.8 AUC was calculated including time-points up to 420 minutes, so that the data could be compared with the human 10 x ADI data.

	Cmax (ng/ml)	Tmax (minutes)	AUC (obs to 420 minutes) (ng x min/ml)	CL/F ml/min
Male Mean	1.29	30	212	1036087
Female Mean	5.92	60	974	225931
Overall Mean	**3.61**	**N/C**	**593**	**631009**

Table 4.8 Pharmacokinetic parameters generated from studies in which mice were dosed with the NOAEL.

As indicated in the concentration time profiles (see figure 4.5), female mice had greater internal exposure following the NOAEL dose than did male mice. Cmax and AUC values were higher in female mice, and CL/F was lower. The concentration-time profiles for both male and female mice show that levels of curcumin increased after 420 minutes, means that the observed AUC values in table 4.8 will underestimate the AUC to infinity. Correspondingly the CL/F values that are reported in table 4.8 would be greater than the true CL/F.

4.9.8 Calculating the chemical-specific inter-species toxicokinetic factor for curcumin

Limited data were available for curcumin in humans and this analysis was undertaken for completeness by comparing human kinetic parameters from the 10 x ADI studies with those from the NOAL mouse studies. The kinetic parameters (excluding CL/F, which is independent of dose) were corrected for dose using the equation given in chapter 2. Table 4.9 shows the resulting chemical specific inter-species factor.

	Cmax (ng/ml)	Tmax (minutes)	AUC (obs) (ng x min/ml)	CL/F (ml/min)
10 x ADI Mean	0.061	75	10.3	1045043
NOAEL Mean	3.605	45	593	631009
Ratio NOAEL/10x ADI	**0.37**	**N/C**	**0.38**	**0.60**

Table 4.9 Chemical specific inter-species factors for curcumin, comparing human data from 10 x ADI studies with animal data from NOAEL studies.

The inter-species factors calculated in table 4.9 are all much lower than the default value of 4.0-fold, despite the fact that the human data is not from the ADI studies, but from the 10 x ADI studies. Due to the limited human database it is difficult to make any general conclusions regarding inter-species differences in the kinetics of curcumin (see later in chapter for discussion).

4.9.9 Studies in which mice were dosed with curcumin at 10 x ADI

Since studies had been conducted in humans at 10 x ADI, a dose-equivalence study was conducted in mice (receiving 10 mg/kg bw, equal to 10 x ADI, rather than 22mg/kg bw, equal to 0.1 x NOAEL), using the same experimental protocol as previously described. Curcumin was not detected in the majority of time points, for the majority of groups of animals. Small amounts of a compound integrated as curcumin were present in the pre-dose samples (100-800 pg/ml) and these precluded measurement of curcumin in the post-dose samples. As with the ADI low concentrations of curcumin present in the samples were the underlying limit to the derivation of pharmacokinetic parameters rather than the interfering peak.

Given the maximum concentrations of curcumin detected in the NOAEL samples (1.29 ng/ml for male mice and 5.92 ng/ml for female mice) the expected concentration in female mice at the lower dose of 10 x <NOAEL (592 pg/ml) should have been quantifiable. It is possible that saturation of first pass metabolism occurred between the NOAEL and 0.1 x NOAEL doses. However, considerably more data would be necessary to demonstrate this, and the overall database for curcumin in animals and humans was too variable to warrant such studies.

4.10 Discussion

4.10.1 Plasma kinetics in which humans received curcumin at the ADI and 10 x ADI

Administration at 10 x ADI meant that curcumin could be detected in plasma samples, but at very low concentrations, in the high picogram range. Observed pharmacokinetic values appeared to suggest that there may be a sex difference in humans, with female volunteers receiving a larger internal dose than male volunteers following dosing at 10 x ADI. Any saturation of kinetics in humans between the ADI and 10 x ADI would have the effect of making the ratio even lower at the ADI. In consequence these limited data indicate that the inter-species default factor would be more than adequate, but a considerably better database would be necessary if a chemical-specific adjustment factor were to be used in place of the default.

Low plasma levels of curcumin in humans following oral dosing have been documented in the literature (Shoba, 1998), and represent a major problem in translating some of the *in vitro* pharmacological effects of curcumin into clinical efficacy. Sharma et al (2001) administered 36, 72, 108, 144 or 180 mg curcumin/day to patients with adenocarcinoma of the colon or rectum. Neither curcumin nor its metabolites were detected in the plasma or urine samples (taken between 0.5 hours after administration to 29 days after administration). Incubation of these samples with glucuronidase or sulphatase did not result in detection of curcumin. Likewise, analysis of blood cell lipoproteins did not reveal detectable levels of

curcumin. The analytical method sensitivity limit was 5 pmol/ml, equivalent to ~2 pg/ml. Curcumin was detected in faecal samples at levels of 53-519 nmol/g-dried faeces (in 144 mg/day group) and 64-1054 nmol/g dried faeces (in 180 mg/day group).

Similarly, Cheng et al. (2001) found low levels of curcumin in patients given up to 12,000 mg/day. Patients eligible for the trial had to have one of the following high risk conditions; recently resected urinary bladder cancer, arsenic Bowen's disease of the skin, uterine cervical intra-epithelial neoplasia (CIN), oral leucoplakia of intestinal or neoplasia of the gastric mucosa. The full dose range used included 500, 1000, 2000, 4000, 8000, 12000 mg/day. A standard pharmacokinetic study was performed where blood and urine samples were taken at the following times after dosage: 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 14 and 24 hours. HPLC analysis of these samples was undertaken (analytical sensitivity was 0.2 μM, equivalent to ~74 pg/ml). Concentration-time profiles were obtained, and table 4.10 shows the pharmacokinetic parameters reported (AUC was calculated using the trapezoid formula).

Dose (mg/day)	**n**	**Cmax (μM) (ng/ml)**	**Tmax (h)**	**AUC_{0-24} (nM x min/ml) (pg x min/ml)**
4000	5	0.51 ± 0.11 (188 ± 41)	1.67 ± 0.58	2.55 ± 1.76 56340 ± 3880
6000	4	0.64 ± 0.06 (236 ± 22)	2.00 ± 1.73	4.80 ± 4.49 106080 ± 99240
8000	2	1.77 ± 1.87 (652 ± 689)	1.75 ± 0.35	13.74 ± 5.63 303720± 124440

Table 4.10 Pharmacokinetic parameters obtained following oral dosing of curcumin to human patients at doses from 4000-8000 mg/day (mw of curcumin is 368.4). Data derived from Cheng et al., (2001).

The data of Cheng et al (2001) indicate non-linear kinetics over the dose-range of 4000-8000 mg/kg/day. Comparison of these data with the results in this chapter indicate that non-linearity occurs at much lower doses as well.

The mean Cmax in the 10 x ADI (average dose of 650 mg) was 61 pg/ml. In the Cheng et al., study a dose of 6000 mg resulted in a Cmax of 236 ng/ml. These more recent studies, highlight the difficulty of measuring plasma levels of curcumin, and support the observation in animal kinetic studies of poor oral bioavailability of curcumin. In the study of Sharma et al., (2001) doses 10-20 times greater than the 10 x ADI dose did not lead to detectable levels of curcumin in the plasma or urine (despite an analytical sensitivity of ~2 pg/ml). This validates the data from the ADI and 10 x ADI studies, where only very low concentrations of curcumin were detected. In the Cheng et al study (Cheng et al., 2001), much larger doses (the 6000 mg/day dose is 10 x higher than the average dose given in the 10 x ADI study) did reveal

concentration time profiles. Based on the Cheng data, we could have expected a Cmax of ~20 ng/ml. Similarly, the 10 x ADI data resulted in a mean AUC of 10287 pg x ml/min, whilst the Cheng data would project an expected AUC of 10608 pg x min/ml (106080 pg x min/ml)/10). These data seem to indicate that less of the 10 x ADI dose (10 mg/kg) was entering the systemic circulation than in the Cheng study. Interestingly these values are very similar, and suggest that the range between the 10 x ADI dose and the 6000 mg/kg bw may be linear. It would be interesting to see if the patient group studies would have given similar kinetic values at 6000 mg/kg bw/day, but there was not time to investigate this within the time constraints of the project.

There were some fundamental differences between the Cheng study and the studies presented in this thesis:

- The different formulation of curcumin used (a tablet was used in the Chang study, compared to a capsule in the 10 x ADI study)
- The patients included in the study are suffering from specific diseases and either the disease process or concurrent medication (possible analgesics, anti-emetics) may be altering the kinetics, particularly bioavailability and clearance.
- This study was conducted in Taiwan, and so fundamental ethnic differences in physiology, and more importantly metabolism may have been operative.

These factors may go some way to explaining why the projected value for Cmax based on the Cheng data are so different from what was observed in the 10 x ADI study. It would have been interesting to compare the Tmax and clearance values from the Cheng study (this may have given more insight into the notion that curcumin has low bioavailability), but unfortunately these were not published.

4.10.2 Conclusions regarding the development of biomarkers of exposure for curcumin based on studies in humans.

The data obtained following dosing with curcumin, demonstrate some of the difficulties of obtaining reliable pharmacokinetic data for compounds with poor oral bioavailability. From the data presented in this thesis, and from supporting literature, doses approximately 10-fold greater than those used in these studies would be needed to derive reliable concentration-time curves from which to obtain PK parameters. Conversely, a much more sensitive analytical technique may be required to quantify curcumin in the plasma of individuals receiving a dose equivalent to the ADI (assuming that the plasma concentrations are linear over the 10-fold range, i.e. that saturation is not occurring at the higher dose, see above).

The curcumin data, particularly the NOAEL data, show that there is a significant sex difference in internal exposure following oral doses of curcumin, with female subjects (mice and humans) having higher Cmax and AUC values, and less rapid CL/F. This phenomenon highlights the importance of using both sexes in the toxicology studies. The NOAEL used to derive the ADI for curcumin was determined using the data for male mice, and because female mice have higher plasma concentrations than male mice there is an extra margin of safety for female members of the human population.

4.10.3 The adequacy of the inter-individual toxicokinetic default factor (3.16-fold) for curcumin

Due to the inability to calculate PK parameters from the ADI studies, the only possible way in which to assess the adequacy of the inter-individual default factor is to analyse the 10 x ADI data. Variability in the PK parameters was low, but the number of subjects was low (n=4), and the reliability of AUC and CL/F values is questionable, since plasma levels have not returned to baseline in all subjects. In the more recent study (Cheng et al., 2001) extended elimination of curcumin was observed, possibly indicative of enter-hepatic re-circulation. In this case, an extended time course as well as a more sensitive method would be required before a valid assessment of the adequacy of the 3.16-fold factor and larger members of subjects at the ADI could be made.

The fact that the elimination phase was not defined would have resulted in underestimation of the AUC (to infinity) and therefore an overestimation in the CL/F. Due to the fact that the terminal elimination phase was not well defined, it is likely that variability in the rate elimination has been underestimated, and so the CoVs reported for the 10 x ADI data in this chapter probably underestimate the true variability. In the Cheng study, variability ranged from 41% to 69% (for AUC values) and 9.4% to 106% (for Cmax values), which were not associated with dose. The last time point was 24 hours post-dose, but the maximum number of subjects/dose group was 5 persons, and the underlying disease state may be affecting variability.

4.10.4 Plasma kinetics in which animals received curcumin at the NOAEL or at 10 x ADI

The dosing of groups of mice with curcumin equivalent to the NOAEL resulted in quantifiable concentrations of curcumin at most time points. Clear concentration time-profiles were evident (see figure 4.5), and a pronounced sex difference was observed. It is not possible to comment on the variability observed in the mouse data, since all time points are independent from one another (there were 5 mice per time point, as terminal sampling was employed). Interestingly, the sex difference seemed to be echoed in the human data (obtained from the 10 x ADI studies), although the number of subjects was insufficient to confirm this possibility. The animal data also support the recommendation that any further

kinetic studies with curcumin using humans, should include later time points, because the animal data clearly show the beginning of a non-exponential terminal elimination curve. Previous studies (discussed in the introduction to this chapter) indicate that this is likely to be due to entero-hepatic circulation of curcumin.

The data presented in this section of my thesis are generally supported by previous studies. The NOAEL studies show rapid absorption and rapid elimination, with most of the curcumin being removed within the time course of the study. There is no directly comparable mouse data, but a study where rats received 2 g/kg, roughly 10-fold higher than the 220 mg/kg NOAEL dose, a Cmax of 1 µg/ml was measured. In this study, the mean Cmax was 0.00361 µg (3.61 ng/ml), 277-fold lower than the 1 µg/ml measured in the rat study.

4.10.5 Intake estimates for curcumin

There is evidence that large sections of the world population regularly consume curcumin at levels above the ADI. Unfortunately, many of these estimates are based on tumeric rather than curcumin. For example a study conducted in India, by the National Nutrition Monitoring Bureau, Hyderabad indicated a range of tumeric intake from 0.1-3.8 g/adult/day, equating to 5 g/day for a 70 kg individual (cited in Shankar et al., 1970). This makes it difficult to comment on the adequacy of the default uncertainty factor concerning pharmacokinetics in relation to intake. Based on the intake data presented above, and assuming curcumin to be present at 1-5%, this would equate to a daily intake of 50-250 mg/day, far in excess of the ADI. Consumption in developed countries is likely to be lower, but is probably growing due to migration and changes in patterns of consumption.

An estimation of the intake for curcumin in France has been made recently (Verger et al., 1998). Using a budget method, the mean intake of curcumin was estimated to be 0.48 mg/kg bw/day, below the ADI of 1mg/kg bw/day. The intake at the 90^{th}, 95^{th} and $97{,}5^{th}$ percentile was also calculated (0.84, 1.052 and 1.26 mg/kg bw/day respectively). Whilst some of these values exceed the ADI, our data and the data from clinical trials (see below) suggest that these, possibly more chronic exposures are unlikely to be of toxicological significance.

In clinical trials where patients received up to 12,000 mg/day curcumin was not associated with apparent side effects despite the presence of measurable plasma levels. One may speculate therefore that poor bioavailability and relatively low toxicity mean that exposure to curcumin could exceed that of the ADI without appreciable side effects. However these trials equate to sub-acute toxicity trials, and further investigation of the kinetics of curcumin, including that of the active metabolites would be required before a chemical-specific adjustment factor could be proposed.

Chapter 5: Studies with propyl gallate

5. Studies with propyl gallate

5.1 Propyl gallate (PG) as a food additive

Propyl gallate (see figure 5.1) has been used as an antioxidant since 1948 in a diverse range of roles such as a stabiliser in cosmetics and food packaging. It is also used as a food additive, and is added to high fat content foods to prevent spoilage. Propyl gallate can be found in pastry, baked goods, confectionary, dried meats and dried milk. Propyl gallate can be used alone, or in combination with butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT) (van der Heijden et al., 1986)

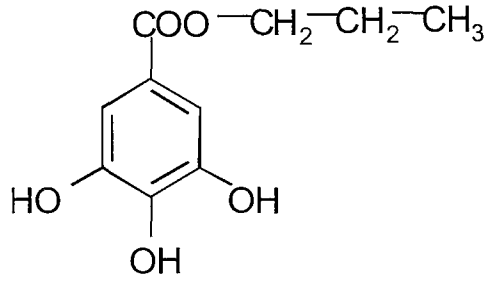


Figure 5.1 Propyl gallate

Historically, the gallates (propyl gallate, octyl gallate and dodecyl gallate) were reviewed by the JECFA as a group, and awarded a group ADI (applicable singly or in combination) of 0.2 mg/kg bw, based on assumed similarity in biotransformation reactions (WHO, 1980). Subsequently, the group ADI was removed, and a numerical ADI of 0-2.5 mg/kg bw was established for PG (there was a lack of adequate data for octyl and dodecyl gallate) (WHO, 1986). Propyl gallate was last reviewed by the JECFA in 1993 When an ADI of 1.4 mg/kg bw/ day was allocated based on a NOAEL of 135 mg/kg bw/day to which a 100-fold safety factor was applied (WHO, 1993).

5.2 The metabolic fate of PG

5.2.1 Animal studies with PG

van Esch (1955) conducted metabolic studies in rats, which were fed 0.035%, 0.2% or 0.5% propyl gallate. Propyl gallate and gallic acid as well as two unidentified spots were detected (in excreta) chromatographically 25 hours following administration of PG. A large proportion of the dose was found as propyl gallate, which is in contrast to octyl and dodecyl gallate where no parent compound was detected and smaller amounts of gallic acid and unidentified compounds were measured. These differences were presumed to be due to reduced solubility and therefore reduced bioavailability of

compound correlated to chain-length. Gallic acid was also found to be excreted as the glucuronide ester. Interestingly, the gallate ester was not hydrolysed by extracts of rat pancreas containing lipase and esterases did not lead to hydrolysis of the gallates and blood esterases could not split the gallates.

When PG was given to rats by stomach tube, the metabolites identified chromatographically were mainly 4-O-methyl gallate and gallic acid (Booth et al., 1959). When 4-O-methyl gallic acid was given to rats orally or via i.p. a glucuronide conjugate could be detected.

Scheline (1966), examined the decarboxylation of gallic and protacatechuic acid in rats. Pyrogallol and 2-O-methylpyrogallol were the major urinary metabolites when gallic acid was given orally but not when PG was given intraperitoneally. Billiary excretions of gallic acid were demonstrated, the main metabolite being 3,5-dihydroxy-4-methoxybenzoic acid. This was shown to undergo decarboxylation in the intestine, forming 2-O-methylpyrogallol. However the amount of being 3,5-dihydroxy-4-methoxybenzoic acid in the bile is not sufficient to account for the levels of 2-O-methylpyrogallol after the administration of gallic acid, and so it seems that pyrogallol absorbed after its formation in the intestine is the source of 2-O-methylpyrogallol.

Analysis of rabbit urine for conjugates following the oral administration of PG demonstrated the presence of a glucuronide as the major metabolite present. Other metabolites identified included 4-O-methyl gallic acid, gallic acid and pyrogallol. Dacre (1960) proposed that the glucuronide is probably 4-O-methyl galloyl-ß:D-glucosiduronic acid based on its chemical reactions. The main metabolic pathway therefore is likely to be cleavage of the ester linkage followed by methylation of the hydroxyl group in the 4 position and/or conjugation with glucuronic acid (see figure 5.2).

To summarise, the major metabolic products of PG are gallic acid and 4-O-methyl gallic acid (Dacre, 1960, Nakagawa et al., 1995, Zong et al., 1999). PG is also converted to a dimer (dipropyl-4,4',5,5',6,6'-hexahydroxydiphenate) and ellagic acid via auto-oxidation. The extent of conversion of the gallates (propyl, octyl and dodecyl gallate) to gallic acid has been shown to be inversely proportional to chain length (de Bie & van Ommen, cited in WHO, 1993)

Further studies have investigated the metabolic fate of gallic acid (Zong et al., 1999). After oral administration of gallic acid to rats, the major metabolite was found to be 4-O-methyl gallate that was detected in the blood and the urine. Both gallic acid and 4-O-gallic acid were detected in far greater quantities in the urine, compared with the blood. The ratio of 4-O-methyl gallate to gallic acid (0.55-0.76) indicate that a substantial proportion of gallic acid is excreted unchanged.

Plasma concentrations of PG were determined up to 40 minutes following intravenous administration of PG with or without pre-treatment with bis-p-nitrophenylphosphate (BNPP) to rats (30 mg/kg PG) and rabbits (15 or 30 mg/kg) (Gui-You & Satoh, 1999 a&b). The half-life, AUC and MRT were considerably higher in rats pre-treated with the BNPP, and clearance was halved. Additionally the concentrations of PG in the plasma and liver from BNPP treated rats were considerably higher at 5, 10 and 20 minutes than those in control groups. These results show that PG is probably hydrolysed by a carboxylesterase.

Despite metabolic turnover and relative lipid solubility differences, it is interesting to examine the results of an animal drug-metabolism pharmacokinetic (DMPK) study conducted with octyl gallate (OG). In a study, where rats received a single oral dose of 15 mg/k ^{14}C-labelled OG, only 20-30% of the administered dose was recovered in the tissues, the majority (60-80%) was found in the gastro-intestinal tract up to 12 hours after administration (Koss & Koransky, 1982). Unchanged ester was identified in the tissues, and the highest concentration of the ester was determined in the liver 10 minutes post-dose.

In a study with catechin and tannic acid (phenolic, "propyl gallate-like" compounds), Wistar rats were given either a control diet, or a diet containing 20g/kg dry matter catechin or tannic acid (TA) (Bravo et al., 1994). Faeces and urine were collected daily during the third experimental week. Only 3.1% and 4.6% of the ingested catechin and TA respectively were excreted in the faeces indicating that absorption and/or degradation of catechin/TA had occurred.

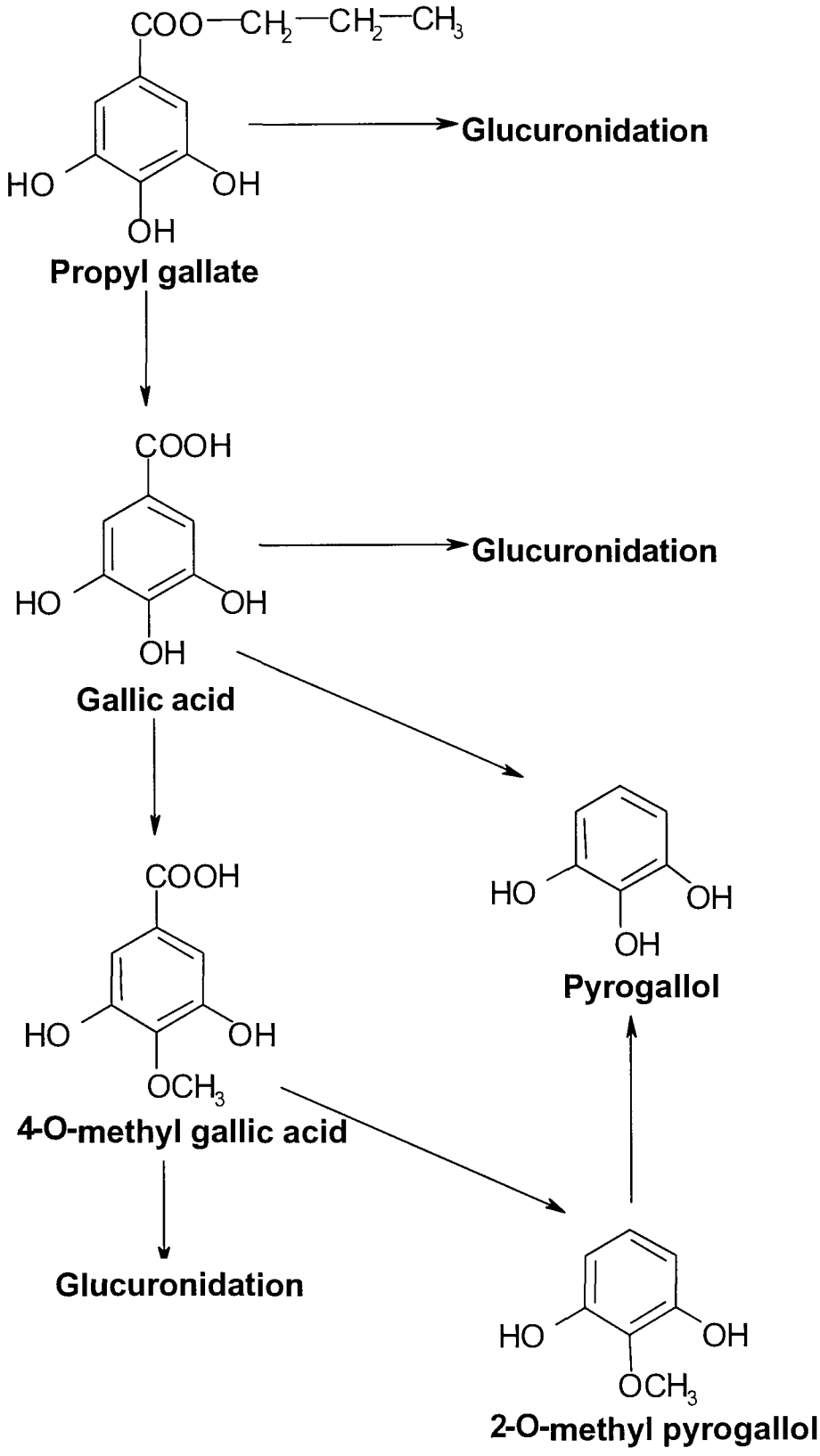


Figure 5.2 Routes of metabolism of PG in the rat

5.2.2 Biochemical effects of PG

The biochemical and biological effects of PG are numerous and have been the subject of an extensive review article (Kahl, 1984). Of particular interest in terms of the toxicological profile of PG is the observation of cytochrome P450 modulation. The major metabolite of PG, gallic acid has been recognised as possessing anti-allergic, anti-inflammatory, antimutagenic and anti-carcinogenic activities (cited in Rajalakshmi et al., 2001). Propyl gallate was found to inhibit the microsomal activity of the mixed function oxidases (i.e. inhibit cytochrome P450 enzymes) at concentrations of 50-500μM in liver microsomal preparations from Sprague Dawley rats (Yang & Strickhart, 1974). Propyl gallate was not found to inhibit electron transfer between NADPH and cytochrome P450 (as suggested by Torrielli et al., 1971), and was found to inhibit the P450s to varying extents.

In vitro studies with mouse liver have revealed that phenolic compounds such as protocatechuic acid, chlorogenic acid, tannic acid, the gallates and silybin could inhibit cytochrome P450 enzymes, with varying specificity (Baer-Dubowska et al., 1998). Tannic acid and dodecyl gallate were more selective against ethoxyresorufin-O-dealkylase (CYP1A1), whilst protocatechuic acid, chlorogenic and silybin were more selective against CYP1A2 and CYP2B.

Similarly, Rahimtula et al., (1979) found that addition of 25 or 125 μmol of propyl gallate to a MFO assay system prepared from the tissues of male Sprague-Dawley rats (liver, kidneys, stomach, colon and small intestine) inhibited the oxidation of benzo(a)pyrene.

PG was added as a 0.3% (w/w) supplement to diets containing various amounts of fats (King & McCay, 1981). Liver weight, liver-to-bodyweight ratio and hepatic microsomal protein level and CYP450 content, as well as specific measures of aniline hydroxylase, aminopurine, N-demethylase and NADPH-cytochrome C reductase were subsequently measured. Unexpectedly, propyl gallate was not found to affect the function of the mixed function oxidase system.

Lipid peroxidation in liver and kidney microsomes of rats treated with propyl gallate was significantly reduced, in a variety of systems tested (Murias et al., 2000). The authors hypothesised that in addition to induction of cytochrome P450 enzymes, PG probably simultaneously increases levels of glutathione peroxidase activity.

Gallic esters (propyl, octyl and dodecyl gallate) were found to inhibit the formation of 5S-hydroxy-6E, 8Z,11Z, 14Z-eicosatetraenoic acid (5-HETE) from arachadonic acid in human polymorphonuclear cells (Christow et al., 1991). Octyl and dodecyl gallate exhibited the greatest inhibition.

5.3 Toxicological effects of PG

5.3.1 Acute studies

The LD_{50} values determined for rats and mice are shown below in table 5.1

Species	Route	LD_{50} (mg/kg bw/day)	Reference
Mouse	oral	2000-3500	Cited in Lehman and Fitzhugh, 1951
Mouse	oral	1,700-3,500	Dacre, 1960
Rat	oral	2,600-3,800	Dacre, 1960
Rat	oral	3,800	Orten et al., 1948
Rat	oral	3,600	FDA, 1950 cited in Lehman and Fitzhugh, 1951
Rat	oral	5000-7000	van Esch, 1955
Rat	i.p.	380	Orten et al., 1948

Table 5.1 LD_{50} values for propyl gallate

5.3.2 Sub-acute studies with PG

Propyl gallate present in the diet of rats at levels of 1.2 and 2.3% caused mild anaemia and decreased bodyweight gain associated with poor food palatability (Orten et al, 1948). Mortality of 40% was experienced within four weeks of feeding 2.3% propyl gallate. The only consistent pathological change was renal tubular damage and the presence of albuminous casts in the kidney. Rats surviving the first four weeks went on to consume propyl gallate in the diet for 10-16 months with no other toxic signs.

Rats were fed 0.035%, 0.2% or 0.5% and pigs were fed 0.2% propyl gallate, octyl gallate and dodecyl gallate in both sub acute and long term feeding studies (van Esch, 1955). No inhibition of growth was observed except in the 0.5% dodecyl gallate study group, which was assumed to be due to reduced food intake. Reproductive function was normal, and no abnormalities were found in organs studies at autopsy.

Weaning rats fed diets containing 20% lard, supplemented with 0, 0.1, 0.2, 0.3, 0.4 or 0.5% propyl gallate for six weeks showed no toxic signs. No effect of propyl gallate was observed against a number of measures including growth rate, liver:bodyweight ratio, adrenal weight, serum cholesterol, adrenal cholesterol, liver lipids, serum sodium or liver cholesterol (Johnson and Hewgill, 1961).

Similarly no effect was seen after feeding of propyl gallate at a level of 0.02% in the fat for 13 weeks. A small inhibition in growth was observed. Following treatment with PG, rats were kept on a starvation diet until death. Rats previously fed on PG had a lower mean survival time than the controls; their total body protein was also reduced over the controls (Bukhan et al., 1962, cited in WHO, 1993). The ethics and

scientific value of this study are questionable.

In rats fed 0, 1000, 5000 or 25,000 mg/kg food for four weeks, several toxic signs were observed in the high dose group. Bodyweight gain, haemoglobin concentration, packed cell volume, red blood cell concentration, mean corpuscular volume and corpuscular haemoglobin were lowered. Increased extramedullary haematopdesis and slightly decreased haemosiderosis were observed in the spleen and were considered to be associated with the observed anaemia. Hyperplastic tubuli in the outer medulla were detected in the kidneys. Rats from the two highest dose groups displayed increased activity of aminopyrine-N-demethylase and glucuronosyl-transferase as well as general increased activity and content of cytochrome P450 enzymes (Strick et al., 1986, cited in WHO, 1993)

Wistar rats fed diets containing 0, 490, 1910, 7455 mg/kg in the food for thirteen weeks displayed toxic signs of the haematopoetic system and morphological changes in the spleen of the high dose animals. Other effects of PG included a decrease in the incidence and increased EROD activity in the high dose group. Increased activity of phase II xenobiotic transformation enzymes (glucuronyl-transferase and glutathione-S-transferase) was observed in the high and mid dose groups. The effects on nephrocalcinosis and the metabolising enzymes were not considered adverse, therefore the NOAEL was determined as 1910 mg PG/kg feed, equal to 135 mg/kg bw/day. This study was used as the pivotal study in the most recent JECFA evaluation of PG in 1993 (Speijers et al., 1993, cited in WHO, 1993).

No adverse effects were observed in guinea-pigs fed 0.02% or in dogs fed 0.1% PG in the diet for 14 months (Orten et al., 1948). Pigs fed 0.2% PG in the diet during sub-acute and long-term studies displayed no observed toxic effects (van Esch, 1955)

5.3.3 Chronic studies with PG

Orten et al (1948) conducted a long-term feeding study in which rats received 0.0017%, 0.0117% and 0.117% PG for two years and of 14-15 months in guinea pigs and dogs (0.0117% PG). No detectable differences were observed in gross appearance, growth or bodyweight, reproduction (guinea pigs), haemaglobin, erythocyte or leukocyte levels in blood (rats), or renal function, reflected in the qualitative composition of the urine (dogs). The histological appearances of the liver, kidney, spleen, stomach, gonads, lungs and hearts were normal. The authors concluded that no detectable toxic effects were produced in animals at a doses equivalent to 100 times the maximal human daily intake of PG (0.0017%). (This intake estimate was based on a 23.3% dietary fat intake, assuming fat to be treated with 0.25 % antioxidant, containing 2% PG.)

There was no significant effect on food and water consumption, body weight or survival time of albino

mice maintained on diets containing 0, 0.5 and 1.0% PG for 21 months (Dacre, 1974). No differences were found in the haematological response of the mice, and all histological findings were comparable in the control and dosed groups. The NOAEL was therefore determined as 1.0%, equivalent to 1.5 g/kg/day (Dacre, 1974).

F344 rats and $B6C3F_1$ mice were fed PG in the diet at levels of 0%, 0.6% or 1.2% for 103 weeks (Abdo et al., 1983). Both rats and mice showed growth retardation and decreased feeding but mortality was not significantly increased in either species at any dose. Observed effects considered to be related to PG intake include an increased incidence of hepatic cytoplasmic vasculorisation and supportive inflammation of the prostate gland in male rats. Whilst there was an increase in tumours of the preputial gland, islet cell tumours of the pancreas and phenochromocytoma of the adrenal gland in the low-dose male rats, these effects were not dose-related and were not considered to be related to PG treatment. Malignant lymphoma occurred with a significantly positive trend in male mice, however the incidence in the high dose male mice was not significantly difference from the historical control rate.

The authors conclude that the evidence for carcinogenicity in male rats is equivocal, whilst there is no indication of carcinogenicity in female rats. PG was not considered to be carcinogenic for B6C3F1 mice although it was recognised that increased incidence in lymphomas could be due to PG treatment,

5.3.4 Hepatotoxicity of PG

The treatment of isolated rat hepatocytes with PG (0.5-2 nM) resulted in concentration-dependent cell death, PG was more toxic than its metabolites (gallic acid, 4-O-methyl gallic acid, PG-dimer, ellagic acid and propyl alcohol were tested) (Nakagawa et al., 1995). PG cytotoxicity was enhanced by the addition of a thiol reductant (dithiothreitol), but the intracellular levels of glutathione and protein thiol were maintained during the incubation period. The authors concluded that PG cytoxicity is mediated via the parent compound, and that the net result is ATP depletion, which is independent of thiol depletion.

PG was found to protect rat hepatocyte cells against oxyradicals generated with xanthine oxidase-hypoxanthine (Wu et al., 1994). This was a dose-dependent effect. Similarly, PG was found to prevent liver injury in rats exposed to a high dose of carbon tetrachloride. PG inhibited the carbon tetrachloride-induced rise in plasma levels of liver enzymes, and PG was found to prevent the accumulation of hepatic tri-glycerides associated with CCl_4 administration (Torrielli & Ugazio, 1975). The uptake of CCl_4 and early steps of free radical generation were not blocked by PG, however PG displayed antioxidant activity, measured as a decrease in the production of malonyl dialdehyde. The decrease in production of malonyl dialdehyde suggests that PG inhibited the MFO system, thereby offering protection to the hepatocytes, by preventing the metabolic activation of CCl_4.

5.3.5 Teratogenicity of PG

Propyl gallate (362-906 mg/kg) was found to ameliorate the embryotoxic effects of a potent teratogen, hydroxyurea (600-750 mg/kg) (deSesso, 1981). Treatment of New Zealand white rabbits with hydroxyurea results in a high percentage of resorptions and severe craniofacial, trunk and limb deformities of all survivors. Injections of a mixture of hydroxurea together with PG lead to dose-dependent reductions in resorptions and specific malformations.

5.3.6 Genotoxicity of PG

Propyl gallate (0.25-1.5mM) was found to induce sister-chromatid exchanges (SCEs) and chromosomal aberrations (CAs) in CHO-K1 cells (Tayama and Nakagawa, 2001). PG underwent autoxidation in the media, to produce the PG dimer and ellagic acid. PG in the presence of S9, resulted in the production of gallic acid which was able to induce SCEs and CAs.

Propyl gallate, gallic acid, ellagic acid and tannic acid were tested in the Ames Salmonella tester strains, TA98 and TA100 (Chen & Chung, 2000). Simultaneously, these compounds were tested for anti-mutagenic activity using 2-nitrofluorene, 4,4'-dinitro-2-biphenylamin, 1-nitropyrene, 1,3-dinitropyrene, 2-nitro-p-phenylenediamine, 3-nitro-o-phenyldiamine and 4-nitro-o-phenylenediamine. None of the tannic acid like compounds was mutagenic, and only tannic acid showed anti-mutagenic activity towards the tested direct mutagens. This suggests that tannic acid, but not is hydrolytic product affects the metabolic activation of the mutagens.

In a variety of genotoxicity assays (including testing for anaphase abnormalities, the *Salmonella typhimurium* revertants assay and the dominant lethal test), propyl gallate was found to be non-genotoxic (Weir and Brusick, 1974. Unpublished report submitted to WHO by the FDA).

5.3.7 Propyl gallate as an initiator or promoter

The majority of studies have shown that PG reduces the genotoxicity and carcinogenicity of other compounds and this has been the subject of an extensive review (Kahl, 1984). Propyl gallate prevents the mutagenic activity of known mutagens including N-methyl-N-'nitro-N-nitroguanidine (MNNG) and N-acetoxy-2-acetylaminofluorene (N-acetoxy-AFF). PG is also able to reduce the mutagenic affect of aflatoxin B_1. Propyl gallate alone has no effect on the spontaneous reversion rate of *S. typhimurium* to hisitidine propotrophy (Rosin & Stich, 1980).

Topical application of PG 30 minutes prior to the application of 12-o-tetradecanoylphorbol-13-acetate (a tumour promoter) resulted in inhibition of promoter-linked ornithine decarboxylase activity (Kozumbo et al., 1983). Using urinary bladder carcinogenesis induced by N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) in rats as a model system, PG was not found to demonstrate a promoting effect (Tamano et al., 1987).

PG, present in the diet at a level of 0.5% was been found to protect Sprague Dawley rats from the carcinogenic effects of 2-acetylamino-fluorene (2-AFF) given at a dose of 0.05% in the diet (Daoud & Griffin, 1985).

F334 rats were treated with 1,2-dimethylhydrazine (DMH) subcutaneously at a dose of 20 mg/kg once a week for four consecutive week (Shirai et al., 1985). After the last injection rats were fed a diet containing 5% sodium L-ascorbate (SA), 0.5% BHA, 0.5% BHT, 0.8% ethoxyquin (EQ) or 1% propyl gallate for 36 weeks. Increases in colon tumours were observed with SA, BHT and EQ. PG and BHA did not modify tumour development. In a similar experiment F334 rats received an intragastric dose (150 mg/kg) of MMNG, and one week later were treated with 0.5% TBHQ (tert-butyl hydroxyquinone), 1% alpha tocopherol or 1% PG in the diet either with or without sodium nitrate (Miyauchi et al., 2002). The results of the study indicated that the co-administration of sodium nitrate and alpha tocopherol, TBHQ or PG promotes forestomach carcinogenesis.

5.3.8 Human toxicity data for PG

A single report is available where the gallates (lauryl gallate) was shown to cause occupational contact dermatitis (Brun, 1970 cited in WHO, 1993). Patch tests with lauryl gallate demonstrated a weak positive response in one individual. There are no other reports in the literature, toxicological or otherwise that reports the effect of propyl gallate administration to human subjects.

5.4 The most recent determination of a NOAEL for PG

The outcomes of the Speijers study, (cited in WHO 1993) (toxic signs of the haematopoetic system and morphological changes in the spleen) in Wistar rats resulted in a NOAEL of 1910 mg/kg feed, equivalent to 135 mg/kg bw. Using a 100-fold uncertainty factor and rounding to one decimal place an ADI for PG of 1.4 mg/kg bw/day was derived.

5.5 Clinical studies with PG

5.5.1 Clinical procedure

The materials and methods used in the clinical studies with PG are given in chapter 2 (materials and methods).

5.5.2 Dose selection and safety

As discussed in the introduction, one of the aims of this project was to compare human and animals' kinetic parameters derived from studies where PG was administered at the no-effect level (the ADI for humans and the NOAEL for animals). These doses are 1.4 mg/kg bw/day and 135 mg/kg bw/day respectively. A secondary aim of the project was to investigate linearity of the kinetic parameters. Doses equivalent to 10 x ADI and 0.1 x NOAEL were administered to humans and animals respectively. In order to gain ethical permission for these studies, the acute reference dose (ArfD) was estimated for PG. In cases of acute exposures to hazardous chemical, the ArfD is estimated based on short-term animal toxicology studies. The ArfD is an estimate of a safe acute exposure, and therefore is normally calculated from no-effect level demonstrated in acute animal toxicity testing. Since our human volunteers in the high dose studies were effectively being exposed to the food additives in much larger quantities, a review of all available acute toxicity data to assess the margin of safety between the ARfD in the animal database and the 10 x ADI dose was undertaken. Table 5.2 lists all the currently available acute toxicity data for PG.

Based on the short-term toxicity data presented in table 5.2, the NOAEL for short-term effect of PG would be estimated to be 1000 mg/kg bw/day or 70000 mg/person/day. Comparing this with the intended single dose for this study (980 mg/person) gives a margin of safety of 71.

Study performed	**Species**	**Duration**	**NOAEL (mg/kg bw/day)**	**Equivalent human intake (mg/day)**	**Reference**
Short-term	Rat	Four weeks	1,000	70,000	Strik et al., 1986*
Short-term	Rat	90 days	135	9, 450	Speijers et al, 1993*
Long-term (1)	Rat	Two years	0.12% = 60	4,200	Orten et al., 1948

(1) 0.12% = 1200ppm. 1200 (ppm) * 0.05 = 60 (mg/kg bw/day)

* cited in WHO, 1997

Table 5.2 Review of the toxicity data available for PG relevant to the derivation of the ARfD.

5.6 Results

5.6.1 Studies in which human volunteers received PG at the ADI.

5.6.1.1 Plasma analyses

A total of 19 volunteers were given PG at the ADI (a dose of 1.4 mg/kg). From these 19 data sets, 11 were used for comparison of pharmacokinetic parameters (see table 5.3) for details of these volunteers). Data sets were excluded because of difficulties experienced in the analysis of some of the sample sets, mostly due to poor coefficient of variation values between triplicate time point samples, which arose from the low concentrations of PG detected. The mean age of the 11 volunteers in the data set was 33 years, (35 years for males and 30 years for females). The mean weight of the male volunteers was 79 kg and for the female volunteers was 61kg.

	Age	Weight	Ethnicity
M18	20	85	Caucasian
M19	54	81	Caucasian
M20	37	74	African
M21	57	90	Caucasian
M22	19	77	Caucasian
M23	20	68	Caucasian
M24	37	81	Indian
Male Mean	**35**	**79**	**N/C**
F16	50	76	Caucasian
F17	27	56	Caucasian
F18	23	56	Caucasian
F19	19	57	Caucasian
Female Mean	**30**	**61**	**N/C**
Overall Mean	**33**	**73**	**N/C**

Table 5.3 Details of volunteers who received propyl gallate at the ADI.

Table 5.3 shows the concentrations of PG, expressed as ng/ml detected at each time point for each individual included in the pharmacokinetic comparisons.

Concentration of PG in plasma samples, ng/ml

Time	Male Data							Female Data			
(minutes)	M18	M19	M20	M21	M22	M23	M24	F16	F17	F18	F19
0	0.0	0.0	0 (0.9)	0(0.8)	0.0	0.0	0.0	0.0	0.0	0 (0.6)	0.0
30	0.4	0.0	2.3	1.0	0.4	0.4	0.0	2.6	0.9	**13.1**	**10.8**
60	2.5	0.0	1.6	2.0	0.2	3.0	**4.6**	1.8	5.5	**5.6**	**7.2**
90	2.9	0.0	**13.3**	**7.6**	2.4	**11.8**	**5.7**	1.7	1.9	0.8	**4.2**
120	**6.2**	0.0	**10.6**	**8.0**	3.0	**6.5**	**8.8**	0.7	1.8	0.4	0.0
150	**10.9**	1.8	1.5	**10.6**	2.8	0.4	**12.4**	**14.3**	0.8	1.3	0.0
180	2.2	**4.3**	2.8	1.9	0.9	0.4	0.0	**10.1**	**6.0**	0.3	0.0
210	1.5	**12.3**	0.6	1.5	0.7	0.2	**10.6**	3.2	**10.6**	1.7	0.0
240	2.3	**32.9**	0.0	2.3	4.0	0.8	0.0	1.9	**4.5**	1.8	2.4
300	**6.4**	**5.4**	0.7	0.6	0.7	0.7	0.0	1.0	2.5	2.4	0.0
360	**5.9**	2.3	0.7	0.1	0.5	0.4	0.0	0.6	0.4	4.2	0.0
420	0.4	0.8	0.7	0.2	0.0	0.0	0.0	1.5	0.7	3.3	0.0

The **red typeface** denotes concentrations that were above the limit of quantification for the method and for which reliable duplicate values were obtained.

Values given are following baseline subtraction; baseline values quantified as PG are shown in parentheses.

Table 5.4 Plasma concentrations of PG from studies in which volunteers received the ADI.

The plasma concentrations shown in table 5.4 show that PG concentrations at many time points was below the limit of quantification for the method of analysis employed. Individual time-concentration profiles were rarely detected, and so they are not shown graphically.

5.6.2 Pharmacokinetic parameters for PG calculated using the data from the ADI studies.

As discussed in the previous section, concentration time curves were not observed in the case of most individuals, therefore the kinetic model (WinNonlin™, described in chapter 2) was not able to predict terminal phase pharmacokinetic values for AUC to infinity, CL/F to infinity or a value for terminal half-life. The pharmacokinetic parameters available for comparison therefore comprise of Cmax, Tmax, AUC (calculated using the trapezoid formula from observed values) and CL/F (calculated as dose/AUC). These parameters are shown below in table 5.5. These values are calculated from the baseline-subtracted data, as shown in table 5.4.

	Cmax (ng/ml)	Tmax (minutes)	AUC (obs) (ng x min/ml)	CL/F (ml/min)
M18	11	150	1652	848
M19	33	240	2518	556
M20	13	90	1088	1287
M21	11	150	1127	1242
M22	4	240	563	2486
M23	12	90	803	1744
M24	12	150	1262	1109
Male Mean	**14**	**159**	**1288**	**1324**
F16	14	150	1258	1113
F17	11	210	1235	1133
F18	13	30	1254	1117
F19	11	30	777	1801
Female Mean	**12**	**105**	**1131**	**1291**
P value (M vs. F)	0.753	0.268	0.656	0.925
Overall Mean	**13**	**139**	**1231**	**1312**
Overall SD	7	78	520	524
Overall CoV	**54**	**N/C**	**42**	**40**

Values in **bold** are the mean data and overall CoV used in later comparisons. P-values compare the male and female data, using unpaired student t-test (2 tails, equal variance)

Table 5.5 Pharmacokinetic parameters (observed) for PG generated from studies in which volunteers received the ADI.

As reflected in the overall CoV values, the variability in pharmacokinetic parameters amongst individuals is relatively small for PG. The Cmax values range from 4-33 ng/ml, and AUC and CL/F values vary little also. However, Tmax values range between 30 minutes to 4 hours. Using a Student's t-test no significant sex differences were detected.

5.6.3 Studies in which human volunteers received PG at 10 x ADI.

5.6.3.1 Plasma analyses

A total of 10 volunteers were given PG at a dose of 14mg/kg (equal to 10 x the ADI) (See table 5.6) From these 10 data sets, 10 were used (5 male data sets and 5 female sets) for comparison of pharmacokinetic parameters. Unlike the ADI studies, PG was more easily detectable in these samples. The mean age of the 10 volunteers in the data set was 24 years old, (25 years old for males and 22 years old for females). The mean weight of the male volunteers was 80 kg and for the female volunteers was

56 kg.

	Age	Weight	Ethnicity
M25	23	63.5	African-Japanese
M26	23	76	Caucasian
M27	25	70	Caucasian
M28	23	93.5	Caucasian
M14	30	96	Caucasian
Male Mean	**25**	**80**	**N/C**
F20	23	53	Caucasian
F21	24	52.5	Caucasian
F22	21	60	Caucasian
F23	21	61.1	Caucasian
F24	23	55	Caucasian
Female Mean	**22**	**56**	**N/C**
Overall Mean	**24**	**68**	**N/C**

Table 5.6 Details of volunteers who received PG at 10 x ADI.

Table 5.7 shows the concentrations of PG, expressed as ng/ml detected at each time point for each individual included in the pharmacokinetic comparisons. Nearly all concentrations were above the limit of quantification for the method.

Concentration of PG in plasma samples, ng/ml

Time	Male Data					Female Data				
(minutes)	M25	M26	M26	M28	M14	F20	F21	F22	F23	F24
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30	89.1	19.4	13.5	122.6	48.4	20.0	0.0	2.2	12.3	5.5
60	77.1	34.0	20.9	208.2	81.7	13.3	0.0	6.2	19.6	10.1
90	30.9	101.9	118.8	92.1	42.5		4.8	15.9	29.4	13.2
120	31.7	131.4	80.2	97.6	70.7	46.1	10.3	42.9	49.5	141.1
150	142.9	118.6	78.6	58.1	64.8		7.4	81.2	70.0	184.3
180	89.4	32.4	136.1	121.4	79.9	85.3	5.6	104.8	45.4	127.0
210	83.2	18.3	206.8	141.2	84.3	240.4	4.8	120.0	156.6	45.9
240	78.4	29.2	86.2	154.8	24.0	150.7	3.2	159.3	111.6	68.9
300	24.2	26.1	35.1	17.5	30.5	19.6	1.8	96.1	18.8	13.3
360	7.8	9.5	13.4	24.4			3.9	11.9	8.8	5.1
420	10.6	8.1	12.1	17.4	11.7		0.0	30.9	6.5	3.5

Values given are following baseline subtraction; baseline values quantified as PG are shown in parentheses. Spaces in the table indicate that a sample was not taken for analysis.

Table 5.7 Plasma concentrations of PG from studies in which volunteers received PG at 10 xADI.

The plasma concentrations shown in table 5.7 show that unlike the ADI studies, PG concentrations at the vast majority of time points were above the limit of quantification for the method of analysis employed. Individual time-concentration profiles were observed, and so these are plotted in figure 5.3.

The concentration-time profiles generated from the 10 x ADI are interesting. Whilst the majority of data points are above the limit of quantification, the profiles do not show uniform characteristics. Cmax values ranged from 10-240 ng/ml and the Tmax varied considerably between individuals, with volunteers M26, F20, F22 and F23 showing delayed absorption. Clearly PG is not simply absorbed and eliminated by a single first-order process, since many subjects showed a double-peak in the plasma-concentration time curve.

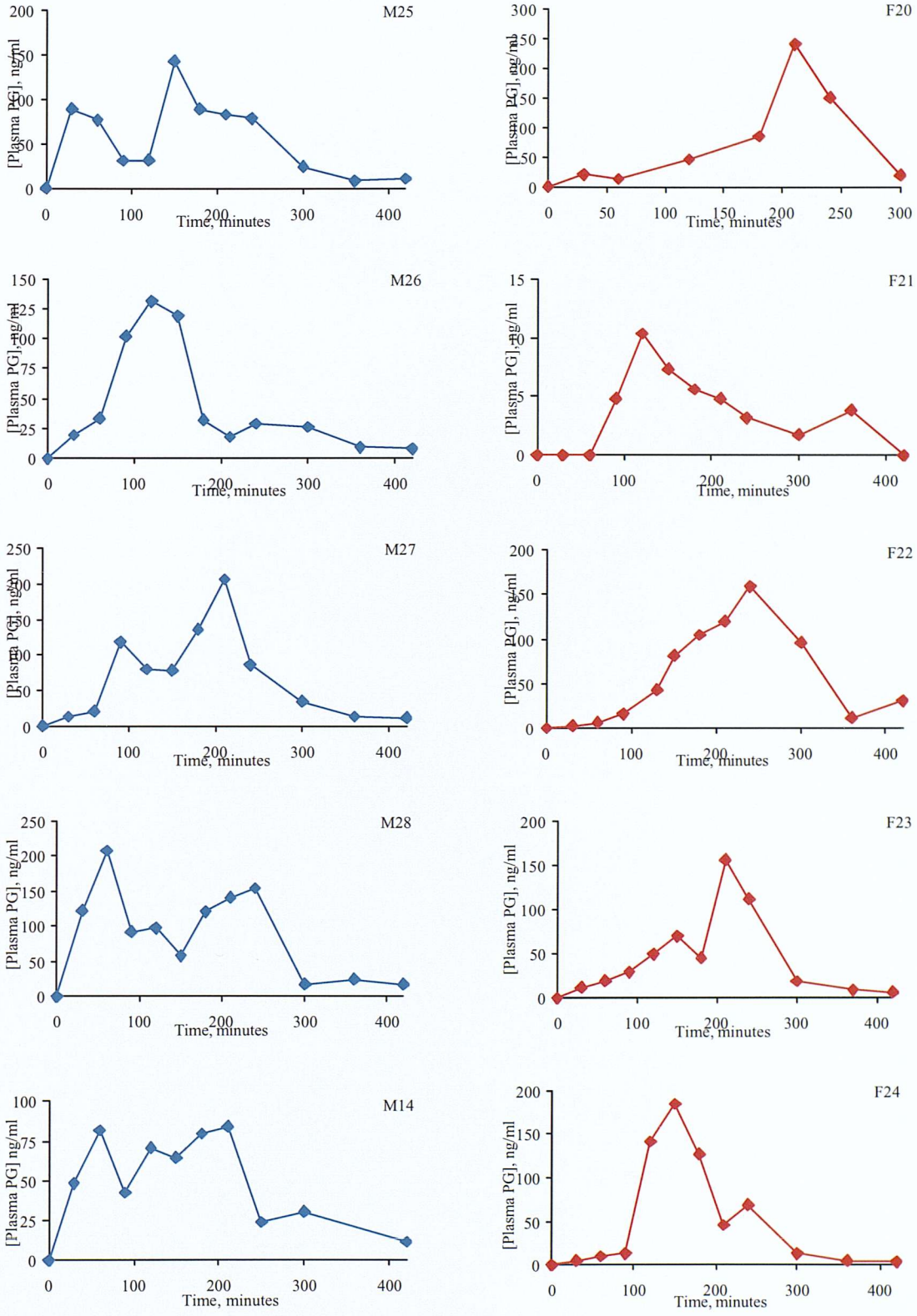


Figure 5.3 Concentration-time profiles for PG in male (blue graphs) and female (red graphs) volunteers after receiving 10 x ADI.

5.6.4 Pharmacokinetic parameters for PG calculated using the data from the 10 x ADI studies

The 10 x ADI plasma data were not suitable for simple mathematical modelling. Figure 5.3 shows that there was not a common concentration-time profile amongst individuals; in particular, there was no consistent clear elimination phase for the model to use for extrapolation. As a result only observed values could be calculated for the studies conducted at 10 x ADI. Values for Cmax, AUC (calculated using the trapezoid formula from observed values) and CL/F (calculated at dose/AUC) are reported (see table 5.8). These data were calculated using the methods applied to the ADI study so that the results were directly comparable with the pharmacokinetic parameters calculated from the ADI studies.

	Cmax (ng/ml)	Tmax (minutes)	AUC (ng x min/ml)	CL/F (ml/min)
M25	143	150	22093	634
M26	131	120	17375	806
M27	207	210	26797	522
M28	208	60	35234	397
M14	84	210	18957	739
Male mean	**155**	**150**	**24091**	**620**
F20	240	210	21662	646
F21	10	120	1473	9502
F22	159	240	25443	550
F23	157	210	18414	760
F24	184	150	20120	696
Female mean	**150**	**186**	**17423**	**2431**
P value (M v. F)	0.913	0.297	0.193	0.286
Overall mean	**152**	**168**	**20757**	**1525**
Overall SD	67	57	8583	2805
Overall CoV	**44**	**N/C**	**41**	**184**
Overall mean - F21	**168**	**173**	**22899**	**639**
Overall SD	47	58	5589	130
Overall CoV	**28**	**33**	**24**	**20**

N/C not calculated. Values in **bold** are the mean data and overall CoV used in later comparisons. P-values compare the male and female data, using unpaired student t-test (2 tails, equal variance).

Table 5.8 Pharmacokinetic parameters (observed) for PG generated from studies in which volunteers received 10 x ADI.

With the exception of one outlying dataset for the volunteer, F21 the data are fairly consistent and show low variability (for example if one compares it with the CoVs seen for PK parameters in the TBZ studies). There is no clear relationship between Cmax and Tmax, i.e. lower Cmax values are not a

consequence of delayed absorption. The AUC and CL/F values are all fairly uniform with the exception of F21. No significant sex differences were observed. To highlight the large impact of F21 the mean values for the PK parameters after removal of F21 from the dataset are shown in table 5.8. It is clear that after removal of this one subject the data demonstrate very low variability (<30% for Cmax, AUC and CL/F). There is no obvious biological or analytical explanation for the abnormal results for subject F21.

5.6.5 Comparing plasma kinetics for PG at the two human doses.

Assuming linear kinetics, a 10-fold increase in concentration-dependent parameters was anticipated in volunteers receiving 10 x ADI (i.e. Cmax and AUC). CL/F however, would be expected to remain the same, as it is a concentration-independent parameter with first order kinetics. Table 5.9 compares the kinetic parameters calculated from observed values as a ratio (10xADI/ADI).

	Cmax (ng/ml)	Tmax (minutes)	AUC (ng x min/ml)	CL/F (ml/min)
ADI Mean	13	139	1231	1312
ADI SD	7	78	520	524
ADI CoV	54	N/C	42	40
10 x ADI Mean	152	168	20757	1525
10 x ADI SD	67	57	8583	2805
10 x ADI CoV	44	N/C	41	184
Ratio 10 x ADI Mean/ADI Mean	**11.6**	N/C	**16.9**	**1.16**

Table 5.9 Comparison of PK parameters for PG from the two studies conducted in humans.

Taken collectively, the ratios in table 5.9 do not support the notion that kinetics are non-linear at the high dose. The Cmax ratio of 11.59 is close enough to 10 to be the result of experimental variability. Similarly, the ratio of 1.16 for CL/F is close enough to 1 to suggest that the kinetics are still linear at this higher dose. The only parameter that suggests the possibility of non-linear kinetics is AUC, the fundamental indicator of internal exposure. The ratio of 16.9 between the high and low dose data suggests that there is a disproportionate increase in internal exposure given a 10-fold increase in dose. (This ratio would be larger with the exclusion of the data for F21). One would expect the increased AUC to have caused a similar difference in CL/F since this is simply dose/AUC. The difference arises from the disproportionate effect of the data for F21. If these data are excluded the AUC ratio is 18.6 (22899/1231) while the CL/F ratio is 2.05 (1312/639). This analysis supports the possibility of non-linear kinetics at the higher dose.

5.6.6 Salivary concentrations from studies where human volunteers received PG at the 10 x ADI.

Due to the very low concentrations of PG observed in the ADI studies the corresponding saliva samples were not analysed. Prior to analysing the saliva samples taken in the 10 x ADI studies, the extent to which PG was protein-bound was determined in protein-binding studies. This meant that analysis of the saliva samples only commenced once the feasibility of detecting PG in the saliva samples was determined.

5.6.7 Protein-binding studies with PG

Protein-binding studies were conducted to determine the level of plasma-protein binding of PG. Albumin was spiked with PG, and using a protocol identical to that described in chapter 2, (Materials and methods), PG was found to be almost 100% protein bound (see table 5.10).

	Ringers	Albumin	% PPB
Ratio PG/EG n = 5	0.003 ± 0.006	0.316 ± 0.370	**97%**

Table 5.10 Results of an experiment to determine the percentage protein binding of PG.

As before the appropriate controls were analysed simultaneously with the samples; where necessary, baseline subtraction of contaminating peaks in Ringers' or albumin solution was executed.

5.6.8 Analysis of saliva samples from 10 x ADI studies

Having ascertained that the protein binding of PG was ~97%, it was decided to only analyse the saliva samples corresponding to the plasma Cmax and a sample either side of the Cmax. The maximal concentrations in plasma at the studies performed at 10 x ADI ranged between 10-240 ng/ml. Based on 97% protein binding, one would therefore expect concentrations in the saliva of between 0.3-7.2 ng/ml. Given the limit of detection of the assay (3.125 ng/ml) it would be unlikely that a concentration time-profile for PG could be followed in the saliva. An analysis of the saliva samples (as detailed above) was conducted for all volunteer samples. The data is shown in table 5.11.

Volunteer	Time point	Concentration of PG in Plasma (ng/ml)	Concentration of PG in Saliva (ng/ml)	CoV	Relative PG in saliva (%[Saliva]/[Plasma])
M25*	**150**	143	4	43	2.8
	180	89	13	25	14.3
	210	83	5	32	6.2
M26	90	102	3	67	3.2
	120	131	7	106	5.2
	150	119	4	17	3.1
M27	180	136	**3**	52	2.2
	210	207	**0**	N/C	0.0
	240	86	**1**	74	1.0
M28	30	123	5	79	4.3
	60	208	15	41	7.1
	90	92	4	12	4.8
M14*	150	65	**1**	2	1.7
	180	80	**1**	89	0.8
	210	84	**0**	N/C	0.0
F20	180	85	4	N/C	4.9
	240	240	18	3	7.6
	300	200	23	6	11.4
F21	90	5	4	40	81.7
	120	10	3	24	31.3
	150	7	8	92	107.6
F22	210	120	4	31	3.2
	240	159	4	28	2.3
	300	96	5	65	4.7
F23	180	45	4	26	9.9
	210	157	**0**	N/C	0.0
	240	112	**1**	53	0.8
F24	120	141	11	3	7.5
	150	184	5	3	2.5
	180	127	11	7	8.4

The timepoint marcated in **bold** was the Cmax in plasma

*The incorrect saliva samples were analysed in error.

The **red typeface** denotes concentrations of PG that were detected, but were below the limit of quantification for the method

Table 5.11 A comparison of plasma and saliva concentrations of PG detected in samples from volunteers receiving PG at a dose of 14 mg/kg bw (equivalent to 10 x ADI). .

Very small amounts of PG were detected in the saliva. In many cases the levels were close to the limit of quantification, and the reported CoV values are high. The Tmax in saliva showed no consistent correlation to the Tmax in plasma. In some individuals the maximal PG in saliva matched that of the Tmax in plasma, in others it did not. In addition, there was no consistent ratio between plasma concentration of PG and salivary concentration of PG either within an individuals samples, or between the samples from different individuals. The mean overall value for PG in saliva compared with plasma was ~15%, which does not support the outcome of the protein-binding study. However, it does not suggest that PG is being retained on the dental roll, and the quality of the saliva data do not allow too much emphasis to be placed on this mean value. In conclusion, salivary analysis of PG was not a reliable marker for PG in the plasma even at a dose of 10 x ADI.

5.6.9 Calculating the chemical-specific inter-individual toxicokinetic factors for PG

The default uncertainty factory, allowing for inter-individual variability in toxicokinetics is 3.16. The coefficient of variation was determined for the three primary kinetic parameters calculated from the human experimental data. The variation observed in the parameters was used to calculate what uncertainty factor would be required to protect 95, 97.5 and 99% of the population (see Chapter 2 Materials and methods). The proportion of the population protected by the current default of 3.16 was also calculated. The results, using parameters derived from analyses of plasma (after dosing at the ADI and 10xADI) are shown in table 5.12.

	ADI PK Paramters			10 x ADI PK Parameters		
	Cmax (ng/ml)	AUC (ng x min/ml)	CL/F (ml/min)	Cmax (ng/ml)	AUC (ng x min/ml)	CL/F (ml/min)
Overall Mean	13	1231	1312	152	20757	1525
Overall SD	7	520	524	67	8583	2805
Overall CoV	**54**	**42**	**40**	**44**	**41**	**184**
Uncertainty factors	Cmax	AUC	CL/F	Cmax	AUC	CL/F
~% Covered by 3.16	**98.9%**	**99.8%**	**99.9%**	**99.7%**	**99.8%**	**82.8%**
95th Percentile	2.30	1.94	1.88	2.00	1.91	**7.39**
97th Percentile	2.70	2.20	2.13	2.28	2.17	**10.84**
99th Percentile	3.24	2.55	2.45	2.66	2.50	**16.92**

The **red typeface** denotes uncertainty factors greater than the default of 3.16-fold.

Table 5.12 Calculating inter-individual toxicokinetic factors for PG using plasma kinetic data derived from the ADI and 10 x ADI studies.

Variability in Cmax and AUC is equally low in both the low and high dose data sets. Data from the low dose (ADI) studies show that based on all pharmacokinetic parameters reported, the default factor of

3.16-fold would be adequate to protect substantial proportions of the population. There is a greater margin between the calculated factors for the 95th, 97.5th, 99th percentile and the default of 3.16 for factors based on AUC and CL/F. In the case of Cmax, to cover the 99th percentile, a factor exceeding 3.16-fold would be required.

As with the ADI data, the variability in Cmax and AUC values from the 10 x ADI data is small, and the 3.16 fold factor is adequate to protect over 99% of the population. However, due to the outlier (F21), the variability in CL/F is dis-proportionately large. Removal of this outlier from the data set would result in all three parameters being adequately covered by the 3.16-fold default. As the data is reported in table 5.12, the 3.16 default would not adequately protect the population, using CL/F as the kinetic parameter of consideration. This is due to the data for one outlier, and such an observation required further study, to examine the reproducibility of this effect and the incidence of the effect in the population. Only then could this observation be interpreted logically.

5.6.10 Studies in which rats were dosed with PG at the NOAEL

Sixteen rats, 8 male and 8 female were dosed with 135mg/kg bw PG, the NOAEL, in 2% ethanol. Dr K. Walton conducted these studies and sample analyses. Tables 5.13 and 5.14 are summaries of the concentrations measured at each time point in each rat. A sample was not taken at 420 minutes in the animal studies, therefore a value was extrapolated from the 240 minute time point using the T½ (calculated using the formula $[420] = C_{240} \times \exp(-((0.693 \times 180)/T1/2))$, where 180 represents the temporal difference between observed and extrapolated values). Using WinNonlin™ (with 1/y weighting) to model the corrected (baseline subtracted data) the mean overall T½ was 96 minutes (123 minutes for the male rats and 69 minutes for the female rats).

Time (minutes)	Concentration of PG in male rat plasma samples in ng/ml							
	M1	M2	M3	M4	M5	M6	M7	M8
0	0 (240)	0	0	0	0	0	0	0
5	8953	11085	2152	10148	2978	3060	2066	1635
10	5304	4499	990	4883	1505	2217	1108	1288
15	1717	1277	653	2129	888	1469	693	486
20	888	991	535	1447	984	1153	537	289
25	564	647	484	890	684	775	297	148
30	524	319	414	617	602	620	244	177
40	106	280	238	519	385	661	197	172
50	45	226	219	454	542	804	201	99
60	36	215	205	723	464	621	130	142
120	0	76	119	485	431	860	59	53
240	0	11	105	340	199	411	53	37

Values given are following baseline subtraction; baseline values quantified as PG are shown in parentheses.

Table 5.13 Plasma concentration of PG from studies in which male rats were dosed with the NOAEL.

Time (minutes)	Concentration of PG in female rat plasma samples in ng/ml							
	F1	F2	F3	F4	F5	F6	F7	F8
0	0 (57)	0 (69)	0 (117)	0	0	0	0	0
5	5302	1939	1970	5697	4334	12825	884	1013
10	1332		720	2280	2549	7602	267	617
15	841	352	383	673	1544	3683	205	383
20	386	228	238	472	706	2553	84	229
25	272	195	223	425	534	1063	82	178
30	221	136	194	344	492	834	89	
40	104	67	56	247	554	517	65	116
50	243	75	85	204	278	300	54	111
60	83	49	22	157	244	242	75	79
120	4	21	0	76	259	311	19	76
240	0	0	0	145	116	98	29	62

Values given are following baseline subtraction; baseline values quantified as PG are shown in parentheses.

Table 5.14 Plasma concentration of PG from studies in which female rats were dosed with the NOAEL.

5.6.10.1 Plasma analyses

PG was readily detectable in the rat plasma samples, because of the large amounts of PG present at all time points. Unlike the human data, the rat data indicated clear absorption, distribution and elimination profiles. Figure 5.4 below shows the average concentration-time profile for the male and female rats. The animals absorbed the dose very rapidly (a consequence of the gavage dosing); PG was being eliminated very rapidly, with very low concentrations of PG remaining at 240 minutes in most animals.

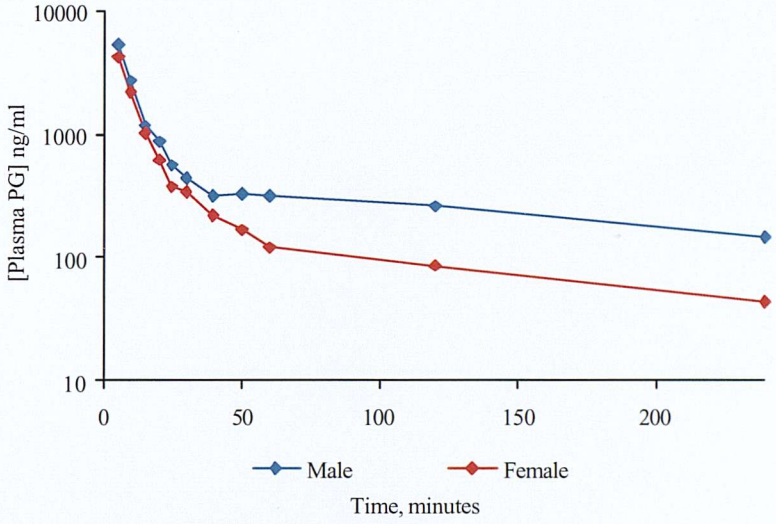


Figure 5.4 Concentration-time profiles for PG generated from mean male and mean female rat data after dosing at the NOAEL.

5.6.11 Pharmacokinetic parameters for PG using data from the NOAEL studies

Pharmacokinetic parameters were calculated for each individual animal using the same method as for human ADI data, i.e. baseline subtraction, followed by manual calculation of AUC using the trapezoid formula (CL/F was calculated as dose/AUC) (see table 5.15). Although the animal data showed clear concentration time-profiles, the data presented were not extrapolated to infinity in order to allow direct comparison with the human PK data.

	Cmax (ng/ml)	Tmax (minutes)	AUC (ng x min/ml)	T 1/2 (minutes)*	CL/F (ml/min)
M1	8953	5	93818	9	1439
M2	11085	5	115961	43	1164
M3	2152	5	70036	182	1928
M4	10148	5	247131	181	546
M5	2978	5	140920	135	958
M6	3060	5	244708	227	552
M7	2066	5	48550	106	2781
M8	1635	5	39595	105	3410
Male mean	**5260**	**5**	**125090**	**123**	**1597**
F1	5302	5	49049	13	2752
F2	1939	5	19257	44	7010
F3	1970	5	21320	11	6332
F4	5697	5	89731	43	1505
F5	4334	5	114210	152	1182
F6	12825	5	207033	109	652
F7	884	5	19388	155	6963
F8	1013	5	38715	492	3487
Female mean	**4246**	**5**	**69838**	**127**	**3735**
P value (M vs. F)	0.62	N/C	0.16	0.95	0.05
Overall mean	**4753**	**5**	**97464**	**125**	**2666**
Overall SD	3896	N/C	77015	119	2248
Overall CoV	**82**	**N/C**	**79**	**95**	**84**

Values in **bold** are the mean data and overall CoV used in later comparisons. P-values compare the male and female data, using unpaired student t-test (2 tails, equal variance)

* T½ is an extrapolated value derived from the mathematical model that the data was fitted to using WinNonlinTM.

Table 5.15 Pharmacokinetic parameters (observed) for PG generated from studies in which rats were dosed at the NOAEL.

The Cmax values in rats range from 884-~13000 ng/ml, whilst the Tmax was consistently the first time point sample (5 mins). There are no statistically significant sex differences; the values for AUC and CL/F varied considerably, with male rats having an overall higher AUC and corresponding lower CL/F. The T½ values (data not shown, values derived from WinNonlin™ modelling of the data extrapolated to infinity) showed no consistent correlation with CL/F, pointing to variability in bioavailability of the dose being the main source of variability between the individual rats. The fact that T½ was not dramatically increased with the higher CL/F values rather suggests that saturation is not occurring in these rats. There was greater variability in the rat data presented in table 5.15 compared to that observed in humans at either the ADI or 10 x ADI. This is unexpected, as rats are a more homogenous population, and so CoV

values were expected be smaller.

5.6.12 Calculating the chemical-specific inter-species toxicokinetic factor for PG

Human and animal kinetic parameters were compared to generate the chemical-specific inter-species toxicokinetic factor for PG. The kinetic parameters (excluding CL/F, which is independent of dose) were corrected for dose using the equation show in chapter 2. In the first instance the inter-species chemical-specific toxicokinetic factor was calculated based on the comparison of the ADI and NOAEL data. (See table 5.16)

	Cmax (ng/ml)	Tmax (minutes)	AUC (ng x ml/min)	CL/F (ml/min)
Human ADI Mean	13	139	1231	1312
Overall SD	7	78	520	524
Overall CoV	54	N/C	42	40
Animal NOAEL Mean	4753	5	97464	2666
Overall SD	3896	N/C	77015	2248
Overall CoV	82	N/C	79	84
Interspecies factor	**0.27**	**N/C**	**1.22**	**2.03**

Table 5.16 Calculating the interspecies toxicokinetic factor for PG using data generated from the ADI and NOAEL studies.

The data in table 5.16 show that based on Cmax, AUC or CL/F, the toxicokinetic default factor of 4.0-fold is more than adequate. There is approximately a 350-fold difference between the rat and human Cmax value, and an 80-fold difference in AUC value, and a 2-fold lower difference in CL/F values. Compared to animals, humans are exposed to a slightly higher internal dose (reflected in the AUC values) but have a lower clearance rate compared to the rats. Interestingly the CoV values were much lower for the kinetic parameters calculated from the human data than the animal data.

5.6.13 Studies in which rats were dosed with PG at 0.1 x NOAEL

As previously reported, studies were conducted in human, where volunteers received 10 x ADI. A dose equivalence study was conducted in rats (receiving 0.1 x NOAEL), using the same experimental protocol as previously described. Table 5.17 reports the summary of the concentrations measured at each time point in the rat.

Time	Male Data						Female Data					
(minutes)	M1	M2	M3	M4	M5	M6	F1	F2	F3	F4	F5	F6
0	0	0	0	0	0	0 (4.9)	0	0	0	0 (10.6)	0	0
5	333.6	79.0	141.2	121.5	59.0	127.5	164.6	75.1	443.2	206.4	13.4	462.0
10	109.9	77.2	83.9	115.1	62.8	79.3	86.2	85.3	191.0	98.1	42.7	62.1
15	116.9	39.5	70.3	74.0	66.1	67.8	51.0	78.6	138.0	35.2	67.9	44.5
20	65.1	55.1	46.3	55.2	52.5	71.9	43.0	68.2	74.5	30.4	57.5	31.8
25	47.6	57.7	47.4	45.2	57.0	65.9	35.3	60.5	33.6	24.3	50.1	30.0
30	46.1	50.9	42.2	38.7	35.3	50.9	28.9	40.7	23.4	21.4	46.4	22.5
40	30.9	49.0		26.4	46.9	45.9	31.8	29.8	20.5	13.0	40.5	8.6
50	29.6	43.7	23.2	33.7	46.4	39.4	26.9	21.8	9.1	5.3	37.0	0.0
60	23.3	27.4	16.0	22.1	67.2	41.1	22.2	16.9	7.3	0.0	31.1	7.5
120	6.7	24.1	14.1	0.0	62.9	8.4	0.0	0.0	0.0	0.0	10.8	0.0
240	5.6	13.8	8.6	0.0	14.1	4.4	0.0	0.0	0.0	0.0	9.6	0.0

Values given are following baseline subtraction; baseline values quantified as PG are shown in parentheses.

Table 5.17 Plasma concentration of PG from studies in which rats were dosed with 0.1 x NOAEL.

5.6.13.1 Plasma analyses

PG was readily detectable in the rat plasma samples after dosing at 0.1 x NOAEL, although much lower concentrations of PG are detected after dosing at the NOAEL. It is noticeable that this difference appears greater than the 10-fold difference in dose (see tables 5.14 and 5.17). Based on the data in table 5.17 the concentration time-profile was expected to be very similar to that generated from the NOAEL data; the mean values for male and female rats dosed at 0.1 x NOAEL data are plotted in figure 5.5.

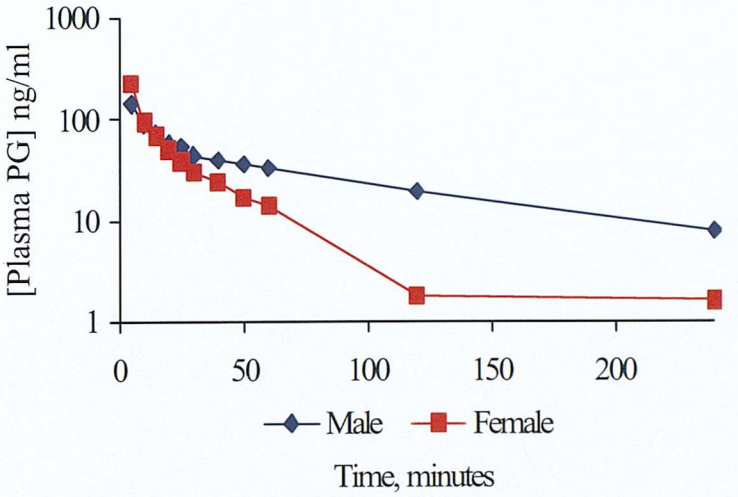


Figure 5.5 Concentration-time profiles for PG generated from mean male and mean female rat data after dosing at 0.1 x NOAEL.

5.6.14 Pharmacokinetic parameters for PG using data from 0.1 x NOAEL studies

Pharmacokinetic parameters were calculated for each individual parameter using the same method as for human ADI data, i.e. baseline subtraction, followed by manual calculation of AUC using the trapezoid formula (CL/F was calculated as dose/AUC). Although the animal data showed clear concentration time-profiles, the data presented are not extrapolated to infinity to allow direct comparison with the human PK data. As for the NOEAL study, the data were extrapolated from 240 to 420 minutes using the terminal half-life for each individual animal.

	Cmax (ng/ml)	Tmax (minutes)	AUC (ng x min/ml)	T 1/2 (minutes)*	CL/F (ml/min)
M1	334	5	6672	75	2023
M2	79	5	8658	174	1559
M3	141	5	6015	191	2244
M4	122	5	3721	26	3628
M5	67	60	13028	71	1036
M6	128	5	6197	60	2179
Male mean	**145**	**14**	**7382**	**99**	**2112**
F1	165	5	3481	38	3879
F2	85	10	3253	24	4150
F3	443	5	5129	16	2632
F4	206	5	2315	9	5832
F5	68	15	6003	92	2249
F6	462	5	3670	18	3679
Female mean	**238**	**8**	**3975**	**33**	**3737**
P value (M vs. F)	0.28	N/C	0.04	0.05	0.03
Overall mean	**192**	**11**	**5678**	**66**	**2924**
Overall SD	143	N/C	2932	60	1339
Overall CoV	**74**	**N/C**	**52**	**91**	**46**

Values in **bold** are the mean data and overall CoV used in later comparisons. P-values compare the male and female data, using unpaired student t-test (2 tails, equal variance)

* T½ is an extrapolated value derived from the mathematical model that the data was fitted to using WinNonlin™

Table 5.18 Pharmacokinetic parameters generated using the data from studies in which rats were dosed with the 0.1 x NOAEL data.

The Cmax values range from 67-~462 ng/ml, whilst Tmax varied between 5 and 60 minutes with a mean of 11 minutes. The sex differences in AUC was statistically significant (a trend had been observed with the NOAEL data), with male rats exposed to a more PG per unit dose. There were two outliers, M5 and F4 that impacted on the mean values and variability for AUC and, particularly CL/F. As for the NOAEL data, T½ values showed no consistent correlation with CL/F, indicating that variability in bioavailability was the source of differences between the individual rats. With the exception of AUC, there is greater variability in the rat data presented in table 5.18 compared to that observed in either the ADI or 10 x ADI data set.

In order to examine the effect of the smaller dose on the kinetics within the rat, table 5.19 was prepared, showing the ratio between the NOAEL and 0.1 x NOAEL pharmacokinetic parameters.

	Cmax (ng/ml)	AUC (ng x min/ml)	CL/F (ml/min)
0.1 x NOAEL Mean	192	5678	2924
SD	143	2932	1339
CoV	74	52	46
NOAEL Mean	4753	97464	2666
SD	3896	77015	2248
CoV	82	79	84
Ratio NOAEL/0.1 x NOAEL	**25**	**17**	**0.91**

Table 5.19 Comparing pharmacokinetic parameters from the two studies in rats.

This comparison shows that differences in concentration-dependent parameters exceed the 10-fold increase in dose. However, CL/F was close to 1, suggesting that little saturation had occurred in the NOAEL study.

5.6.15 The magnitude of the inter-species factor at different dose levels

The inter-species toxicokinetic differences were calculated using all possible combinations of data (see table 5.20). Differences are calculated as shown in chapter 2 except in the cases of dose equivalence, and in the case of CL/F, which is calculated as animal value/human value, without correction for dose.

	I.S factors based on PK parameter means		
	Cmax	AUC	CL/F
ADI/NOAEL	**0.27**	**1.22**	**2.03**
ADI/0.1 x <NOAEL	0.66	2.09	2.23
10 x ADI/NOAEL	0.31	2.05	1.75
10 x ADI/0.1 x <NOAEL	0.77	3.52	1.92

Table 5.20 Inter-species differences for PG based on observed PK values from human and animal studies.

The comparisons in table 5.20 show that differences between animal and human kinetic values, after correction for dose are small. At dose equivalence, the difference in Cmax is close to 1, and all the calculated factors are within a small range, reflecting little saturation in either species at the higher dose. Taken together these differences show that using applying a 4-fold uncertainty factor for extrapolation from rat kinetics (from doses within the range studied) to the likely value for human kinetics will result in underestimation of the true human kinetic capacity to remove PG from the system.

5.7 Discussion

5.7.1 Plasma kinetics from studies in which humans received PG at the ADI.

The LoD (limit of detection) of ~4 ng/ml for the PG assay did not prove to be adequately sensitive to quantify PG detected in the ADI studies. PG was not detected at many of the time points, and together with high CoV values in the samples with concentrations less than 10 ng/ml meant that clear concentration time profiles were not attained from the PG ADI studies.

The lack of clear concentration time profiles meant that analysis of the data with WinNonlin™ was not possible. The observed PK values generally showed relatively limited variability, however there were two volunteers (F18 and F19) who had noticeably lower Tmax values. This suggests possible differences in rate of absorption, although AUC values, and indirectly, bioavailability seem to be unrelated to Tmax. No sex differences were observed and there was no obvious differences in PK parameters related to ethnicity.

5.7.2 Plasma kinetics in which humans received PG at 10 x ADI

Dosing of human volunteers at 10 x ADI resulted in higher plasma concentrations of PG, and most time points had quantifiable concentrations of PG. This allowed more reliable determination of concentration time profiles (see figure 5.3). These profiles showed clear absorption and elimination phases, although interestingly in nearly all cases, "double absorption" peaks were observed. There is evidence (from animal studies) in the literature (see section 5) suggesting that propyl gallate undergoes glucuronidation. It is possible therefore that propyl gallate undergoes enterohepatic re-circulation of the PG-glucuronide. Certainly the PG-glucuronide would be in the correct molecular weight cut-off for biliary excretion (Smith, 1973). However secondary peaks due to enterohepatic circulation usually appear after 8 hours of more. The time courses in figure 5.3 suggest some influence of gastric emptying.

In order to truly ascertain whether or not enterohepatic re-circulation of the PG-glucuronide is occurring, further studies with longer time courses would be present. The results of such studies are not presented in this thesis due to the time constraints of the project. Should such studies be attempted in the future, it may be advisable to use a dose greater than 10 x ADI in order, to allow adequate determination of the terminal elimination slope. Conversely, this may not be necessary should a more sensitive analytical method be established.

There was one obvious outlier in the 10 x ADI PG data. The concentration-time profile for volunteer F21 was very similar to all the others, but the concentrations present in the time points were very low in

comparison to the other data sets. This single data set skews the PK data, so that, (as shown in table 5.8) the CoVs are considerably reduced if F21 is not included in the derivation of the mean and standard deviations. This outlier particularly affects the measured CoV in CL/F. It would desirable to repeat this 10 x ADI study in this particular individual to rule out experimental error (analytical error can be excluded because data for standard curve determined by HPLC concomitantly with the samples were within the normal range). Additionally, one may decide to conduct more PG 10 x ADI studies, to see if there was a significant incidence of outliers. Both of these possibilities were considered, but the time constraints of the project and ethical considerations (contacting volunteers to take part in the study for a second time) prevented either of these options being executed. Once the repeatability and incidence had been defined, it would be possible to investigate possible causes.

Generally there was low variability in the PK parameters (with the exclusion of CL/F, caused solely by F21). In particular low variability in Cmax and Tmax values, suggests relatively uniform absorption of PG dose.

When one compares the ADI and 10 x ADI data, it is clear that proportionally more of the higher dose reached the plasma (reflected in >10-fold increases in Cmax and AUC values). However, CL/F values were fairly constant (r is 1.16) indicating that significant saturation had not occurred at the higher dose of 10 x ADI. In the case of accidental or transient exposure above the ADI, however, one may consider Cmax the most relevant PK parameter for consideration. In this case, one cannot simply extrapolate 10-fold from the ADI value. It is unfortunate that T ½ could not be determined for either the ADI or 10 x ADI studies, since consideration of the difference in Cmax together with the T½ values would give a better insight into the possible increased exposure from a larger dose of PG.

5.7.3 Salivary concentrations of PG as a biomarker of internal dose

The estimated plasma protein binding of PG was 97%, and so analysis of PG in the saliva samples from individuals receiving PG at the ADI dose was not undertaken. The analytical range required to be able to monitor PG in these samples would be in the very low nanogram/hundreds of picogram range. Together with the notion that it is unlikely that the population at large would consume bolus doses of PG at the ADI, a considerably more sensitive analytical method would be required to monitor "normal" intakes

Based on the mean plasma Cmax value of 152 ng/ml from the 10 x ADI experiments, saliva concentrations of PG would be expected to be around 5 ng/ml, which is just on the limit of detection. For the majority of samples analysed, this is the case, although there are some samples in which more or less PG is quantified that one would expect. In most cases with high Cmax values (M28, F20 and F24), there were measurable concentrations in saliva, but PG was not detected in one subject (M27), so that this

apparent trend was not observed consistently. A more sensitive analytical technique or the administration of larger bolus doses would be required to examine the possibility to use saliva as a biomarker of internal exposure of PG.

5.7.4 The adequacy of the inter-individual toxicokinetic default factor (3.16 fold) for PG

The default factor of 3.16-fold adequately protects significant proportions of the population based on the ADI data. This is the case for the high dose data too, except if the variability in CL/F is used to estimate proportions of the population covered, in this instance only 83% of the population would be adequately protected. However, as discussed earlier, Cmax can be considered to be the relevant parameter for consideration at doses above the ADI.

5.7.5 Sources of variability in the human PG data

As outlined in previous results sections, there are 4 main pharmacokinetic processes which one can consider as potential sources of variability; absorption of the compound, distribution of the compound, metabolism of the compound and excretion of the compound. Humans have been shown to express esterases in the liver, plasma, small intestine, brain, stomach, colon, macrophage and monocytes (reviewed by Satoh & Hosokawa, 1998). Human carboxyleterases are generally regarded as having low substrate specificity, and so there are numerous sources of potential variability.

There was no evidence for the existence of an active transport mechanism for PG, since the higher dose did not prolong the absorption phase, and the Cmax was higher that the anticipated 10-fold increase. Bioavailability cannot be estimated from the data presented in this chapter; an i.v. bolus dose would be required, and these experiments were outside the scope of this study. Similarly, distribution of PG in the body could not be determined. The metabolism of PG was determined indirectly via the disappearance of PG from the plasma, and is reflected in the parameter, CL/F. The rate of clearance is due to two variables; the excretion of unchanged PG in the urine, and faeces and secondly, the biotransformation of PG to metabolites. Urine samples were taken from volunteers, but due to time constraints it was not possible to analyse these samples for the parent compound. It may also have been possible to incubate the plasma samples (and possibly urine samples) with glucuronidases to determine the extent of PG glucuronidation in humans. Additionally, analysis of the plasma samples for gallic acid (an analytical method for gallic acid was under validation) would have generated some information to the fate of propyl gallate in humans, an area of investigation that is not extensively reported in the literature.

A single study has investigated the sulphation of PG, in a human liver assay (Bamforth et al., 1993). PG was found to be sulphated but at very low rates, and there has been no mention in the literature relating to

animal studies of a sulphate conjugate of PG. However, PG was shown to inhibit the sulphation of other compounds (estradiol, dopamine, 1-naphthol, estrone and to a lesser extent DHEA (dehydroepiandosterone)).

Using animal data for comparison allows some interpretation of the human data presented here. The animal data (see earlier in this chapter) are also limited, but generally suggest extensive metabolism (presumably by ubiquitously expressed esterases in intestine, plasma and liver); only 9% of an oral dose of octyl gallate was present in the excreta of rats 24 hours after administration of an oral dose (Koss & Koransky, 1982). Several authors report that the major metabolic product of PG is gallic acid, and both PG and gallic acid have been shown to be glucuronidated. The ubiquitous expression of esterases in intestine, plasma and liver, together with overlapping/non-specific substrate specificity is likely to result in the primary biotransformation step (propyl gallate hydrolysis to gallic acid) having a very high capacity. Therefore it is not surprising that saturation is not observed in the 10 x ADI studies.

There are very few accessible data in the literature documenting the extent of human variability in esterase activity. One study (Williams et al., 1989) explored human liver and plasma esterase activity using aspirin as a substrate. Two-fold variation was observed in both liver and plasma esterase activity (determined *in vitro* from wedge biopsy samples and venous blood samples), and interestingly, there was a correlation between individual plasma and liver activities. The results indicated that the esterases in blood had approximately equal hydrolysis capacities, suggesting that subsequent to first-pass metabolism in the liver, the blood may be an important site for aspirin hydrolysis (and possibly for other xenobiotics) in humans. A twofold variability is relatively small, and would support the data presented here, where variability in the PK parameters presented is generally small.

A recent meta-analysis of pharmacokinetic data for esterase substrates (where >60% of metabolism was via esterases) was conducted using data from humans (Dorne et al., 2004). Generally a CoV of 25-30% was observed for PK parameters (including AUC and clearance). The CoVs for the data presented in this chapter (see tables 5.9 and 5.19) are higher than this, this is partly due to the fact that AUC was not extrapolated to infinity and that CL/F results in more variable data, as the extent of absorption cannot be estimated. Dorne et al found no major differences were between healthy adults and either children or the elderly, although clearance and variability in PK parameters was higher in both these groups. Patients with liver disease had lower i.v. clearance compared to healthy adults, although the data were inconsistent across different parameters. The same was true for patients with renal disease, but the variability was reduced. Analysis of data from Chinese subjects suggested that this population would require a higher pathway related factor for compounds metabolised via hydrolysis.

It would have been interesting to determine plasma esterase activity in the volunteers receiving PG. Due

to the time constraints of the study these studies were not performed, but if one could correlate PK parameters to plasma esterase activity, plasma esterase activity could be used as a biomarker of metabolic potential for the gallates.

There is evidence in the literature of polymorphisms of esterase D, a red cell esterase. Three allelic phenotypes exist, EsD 1-1, EsD 2-1 and EsD 2-2. In two separate studies the frequency of these alleles was determined. Masala et al., (1986) reported gene frequencies of 0.883 (EsD 1) and 0.117 (EsD 2) in a Sardinian population. Similar gene frequencies, 0.911 (EsD 1) and 0.089 (EsD2) were observed in a Yugoslavian population (Lemic et al., 1988), and this study correlated phenotype to enzyme activity. The group (n=290) with the EsD 1 phenotype had approximately 2-fold higher esterase activity (using 4-methylumbelliferyl acetate as a substrate) than the group (n=60) with the EsD 2 phenotype. Whilst this evidence points to a specific esterase in particular populations, it is likely that other such polymorphisms exist for other esterases.

There was not a big enough age range in the volunteers that took part in the study to examine the effect of age upon kinetic parameters. Studies using aspirin as a substrate do not support the general notion that metabolism is impaired with age (Williams et al., 1989, Yelland et al., 1991).

Esterases purified from human intestinal mucosa and liver were compared for their relative substrate specificities and enzyme properties. The substrate specificities were found to be very similar, and both esterases were found to be "B-esterases" (according the classification by Aldridge (1953)) because they were inhibited by organophosphates. The molecular weights and isoelectric points of the two enzymes were different, and the intestinal enzyme was more stable. The specific activity of the intestinal enzyme was found to be three times higher than the hepatic esterase.

Williams et al., (1986) studied esterase activity in the plasma of Ghanaian and British subjects. Aspirin esterase, paraoxonase and phenylacetate esterase activities were lower in Ghanaian subjects than those in British subjects, however plasma cholinesterase activity was similar in both groups.

5.7.6 Plasma kinetics which rats were dosed at the NOAEL and 0.1 x NOAEL

Examination of the observed/calculated PK parameters at the NOAEL revealed a statistically significant sex difference in CL/F, with female rats having a more rapid CL/F than the male rats. Similarly the 0.1 x NOAEL data reveal sex differences in AUC and CL/F values. This may suggest that there was a sex difference in a biological process related to elimination (liver blood flow, enzyme activity). There is evidence in the literature that male and female rats express different isoezymes of hepatic carboxylesterases (reviewed by Satoh & Hosakawa, 1998). It is thought that carboxylesterase expression

may be hormone linked as expression has been shown to be reduced in castrated males (and testosterone proprionate reversed this effect). Additionally, the activities of some carboxylesterases were increased in ovariectomized female compared to normal female rats (Satoh and Hosokawa, 1998).

Whilst the ratio for Cmax and AUC between the NOAEL and 0.1 x NOAEL is greater than 10 (more of the higher dose is reaching the systemic circulation), the ratio of close to 1 for CL/F suggests that saturation was not occurring to any extent at the NOAEL.

Interestingly variability in PK parameters varies between the doses, with less variability present in the 0.1 x NOAEL data, despite a greater number of animals per group in the NOAEL studies (8 male and 8 female rats in the NOAEL studies, and 6 male and 6 female rats in the 0.1 x NOAEL studies). There are several possible reasons behind this apparent dose-dependent difference. It is feasible that just by chance the animals in the 0.1 x NOAEL group were more homogenous than those in the NOAEL group, this is supported by the CoV values in table 5.19. It is also possible that there is a dose-dependent shift in underlying kinetic processes (relating to absorption, distribution, metabolism, elimination) resulting in greater variability in the high dose group.

Surprisingly, the variability in the animal PK parameters was greater than that in the human data (tables 5.9 and 5.19). This is unexpected, as generally, animals are considered to more homogenous, and are thought to have a greater metabolic capacity than humans. Possible reasons for these differences in variability include the fact that esterase activity has been demonstrated in the rat in the kidney proximal tubules, small intestine, skin, heart, muscle , blood, lung, testis, nasal and respiratory tissues, adipose tissue, leukocytes and the central nervous system (Kendall et al., 1983, McCracken et al., 1993 and reviewed by Satoh & Hosokawa 1998). It is therefore possible that at the lower dose of 0.1 x NOAEL, a greater proportion of the absorbed dose is hydrolysed by the liver esterases, and a smaller proportion reaches the extrahepatic esterases, resulting in lower variability. Following this hypothesis, at the higher dose, a smaller proportion of the absorbed dose would be hydrolysed by the liver esterases (given a constant hepatic extraction ratio), resulting in a greater proportion of the dose reaching the extra-hepatic esterases, possibly leading to greater variability. Continuing with this hypothesis one would presume that humans have a greater capacity to metabolise propyl gallate in first pass metabolism via the esterases described above.

5.7.7 Species differences in esterase activity

A number of studies have highlighted significant species differences in esterase activity (Walker & Mackness, 1983, Williams, 1985, Leinweber, 1987). The general trend is that rodents tend to metabolise ester-containing drugs much faster than humans. In addition to this complication when extrapolating

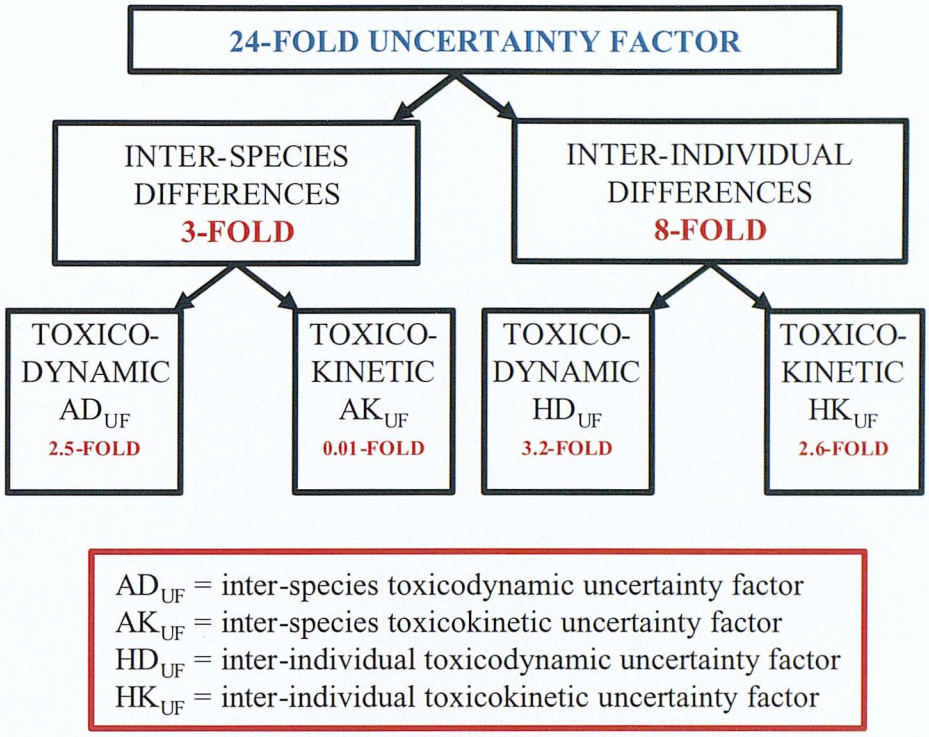
from animal data to humans, there is evidence of different phenotypes within animals. Bimodal distribution of esterase activity has been observed in New Zealand white rabbits (Stampfli & Quon, 1995) and ester hydrolysis rates in the blood showed 70% with fast and 30% with slow esterase activity. These authors did not find bimodal distribution in rats, dogs and humans. Similarly, other polymorphisms have been discovered in the rat and rabbit (Kendall, 1983, van Zutphen et al., 1983 and Bellen et al., 1984). In the rabbit, one locus, with three alleles was responsible for large inter-animal variation resulting in the number of PAGE bands in the Es-1 system ranging from 2-16 (Bellen et al., 1984). Each protein arising from an allele was thought to undergo post-translational modifications giving rise to between 2 and 5 monomeric enzymes which combine to form 1-11 dimers. Interestingly allelic variation in the rabbit Es-4 and Es-5 system was confined to certain organs, indicative of another layer of complexity in the variability of the esterase enzymes (van Zutphen et al., 1983).

5.7.8 The adequacy of the inter-species toxicokinetic default factor (4.0-fold) for PG

The default factor of 4.0-fold adequately covers species differences in kinetics based on all PK parameters at the ADI and NOAEL data. In fact the differences between rats and humans are less than 4.0-fold when comparing any of the data sets, suggesting that species differences are smaller than predicted. This is unexpected given the very high rates of ester hydrolysis reported in rats (see above). In cases of acute exposure (reflected in the differences in Cmax values), the data presented here indicate that humans would still be adequately protected at doses up to 10 x ADI (based on NOAEL data).

5.7.9 Calculating the chemical specific adjustment factor for PG

As explained previously, the aim of the revised IPCS framework (WHO, 1994) is to allow (and encourage) the inclusion of chemical-specific data in the derivation of the ADI. Figure 5.6 below shows the derivation of the safety factor that would result if the inter-species factor using the mean values for AUC (incorporating both sexes for the rats) from the NOAEL and ADI data. There was a significant difference in the AUC values calculated from male and female rat data from the NOAEL studies. Using the female rat data the inter-species factor is 1.7, resulting in an overall composite factor of 35-fold. Using the male data the inter-species factor is 0.95 resulting in a composite uncertainty factor of 19-fold. The factor for inter-individual differences in toxicokinetics is based on covering the 99th percentile of the population, using the CoV for AUC from the ADI data.



NB – the inter-species factor and the inter-individual factors have been rounded to the nearest whole number

Figure 5.6 Substituting data-derived toxicokinetic values into the IPCS framework, resulting in a chemical specific adjustment factor for PG.

The sex difference in rats affects the composite uncertainty factor, and it is likely that the regulatory assessment would be to use the data that gives the largest safety margin (i.e. use the female rat data). It is unlikely however, that the data presented in this chapter would be considered sufficiently robust to use as a basis for the derivation of a chemical-specific adjustment factor. This is mostly due to the quality of the human ADI data, and in particular the one outlying data set that would require explanation and interpretation.

As with the other data sets presented in this thesis the human variability estimates reported in this chapter are likely to be underestimating the true variability in the wider populations. Repeat studies in humans using a more sensitive analytical method would strengthen the database, and possibly allow determination of the T½ at the ADI for PG. This would require a more sensitive analytical method and increased study duration to define the terminal phase. An extended population study would be recommended prior to the replacement of the default 100-fold uncertainty factor.

No data relating to PG intake were found for discussion.

Chapter 6: Studies with TBZ

6. Studies with thiabendazole (TBZ)

6.1 TBZ as a food additive

Thiabendazole (TBZ, see figure 6.1) belongs to a group of antihelminthic compounds (including mebendazole and albendazole) know as the benzimidazoles. TBZ has a broad spectrum of antihelminthic action and was originally introduced as a therapeutic drug for the treatment of roundworm infestations in man and animals (Robinson et al, 1978). In humans it is indicated for the treatment of (threadworm), cutaneous *larva migrans* (creeping eruption) and also kills the larvae of *Trichinella spiralis* and other nematode infections including *Enterobius vermicularis* (pinworm) and *Necator amercicanus* (Council on Drugs, 1968 and Dollery, 1999). Daily doses of 25-50 mg/kg bw/day are used for the treatment of *Strongyloides stercoralis* (British National Formulary 45, 2003).

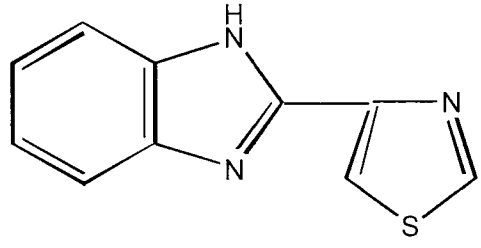


Figure 6.1Chemical structure of thiabendazole

More recently TBZ was found to be effective against a wide range of fungal diseases (mould, rot, blight and stain, storage disease and Dutch elm disease) effecting vegetables, fruits, nuts and other crops and is now used as a food preservative (E233) and an agricultural fungicide (Robinson et al., 1978, Arenas & Johnson, 1995).

TBZ was first evaluated as a pesticide at the 1970 and 1977 Joint FAO/WHO meetings on Pesticide Residues (JMPR) (FAO/WHO, 1971, 1978 cited in WHO, 1997). At the 1977 Joint Meeting, an ADI of 0-0.3 mg/kg bw was established. In the most recent evaluation in 1997, (WHO, 1997) an ADI of 0-0.1 mg/kg was established based on a NOAEL of 10 mg/kg observed in reproductive toxicity and in chronic studies where the critical effect was reduced bodyweight gain.

6.2 The metabolic fate of TBZ

6.2.1 Animal studies with TBZ

Due to its use as a veterinary drug, many of the early ADME studies on TBZ were conducted in farm animals. Using radioactively labelled (^{14}C or ^{35}S) TBZ doses (50-200 mg/kg bw) studies were conducted in calves, pigs, goats and lambs (Tocco, 1964 and Tocco et al., 1965). Absorption was rapid, with maximal plasma concentrations observed within 4-7 hours. Excretion of TBZ was mainly via the urine and was rapid, with elimination more or less complete within 3 days (90% within 48 hours in the case of lambs). Trace amounts of the radiolabel appeared in the milk of lactating animals (<1% in goats, and 0.1% in dairy cows) with maximal concentrations observed within 24 hours. Hydroxylated TBZ (5-OH-TBZ) and its conjugates were the only metabolites detected. Similar kinetic characteristics were observed following oral doses of 44 or 440 mg/kg bw to horses (Hardee et al., 1987). The higher dose resulted in non-linear kinetics, suggesting saturation or end product inhibition by 5-OH TBZ.

Studies have also been conducted in species more frequently used for toxicological studies. In dogs a single oral dose (50 mg/kg bw) of radio-labelled TBZ resulted in maximal plasma concentrations within 2 hours. Excretion was complete within 8 days with 35% of the dose appearing in the urine and 47% appearing in the faeces (Tocco et. al, 1966). Similarly administration of TBZ to rats resulted in rapid absorption (maximal plasma concentrations within 23 hours) and excretion (Tocco et al., 1966 and FAO/WHO, 1971 cited in WHO, 1997). Excretion rates were dose-dependent, with the former study reporting the major excretory product as 5-OH TBZ (67% of the dose within 48 hours). The later study recovered 80-90% of the dose in the urine and faeces within 72 hours, and identified the major metabolites as 5-OH-TBZ glucuronide (50% of the label) and 5-OH-TBZ sulphate (40% of the label) 24 hours after dose administration. Studies in F344/DuCrj rats (Fujitani et al., 1991) , where rats received a single oral dose of 800 mg/kg bw TBZ led to the identification, using mass spectroscopy, of two novel metabolites, 4-hydroxythiabendazole (4-OH TBZ) and 2-acetylbenzimidazole (ABI) (see figure 6.2).

The choice of vehicle has been shown to affect the kinetics of TBZ in pregnant Jcl:ICR mice (Yoneyama et al., 1984 and Tsuchiya et al., 1987). Use of olive oil as a dosing vehicle resulted in more rapid absorption and urinary excretion than in gum arabic oil, but other kinetic parameters were comparable. Most of the radiolabel of a 1300mg/kg dose was recovered in the urine (60%) and faeces (37%) within 7 days (Tsuchiya et al., 1987). Several different metabolites were identified in the 24-hour urine samples; 12-15% as unchanged TBZ, 22-24% as 5-OH-TBZ glucuronide, 30-31% as 5-OH-TBZ sulphate and a small amount of N-methylated TBZ was detected in the urine and faeces (Tsuchiya et al., 1987). Following oral dosing (1000mg/kg bw/day) of pregnant Crj:CD-1 (ICR) mice on day 10 of gestation, TBZ and 5-hydroxythiabendazole and the glucuronide and sulphate of 5-OH TBZ were detected in mouse

urine. Two other metabolites were observed but not identified (Fujitani et al., 1991).

6.2.2 Observations in humans with TBZ

Results of ADME studies in humans are comparable with those in most animal species. After oral administration of 1-2g of radioactively labelled TBZ, absorption was rapid, with maximal plasma concentrations observed within 1-3 hours (Tocco et al., 1996 and FAO-WHO 1971, cited in WHO 1997). TBZ was eliminated rapidly, reaching undetectable levels within 48 hours of the dose in the earlier study, and with 80% of the label excreted within 24 hours in the later study. The majority of the label was excreted in the urine (~90%) and the metabolites were identified as the glucuronide and sulphate esters of 5-OH-TBZ.

A single patient received 25 mg/kg TBZ every 12 hours for 5 days (Bauer et al., 1982). The treatment was administered following cadaveric renal transplant, and the patient was haemodialysed for 4 hours 2 times per week. (1 treatment occurred during TBZ treatment.) The T½ of TBZ was calculated to be 1.2 hours with no sign of accumulation of TBZ in the body, although accumulation of the conjugates of 5-OH-TBZ was observed.

6.2.3 Observed species differences in metabolism/excretion of TBZ

In the studies by Tocco, 1964 and Tocco et al., 1966 there were marked species differences in the extent to which 5-OH conjugation occurred (see table 6.1).

Species	**% of radiolabel appearing in the urine as conjugated 5-OH TBZ within 72 hours**
Human	50
Rat	74
Dog	23

Table 6.1 Species differences in excretion of 5-OH TBZ conjugates following oral dosing of ^{14}C – TBZ

The difference in the relative amounts of 5-OH-TBZ present in the urine of different species may indicate varying capacities for different species to hydroxylate TBZ. Differential urinary concentrations of TBZ between the species would be expected to reflect plasma concentrations of TBZ, and therefore would impact significantly on kinetic parameters. Different kinetic parameters between rats (the test species) and humans would affect the chemical-specific inter-species factor derived from the present studies. Similarly differences in hydroxylation may be a major source contributing to the overall variability in

kinetic parameters observed within humans, thus affecting the chemical specific inter-individual factor.

6.3 Enzymes involved in the hydroxylation of TBZ

The early ADME studies, as described above identified the major metabolic products of TBZ. The monoxygenase class of enzymes (CYP450 enzymes) is known to hydroxylate a wide range of endogenous and xenobiotic compounds (Gibson & Skett, 2001 in "Introduction to Drug Metabolism). The CYP450 "family" consists of several different classes of enzyme, and a number of these enzymes are polymorphic and/or subject to wide ethnic variation (Pacifici et al., 2001). Additionally, UDP-glucuronosyltransferase (UGT) enzymes and sulfotransferases, responsible for the conjugation of 5-OH-TBZ to glucuronide and sulphate moieties exist in several different isoforms (Pacifici et al., 2001). Currently the specific UGT or sulfotransferases involved in the conjugation of 5-OH-TBZ has not been elucidated. However, several *in vivo* and *in vitro* studies have been conducted to and define the cytochrome P450 enzyme(s) responsible for the hydroxylation of TBZ.

Many of the benzimidazoles have been reported to induce CYP1A1 and CYP1A2 in a variety of *in vitro* and *in vivo* situations. Omeprazole has been shown to induce CYP1A1 and CYP1A2 in human hepatocytes (Diaz et al., 1990), whilst albenadzole and oxfendazole induce P450s in rat and rabbit (El Amri et al., 1987 and Gleizes et al., 1991).

The liver from rats pre-treated with the microsomal hydroxylation inhibitors ethyoxyquin or desmethylimipramine was used for TBZ incubations (Wilson et al., 1979). Pre-treatment with desmethylimipramine led to a 45% inhibition of hepatic microsomal hydroxylation of thiabendazole. Pre-treatment with ethoxyquin resulted in a 65% inhibition of hepatic microsomal hydroxylation of thiabendazole. Importantly, these inhibitors show similar affinity for microsomal enzymes as TBZ (unlike SKF-525A that has affinity several orders of magnitude higher).

Using hepatocytes prepared from rabbits dosed with 30, 60 100 or 200μM TBZ 24 hours previously, TBZ was shown to significantly increase P450 activity in general, and specifically CYP450 1A1 and ethoxyresorufin-O-deethylase (EROD) activities (Aix et al., 1994). Interestingly, TBZ did not compete with TCDD for the Ah receptor (competition studies with 3H-TCDD compared to those with the positive control TCDF). The structural properties of TBZ would predict this behaviour; TBZ is not a planar aromatic chemical.

Rey-Grobellet et al., (1996) demonstrated that TBZ increased EROD activity, CYP1A1 &1A2 protein and mRNA levels in a dose-dependent manner in cultured rabbit hepatocytes. Actinomycin D (a transcription inhibitor) had the effect of decreasing protein production of CY1A1 and CYP1A2.

Cycloheximide, (an inhibitor of protein synthesis) had a similar effect. Comparison of these results with results from experiments with a wide range of benzimidazoles show that sulphur is not required to induce CYP1A1 transcription (comparing induction activity of a sulphur free compound, carbendazim with sulphur-containing compounds TBZ and camendazole). However, a substituent at C2 is required for induction potency (benzimidazole, with no C2 substitution is inactive), whilst 5-hydroxylation results in non-inducing derivatives. TBZ was also shown to induce coumarin 7-hydroxylase activity, a monooyxygenase mediated via CYP2A isoenzymes. The precise CYP2A involved was not elucidated, and the molecular mechanism of the induction is not yet understood.

Coulet et al., (1998a) used cultured hepatocytes from rat, rabbit, calf, pig and sheep to demonstrate that the rabbit was the most extensive and rapid metaboliser of TBZ to 5-OH-TBZ. The other species converted TBZ to 5-OH-TBZ at slower rates. Another metabolite produced by all species, M1 was also observed. The sheep was the only species to produce significant amounts of another metabolite M2. Interestingly, 5-OH-TBZ production correlated with protein-bound residue formation, implicating the CYP monooxygenase system in the metabolic activation and subsequent protein binding of TBZ.

The ability to induce 5-OH-TBZ production in cultured rabbit hepatocytes was determined for a number of inducers; ß-napthoflavone (BNF), phenobarbitone (PB), clofibrate (CLO) and rifampicine (RIF) (Coulet et. al 1998b). Only treatment with BNF or CLO resulted in increased hydroxylation. These compounds are known to induce CYP1A1, CYP1A2 and CYP4A. In cultured human cell lines, CYP1A2 expression was required for hydroxylation of TBZ. In addition, a correlation was made between hydroxylase activity and both ethoxyresorufin and methoxyresorufin O-dealkylation (EROD or MROD) activities in rabbit hepatocytes. EROD and MROD are known to be mediated by CYP1A1 and CYP1A2 (Coulet et. al 1998b). Using phenacetin-O-deethylase as a marker reaction, a recent study using rat and human liver microsomes (Li et al., 2003) and recombinant CYP enzymes concluded that CYP1A2 was responsible for the hydroxylation of TBZ. In agreement with this study, Coulet et al., (2000) were able to demonstrate that a CYP1A2 mediated reaction was required to activate [^{14}-C] TBZ in cultured rabbit hepatocytes, enabling the hydroxylated form to bind to microsomal proteins. Using human liver cells (THLE-5), bronchial cells (BEAS-2B) and intestinal (Caco-2) cells the main sites of covalent protein binding were identified as the liver and lung. Low levels of metabolic activation in the intestines would explain relatively low toxicity of TBZ at this site. The addition of ß-napthoflavone (a known CYP1A1/1A2 inducer) had the effect of increasing the rate and extent of TBZ-OH protein binding.

6.4 Conclusions: the metabolic profile of TBZ

In all species studied, TBZ is absorbed rapidly following oral dosing, however is has been observed that pharmacokinetic parameters may be dependent on the vehicle used to administer the dose. The major

metabolites of TBZ have been identified as 5-OH TBZ, and its glucuronide and sulphate conjugates. Other metabolites that have been identified include 4-OH TBZ and ABI in mice. TBZ and its metabolites are rapidly excreted in the urine and faeces (proportions vary between studies) and elimination is complete (~90% excretion) within 2-8 days. There is no evidence in the literature for biliary excretion or enterohepatic recirculation of TBZ. A series of *in vitro* experiments using cultured hepatocytes and human cell lines have shown that inducible CYP1A enzymes are primarily responsible for the hydroxylation of TBZ to 5-OH-TBZ. Constitutive CYP4A may be involved and some studies indicate a possible role for CYP2A.

6.5 Toxicological effects of TBZ

Generally it is accepted that TBZ has low toxicity, and is well tolerated in all species tested (Robinson et al., 1978). It has been extensively studied in 25 animal species including animals used for classical toxicity studies (mouse, rat, dog, rabbit), husbandry animals (cattle , sheep, goats, horses, pigs) as well as less extensively studied species, including zebu, dromedaries, chinchillas, gerbils and antelopes (Robinson et al., 1969). Many of these studies were conducted in the early 1960's and only studies contributing to the JECFA toxicological database will be considered further.

6.5.1 Acute toxicity of TBZ

The acute toxicity of TBZ has been investigated in a number of species. LD_{50} values ranged between 130-3850 mg/kg bw for a variety of animals/routes (see table 6.2).

Species	**Sex**	**Route**	**LD_{50} (mg/kg bw)**
Mouse	M&F	Oral	2400-3810
	M&F	i.p	430
	M&F	i.v	150
Rat	M&F	Oral	3330-3600
	M&F	i.p	1850
	M&F	i.v	130
	M&F	inhalation	>397 mg/m^3
Rabbit	M&F	inhalation	3850 mg/m^3
	M&F	dermal	>2000 mg/m^3
Sheep	NR	drench	2000
Goat	NR	drench	>2000

Table 6.2 The acute toxicity of TBZ (Taken from WHO, 1997) (NR, not recorded).

6.5.2 Carcinogenicity, reproductive and teratogenic studies with TBZ

A large majority of these studies were submitted as unpublished reports to the JMPR 1970 meeting, the

details of the studies and their outcome are reported using the JMPR summaries are presented in table 6.3.

Two studies have reported that TBZ is not carcinogenic to rats or mice. Male mice were fed diets containing 0, 220, 660, 2000 ppm of TBZ and female mice were fed 0, 660, 2000, 5330 ppm TBZ for up to 2 years (Bagdon et al., 1980 (unpublished report cited in WHO, 1997)). Tumour incidences were unrelated to treatment, and the only other significant effects observed were depressed body-weight gain, increased liver weight, and decreased kidney weight. The NOAEL was 660 ppm, or 6 mg/kg bw/day. In a chronic feeding study in rats (Woodard, 1964 (unpublished report cited in WHO, 1997)) the observed toxic effect of reduced bodyweight gain were seen at doses of 40 or 160 mg/kg bw, but not at 10 mg/kg bw day. No increased incidence of tumours was reported. The NOAEL of TBZ in this study was established as 10 mg/kg bw/day.

Studies in rats included the observation that feeding 0.8% TBZ in the diet for 13 weeks led to transitional cell hyperplasia of the urinary bladder epithelium in rats and that TBZ increased the carcinogenic potential of sodium-O-phenylphenate in rat urinary bladder (Fujii et al., 1986 cited in Fujii et al., 1991).

Subsequently Fujii et al., (1991) failed to demonstrate that TBZ is carcinogenic to rats at levels of 4% in the diet for 104 weeks. In contrast to the previous study where hyperplasia of the bladder was observed, hyperplasia of the renal pelvis and papilla in both male and female rats was observed in this study. Due to the low incidence of these effects it was difficult to demonstrate a dose-effect relationship. TBZ was also found to be protective against pituitary and mammary tumours, although a mechanism was not proposed.

Study performed	Species	Duration	Effect at doses above the NOAEL	NOAEL mg/kg bw/day	Reference
Long-term	Mouse	2 years	Liver weight and depressed body weight gain.	6	Bagdon et al. 1980 (cited in WHO, 1997)
Long-term	Rat	2 years	Depression in growth rate	10	Woodard, 1964 (cited in WHO, 1997)
Long-term	Rat	2 years	Preputitial gland adenomas	20	Fujii et al 1991
Reproduction	Mouse	5 generations	Decreased weights of weanlings	30	FAO/WHO 1971 (cited in WHO, 1997)
Reproduction	Rat	3-generation, 2 litter reproduction study (NOAEL for reproduction effects)	Decreased bodyweight and food consumption.	80	Vogin et al, 1968 (cited in WHO, 1997)
Embryotoxicity/ teratogenicity	Mouse	Dosed on gestation day 9.	Depressed maternal weight gain. Reduced foetal weights and malformations.	30	Ogata et al, 1984
Embryotoxicity/ teratogenicity	Rat	Dosed on gestation days 6-17. Dams killed on gestation day 20.	Food intake and bodyweight gain.	10	Lankas and Wise, 1990 (cited in WHO, 1997)
Embryotoxicity/ teratogenicity	Rabbits	Dosed on gestation days 8-16. Does killed on day 29 or 30.	Maternal bodyweight decreased. Resorption rate increased.	100	Peck, 1966 (cited in WHO, 1997)
Embryotoxicity/ teratogenicity	Rabbits	Dosed on gestation days 6-18. Does killed on day 29.	Increased resorption rate and malformations.	24	Hoberman and Lankas, 1990 (cited in WHO, 1997)
Embryotoxicity/ teratogenicity	Rabbits	Dosed on gestation days 6-18. Does killed on day 28.	Food intake and bodyweight gain reduced. Resorption rate increased and malformations.	150	Lankas and Wise, 1990 (cited in WHO, 1997)
Observational study (double-blind placebo study)	Human	24 weeks (NOAEL not established – only one dose level was administered)	None – doses used produced no effects	3-4	Colmore, 1965 (cited in WHO, 1997)

Table 6.3 Summary of toxicological studies conducted with TBZ, detailing main toxicological effects and established NOAELs

Ogata et al (1984) fed groups of pregnant ICR mice TBZ throughout different periods of the gestation. A number of teratogenic effects displayed dose-response relationships. The types of effects observed depended on the timing of exposure, suggesting that TBZ is a non-specific teratogen. The ED_{01} was calculated to be 362 mg/kg bw/day for reduction deformity of the limbs and 26.4 mg/kg bw/day for skeletal malformations. (In an earlier reproduction study teratogenic effects were not observed in rats administered at 250 mg/kg (Khera et al., 1979). Interestingly the recommended maximum daily intake of TBZ as an anthelmintic is 3 g daily, which assuming an average human bodyweight of 60kg is equal to a dose of 50 mg/kg bw/day. According to the results of this study this represents a dose which would be expected to induce skeletal malformations. However, based on the ADI of 0.1 mg/kg bw, there is a 264-fold safety margin for skeletal malformations and a 3620-fold margin of safety for reduction limb deformity.

To determine the teratogenic potential of TBZ and its metabolites in the mouse, pregnant Jcl:ICR mice were treated with SKF-525A or phenobarbital (an inhibitor and an inducer of CYP2B1 CYP2B2 and CYP2C respectively) prior to administration of TBZ (250-1000 mg/kg bw) (Ogata et al., 1987). There was an increased TBZ-induced incidence of reduction-deformed limbs associated with pre-treatment with SKF-525A. Conversely there was an associated decrease in the TBZ-induced incidence of reduction-deformed limbs associated with pre-treatment with PB. Reduction-deformed limbs were only observed at doses greater then 250 mg/kg bw/day (i.e. in the 500 and 1000 mg/kg bw dose groups). This is in agreement with the ED_{01} of 362.0 mg/kg bw/day described by Ogata et al., 1984. These data suggest that TBZ is more toxic (in this assay) than the metabolic products, i.e. TBZ is the proximate teratogen.

The findings of another experiment (Ogata et al. 1989) suggest that TBZ is likely to be the proximate teratogen. Mice were pre-treated with an intraperitoneal injection of either cysteine, reduced glutathione (GSH) or di-ethylmaleate (DM) (a known GSH-depleting substance). One hour later the mice received TBZ at doses of 25, 500 or 1000 mg/kg bw. Pre-treatment with cysteine or GSH lead to increased teratogenicity (measured as a number of parameters, e.g. reproductive parameters, skeletal malformations, and the kinetic profile of TBZ). Pre-treatment with DM resulted in a decreased teratogenic effect of TBZ. *In vitro* studies have shown that cysteine and GSH inhibit the binding of TBZ to liver microsomes *in vitro*, and thus may inhibit the oxidation of TBZ. DM will promote the binding of TBZ to liver microsomes and subsequent metabolism. These findings suggest that TBZ is the proximate teratogen, and that TBZ undergoes metabolic deactivation in liver microsomes. Further evidence that TBZ is the proximate teratogen comes from an assay of cartilage proteoglycan in the limb bud cell culture system. The dose of TBZ and its metabolites to reduce alcian blue staining by 50% was taken as a measure of teratogenic potential of the chemical (see table 6.4). Further *in vitro* studies with 5-OH demonstrated that it is not teratogenic, i.e. TBZ is the proximate teratogen (Tsuchiya et al., 1987).

Substance	EC_{50} value (mM)
TBZ	0.09
5-OH-TBZ	0.09
N-methylated-TBZ	0.24
5-OH-TBZ glucuronide	2.54
5-OH-TBZ-sulphate	3.37

Table 6.4 The relative EC_{50} of TBZ and its metabolites for limb-reduction deformity. Taken from Tsuchiya et al., (1987).

The reproductive effects of TBZ in the rat were investigated (Lankas & Wise, 1990 (unpublished report, cited in WHO, 1997)) using doses of 0, 10, 40, 80 mg/kg bw administered to pregnant rats on days 6-17 of gestation. Doses of 40 or 80 mg/kg bw/day resulted in decreased bodyweight gain and depressed food intake in dams. Ptosis was observed in the 80 mg/kg bw/day group. No increased mortality, abortions or resorption rate was observed at any dose. Foetal abnormalities were not observed in any dose group, but statistically significant decreases in foetal weight were observed at 40 and 80mg/kg bw/day. The NOAEL of TBZ in this study was therefore 10 mg/kg bw/day. This study was used as the pivotal study in the derivation of the current ADI of 0.1 mg/kg. (The NOAEL of 6 mg/kg bw/day that was determined in the 2 year mouse study (see table 5) (Bagdon et al. 1980 (cited in WHO, 1997) was not used to determine the ADI. The next dose (60 mg/kg bw/day) used in this study was considered to large an increment; i.e. the precision of the NOAEL was disputed.

Yoneyama et al., (1984) and Tsuchiya et al., (1987)., investigated the effects of dosing vehicle on pharmacokinetic parameters and maternal-placental transfer in Jcl:ICR mice. TBZ was given as a radiolabelled dose of 1g/kg either olive oil or gum arabic oil on day 9 gestation (Tsuchiya et al., gave 1.3 g/kg on day 11 gestation). The mice were killed at intervals up to 72 hours post-dose. The maximal concentrations measured in maternal plasma and conceptuses were significantly higher in the group dosed with olive oil as the vehicle; the Tmax was earlier in this group. Placental transfer of the radiolabel to embryos (studies were conducted on gestation day 16, so that the embryo could be analysed rather than the entire conceptus) was greater in the olive-oil vehicle group, suggesting that choice of vehicle may alter the quantitative but not qualitative teratogenicity of TBZ. The difference between Cmax values was not as large as that observed in the maternal plasma or conceptuses dosed on day 9, suggesting a placental block to TBZ transfer or differential maternal/foetal protein binding (Tsuchiya et al., 1987).

A two generation feeding study in which mice were fed from the age of 5 weeks in the F_0 generation throughout until age 9 weeks in the F_1 generation revealed several adverse reproductive and neurological effects in the F_1 generation at the highest dose (equivalent to 700-1800 mg/bw/day) (Tanaka et al., 2001). The effects included delayed self-righting, decreased swimming limb movement and decreased litter size and birth weight. Slight dose-related delays in self-righting were observed at doses equivalent to 50 and

180 mg/kg bw. A 500-fold margin of safety still remains between the LOAEL determined in this study and the ADI of 0.1 mg/kg/day.

6.5.3 Nephrotoxicity studies with TBZ

Using male mice depleted of glutathione (GSH) through treatment with DL-buthionine sulphoximine (BSO), an inhibitor of GSH synthesis, Mizutani et al., (1990) showed that TBZ was able to cause kidney damage. The damage was characterised by increased relative kidney weight and increased serum urea nitrogen (SUN) and by extensive tubular necrosis. These effects were prevented when a GSH-ester was given prior to TBZ administration, suggesting that GSH depletion is a major factor in the toxicity of TBZ. It was also found that TBZ required metabolic activation to cause these nephrotoxixc effects, because treatment with inducers of GSH (piperonyl butoxide, methoxalen and carbon disulphide) gave protection against TBZ plus BSO induced damage. The role of CYP450 enzymes was highlighted; treatment with polychorinated biphenyls (PCB), beta napthaflavone (ß-NF) and phenobarbitone (PB) resulted in protection, presumed to be due to increased first pass metabolism. When TBZ was given after BSO, there was further decrease in GSH levels, suggesting that TBZ was generating an active metabolite that reacted with GSH. Addition of TBZ alone had little effects on GSH levels, suggesting that the metabolite is formed slowly enough for GSH to be restored.

Further investigations by Mizutani et al., (1992) revealed that several thiazole and benzimidazole compounds related to TBZ followed similar patterns of nephrotoxicity. In the absence of BSO no nephrotoxicity was observed, and P450 inhibitors prevented toxicity in the presence of BSO. The nephrotoxicity was only observed in male mice. The structural requirements for toxicity were also explored (Mizutani et al., 1993). Unsubstituted thiazole and thiazoles with 4- and/or 5- and no 2- substituents were nephrotoxic, whilst the potency decreased with increasing number and bulk of 4- or 5- substituents. (Thioformamide and tert-butylglyoxal were identified as urinary metabolites in mice dosed with 4-tert-butylthiazole.) The toxic potential was markedly increased by substituents at the 2- position. A scheme was proposed where nephrotoxic thiazoles which lack 2- substituents would undergo microsomal epoxidation of the C-4/5 bond and after being hydrolysed, the resulting epoxide would then form thioformamide, a possibly toxic metabolite (see figure 6.2). Thioformamide was able to increase SUN levels in mice pre-treated with BSO (Mizutani et al., 1996), again this was a male-specific phenomenon, and the signs of nephrotoxicity were identical to those of TBZ and other nephrotoxic thiazoles.

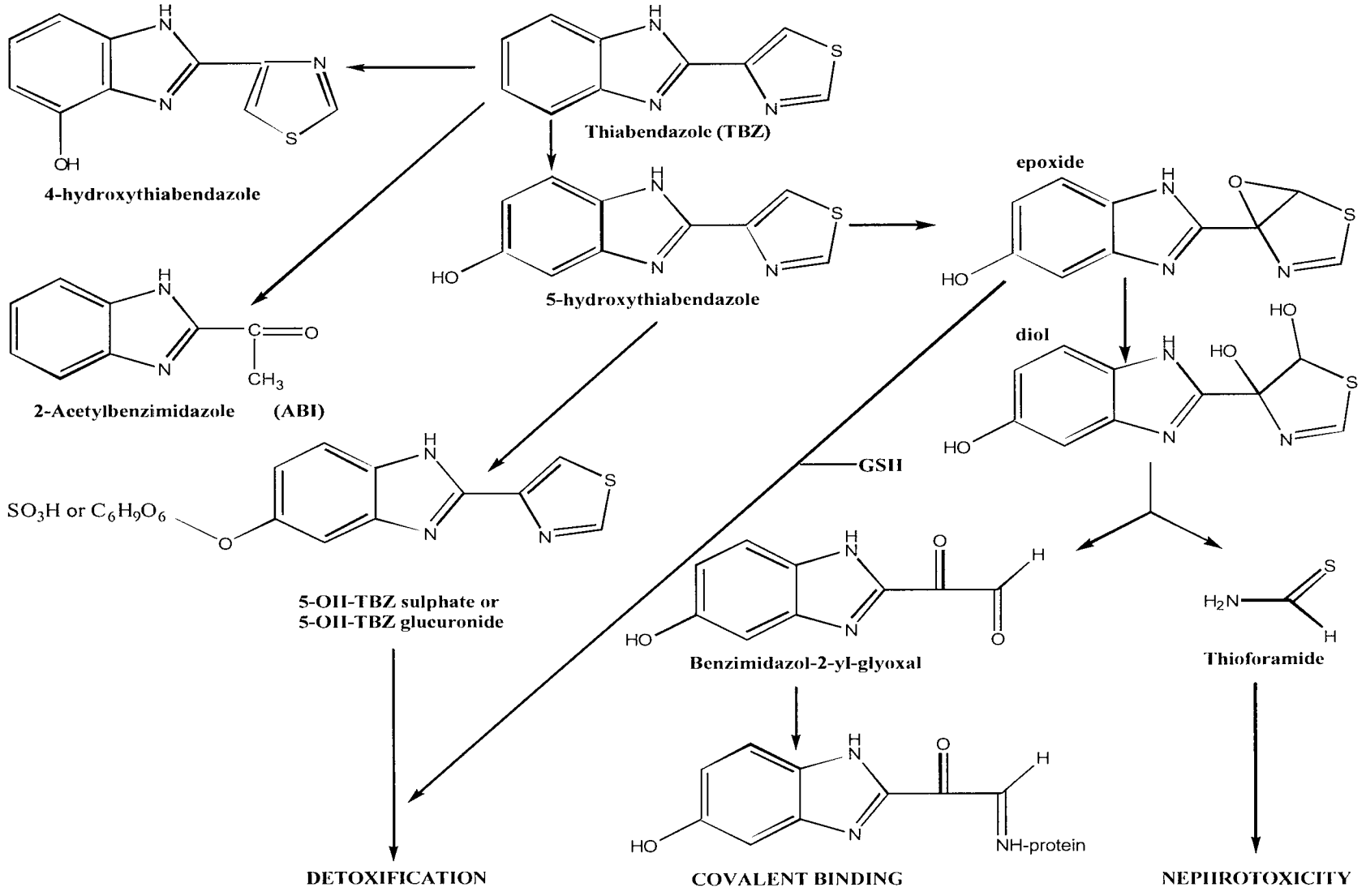


Figure 6.2 Proposed metabolism of TBZ resulting in the production of protein bound and nephrotoxic metabolites

6.5.4 Toxicological observations in humans

Observations in humans documented in the literature are often limited to clinical observations following treatment. Side effects reported after oral doses of TBZ include nausea, vomiting, dizziness and drowsiness. All effects were noted to be transient (Franz, 1965). The adverse effects of 50 mg/day-3 g/day TBZ administered to 23 patients with trichinosis for up to 10 days included exanthema, impotence, diarrhoea, liver damage, fever and dizziness (Hennekeuser, 1969 cited in WHO, 1997). Other side effects reported following treatment with TBZ include anorexia, nausea and vomiting, abdominal discomfort, diarrhoea, dizziness, occasional hypotension, neurotoxicity, leukopenia and elevated SGOT serum (glutamate oxaloacetate transanimase) levels. It is reported that these effects were dose-related, and were usually seen after prolonged administration (Schumaker et al., 1978). Since the doses that are given in the present experiment were much lower than the therapeutic dose, side effects to TBZ were not anticipated

A 24-week double-blind, placebo-controlled study where 100 male volunteers were given a placebo or 250 mg/day TBZ was conducted (Colmore, 1965; cited in WHO, 1997). As well as a general physical examination, measurements of cholesterol, glucose, urea, alkaline phosphatase, thymol turbidity, bilirubin measurements were made at 0, 12 and 24 weeks. In addition protein-bound iodine (PBI) in serum was determined and an ECG (electrocardiogram) was conducted at 0 and 24 weeks. No adverse effects were seen in any of the parameters studied at this dose.

6.6 The most recent determination of a NOAEL for TBZ

The outcomes of the Lankas & Wise study, (cited WHO, 1997) (statistically significant decreases in foetal weight) were used to derive the NOAEL for TBZ of 10 mg/kg bw/day. A 100-fold uncertainty factor was applied to this, resulting in an ADI of 0.1 mg/k bw/day (WHO, 1997).

6.7 Clinical studies with TBZ

6.7.1 Clinical procedure

The materials and methods used in the clinical studies with TBZ are given in chapter 2 (materials and methods).

6.7.2 Dose selection and safety

As discussed in the introduction, one of the aims of this project was to compare human and animal kinetic parameters derived from studies where TBZ was administered at the no-effect level (the ADI for humans and the NOAEL for animals). These doses are 0.1 mg/kg bw/day and 10 mg/kg bw/day respectively. A secondary aim of the project was to investigate linearity of the kinetic parameters. Doses equivalent to 10 x ADI and 0.1 x NOAEL were administered to humans and animals respectively. In order to gain ethical permission for these studies, the acute reference dose (ArfD) was estimated for TBZ. In cases of acute exposures to a hazardous chemical, the ArfD is estimated based on short-term animal toxicology studies. The ArfD is an estimate of a safe acute exposure, and therefore is normally calculated from no-effect level demonstrated in acute animal toxicity testing. Since our human volunteers in the high dose studies were effectively being exposed to the food additives in much larger quantities, a review of all available acute toxicity data to assess the margin of safety between the ARfD in the animal database and the 10 x ADI dose. Table 6 lists all the currently available acute toxicity data for TBZ.

Based on the short-term toxicity data presented in table 6.5, the NOAEL for short-term effects for TBZ would be estimated to be >25 mg/kg/day or >1750 mg/person/day. Comparing this with the intended single dose for this study (70 mg/person) gives a margin of safety of >25.

Study performed	Species	Duration	NOAEL (mg/kg bw/day)	Equivalent human intake (mg/day)	Reference**
Short-term toxicity study	**Rat**	**13 weeks**	**25**	**1,750**	**Kangas et al., 1989**
Short-term toxicity study (*)	Rat	13 weeks	9	630	Myers and Lankas, 1990
Short-term toxicity study	Rat	180 days	25	1,750	FAO-WHO, 1971; Robinson, 1964
Short-term toxicity study	Dogs	14 weeks	35	2,450	Batham et al., 1989
Short-term toxicity study	Dogs	2 years	20	1,400	FAO-WHO, 1971; Robinson, 1964
Short-term toxicity study	Dogs	2 years	20	1,400	FAO-WHO, 1971; Woodard, 1964
Short-term toxicity study	Sheep	16 weeks	10	700	FAO/WHO, 1971
Long-term toxicity study	Mouse	2 years	6	420	Bagdon et al. 1980
Long-term toxicity study	Rat	2 years	10	700	FAO/WHO 1971 Woodard, 1964

*Decreased body-weight gain and food intake was observed at the next highest dose (37 mg/kg bodyweight, equivalent to 2,590 mg/day in humans). There is some evidence to suggest that palatability issues rather than compound toxicity produce these effects.

**All references cited in WHO, 1997

Table 6.5 A summary of all short-term animal toxicity data available forTBZ. The study highlighted in bold is the study that would be considered relevant for derivation of the ArfD

6.8 Results

6.8.1 Studies in which human volunteers received TBZ at the ADI.

6.8.1.1 Plasma analyses

A total of 19 volunteers were given ADI at a dose of 0.1mg/kg. From these 19 data sets, 11 were used for comparison of pharmacokinetic parameters. This was due to difficulties experienced in the analysis of the sample sets, mostly due to poor coefficient of variation values between triplicate time point samples, where very little TBZ was detected. Details of the volunteers are given in table 6.6. The mean age of the 11 volunteers in the data set was 28 years old, (30 years old for males and 26 years old for females). The mean weight of the male volunteers was 80 kg and for the female volunteers was 62kg.

	Age	Weight	Ethnicity
M29	24	70	Caucasian
M30	47	80	Chinese
M31	33	92	Caucasian (Greek Cypriot)
M14	29	89	Caucasian
M32	22	73	Caucasian
M33	26	77	Caucasian
Male Mean	**30**	**80**	**N/C**
F25	20	65	Caucasian
F26	19	69	Caucasian
F27	21	50	Caucasian/Black Carribean
F28	45	63	Caucasian
F29	23	62	Caucasian (Hungarian)
Female Mean	**26**	**62**	**N/C**
Overall Mean	**28**	**72**	**N/C**

Table 6.6 Details of volunteers who received thiabendazole at the ADI.

Table 6.7 shows the concentrations of TBZ in plasma, expressed as pg/ml detected at each time point for each individual included in the pharmacokinetic comparisons. The red bold typeface denotes concentrations that were below the limit of quantification for the method. Values given are following baseline subtraction; baseline values quantified as TBZ are shown in parentheses at time zero.

The plasma concentrations shown in table 6.7 show that TBZ concentrations at many time points was below the limit of quantification for the method of analysis employed. Individual concentration-time profiles were rarely detected, and they are not plotted graphically.

	Concentration of TBZ in plasma samples, pg/ml										
Time	Male Data						Female Data				
(minutes)	M1	M2	M3	M4	M5	M6	F1	F2	F3	F4	F5
0	0 (259)	0 (78)	0 (156)	0 (106)	0 (0)	0 (116)	0 (202)	0 (0)	0 (255)	0 (116)	0 (52)
10	59						51				
15		4	0	0	**764**			0	48		
20	17						118				
30	170	22	0	18	**836**	9	111	0	4	53	13
45	103						**394**				
60	88	77	**289**	**446**	**382**	158	**397**	0		119	144
90	203	121	218	187	**299**	**501**	**774**	69	59	173	105
120	208	183	**359**	178	**302**	**495**	**1121**	**438**	20	213	204
150		146	241	136	201	**471**	**1600**	**944**	243	136	140
180	157	114		95	0	207	**1677**	**644**	**356**	76	104
210		23	210	53	0	61		**273**	**614**	177	260
240	95	11	0	24	0	39	234	127	168	57	72
300	33	0	0	0	0	4	222	74		0	67
360	40	0	0	0	0	0	177	39		8	97
420	44	0	0		0	27	**264**	47		0	46

Values given are following baseline subtraction; baseline values quantified as TBZ are shown in parentheses.

Figures in **red text** denote concentrations above the limit of detection.

Table 6.7 Plasma concentrations of TBZ from studies in which volunteers received the ADI.

6.8.2 Pharmacokinetic parameters for TBZ calculated using the data from the ADI studies

As discussed in the previous section, concentration-time curves were not observed in the case of most individuals. Therefore the kinetic model (WinNonlin™, described in chapter 2), could not be used to estimate a value for terminal half-life or to estimate the terminal phase pharmacokinetic values for AUC to infinity or CL/F. The pharmacokinetic parameters available for comparison therefore comprise of Cmax, Tmax, AUC (calculated using the trapezoid formula from observed values) and CL/F (calculated as dose/AUC). These parameters are shown below in table 6.8.

	Cmax (pg/ml)	Tmax (minutes)	AUC (pgxmin/ml)	Clearance (dose/AUC)
M29	208	120	42668	2344
M30	183	120	20949	4773
M31	359	120	46271	2161
M13	446	60	34351	2911
M32	836	30	65796	1520
M33	501	90	59828	1671
Male Mean	**422**	**90**	**44977**	**2563**
F25	1677	180	244132	410
F26	944	150	84933	1177
F27	614	215	43080	2321
F28	213	120	31455	3179
F29	260	210	43564	2295
Female Mean	**742**	**175**	**89433**	**1877**
P value (M vs. F)	0.26	0.0058	0.26	0.35
Overall Mean	**567**	**129**	**65184**	**2251**
Overall SD	448	58	61893	1144
Overall CoV	**79**	**45**	**95**	**51**

Values in **bold** are the mean data and overall CoV used in later comparisons. P-values compare the male and female data, using unpaired student t-test (2 tails, equal variance)

Table 6.8 Pharmacokinetic parameters for TBZ generated from using the data from studies in which volunteers received the ADI.

There were four volunteers with noticeably higher Cmax values, M32, M33, F25 and F26. This seems to be unrelated to rate of absorption, as the Tmax ranged from 30 minutes to 180 minutes in these subjects. The overall mean Tmax mean is a little over 2 hours, suggesting fairly rapid absorption. The AUC and CL/F values are fairly uniform, with the exception of F25 who was an outlier for these two parameters. CL/F would normally be calculated/extrapolated to infinity, but these values are calculated on observed values and therefore these results for CL/F are approximate values.

A Student's t-test was performed (using 2 tails, equal variance) to investigate any sex differences in the pharmacokinetic parameters. The only significant difference observed was between the Tmax values ($p=0.0058$), which would suggest that the male volunteers absorbed the dose more rapidly than the female volunteers.

6.8.3 Studies in which human volunteers received TBZ at 10 x ADI.

6.8.3.1 Plasma analyses

A total of 10 volunteers were given TBZ at a dose of 1mg/kg (equal to 10 x the ADI). From these 10 data sets, 9 were used (5 male data sets and 4 female sets, see table 6.9) for comparison of pharmacokinetic parameters. This was due to one sample set (from a female volunteer) accidentally being left out of the freezer for an indeterminable length of time. Unlike the ADI studies (see earlier), TBZ was measured more reliably in these samples. The mean age of the 10 volunteers in the data set was 25 years old, (28 years old for males and 22 years old for females). The mean weight of the male volunteers was 73 kg and for the female volunteers was 64 kg.

	Age	Weight	Ethnicity
M34	50	70	Caucasian
M35	24	69	Caucasian
M36	19	79	Caucasian
M37	26	67	Malaysian
M18	21	81	Caucasian
Male Mean	**28**	**73**	**N/C**
F30	27	57	South asian
F31	24	65	Caucasian
F32	19	63	Caucasian
F26	20	67	Caucasian
F33	19	68	Caucasian
Female Mean	**22**	**64**	**N/C**
Overall Mean	**25**	**68**	**N/C**

Table 6.9 Details of volunteers who received TBZ at 10 x ADI.

Table 6.10 shows the concentrations of TBZ, expressed as ng/ml (not pg/ml) detected at each time point for each individual included in the pharmacokinetic comparisons. The vast majority of plasma concentrations shown in table 6.10 were above the limit of quantification for the method of analysis employed. Individual time-concentration profiles were observed, and so these are plotted graphically in figure 6.3.

The graphs show clear concentration-time profiles, with similar characteristics. The Cmax values ranged from ~20 ng/ml- ~300 ng/ml. The Tmax values are very similar for male and female subjects. All subjects showed a return to baseline (or very close to baseline) within the time course of the study.

	Concentration of TBZ is plasma samples (**ng**/ml)								
Time	Male Data					Female Data			
(minutes)	M33	M34	M35	M36	M17	F31	F32	F33	F34
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30	0.0	0.0	0.4	0.6	0.0	0.1	0.0	0.2	3.4
60	19.6	0.1	0.9	0.6	18.5	0.5	34.5	7.3	4.6
90	20.5	0.1	1.6	0.5	5.5	16.7	303.3	50.9	79.2
120	26.5	0.3	51.9	356.0	3.9	80.4	201.8	17.3	186.5
150	12.4	133.5	32.2	315.3	1.9	26.3	144.7	21.1	283.8
180	9.6		20.0	157.4	1.3	21.4	105.0	11.4	303.6
210	5.4	55.2		50.5	0.0	12.8	46.2	5.1	214.8
240	1.3	22.1	4.6	34.6	0.0	5.8	23.1	3.0	85.9
300	0.4	12.2	2.5	18.8	0.0	3.0	15.3	1.8	44.2
360	0.0	5.9	1.5	9.7	0.0	1.7	6.5	1.0	27.7
420	0.0	3.6		6.4	0.0	1.2			28.7

Table 6.10 Plasma concentrations of TBZ from studies in which volunteers received 10 x ADI. Spaces in the table indicate that a sample was not taken for analysis.

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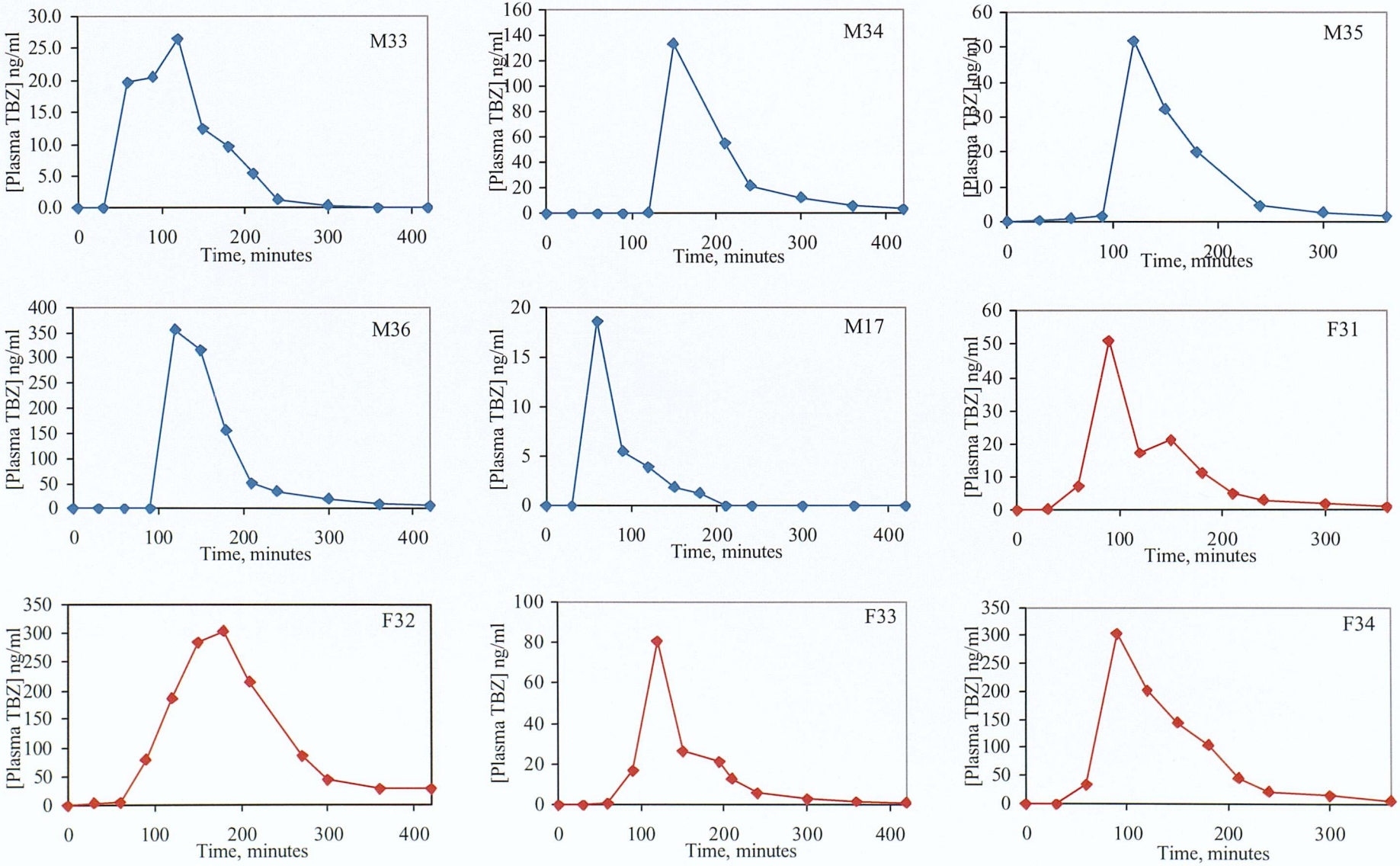


Figure 6.3 Concentration-time profiles for TBZ in male (blue graphs) and female (red graphs) volunteers after dosing at 10 x ADI.

6.8.4 Pharmacokinetic parameters for TBZ calculated using the data from the 10 x ADI studies

Two pharmacokinetic analyses of the data were undertaken. In the first instance, observed values for Cmax, AUC (calculated using the trapezoid formula from observed values) and CL/F (calculated at dose/AUC) were reported (see table 6.11). These data were calculated using the methods applied to the ADI study so that the results were directly comparable with the pharmacokinetic parameters calculated from the ADI studies. The majority, but not all of the concentration-profiles plotted from studies at 10 x ADI allowed the terminal elimination phase to be identified. The mathematical modelling system described in chapter 2, WinNonlin™ (using a 1/y weighting) was used to calculate the terminal half-life and parameters extrapolated to infinity (see table 6.11).

	Observed values				Parameters extrapolated to infinity			
	Cmax (ng/ml)	Tmax (minute)	AUC (**ng** x min/ml)	CL/F (ml/min)	AUC (**ng** x min/ml)	T1/2 (minutes)	?z values used to determine T1/2	CL/F (ml/min)
M33	27	120	3071	326	2907	29	180-300	344
M34	134	150	10835	92	11054	69	240-420	90
M35	52	120	4178	239	4349	77	240-360	230
M36	356	120	29885	33	30562	73	210-420	33
M17	19	60	935	1069	991	42	90-180	1011
Male mean	**117**	**114**	**9781**	**352**	**9973**	**58**	**N/C**	**342**
F31	51	90	3679	272	3780	75	240-360	265
F32	304	180	41394	24				
F33	80	120	5321	188	5459	82	240-420	183
F34	303	90	27216	37				
Female mean	**185**	**120**	**19403**	**130**	**4619**	**79**	**N/C**	**224**
P value (M vs. F)	0.50	0.82	0.37	0.34	0.58	N/C	N/C	0.71
Overall Mean	**147**	**117**	**14057**	**253**	**8443**	**64**	**N/C**	**308**
Overall SD	135	N/C	14815	326	10244	20	N/C	327
Overall CoV	**92**	**N/C**	**105**	**129**	**121**	**32**	**N/C**	**106**

Values in **red bold** are the mean data and overall CoV used in later comparisons. P-values compare the male and female data, using unpaired student t-test (2 tails, equal variance)

Table 6.11 Pharmacokinetic parameters (observed and extrapolated to infinity using WinNonlin ™) for TBZ generated using the data from studies in which volunteers received 10 x ADI plasma data. For volunteers F32 and F34 WinNonlin™ could not fit the data to allow extrapolation of these data to infinity.

No significant sex differences were observed for any of the pharmacokinetic parameters. There was a 10-fold range of values for Cmax, but Tmax values were all very similar at around 2 hours. There were a few outliers in the group, contributing to the wide-ranging AUC and CL values observed. The coefficient of variation for Cmax, AUC and CL were large, all around or above 90%.

There was not a large difference between the AUC values (and therefore the CL/F values) reported as observed or extrapolated values in table 6.11, i.e. the use of the kinetic model and extrapolation to infinity did not alter parameters significantly. This is primarily due to the fact that in most cases, the concentrations of TBZ in the plasma had returned to baseline within the time-course of the study, and so the mathematical model had little temporal extrapolation to do. These observations validate the use of the overall mean pharmacokinetic parameter values in table 6.11 for inter-individual and inter-species comparisons (see later in this chapter). The terminal half-life T½ is the parameter with the least variability, with a range of 25-77 minutes. The mean value of 63 minutes suggests rapid elimination of the compound, and whilst the AUC and CL/F values vary considerably, T½ stays more constant. This suggests large differences in the bioavailability of the TBZ in individuals, i.e. some individuals are absorbing more or less of the dose. The mean T½ value reported in table 6.11 of 64 minutes was similar to that reported by Bauer et al., 1982, of 1.2 hours, where volunteers received a much higher dose (25mg/kg).

6.8.5 Comparing plasma kinetics for TBZ in humans at two doses.

Assuming linear kinetics, a 10-fold increase in concentration dependent parameters was anticipated in volunteers receiving 10 x ADI (i.e. Cmax and AUC). CL/F however, would be expected to remain the same, as a concentration-independent parameter. Table 6.12 compares the kinetic parameters calculated from observed values as a ratio (10xADI/ADI).

	Cmax (ng/ml)	Tmax (minutes)	AUC (ng x ml/min)	CL/F (ml/min)
Mean ADI Value	0.57	129	65	2251
SD	0.45	58	62	1144
CoV	79	45	95	51
Mean 10 x ADI Value	147	117	14057	253
SD	135	N/C	14815	326
CoV	92	N/C	105	129
Ratio 10xADI/ADI	**259**	**N/C**	**216**	**0.11**
P value	0.05	N/C	<0.01	<0.01

Table 6.12 Comparing pharmacokinetic parameters for TBZ generated from the two human studies.

Taken collectively, the ratios in table 6.12 suggest that the pharmacokinetics are non-linear between the ADI dose and the 10 x ADI dose. Instead of 10-fold increases in Cmax and AUC, 259-fold and 216-fold differences were observed. Tmax did not change significantly, suggesting that absorption was not impaired in either group. Most interestingly, the CL/F ratio value, which would be ~1 for linear kinetics, was calculated as 0.11-fold, which taking the inverse means that CL/F was 8.88-fold lower than in the

high dose group compared to that in the low dose group. This result suggests that a biological process is becoming saturated, resulting in dose-dependent absorption or clearance (see discussion of this chapter).

6.8.6 Salivary concentrations results from studies in which human volunteers received TBZ at 10 x ADI.

One of the aims of this project was to evaluate the possibility of using saliva as an alternative method of determining the internal exposure of a food additive. TBZ was present in the plasma as two forms, either bound to plasma proteins (specifically to albumin), or free in the plasma as unbound chemical. In contrast, saliva, which does not contain the same proteins as the plasma, would contain only the unbound (free) TBZ. Due to the very low concentrations of TBZ observed in the ADI studies the corresponding saliva samples were not analysed. Prior to analysing the saliva samples taken in the 10 x ADI studies, the extent to which TBZ was protein-bound was determined in protein-binding studies. This meant that analysis of the saliva samples only commenced once the feasibility of detecting TBZ in the saliva samples was determined.

6.8.6.1 Protein-binding studies with TBZ

Protein-binding studies were conducted to determine the level of plasma-protein binding of TBZ. In experiment I, the Ringers' solution was spiked with TBZ, whilst in experiment 2, the albumin was spiked with TBZ. The appropriate controls were analysed simultaneous to the samples; where necessary, baseline subtraction of contaminating peaks in clean Ringers' or clean albumin was executed. The results of studies with TBZ (see table 6.13) revealed that TBZ was approximately 50% protein-bound.

	Ringers	Albumin	% Protein-binding
Experiment 1 Ratio TBZ/MPBZ, n= 4	0.025 ± 0.0016	0.071 ± 0.0043	**48%**
Experiment 2 Ratio TBZ/MPBZ, n= 5	0.444 ± 0.046	0.896 ± 0.19	**49%**

Table 6.13 Results of an experiment to determine the percentage protein binding of PG.

Because the estimated extent of protein-binding of TBZ was ~50%, it was anticipated that detectable concentrations of TBZ would be present in the saliva samples from volunteers who received the higher dose of TBZ, (1mg/kg TBZ). The salivary concentrations of TBZ were determined for 6 individuals, the first 3 male and first 3 female volunteers to participate in the study. Table 6.14 shows the concentrations of TBZ in saliva expressed as ng/ml detected at each time point in all analyses.

	Concentration of TBZ, ng/ml					
Time	Male Data			Female Data		
(mintues)	M33	M34	M35	F31	F32	F33
0	0.00	0.00	0.00	0.00	0.00	0.00
30	1.48	**0.07**	0.46	**0.06**	0.41	0.00
60	1.64	0.13	0.14	1.46	0.81	0.00
90	1.62	**0.05**	0.14	4.88	10.20	0.00
120	1.04	**0.07**	5.45	2.87	19.76	4.44
150	1.01	6.87	1.47	1.41	22.22	0.47
180	0.71	4.67	1.28	1.07	14.41	0.84
210	0.55	1.21		0.57	6.03	0.00
240	0.17	4.67	0.49	0.57	7.12	0.00
300	0.20	0.47	**0.10**	0.38	4.58	0.00
360	**0.09**	0.22	0.70	0.21	2.10	0.74
420	0.03	**0.08**			1.48	0.00

Time points below the limit of quantification are shown in **red typeface**.

Table 6.14 Salivary concentrations of TBZ from studies in which volunteers received TBZ at 10 x ADI.

The concentrations in saliva are shown in table 6.14. The concentrations at the vast majority of time points were above the limit of quantification for the method of analysis employed. Individual concentration-time profiles were observed, and so these are plotted graphically. Figures 6.4 and 6.5 show the male and female concentration time curves respectively.

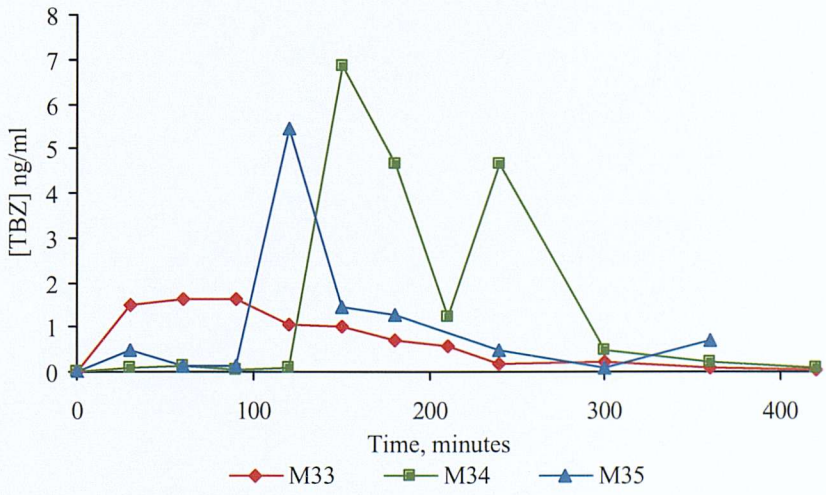


Figure 6.4 Salivary concentration-time profiles for TBZ in male volunteers after dosing at 10 x ADI

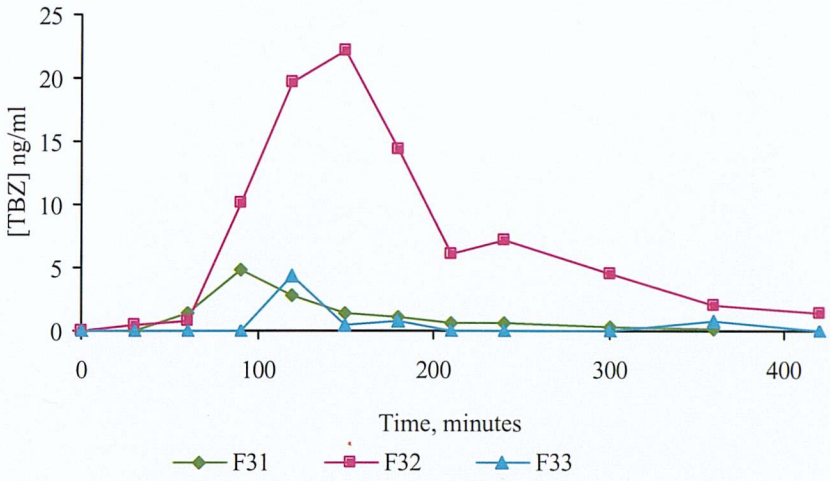


Figure 6.5 Salivary concentration-time profiles forTBZ in female volunteers after dosing at 10 x ADI.

Plasma and saliva concentration-time curves were compared for individuals where both analyses were undertaken. These are shown collectively as figure 6.6. Clearly most of the saliva data reflect the plasma profile of TBZ absorption and elimination. However, the salivary Cmax values are clearly not 50% of the plasma Cmax values and the slope of the elimination phase for saliva and the slope of the plasma elimination phase vary in the same individuals.

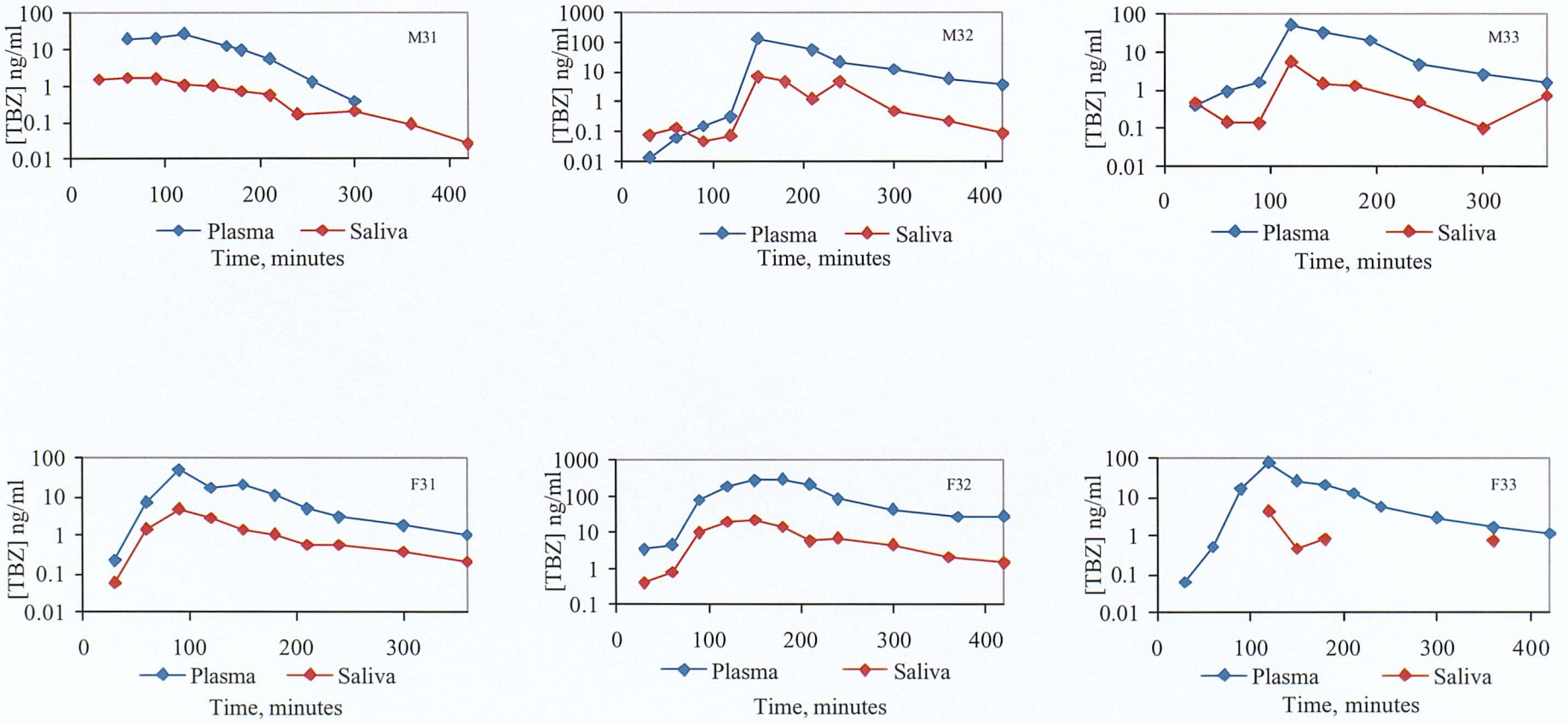


Figure 6.6 Comparing plasma and saliva concentration-time curves for male and female volunteers, following dosing at 10 x ADI.

NB – The saliva profile for F33 is incomplete because after baseline subtraction, the majority of timepoints contained no detectable TBZ. (See table 6.14.)

6.8.7 Pharmacokinetic parameters for TBZ calculated using the saliva data from 10 x ADI studies

Although concentration time-curves were observed in most data sets, it was not possible to identify the terminal elimination phase in the majority of cases. Therefore the kinetic model (WinNonlin™, described in chapter 2), could not be used to estimate the terminal phase pharmacokinetic values. The pharmacokinetic parameters together with a comparison of plasma and salivary AUCs are shown in table 6.15. Figure 6.6 depicts the plasma and salivary concentration-time profiles for the 6 individuals.

	Cmax (ng/ml)	Tmax (minutes)	AUC (ng*min/L)	AUC Saliva/ AUC Plasma	CL/F (ml/min)
M31	1.64	60	268	0.09	3732
M32	6.87	150	646	0.06	1548
M33	5.45	120	298	0.07	3356
Male mean	**4.65**	**110**	**404**	**0.08**	**2879**
F31	4.88	90	425	0.12	2353
F32	22.22	150	2980	0.07	336
F33	4.44	120	217	0.04	4608
Female mean	**10.51**	**120**	**1207**	**0.08**	**2432**
P value (M vs. F)	0.388	0.768	0.421	0.989	0.767
Overall mean	**7.58**	**115**	**806**	**0.08**	**2655**
Overall SD	7.37	35	1076	0.03	1560
Overall CoV	**97**	**N/C**	**134**	**34**	**59**

Values in **bold** are the mean data and overall CoV used in later comparisons. P-values compare the male and female data, using unpaired student t-test (2 tails, equal variance)

Table 6.15 Pharmacokinetic parameters (observed values) for TBZ generated from studies in which volunteers received 10 x ADI.

No significant sex differences were observed for any of the pharmacokinetic parameters. There was an approximate 20-fold range of values for Cmax, but Tmax values were all very similar, and were consistent with the plasma Tmax at around 2 hours. As previously described there were a few outliers (F32 and F33) in the group, contributing to the wide-ranging AUC and CL values observed. The coefficient of variation for Cmax, AUC and CL were large, all around or above 90%, which again was similar to the variation observed in the plasma data. The ratio between the observed AUC for saliva and those calculated from the corresponding plasma samples was determined. The ratios are reasonably constant, but were <0.1, while a value of 0.5 would be expected based on the *in vitro* binding study. This suggests that saliva samples do not represent a robust reliable method of determining pharmacokinetic

parameters. However it is clear from figure 6.6 that the concentration-time curves in the plasma adequately reflect the profiles in the plasma. The Cmax and AUC values for saliva are clearly not 50% of those for plasma, and the slope of the elimination curve appears to differ between plasma and saliva in the same subject.

6.8.8 Comparing plasma and salivary kinetics at 10 x ADI

Based on protein binding of 50%, one would have expected that the concentration of TBZ in saliva would be one half of that observed in the plasma. Table 6.16 compares the kinetic parameters calculated from observed kinetic value in the plasma with that in the saliva as a percentage ([Saliva]/[Plasma] x 100).

	Cmax (ng/ml)	Tmax (minutes)	AUC (ng x min/ml)
Saliva Mean	7.6	115	806
Plasma Mean	147	117	14057
Saliva as a % of plasma value	**5.2**	**N/C**	**5.7**

Table 6.16 Comparison of plasma and saliva pharmacokinetic parameters for TBZ.

Significantly less TBZ was measured in the saliva compared to the plasma. The ratios for Cmax and AUC suggest approximately 95% protein-binding of TBZ. The Tmax values were essentially identical, suggesting rapid equilibrium between plasma and saliva. Following the analyses of the saliva samples, and the apparent contradiction between the salivary concentrations detected, the corresponding plasma concentrations, based on protein binding of ~50%, an experiment was conducted to determine if there was low recovery of TBZ from saliva once it had passed through a dental roll. The experimental protocol is detailed in chapter 2. The results, shown in table 6.17., clearly show that TBZ is being retained on the dental roll during the preliminary extraction process.

	Mean TBZ/MPBZ ratio
Unspiked Saliva n=5	0.000835 ± 0.000966
Spiked Saliva - **dental roll** n=5	0.261 ± 0.0190
Spiked Saliva + **dental roll** n=5	0.116 ± 0.00677
% TBZ recovered from dental roll	**44**

Table 6.17 Recovery of TBZ from the dental roll used for saliva collection.

Approximately 60% of the TBZ in the saliva sample was retained on the dental roll. Because TBZ is 50% protein bound, but only 50% of the 40% available TBZ would be present in saliva. Using the example of volunteer M34, at 150 minutes post-dose plasma concentrations of TBZ were quantified as

134 ng/ml (see table 6.11) giving an expected concentration in the saliva of approximately 67 ng/ml. However, allowing for the loss of ~60% of the TBZ due to the collection technique, this would result in an expected concentration of ~27 ng/ml. The actual concentration detected was 6.87 pg/ml, which is still less than the expected concentration, after taking into account all losses.

6.8.9 Calculating the chemical-specific inter-individual toxicokinetic factors for TBZ

The default uncertainty factory, allowing for inter-individual variability in toxicokinetics is 3.16. The variation observed in the parameters was used to calculate what uncertainty factor would be required to protect 95, 97.5 and 99% of the population. The proportion of the population protected by the current default of 3.16 was also calculated. The results, using parameters derived from analyses of plasma (and saliva, in the case of the 10xADI studies) are shown in tables 6.18 and 6.19.

	Cmax (ng/ml)	AUC (ng x min/ml)	CL/F (min/ml)
Mean	0.57	65	2251
SD	0.45	62	1144
CoV	**79**	**95**	**51**
Uncertainty Factors	Cmax	AUC	Clearance
95th Percentile	3.14	**3.74**	2.21
97.5th Percentile	**3.92**	**4.82**	2.57
99th Percentile	**5.05**	**6.46**	3.06
% Covered by 3.16	**95.0**	**92.4**	**99.1**

Uncertainty factors in **red typeface** denote those greater than the default of 3.16-fold.

Table 6.18 Calculating the inter-individual toxicokinetic factors for TBZ using plasma kinetic data derived from the ADI studies.

	Observed values			Extrapolated parameters	
	Cmax (ng/ml)	AUC (ng x min/ml)	CL/F (ml/min)	AUC (ng x min/ml)	CL/F (ml/min)
Overall Mean	**147**	**14057**	**253**	**8443**	**308**
Overall SD	135	14815	326	10244	327
Overall CoV	**92**	**105**	**129**	**121**	**106**
95th Percentile	3.63	4.13	5.09	4.77	4.17
97.5th Percentile	4.64	5.42	6.96	6.43	5.48
99th Percentile	6.18	7.43	10.00	9.11	7.53
~% Covered by 3.16	**93**	**91**	**88**	**89**	**91**

Table 6.19 Calculating the inter-individual toxicokinetic factors for TBZ using kinetic data derived from the 10 x ADI studies.

Variability in kinetic parameters was large in both the high and low dose groups; the CL/F CoV in the low dose represents the least variable parameter. The data from the low dose studies show that based on AUC, safety factors greater than 3.16-fold would be required to protect substantial proportions (>95%) of the population. Using Cmax or CL/F the 3.16-fold default uncertainty factor would be adequate. The high dose pharmacokinetic data, show that the default uncertainty factor would be less than adequate for TBZ in persons consuming 10 x ADI.

6.8.10 Studies in which rats were dosed with TBZ at the NOAEL

Twelve rats, 6 male and 6 female were dosed with 10mg/kg bw TBZ, the NOAEL, as a suspension in 5% carboxymethylcellulose. Dr K. Walton conducted these studies and sample analyses. Table 6.20 is a summary of the concentrations measured at each time point in each rat. A sample was not taken at 420 minutes in the animal studies, therefore a value was extrapolated from the 240 minute time point using the T½ (calculated using the formula [420] = exp(-((0.693 x 180)/T½)), where 180 represents the temporal difference between observed and extrapolated values). Using WinNonlin™ (with 1/y weighting) to model the corrected (baseline subtracted data) the mean overall T½ max was 56 minutes (60 minutes mean for the male rats and 53 minutes mean for the female rats). The mean male and female data are plotted in figure 6.7.

6.8.11 Plasma analyses

	Concentration of TBZ in rat plasma samples, ng/ml												
Time	Male Rat Data						Female Rat Data						
(minutes)	M1	M2	M3	M4	M5	M6	F1	F2	F3	F4	F5	F6	F7
0	0	0	0	0	0	0	0	0 (8.5)	0	0	0 (2.3)	0	0
10	11	327	20	444	266	117	250	1689	1061	197	335	141	222
20	375	1761	138	1474	1630	608	763	3548		1319	2222	921	1480
30	1323	2218	544	2308	2599	1595	1234	3650	2383	1488	2095	1757	1906
45	2304	2372	1434	2665	2859	2227	1629	3462	3486	1834	3694	1647	2864
60	2506	2289	1702	2881	3217	2535	1644	3371	3947	1591	4228	1631	3103
90	2994	2321	1978	2737	3207	2740	1512	2826	4087	1465	3567	1234	3213
150	1969	2034	1262	2676	2046	2694	1001	1578	3002	537	2002	754	1909
240	1564	2010	869	2052	812	1739	329	374	312	69	288	497	333
480	19	34	93	66	18	97	4	13	0	7	14	0	4
1440	0	0	0	0	0	0	0	0	0	0	0	0	0

Values given are following baseline subtraction; baseline values quantified as PG are shown in parentheses.

Table 6.20 Plasma concentrations of TBZ from studies in which rats were dosed with the NOAEL.

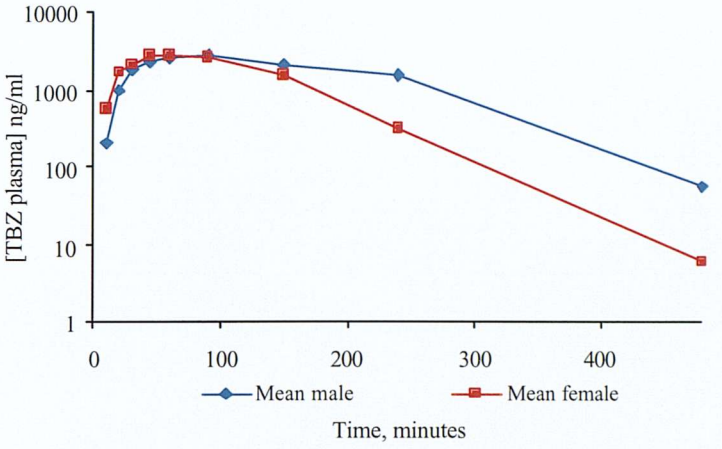


Figure 6.7 Concentration profiles for TBZ generated from mean male and mean female rat data after dosing at the NOAEL.

Immediately it is noticeable that the plasma concentration values in rats are much larger than those observed in humans, even at the higher dose of 10 x ADI. The animals are absorbing the dose more quickly, which is not surprising, as the dose is being directly placed in the stomach via the gavage technique of dosing. As with the 10 x ADI human data, 2 pharmacokinetic analyses were performed using the NOAEL data.

6.8.12 Pharmacokinetic parameters for TBZ calculated using data from the NOAEL studies

Pharmacokinetic parameters were calculated for each individual parameter using the same method as for the human ADI data, i.e. baseline subtraction, followed by manual calculation of AUC using the trapezoid formula. CL/F was calculated as dose/AUC. (See table 6.21.) The animal data showed clear concentration-time profiles, as so the data were extrapolated to infinity, although the later time points (480 and 1440 minutes) were not used in the extrapolation of kinetic parameters to keep the comparison equal. (Similarly the latter timepoints were not used to derive the observed values). Similar to the scenario in humans, TBZ is being rapidly cleared, (expressed as CL/F), with very small amounts of TBZ remaining 8 hours (480 minutes) after dose. As previously described, a secondary analysis of the NOAEL data, using WinNonlin ™ to extrapolate to infinity was performed. Here, the later time point data (480 and 1440 minutes) were used in the model. As before, the use of the model makes very little difference to the PK parameter values, validating the use of the non-modelled data to calculate the inter-species factors.

	Cmax (ng/ml)	Tmax (minutes)	AUC (ng x min/ml)	CL/F (ml/min)	T1/2* (minutes)	?z values used to determine T1/2
M1	2994	90	614899	16	47	90-480
M2	2372	45	690264	14	65	30-480
M3	1978	90	386899	26	84	90-480
M4	2881	60	773287	13	56	150-480
M5	3217	60	578827	17	45	150-480
M6	2740	90	699693	14	63	150-480
Male mean	**2697**	**73**	**623978**	**17**	**60**	**N/C**
F1	1644	60	275712	36	39	150-480
F2	3650	30	521892	19	19	90-480
F3	4087	90	650740	15	35	90-240
F4	1834	45	213607	47	68	150-480
F5	4228	60	554147	18	52	60-480
F6	1757	30	289113	35	121	90-240
F7	3213	90	487481	21	38	150-480
Female mean	**2916**	**58**	**427528**	**27**	**53**	**N/C**
P value (M vs. F)	0.67	0.28	0.04	0.07	0.65	N/C
Overall mean	**2815**	**65**	**518197**	**22**	**56**	N/C
Overall SD	867	23	178362	10	26	N/C
Overall CoV	**31**	**36**	**34**	**47**	**46**	**N/C**

Values in **bold** are the mean data and overall CoV used in later comparisons. P-values compare the male and female data, using unpaired student t-test (2 tails, equal variance)

*T½ values were derived using the mathematical model WinNonlin™. All other PK parameters reported are observed values.

Table 6.21 Pharmacokinetic parameters for TBZ generated from studies in which rats received the NOAEL.

The PK data for rats dosed at the NOAEL show a slight sex difference in AUC and CL/F values, the former achieving statistical significance with higher levels in male rats. The overall concentration-time profiles were the same for male and female rats, and there is no difference between the sexes in T½ values, suggesting that the difference in AUC is due to differences in bioavailability.

Human and animal kinetic parameters were compared to generate the chemical-specific inter-species toxicokinetic factor for TBZ. The kinetic parameters (excluding CL/F, which is independent of dose) were corrected for dose using the equation given in chapter 2.

	Cmax (ng/ml)	AUC (ng x min/ml)	CL/F (ml/min)
Human ADI Mean	0.57	65	2251
SD	0.45	62	1144
CoV	79	95	51
Animal NOAEL Mean	2815	518197	22
SD	867	178362	10
CoV	31	34	47
Interspecies factor	**0.020**	**0.013**	**0.010**

Table 6.22 Calculating the interspecies toxicokinetic factor comparing ADI and NOAEL data.

The data in table 6.22 show that based on Cmax, AUC or CL/F, the toxicokinetic default factor of 4.0-fold is more than adequate. There is approximately a 5000-fold difference between the rat and human Cmax value, an 8000-fold difference in AUC value, and a 100-fold difference in CL/F values. Compared to animals, humans are being exposed to a much smaller internal dose (reflected in the AUC values) and are able to eliminate TBZ much more rapidly. The CoV values were much lower for the kinetic parameters calculated from the animal data. This is due to the inherent lower variability in this more homogenous study group.

6.8.13 Studies in which rats were dosed with TBZ at 0.1 x NOAEL

As previously reported (see earlier), studies were conducted in human, where volunteers received 10 x ADI. A dose equivalence study was conducted in rats (receiving 0.1 x NOAEL), using the same experimental protocol as previously described. Table 6.23 reports the summary of the concentrations measured at each time point in the rat. Figure 6.8 shows the mean male and female concentration-time profiles.

Time (minutes)	Concentration of TBZ in rat plasma samples, ng/ml											
	Male Rat Data						Female Rat Data					
	M1	M2	M3	M4	M5	M6	F1	F2	F3	F4	F5	F6
0	0 (5.5)	0 (5.0)	0 (1.6)	0 (1.3)	0 (0.94)	0 (1.1)	0	0	0	0	0	0
10	11	36	41	31	38	50	50	34	88	93	90	41
20	39	120	80	73	171	87	59	12	44	40	94	36
30	31	111	69	61	184	88	51	12	31	31	80	34
45	21	50	31	31	129	78	30	5.9	26	23	47	21
60	15	17	18	24	40	68	35	3.1	16	19	29	14
90	7.0	13	8.2	18	36	43	14	1.6	14	10	15	6.4
150	2.9	2.4	2.8	16	6.0	4.6	3.3	0.5	3.1	3.3	3.7	4.5
240	1.7	1.2	1.9	13	1.5	2.1	0.8	1.1	0.0	0.0	4.2	0.7
360	0.6	0.9	0.0	1.9	0.5	0.6	0.0	1.5	12	16	2.6	1.9
480	1.3	1.8	0.0	0.7	0.6	0.9	1.7	1.3	7.9	0.0	0.8	0.3

Values given are following baseline subtraction; baseline values quantified as PG are shown in parentheses.

Table 6.23 Plasma concentrations of TBZ from studies in which rats were dosed with 0.1 x NOAEL.

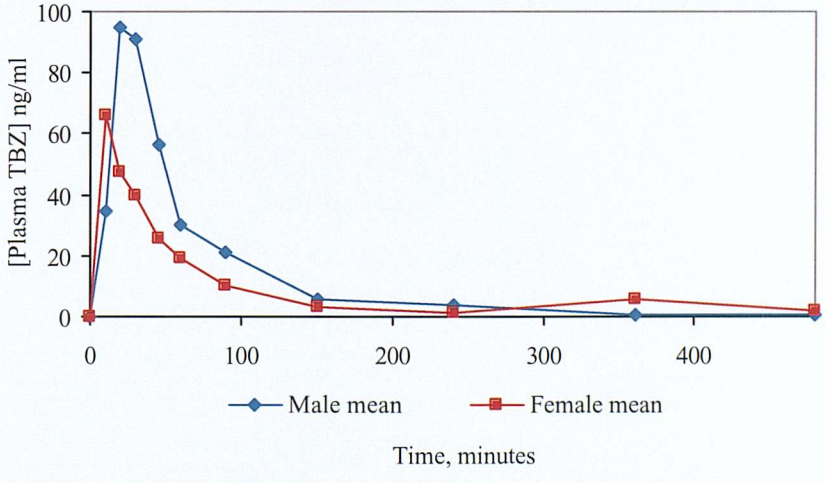


Figure 6.8 Concentration-time profiles for TBZ generated from mean male and mean female rat data after dosing at 0.1 x NOAEL

Comparing these data with those obtained after dosing at the NOAEL, one can see that much lower concentrations of TBZ are detected after dosing at 10 x less the NOAEL, and that this difference is greater than the 10-fold difference in dose. The maximal plasma concentrations are reached within 30 minutes, which is slightly faster than that observed with the higher dose of the NOAEL.

6.8.14 Pharmacokinetic parameters for TBZ calculated using data from the 0.1 x NOAEL studies

Pharmacokinetic parameters were calculated for each individual parameter using the same method as for the human ADI data, i.e. baseline subtraction, followed by manual calculation of AUC using the trapezoid formula (See table 6.24). CL/F was calculated as dose/AUC. The data were also modelled in WinNonlin™ and the extrapolated data are presented for comparison. The 480 time point was not used in the calculation of observed kinetic parameters to keep the comparison with human data equal.

	Cmax (**ng**/ml)	Tmax (minutes)	AUC (**ng** x ml/min)	CL/F (ml/min)	T 1/2* (minutes)	?z values used to determine T1/2
M1	39	20	2250	445	38	20-150
M2	120	20	5060	198	25	30-150
M3	80	20	3705	270	35	45-150
M4	74	20	6397	156	91	45-480
M5	184	30	9856	101	33	60-240
M6	88	30	8149	123	24	60-150
Male mean	**98**	**23**	**5903**	**215**	**41**	**N/C**
F1	59	20	3923	255	38	45-360
F2	34	10	1140	877	32	30-150
F3	88	10	4544	220	35	30-150
F4	93	10	4570	219	37	30-150
F5	94	20	5829	172	29	30-150
F6	41	10	2720	368	25	30-90
Female mean	**68**	**13**	**3788**	**352**	**33**	**N/C**
P value (M vs. F)	0.233	0.007	0.144	0.284	0.452	N/C
Overall mean	**83**	**18**	**4845**	**284**	**37**	**N/C**
Overall SD	41	7	2463	211	18	N/C
Overall CoV	**50**	**39**	**51**	**74**	**49**	**N/C**

Values in **bold** are the mean data and overall CoV used in later comparisons. P-values compare the male and female data, using unpaired student t-test (2 tails, equal variance)

*T½ values were derived using the mathematical model WinNonlin™. All other PK parameters reported are observed values.

Table 6.24 Pharmacokinetic parameters for TBZ generated from studies in which rats were dosed at 0.1 x NOAEL data.

As with the NOAEL data there was a slight sex difference in AUC and CL/F values, however at 0.1 x

NOAEL these differences were not significant. Interestingly there was a significant sex difference in the Tmax value, with female rats reaching Cmax approximately 30 minutes earlier than the male rats. CoV values were generally similar to those observed in the NOAEL data, the only exception being CL/F, where variability was higher in the lower dose data. The T½ at the lower dose is shorter by approximately 50%. This together with the observation that CL/F is increased 10-fold at the lower dose suggests that saturation of first pass metabolism is occurring at the high dose. The mean PK parameters for the two doses are compared below in table 6.25.

	Cmax (**ng**/ml)	Tmax (minutes)	AUC (**ng** x min/ml)	CL/F (ml/min)	T 1/2 (mintues)
Mean 0.1 x NOAEL value	83	18	4845	284	37
Overall SD	41	7	2463	211	18
Overall CoV	50	39	51	74	49
Mean NOAEL value	2815	65	518197	22	56
SD	867	23	178362	10	26
CoV	31	36	34	47	46
Ratio NOAEL/10 x <NOAEL	**34**	**N/C**	**107**	**0.08**	**1.53**
P value	<0.01	N/C	<0.01	<0.01	0.04

Table 6.25 Comparison of pharmacokinetic parameters for TBZ from the two studies conducted in rats.

Interestingly, the rats are also showing signs that saturation of first pass metabolism is occurring at the higher dose. As with the humans, a 10-fold increase in dose would be expected to have the effect of raising the PK parameter value 10-fold. These data clearly show statistically significant increases of greater than 10-fold, and as before CL/F, which should be dose independent is also increased above the expected ratio of 1.

6.8.15 The magnitude of the chemical-specific inter-species toxicokinetics factor at different doses.

The inter-species toxicokinetic factors were calculated using all possible combinations of data (see table 6.26). This allows examination of the effects of the non-linear kinetics on the magnitude of the IS TK factor to be explored. Factors are calculated as shown in figure 2.15, except in the cases of dose equivalence, and in the case of CL/F, which is calculated as animal value/human value, without correction for dose.

	Cmax (obs)	AUC (obs)	CL/F (obs)	AUC (extr)	CL/F (extr)
ADI/NOAEL	**0.020**	**0.01**	**0.010**	**N/C**	**N/C**
ADI/0.1 x NOAEL	0.068	0.135	0.13	N/C	N/C
10 x ADI/NOAEL	0.52	0.27	0.09	0.16	0.07
10 x ADI/0.1 x NOAEL	1.8	2.9	1.1	1.69	0.85

N/C – These ratios could not be calculated, as the ADI data were not modelled using WinNonlin™. (See section 6.8.2.)

Table 6.26 Inter-species factors vary depending on the dose used to calculate the pharmacokinetic parameters.

The effect of non-linear kinetics observed in both the rats and human subjects is clearly seen in table 6.26. The correct comparison (ADI vs. NOAEL) gives a factor two orders of magnitude from the default factor of 4.0-fold. However, there are marked differences in the calculated factors when data from other doses are used. The highest factors are produced when the dose equivalent (10 x ADI vs. 0.1 x NOAEL) data are compared, and they are ~100-fold different to the factor based on ADI vs. NOAEL. These data emphasise the importance of selecting the correct dose for animal and human studies in the estimation of chemical-specific adjustment factors. This is examined in more detail in the discussion of this chapter.

6.9 Discussion

6.9.1 Plasma kinetics from studies in which human volunteers received TBZ at the ADI

Very low concentrations of TBZ were quantified in the plasma of volunteers following a single oral dose of TBZ at the ADI (0.1 mg/kg). Despite a relatively sensitive assay (LoD ~250 pg/ml) reliable kinetic data were not obtained. This highlights the difficulties one might experience if further research were undertaken where dietary exposure to TBZ was intended to be quantified. It would also make the study of subjects in sensitive subgroups, where exposure to larger doses may not be ethically acceptable, unreliable and therefore unethical.

The usefulness of the human ADI data in measuring variability in PK parameters is limited, because many measurements of TBZ were at or below the limit of detection. However, one can see that overall, there is greater observable variation in Cmax and AUC (CoV values of 79% and 95% respectively) compared to that observed with CL/F (CoV of 51%). The lower variability observed in CL/F suggests that bioavailability at the ADI is not making a particularly large impact on the variability of the data. However as CL/F is a function of Dose/AUC, these data suggest that inter-subject variability in first pass metabolism is the most likely sources of variability in the PK parameters.

A considerably more sensitive analytical technique would be necessary to obtain clearer concentration-time profiles, and therefore more reliable PK parameters. A method with analytical sensitivity in the range of low picograms may have provided this extra sensitivity, and possibly could have been achieved via LC-MS-MS.

6.9.2 Plasma kinetics from studies in which human volunteers received TBZ at 10 x ADI

Dosing of human volunteers at 10 x ADI resulted in measurable plasma concentrations of TBZ. Clearly detectable TBZ in all sample sets led to two separate calculations of PK parameters (as described earlier in this chapter). The impact of the use of the mathematical model on the variability is demonstrated in tables 6.11, 6.19 and 6.24.. The observed and extrapolated values were directly compared for volunteers where the concentration-time curves allowed determination of the terminal elimination phase. The use of the model did not have a major impact on AUC of CL/F values or variability. This validated the subsequent use of the non-extrapolated data in the human dose-comparison and the inter-species comparisons. Greater variability was observed in the PK parameters calculated from the high dose data, compared to those following the ADI dose.

On consideration of the PK parameters calculated from the two dose groups, ADI and 10 x ADI, there is clear evidence of non-linear kinetics. Changes in dose-dependent PK parameters (Cmax and AUC) in the high dose group were far larger than the 10-fold predicted based on the dose increment. CL/F is amplified nearly 9-fold; this result suggests that saturation of a biological process occurs at the higher dose. Taken together with the greater variability observed at the high dose, one can hypothesise that saturation may occur at different points (between the ADI and 10 x ADI dose) for each individual. Correspondingly, the extent of saturation at the 10 x ADI dose is different for different individuals, resulting in greater variability observed at the higher dose.

Saturation occurs when an individual's capacity to remove the compound on its first-pass through the gut wall and/or the liver (before the compound enters the systemic circulation) is overwhelmed. The lower CL/F values observed in the 10 x ADI data (nearly 9-fold lower CL/F than in the ADI data) suggest that saturation of first-pass metabolism is occurring at the higher dose. If saturation was occurring in the liver, one would expect T½ values to increase with lowered CL/F. However this is not apparent in the data (see tables 6.11 and 6.19). Although T½ could not be estimated from the ADI data, the graphs in figure 6.3 show that similar elimination-phase profiles are generated from the ADI and 10 x ADI data. Instead it seems likely that saturation is occurring in the gut wall; more dose is entering the systemic circulation, F is increased and CL/F is reduced at the high dose. Interestingly the wide values for all the PK parameters at the 10 x ADI dose demonstrate that the point of saturation in each individual is not the same. For example M17, shows PK values very similar to those obtained from the ADI studies.

6.9.3 Salivary concentrations of TBZ as a biomarker on internal dose.

Analyses of saliva samples taken in the ADI studies were not undertaken, because the plasma concentrations of TBZ were close to the LoQ (limit of quantification defined as the lowest standard used in the construction of the standard curve). Based on protein binding of ~50%, it would be predicted that an assay with sensitivity in the sub-picogram range would be required to adequately monitor the salivary concentrations of TBZ. This highlights the difficulty of using this approach for possible wide-scale screening programmes, for research purposes.

The results of two different experiments demonstrated that TBZ plasma protein binding (PPB studies) was approximately 50%. Based on the mean Cmax values observed in the plasma from the 10 x ADI studies one would expect the mean saliva concentrations of TBZ to be ~50 ng/ml. However the observed mean was ~8 ng/ml, which is not in agreement with the PPB studies. Part of the discrepancy was because the recovery of TBZ from the dental roll was less than 100%. However, the saliva concentration was still less than would be expected. The impact of the collection method on the validity of the samples can also be considered. It is possible that there is not sufficient time for the internal dose to equilibrate between the plasma and the saliva. This would be unlikely to be the case for all volunteers at all time points and is also unlikely given that Tmax values are similar in saliva and plasma. The composition of the saliva is known to vary, depending on factors such as age, sex, time of day, diet and changes in flow rate (Drobitch & Svenson, 1992). All of these factors could contribute to variability seen in the PK parameters themselves, but they do not help explain the observed inconsistency between plasma concentrations and saliva concentrations.

Saliva secretion by the three salivary glands (parotid, sublingual and submaximillary) is innervated by the sympathetic and parasympathetic nervous systems. The two systems have overlapping roles, but the sympathetic nervous system generally increases amylase, and potassium secretion, whilst the parasympathetic system initiates and maintains secretion (Drobitch & Svensson, 1992). This is achieved through selective parasympathetic or sympathetic innervation of particular glands (Gannong, 1995). The net result of stimulated saliva production is that bicarbonate is produced from CO_2 in the salivary duct, and that the pH rises from a basal level of approximately pH 5-7 to a pH of approximately pH7-7.8 (Wolff et al., 1999). For a weak base such as TBZ the degree of ionisation (and hence salivary concentration) will decrease with a rise in pH value. Less ionised drug should correlate to higher salivary drug concentrations, but this was not observed. These observations suggest either a problem of TBZ stability in saliva, or some metabolism of TBZ in saliva so that it was not detected.

In the method of collection described in chapter 2, the volunteer was given the dental roll to place in their mouths simultaneous to the blood sample being taken. The dental roll remained in their mouth until after

blood collection was complete, and was then taken and the sample extracted from the dental roll. Using this protocol it is possible therefore that nervous stimulation of saliva may have occurred, resulting in increased ionisation of TBZ, and therefore partitioning of TBZ in the plasma.

Despite the problems with the recovery of TBZ from the dental rolls, it was still possible to evaluate the validity of using saliva as a biomarker of internal exposure. Table 6.16 compares AUCs from saliva and plasma, showing that generally, the ratio is constant at ~0.07. There is some variation (CoV is 46%), but this may be due to variation in the precise percentage of TBZ recovered from the dental roll from each individual, or could be due to variation in one of the unaccounted for processes discussed in the previous paragraphs.

6.9.4 Conclusions regarding the feasibility of pharmacokinetic human studies with TBZ

In the case of TBZ, either doses larger than the ADI are required to allow adequate monitoring of the plasma concentrations, or a more sensitive analytical method is required. When one considers that a human would rarely consume the ADI for TBZ as a bolus dose, the possibility of monitoring exposure of the population to TBZ using the analytical method described here is not feasible. Saliva would probably be a reasonable biomarker of exposure to TBZ, although further validation of different saliva collection methods would be necessary. Furthermore, saliva would only be reliable (given our analytical LoD) when doses/amounts of TBZ above the ADI were administered/consumed. Given the observed non-linear pharmacokinetics this may be of limited value in extrapolating to doses/amounts of TBZ to which the population would generally be exposed. Except in the case of Tmax in the ADI studies, no statistical differences based on sex were observed. No difference between the sexes was observed at the 10 x ADI dose, where there was greater data precision. One may conclude, therefore that the sex difference in Tmax seen at the ADI is unlikely to be of biological significance, and that any future studies would not need to select one sex only in human studies.

6.9.5 Comment on the adequacy of the inter-individual toxicokinetic default factor (3.16-fold) for TBZ

In summary, based on the two fundamental indicators of chronic exposure (AUC and CL/F), the default factor of 3.16 adequately protects a greater proportion of the population exposed to TBZ at the ADI than at 10 x ADI. This can be explained; because there is greater variability in the 10 x ADI PK data compared to the ADI data, for reasons previously discussed. The data from one volunteer, M17 are particularly interesting, as this volunteer received a dose equivalent to 10 x ADI, yet the kinetic parameters are comparable with the ADI data set. Together with the observation of saturation occurring

at the higher dose, this observation warrants a more thorough investigation of human variability in the ADI-10 x ADI range.

Emphasis on the outcome of the ADI data must be carefully weighed against the reliability of the data at this lower dose. Here, the most important chronic indicator, CL/F results in an adequate proportion (99%) of the population being "covered" by the 3.16-fold default. In the case of the 10 x ADI data, one might consider the inter-individual factor based on Cmax more useful, since exposure to 10 x ADI TBZ is unlikely to be sustained for long periods of time. Using Cmax values from plasma, significant proportions of the population are still adequately protected (93%).

In conclusion, based on the data presented, and in consideration of the reliability of the data presented, the default of 3.16-fold for chronic exposure at the ADI is not under-protective. At higher exposure levels where greater variability is observed, based on a marker of acute exposure, Cmax, the default is not as adequate (covering 93% of the population). Considered in light of lower CL/F values at the higher dose, there may be some concern, if evidence is available indicating chronic exposure to levels of TBZ above the ADI for extended periods of time.

In support of the findings of the human studies, that the 3.16-default is adequate, are analyses by Dorne et al. (2001) in which PK derived from human studies with CYP1A2 substrates were used to assess the adequacy of the toxicokinetic default of 3.16-fold. These analyses showed that variability in CYP1A2 activity in healthy adults based on data after intravenous and oral dosage (used the PK parameters CLm, CL and AUC) ranged from 34-42%. The variability in Cmax was 21%. The conclusion to the analysis was that the 3.16-fold default uncertainty factor would cover over 99% of healthy adults, but would give lower protection to some subgroups (including pregnant women at term, healthy elderly, patients with liver disease) and would be inadequate for neonates. The variability reported by Dorne et al. is considerably smaller than reported in this chapter. This may have been a function of the specific substrates used for the analyses; that they were more specific substrates for CYP1A2, or that the doses used meant that all subjects under study were definitely not or definitely were induced, thus reducing variability.

6.9.5.1 Sources of variability in the human TBZ data.

There are 4 main pharmacokinetic processes in which individuals may demonstrate inter-individual differences; absorption of the compound, distribution of the compound, metabolism of the compound and excretion of the compound. Most compounds will cross the intestinal epithelium via passive diffusion, and because of the high perfusion of the gut, blood flow is not likely to be a rate limiting process. If an active transport mechanism were in place, then saturation of this process may occur, and at increasing

doses of TBZ, proportionally less TBZ would enter the systemic circulation. The data for TBZ do not support this notion, and it can be assumed that passive diffusion is responsible for TBZ transfer into the systemic circulation. P-glycoprotein is a transporter that transfers basic drugs from enterocytes back into the gut lumen. Saturation of P-glycoprotein at high doses would result in an increase in AUC in plasma, as there would be a reduction in transfer back into the gut lumen. The bioavailability of TBZ, that is, the fraction of the administered dose reaching the systemic circulation cannot be estimated from the data presented here. A study in which TBZ was given as an i.v. bolus dose would be required to calculate bioavailability, a regimen which although of scientific interest was outside the scope of this study. Similarly the distribution of TBZ in the tissues could not be calculated from the oral dosing data generated. The metabolism of TBZ though, was recorded, albeit indirectly, through the disappearance of the compound, and is reflected in the reported parameter, CL/F. (Sequestration into tissues would also affect the apparent rate of clearance, although this is unlikely to be a major factor in TBZ elimination due to its relative water solubility.) The rate of clearance of TBZ from the body was due to two factors, firstly excretion of unchanged drug in the urine, and secondly biotransformation to metabolites. In animals, very small amounts of TBZ are excreted unchanged, even at doses higher than the NOAEL doses (see the introduction to this chapter), suggesting that metabolism (in the first instance by CYP450 enzymes) is likely to be a major route of elimination. Unchanged TBZ was not measured in the urine of volunteers dosed with the ADI or 10 x ADI; urine samples were taken, but priority was given to analysis of plasma and saliva samples. An attempt was made to obtain the metabolites of TBZ from Merck Sharpe and Dohme, but this was not possible.

Once saturation kinetics is observed, there are two critical processes which govern the rate of elimination. Firstly the ability of the organs to extract and remove the substrate by metabolism or excretion, and secondly the extent to which the chemical remains in the circulation and is available for elimination rather than becoming sequestered by tissues.

As introduced earlier, the primary cytochrome P450 enzyme involved in the metabolism of TBZ is CYP1A2. CYP1A2 is mainly expressed in human liver, contributing 13% of the total CYP protein content in the liver (Ou-Yang et al., 2000). There is known to be wide inter-individual variation in the expression of CYP1A2 in the human liver. Using immunoblot assays, this has been estimated to be approximately 40-fold (Schweikl et al., 1993, Guengerich et al., 1999), although the experiment in question included individuals that smoked, thus augmenting the observed variation (see table 6.27). In a separate experiment, Zhou et al., (2003) observed differences of one order of magnitude in the expression of CYP1A2 in samples from a human liver bank. There is no indication of ethnic difference in the relative amounts of CYP1A2 present in the liver (shown in Caucasian and Japanese populations, Shimada et al., 1994).

Quite recently, and of importance, Lindell et al., (2003) have demonstrated low, but highly variable (CoV of 59%) levels of CYP1A2 in human duodenal biopsy samples. In the case of low-level exposure to substrate (e.g. in the case of food additives at the ADI), this pathway of first pass metabolism may contribute greatly to variability in bioavailability and subsequently other PK parameters.

CYP1A2 is implicated in the metabolism of a diverse range of chemicals including the activation of several carcinogens such as heterocyclic amines (Butler et al., 1989, Boobis et al., 1996, Zhao et al., 1994) (of particular interest is the activating role of CYP1A2 of PhIP (2-amino-1-methyl—6-phenylimidazo (4,5-b) pyridine) and MeIQx (2-amino-3,8-dimethylimidazo (4,5-f) quinoxaline)), nitroaromatic compounds, mycotoxins, estrogens, (Shou et al., 1997, Landi et al., 1999) and aflatoxin B1 (Gallagher et al., 1994).

Due to the importance of CYP1A2 from an environmental health perspective, and because of the high observed variability in expression of CYP1A2, there have been specific investigations, attempting to identify the causal factor. Many probe substrates have been documented for use in CYP1A phenotyping studies e.g. phenacetin, theophylline and caffeine. However there has been some criticism that due to overlapping substrate specificity of these enzymes, the test substrates do not discriminate between inducibility of CYP1A1 or CYP1A2 (Tassaneeyakul et al., 1993, Eugster et al., 1993).

One method that has been robustly validated is the caffeine assay for determining phenotype (Fuhr et al., 1996). Butler et al., (1992) developed a method using caffeine urinary metabolite ratios as a marker for CYP1A2 activity. Caffeine undergoes 3-demethylation by CYP1A2, and is then further acetylated by NAT. For the CYP1A2 phenotype, the urinary molar ratio of [1,7-dimethylxanthine + 1,7-dimethyluric acid]/caffeine was found to be correlated well with the rate constant for caffeine 3-demethylation.

Several factors (mostly environmental) have been found to affect CYP1A2 activity *in vivo*, and these are summarised in table 6.27. Clearly any phenotypic variation in observed CYP1A2 activity will be multi-factorial in origin. The PK parameters from the present study were compared against sex, and no significant differences were observed. All of our subjects were non-smokers. Diet was recorded in the diary sheets prior to the study, but the number of individuals in the study groups were considered too small to draw any meaningful conclusions, and so the data are not discussed here. It is possible that dietary and environmental factors may have impacted on the variability in the results obtained.

Factor	Effect on CYP1A2 activity	Reference
Age	↔ no change observed	Lindell et al., 2003
Sex	↑ CYP1A2 activity in male subjects ↔ no change observed	Schrenk et al., 1998, Tantcheva-Poor et al., 1999 Kashuba et al., 1998, Welfare et al., 1999, Simon et al., 2001, Vistisen et al., 2002. Beierle et al., 1999
Body mass index	↑ CYP1A2 activity with increasing BMI	Tantcheva-Poor et al., 1999
Oral Contraceptive	↔ no change observed ↓ CYP1A2 activity ↓ activity of CYP1A2 lower clearance of caffeine	Schrenk et al., 1998 Catteau et al., 1995 Tantcheva-Poor et al., 1999 Kalow et al., 1991 Vistisen et al., 1992 Balogh et al., 1995
Exercise	↑ CYP1A2 activity by 70%	Vistisen et al., 1992
Smoking	↑ CYP1A2 activity in smoking subjects	Schrenk et al., 1998 Nordmark et al., 1999 Catteauet al., 1995 Tantcheva-Poor et al., 1999 Kalow et al., 1991 Vitisan et al., 1992
Menstrual cycle	↓ slower clearance of caffeine in the luteal phase – linked to ↑ progesterone ↔ no change observed	Lane et al., 1992 Kashuba et al., 1998
Bioflavenoids	↓ - these compounds inhibit CYP1A2	Doostdar et al., 2000
Charcoal-broiled beef	Intake ↑ CYP1A2 activity	Pantuck et al., 1976
Cruciferous vegetables	Intake ↑ CYP1A2 activity	Vistisen et al., 1992

Table 6.27 Individual, environmental and dietary factors contributing to inter-individual variability.

Several studies have been conducted, looking at the population distribution of poor, intermediate and extensive metaboliser phenotypes. Butler et al., 1992 examined CYP1A2 phenotype for subjects from Arkansas, Italy and China (n=100/group). Using the data from non-smokers, a trimodal distribution seemed apparent, suggesting poor, intermediate and extensive phenotypes. The proportions of these phenotypes in the population ranged from 12-13% (slow), 51-67% (intermediate) and 20-37% (rapid). A reproducibility experiment with 13 subjects showed that with the exception of one volunteer , intra-individual variation did not alter phenotype of the 5-day and 5-week study periods.

Illet et al., (1993) investigated the distribution of the CYP1A2 phenotype in 90 Australian volunteers, using the caffeine method developed by Butler et al., (1992). The distribution in non-smokers, was 5% classified as poor metabolisers, and 95% as extensive metabolisers.

Nakajima et al., (1994) examined the prevalence and distribution of CYP1A2 phenotypes in the Japanese population. CYP1A2 activity appeared to be bimodally distributed, suggesting the existence of poor and

extensive metaboliser phenotypes (PM and EM respectively). By their classification, 14.1% of the Japanese population studied had the poor metaboliser phenotype. Family studies suggested that the poor phenotype of CYP1A2 is an autosomal recessive trait. A later study by Yokoi et al., (1995) performed caffeine phenotyping in 205 Japanese volunteers. Analysis of the urinary metabolite ratios again indicated that 86% of the population studied were extensive metabolisers.

The presence of bimodal or trimodal distributions of CYP1A2 activity suggests that genetic polymorphisms may be occurring in the CYP1A2 gene, and some research has been undertaken, with limited success to try and identify functional mutations of the gene. Several investigators have identified mutations in the 5'-flanking region of CYP1A2, but no significant correlation was found with function, i.e. phenotype (Chida et al., 1999, Aitchison et al., 2000, Han et al., 2000). Nakajima et al., 1999 identified the existence of a point mutation (from guanine in the wild type to adenine in the mutated type) at residue –2964 in the gene. This was shown to be genetically inherited, and the frequency of the allele in 116 Japanese volunteers was 0.77 and 0.23 for the wild and mutated types respectively. The point mutation caused a significant decrease of CYP1A2 activity (as determined by the caffeine-3-demethylation assay) in Japanese smokers, suggesting that the polymorphism is a causal factor in decreased CYP1A2 inducibility. Genomic differences between PMs and EMs have been investigated using DNA sequencing, but no difference in nucleotide sequence were observed between PMs and EMs (Yokoi et al, 1995).

Sachse et al. (1999) identified a polymorphism in intron 1 of CYP1A2. Using caffeine as a probe substrate an investigation of the functional consequences of this C-A polymorphism was conducted using groups of Caucasian smokers and non-smokers. The incidence of the heterozygous genotype was 44%, that of the C/C homozygous combination 10% and of the A/A genotype 46%. There was no significant difference in CYP1A2 activity between the genotypes in non-smoking individuals, but a 1.6-fold higher metabolic activity was observed in subjects homozygous for the A allele compared to other genotypes in the smoking groups. However whether the expression of this allele directly or indirectly (via a polymorphism) confers high inducibility has not yet been determined.

The topology of the active site of CYP1A2 has been the subject of limited research. Techniques have focused on induction of mutations in the active site of CYP1A2, but this has not been linked to the existence of these mutations *in vivo* (Tuck et al., 1993).

Whilst polymorphisms have been suggested as a reason for the tri-modal distribution in CYP1A2 activity reported by some individuals (Butler et al., 1992), functionally significant polymorphisms have not been discovered. One possible alternative explanation is that inter-individual differences occur at the levels of gene regulation of CYP1A2, resulting in high human variability in response to pharmaceutics, toxins etc.

Two xenobiotic response elements have been identified, as well as an antioxidant response element in the CYP1A2 gene (Eaton et al., 1995). The general consensus is that CYP1A2 is transcriptionally activated via the Ah-dioxin receptor (Quattrochi et al., 1994, Shih et al., 1999, Scheel et al., 2002). The ARNT (aryl hydrocarbon nuclear translocator) itself is polymorphic, although no correlation was found between genetic variations in ARNT and the CYP1A2 phenotype (Scheel et al., 2002).

Another area of research has focused on the regulation of CYP1A2 activity by external environmental factors, such as the intake of therapeutic drugs and the simultaneous up or down-regulation of CYP1A2 and other xenobiotic metabolising enzymes. For example, Rost et al. (1992), phenotypically classified volunteers to groups of extensive, intermediate and poor metabolisers of CYP2C19 using S-mephenytoin. Using omeprazole as a probe substrate, increases in CYP1A2 activity of 39% were measured from poor and intermediate metabolisers, but only of 12% in extensive metabolisers, suggesting that omeprazole induces CYP1A2 more effectively in the poor/intermediate phenotype. The results of this study suggest that CYP2C19 phenotype co-segregates with CYP1A2 inducibility. Bock et al., (1994) found that CYP1A2 activity was significantly correlated to paracetamol glucuronidation in male smokers, suggesting some co-regulation of CYP1A2 and of UDP-glucuronosyltransferase isoenzymes. MacLeod et al. (1997) investigated the correlation between CYP1A1 and GSTM1 phenotypes and CYP1A2 activity in human volunteers. It was found that individuals possessing the CYP1A1 genotype Ile/Ile had a greater CYP1A2 activity than those with the heterozygous allelic variant Ile/Val. Upon exposure to cigarette smoke or high-temperature cooked meat, individuals with the heterozygous allele had significantly increased CYP1A2 activity compared to the homozygous Ile/Ile variant. A null GSTM1 genotype resulted in increased CYP1A2 activity both from baseline activity levels and in the induced state. In individuals who conferred the CYP1A1 Ile/Val genotype and the GSTM1 null genotype and intermediate value of activity was observed (compared to the other possible combinations). This suggests that the two genotypes are not synergistic in their ability to elevate CYP1A2 activity.

In summary there are a multitude of factors contributing to inter-individual variability in the regulation of constitutive levels of CYP1A2, the inducibility of CYP1A2, the catalytic efficiency of the protein itself. It would have been interesting to phenotype (using the caffeine assay) the volunteers in our study, and categorise them accordingly, to see how well TBZ disappearance correlated with caffeine metabolite ratio. However, phenotyping was not a primary objective of study. In the future, if functionally significant polymorphisms are elucidated, blood samples could be used to genotypically determine CYP1A2 activity.

6.9.6 Comments on the adequacy of the inter-species toxicokinetic default factor (4.0-fold) for TBZ

Based on the appropriate comparison of NOAEL compared to ADI data, the inter-species toxicokinetic default of 4.0-fold is more than adequate for all indicators measured. All the values for the chemical specific inter-species factor are very similar, and show 200, 308, and 400-fold margins of safety in addition to the 4.0-fold already provided to allow for inter-species differences. Table 6.25 shows the differences between the PK parameters in the rat at the high and low dose. Clearly the rat shows saturation kinetics at the higher dose, resulting in larger PK values than expected from the NOAEL data. These impacts on the inter-species comparison, exaggerating the observed differences, and adding to the margin of safety. However the amplification of the NOAEL PK parameters as a result of saturation does not itself account for the large species difference; humans have a smaller internal exposure and much more rapid clearance than expected.

Although there is clearly non-linear pharmacokinetics at the higher dose, there is greater variability in PK parameters at the lower dose. This is partly due to much smaller concentration s of TBZ detected at the lower dose, resulting in less reliable, more variable data. The magnitude of the difference in PK values between the low and high dose in animals is not as large for Cmax and AUC as it is in humans, but it is larger for CL/F. There is a 9-fold difference in CL/F between the low and the high dose in humans and a 13-fold difference in CL/F between the low and high dose in rats. This suggests that rats administered the NOAEL dose were more saturated than humans receiving the 10 x ADI dose. The relationship between dose and changes in PK parameter are not parallel between species.

Clearly the particular doses chosen for inter-species comparison are critical, since, the underlying assumption that at 100-fold dose interval (i.e. NOAEL vs ADI) both PK-dose curves for TBZ are on a linear part of the curve is not the case in reality. It is necessary to compare linear, non-saturated parts of this curve, otherwise safety factors could be grossly over or under estimated. This concept is discussed further later in this discussion.

6.9.6.1 Sources of interspecies differences between the human and rat data.

The major sources of inter-species differences, blood flow, (hepatic and renal), body mass to surface area differences, are already taken into account in the default factor of 4.0-fold (Renwick, 1993). Bioavailability and volume of distribution were not measured in either species, and so further comment on species specific transporters/proteins/ etc. is not useful. Metabolites were not determined either, so examination of the metabolite ratios is not possible. It is not clear why humans are much more capable at

preventing the absorption of TBZ by p-glycoprotein or first pass metabolism or of clearing TBZ from the system. Inter-species adjustment factors for a number of species were calculated from published data for a number of known CYP1A2 substrates (including caffeine, theobromine, theophylline and paraxanthine) (Walton et al., 2001b). The inter-species adjustment factor based on extrapolation of internal doses from rat data to human data was on average 5.4-fold, i.e. exceeded (but not greatly) the 4.0-fold inter-species toxicokinetic default. The need for chemical-specific data was highlighted, as the adjustment factors varied considerably between species 10.6 for the mouse, 2.6 for the rabbit and 1.6 for the dog.

Li et al., (2003) observed a longer half-life of 1μM (equivalent to ~200 ng/ml) TBZ in rats vs. human microsomal incubations (69.0 vs. 39.6 mins). In contrast to Li et al., the results of the animal studies show a shorter T½ in rats (37 minutes in 0.1 x NOAEL studies) compared with humans at equivalent dose (64 minutes in the 10 x ADI studies). Li et al., reported an extrapolated human hepatic clearance of TBZ of ~18 ml/min/kg. CL/F values reported for humans in this chapter were 2251 ml/min/kg (ADI dose) and 253 ml/min/kg (10 x ADI dose). Clearly there is a large discrepancy between our data and the data in the literature. However, we are not reporting hepatic clearance, and not even total clearance, but CL/F. Hence our reported values are likely to be greater than the hepatic clearance reported by Li et al.

One of the variables known to be implicated in the clearance of TBZ from both species is the constitutive levels, inducibility and general activity of CYP1A2. Generally, the expression of CYP1A is the same between rat and human in hepatic and extrahepatic tissues. Exposure to PAHs results in induction of CYP1A2 in the liver, but CYP1A1 in extrahepatic tissues (Sesardic et al., 1990).

Despite a high (72%) sequence homology, there are clear differences in activity between human and murine CYP1A2 activity *in vitro*. Several authors have noted that the metabolite profile generated in studies using typical substrates (caffeine, paraxanthine, polyaromatic hydrocarbons, MeIQx and PhIP) is species dependent (Labedzski et al., 2002, Sesardic et al., 1990, Fuhr et al., 1993, Xu et al., 2000, Turesky et al., 2002, Zhao et al., 1994). Additionally, the activity of CYP1A2 is species dependent. Shimada et al., (1997) examined the oxidation activity of hepatic CYP1A2 using known CYP1A2 substrates, and discovered that whilst humans and primates shared similar activity, oxidation rates in dogs, guinea pigs and rat were distinctly different. Other authors have used a variety of substrates, MeIQx, PhIP, TCDD, and have observed opposing activities. Xu et al., (2000) studied the effects of TCDD on CYP1A induction using primary rat and human cell cultures. TCDD-induction of EROD activity was ~10-fold lower in humans than in rats, and measured AhR (aryl-hydrocarbon receptor) was 4-fold higher in rats vs. humans. Interestingly, TCDD predominantly induced CYP1A1 in rats and CYP1A2 in humans.

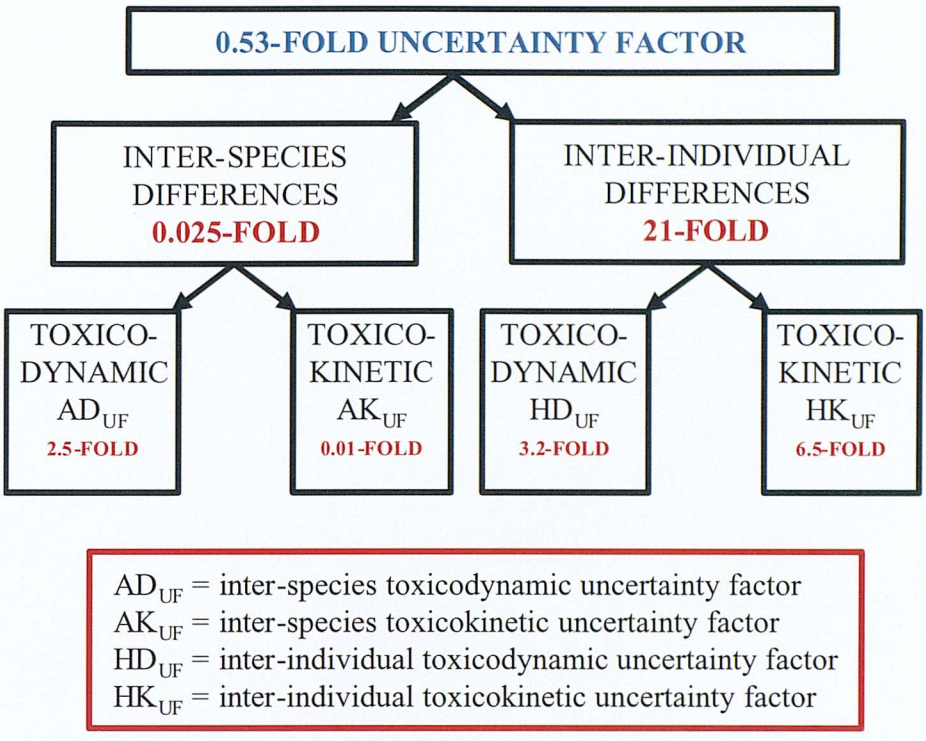
In another example, Turesky et al., (1998) showed that PCB-induced rats and (non-induced) human liver microsomes displayed roughly the same activity in N-oxidation of MeIQx. Paradoxically, most PCB-

treated rat microsomes showed greater activity than the human samples in the N-oxidation of PhIP. Recombinant human CYP450 1A2 showed catalytic efficiencies of MeIQx and PhIP N-oxidation that were 10-19-fold higher than purified rat CYP450 1A2. Both expression and substrate-dependent activation varied considerably between the two species. Using MeIQX and PhIP as substrates elevated levels of the glucuronidated metabolic products were observed in human hepatocytes (consistent with findings from 1998) compared with rat hepatocytes (Turesky et al., 2002). The major oxidation products differed between the species – demonstrating catalytic and enzymatic differences. DNA adduct formation was higher in humans than in rats exposed to comparable levels of MeIQx., and significantly higher levels of the N2 metabolite were observed in human vs. rat urine, reflecting of higher catalytic activity humans.

These observations highlight the complexity in inter-species extrapolation, where constitutive enzyme activity, the inducibility of the enzyme and the specificity of the enzyme are independent variables. Ideally any animal study for a CYP1A2 substrate would need to be carefully validated, so far as possible in humans.

6.10 Calculating the chemical-specific adjustment factor for TBZ

The PK parameters calculated (and/or extrapolated) from the data presented in this thesis could be used to refine the overall safety factor assigned to TBZ. CL/F would be the parameter of choice, as it most accurately reflects the bodies overall ability to remove a dose of TBZ from the system. Consequently the overall safety factor would be calculated as per figure 6.9. The factor for inter-individual differences in toxicokinetics is based on covering the 99^{th} percentile of the population, using the CoV for AUC from the ADI data.



NB – the inter-species factor and the inter-individual factors have been rounded to the nearest whole number

Figure 6.9 Substituting data-derived toxicokinetic values into the IPCS framework, resulting in a chemical specific adjustment factor for TBZ.

By using the actual in-vivo derived data, the overall composite factor is reduced from 100 to a factor of 0.53-fold (see figure 6.9). This would have the impact of increasing the ADI from 0.1 mg/kg/day to approximately 19 mg/kg/day, nearly the therapeutic dose. However, this calculation assumes that the kinetics in humans are linear over the range 0.1 to 19mg/kg, which we have demonstrated is not the case. Using the data at 1mg/kg in humans (which is closer to the projected ADI of 19 mg/kg) and 10mg/kg in rats the interspecies-adjustment factor would be about 0.68, not 0.025, and the correct total chemical-specific adjustment factor based on the scheme in figure 6.9 would be [0.27 x 2.5 x 6.5 x 3.16] or 14. This would give an "ADI" of 0.7 mg/kg (10mg/kg bwt in rats divided by 14) and this would be a more correct value because it is based on the data for animals treated at the NOAEL and humans given a dose similar to the predicted ADI. Given the severe non-linearity in TBZ kinetics in humans, it probable that the AUC at 0.7 mg/kg in humans would be unpredictable and could not be based on the AUC measured at 1mg/kg, and therefore further data would be required before the ADI could be established with confidence.

Non-linear kinetics, as found for TBZ would also be important in consideration of possible health effects at intakes above the ADI, because for example a two-fold increase in external dose would produce a

much greater increase in the internal dose.

6.10.1 Intake estimates for TBZ

The whole process of the risk assessment of food chemicals is meaningless unless one relates likely exposure levels to scientifically derived "safe intake" levels. Compared with the other compounds, relatively few reports were available in the literature commenting on the intake of TBZ. An official governmental study published with the Japanese government (Ishiwata et al., 2002b), reports that in the fiscal year 1998, TBZ was detected in 53% of citrus samples and 3.6% of banana samples tested. The mean concentration in these samples was 1.39 mg/kg (13.9% of the 10mg/kg limit) and 0.45 mg/kg (15.1% of the 3.0 mg/kg limit) respectively. Moreover TBZ was detected in non-permissible items such as non-alcoholic beverages and dried fruits. It was not however found in meat, milk or milk products. The precise impact of these data on the significance of the findings in this thesis is difficult to elucidate. Certainly it would appear that TBZ is not detected in amounts exceeding the limits on the fruits tested, especially when one considers that whole fruit is used in these analyses. The fact that TBZ is found in other items, perhaps where it is not expected may mean that estimated intakes of TBZ are sometimes underestimates.

The Committee on Toxicity (COT, 2002) reported the intake of TBZ in the years 1984-85, 1989-90, 1996-97. The levels of TBZ were undetectable in the first two intake surveys, but was detectable in the later survey (1996-97) at levels of 19.7μg/person/day, which equates to 0.3% of the ADI based on an average bodyweight of 60 kg. This report highlights the very low levels of TBZ detected in dietary components (principally fruit and vegetables), and suggests that intakes are unlikely to exceed the ADI in the case of most individuals.

At the moment, with the current ADI of 0.1mg/kg/day, generated using the default paradigm, it is unlikely that dietary exposure to TBZ represents a risk to human health, when consumed within the normal range. This is because the default inter-species factor of 10-fold grossly overestimates the true in-vivo derived chemical-specific factor of 0.025. The excessive nature of the inter-species 10-fold factor, actually includes another margin of safety because of the toxicokinetic differences reported in this chapter.

Chapter 7 : Final discussion

7. Final discussion

7.1 General summary

As discussed in chapter 1, there are a variety of approaches to the risk assessment of food additives (and chemicals in general) and the IPCS framework, leading to the derivation of chemical-specific adjustment factors is only one such approach. Whilst the chemical-specific approach may be the most desirable by virtue of the fact that the data is precisely relevant to the situation under assessment, the feasibility of the generation of such data leaves the method open to criticism. Other approaches (discussed in chapter 1), are also evolving, such as probabilistic modelling and the use of physiologically-based pharmacokinetic models. The diversity in approach to risk assessment should be appreciated, as the diversity of the task of chemical risk assessment relies on risk assessors being able to employ a range of approaches.

7.2 Feasibility of *in vivo* studies to generate data to replace the kinetic toxicokinetic default factors

The deconstruction of the traditional 10 x 10 (inter-species differences x human variability) uncertainty factor framework has allowed the inclusion of chemical specific data into risk assessment. Supporting scientific data for the allocation of the new numerical defaults have been from pharmaceutical data, which for reasons outlined in chapter 1 is not ideal. Despite the new framework being accepted by the IPCS in 1994, few risk assessments have been able to modify the standard 100-fold default used in the extrapolation from a no-effect level derived from animal studies to a no-effect level in humans. There have been some examples where the necessary data have been available (e.g. boron, dioxins, cyclamate), as outlined in chapter 1, but for the majority of food additives, the necessary chemical-specific data are not available.

The data presented in this thesis are therefore novel in a number of ways. They demonstrate that the derivation of human pharmacokinetic data is possible, but will mostly be limited by the sensitivity of the assays used to determine the concentration of food additive in the biological fluid. Interestingly, the compounds with the higher ADI values (PG and curcumin) were not significantly more easy to detect than the compounds with lower ADI values (BHT and TBZ). In fact the pathways of metabolism appear to be the most important determinants of whether or not a substrate can be monitored adequately in biological fluids. The compounds believed to undergo "non-specific" hydrolysis reactions, curcumin and PG, were very difficult to detect at the ADI, whilst compounds undergoing hydroxylation via CYP450 enzymes (BHT and TBZ) were more readily detectable. The use of saliva as a biomarker of internal concentrations of substrate is possible as the TBZ 10 x ADI data generate concentration-time profiles which mirror the plasma concentration time profiles. However, the sensitivity of the analytical method and the extent of protein binding of the food additive will be the major factors in determining whether or

not this is feasible for the food additive under consideration. The data also show, interestingly, that for some compounds, humans display signs of non-linear kinetics within a 10-fold range of dose (see table 7.5)

7.3 Compound-specific differences in human variability

Tables 7 .1 and 7.2 are summary tables of human variability, expressed as CoV for the ADI and 10 x ADI studies for all the substrates.

	Cmax	AUC	CL/F
BHT*	80%	65%	62%
Curcumin	-	-	-
PG	54%	42%	40%
TBZ	79%	95%	51%

* Using CoV from data extrapolated to infinity

Table 7.1 Summary of variability in pharmacokinetic parameters for humans dosed with the substrates at doses equivalent to the ADI

	Cmax	AUC	CL/F
BHT*	42%	50%	48%
Curcumin	38%	31%	30%
PG	28%	24%	20%
TBZ*	92%	121%	106%

* Using CoV from data extrapolated to infinity

Table 7.2 Summary of variability in pharmacokinetic parameters for humans dosed with the substrates at doses equivalent to 10 x ADI.

Comments regarding compound-specific factors resulting from measured human variability have been discussed previously and will not be re-examined here. Generally speaking the data sets for BHT, curcumin and propyl gallate show less variability at the higher dose of 10 x ADI than at the lower dose of the ADI. The variability in PK parameters observed in the PG data is generally small indicative perhaps of the ubiquitous nature of the esterases. The lower variability at the higher dose can be explained in many ways (see chapter 5 discussion), but it is likely that the higher concentrations at the 10 x ADI studies result in inherently lower analytical variability as a function of more accurate concentration-time profiles. Interestingly, in the case of TBZ, greater variability is seen at the high dose compared to the low dose. This is probably associated with the saturation that is occurring at the high dose, the extent of which is probably variable in between subjects.

Generally speaking, the human variability kinetic default of 3.16-fold is adequate for the substrates studied. However, it is important to consider the limitations of the current experiment. The subjects under study were from a restricted age group (18-65), were non-smokers and were not taking other medication. Both sexes and ethnic minorities were represented, but the study was not of sufficient size to determine any possible sex or ethnic differences.

7.3.1 General sources of human variability

Compound-specific sources of human variability have been discussed in the chapters for the relevant substrates, and will not be re-iterated here. Instead, broader issues relating to possible sources of variability will be highlighted.

Variability in the alleles, gene copy number, expression and activity of phase I enzymes involved in the initial biotransformations of xenobiotics have been the subject of intense research and review (for example, Snawder & Lipscomb, 2000, Shu et al., 2001, Evans & Johnson, 2001). Induction of CYP450s can also be a source of variability (reviewed by Lin & Lu, 2001). This is particularly important in cases where intake above the ADI occurs and induction of CYP450 enzymes may occur in some individuals. This may have implications in terms of increased bioactivation of toxic species, and would mean that safety margins for other substrates may be compromised.

One of the criticisms of the use of the mathematical model to predict the proportion of the population covered by the 3.16-fold factor, is that it is assumed that variability in single measurements will reflect that in chronic exposure. The assumption is that outliers on one occasion will remain outliers in subsequent measurements. Renwick & Lazarus (1998) considered this, but very little data is available to investigate intra-individual variability. It is unlikely however, that outliers will be constant upon repeat administration unless there is a specific reason (e.g. genetic differences affecting variability). It would have been advantageous to conduct repeat dose studies (RDS), as advocated by Kalow et al., (1999). Traditionally twin studies have been used to determine the genetic component of variability, but a more efficient method may be to measure variability both between individuals and within an individual upon repeat administration (the precise number of repeat administrations necessary has not been decided). The variability could then be estimated using the standard deviations of the inter-individual and intra-individual variability. This would have been a particularly useful exercise to conduct with the volunteers presenting for this study, since other constant factors that might otherwise be mistaken for the genetic component of variability (smoking, contraceptive use) would be excluded. Intra-individual variability is certainly a phenomenon worth considering if human kinetic data become a pre-requisite for risk assessment databases.

The role of a variety of transporters (including multi-drug transporters) in bioavailability, metabolism and toxicity of xenobiotics is a relatively new area of investigation (Xie et al., 2001, Kim, 2002, Lindell et al., 2003 and Bodó et al., 2003). Multi-drug transporter proteins are members of the ABC transporter group and include the multi-drug resistance protein p-glycoprotein (MDR1) and the multidrug resistance associated proteins MRP1, MRP2 and possibly MRP3, 4 and 5 and the ABCG2 protein. MDR1 and ABCG2 protein have been shown to extrude large hydrophobic positively charged molecules, whilst members of the MRP family can additionally extrude water-soluble anionic compounds. Possible co-expression of CYP3A4 and MDR1 in human duodenal biopsies has been reported (Lindell et al., 2003). This is of potential importance, since MDR-1 may modulate the biotransformation or bioactivation of substrates of CYP3A4, with possible toxicological consequences. Differential expression of transporter proteins may add another layer of complexity to inter-species differences and human variability. Organic anion transporter proteins (OATPs) are differentially expressed in different parts of the body (Kim, 2002), and are thought to be co-expressed with MDR-1 and CYP3A4, demonstrating that drug disposition is not always just a simple matter of passive diffusion!

Polymorphisms have been reported for the MDR-1 gene (discussed in Xie et al., 2001), and have been correlated with clinical outcome, and possible ethnic differences have been hypothesised (black individuals having greater MDR-1 mediated transport than white individuals).

The continuing elucidation of the role of transporter proteins may help explain a possible source of human variability that cannot be accounted for due to metabolic differences alone. Of particular interest is the role of MDR-1, expression of which may result in decreased exposure to a xenobiotic (but one may need to take into account co-expression with CYP3A4). Situations in which MDR-1 is known to be up-regulated, for example in patients with cancer, may in light of further scientific findings, require specific risk management advice.

7.3.2 Potential of pharmacogenomics in risk assessment

As more information becomes available regarding polymorphisms in metabolising and transporting proteins, it is possible to envisage that in years to come, wide-scale screening of the population will become commonplace. As is already happening in the case of some genotypes (CYP2D6 and CYP2C19 polymorphism) for example, the genotype of individuals is being determined so as to inform the prescribing process for anti-psychotic medication. As this procedure becomes more commonplace, the resulting distribution data may help risk assessors, particularly if chemical-specific factors are developed (see later). Eventually one might envisage screening of representative proportions of the population purely to determine the distribution of proteins more relevant to risk assessment/risk management (for examples CYP1A1).

7.3.3 Compound-specific inter-species differences

There are many articles in the literature describing inter-species differences in the metabolism of xenobiotics, mostly relating to pharmaceutical compounds, and these were subject to a limited review by Renwick (1993) prior to the deconstruction of the 10 x 10 uncertainty factor paradigm. Research on inter-species differences from a molecular to a whole-body system level is ongoing (for example Shimada et al., 1997, Ulrich et al., 2002). Table 7.3 summarises the inter-species differences observed in this thesis based on kinetic data derived at the NOAEL in animals and at the ADI in humans.

	Cmax	**AUC**	**CL/F**
BHT	26.27	5.14	3.97
Curcumin	5.78	1.41	0.20
PG	0.27	1.22	2.03
TBZ	0.020	0.013	0.010

Table 7.3 A summary of inter-species factors calculated for the substrates under investigation (based on ADI/NOAEL data).

Based on indicators of chronic exposure, AUC and CL/F, the 4.0-fold default factor for inter-species differences in kinetics is generally adequate. There are outliers from the default of 4 in both directions; the interspecies factor for BHT, based on Cmax is particularly high, indicative that humans have greater acute exposure to a dose of BHT than animals. The chemical-specific factors for TBZ however, indicate that humans have a much smaller internal dose of TBZ than animals. This highlights the difficulties of using animal kinetic data as a basis to extrapolate to the likely kinetic characteristics in humans. Compounding this marked species difference for TBZ is the fact that both animals and humans are displaying non-linear kinetics within the 10-fold dose ranges of the experiments. The data in table 7.3 emphasise the simplicity of using a single default for inter-species differences that is applied to different animals' species and different pathways of metabolism.

The research area of general sources of inter-species differences in kinetics is vast, and beyond discussion here. Possible reasons for chemical specific inter-species are discussed in the previous result chapters (3,4,5, and 6) and included higher values for liver and kidney blood flow per kg bw in animals. Another possible source of generic inter-species differences includes possible differences in the extent of protein binding of the substrates. These would have been relatively simple experiments to conduct, but due to the time limits of the project were not undertaken. It is clear that chemical-specific data should be generated wherever possible, either by direct experimentation or by the use of a PB-PK model.

7.3.4 Interactions between food additives

Curcumin, BHT, PG and TBZ were identified as possibly having additive/synergistic effects on the liver in a review that was designed to identify possible interactions/joint actions between food additives (Groten et al., 2000). The conclusions of the paper were that due to the low numerical ADI values and low intakes, simple interactions were unlikely. Two or more additives would have to be consumed at or above the ADI before a simple metabolic interaction would be likely to occur. The extent of variability in pharmacokinetic parameters such as clearance would also be expected to predict the likelihood of possible interactions. It is possible to imagine that a broader distribution in a parameter such as clearance (assuming that the same pathways, physiological and/or metabolic), would indicate the presence in the population of individuals with low levels of enzyme activity, that is a low Vmax. Saturation of metabolism by the presence of high concentrations of two substrates could make an interaction more likely in such individuals. The variability in the data presented in this thesis was varied and unpredictable based on dose. It follows then, that one would need to consider variability in the context of the relevant dose in order to examine the possible chance of interaction. It is anticipated that intake of BHT and curcumin could, quite possibly be approaching/exceeding the ADI on a regular basis, and in light of evidence suggesting excursions above the ADI, risk assessors may need to consider the implications of intake above the ADI and possible interactions.

7.3.5 Dose-dependent or non-linear kinetics

Saturation occurs when the capacity of one or more of the physiological/metabolic processes in volved in the removal of a xenobiotic from the body system is exceeded. The effect of one system becoming saturated can impact other systems responsible for removal, for example, saturation of protein binding may result in more free drug being excreted via the kidneys. The consequences of saturation on pharmacokinetic process are outlined in table 7.4.

Site	Interaction	Possible consequences of saturation at high dose
Absorption	Active uptake	Reduced plasma levels and AUC after oral dose
	First pass metabolism	Increased plasma levels and AUC after oral dose
Distribution	Plasma protein	Increased volume of distribution, increased glomerular filtration, increased hepatic clearance if extraction ratio is low.
	Tissue protein	Decreased volume of distribution
Metabolism	Metabolising enzyme (saturation by substrate, depletion of co-factors, end product inhibition)	Decreased clearance, AUC/dose ratio increases for parent compound. AUC of metabolite/dose ration may decrease after an oral dose. Enzymes with a higher Km may handle a larger proportion of the dose.
Excretion	Renal tubular secretion	Decreased renal clearance. AUC/dose ratio increases for oral dose. Normal routes of elimination become more important. Total excretion I urine per dose may decrease depending on other routes of elimination.
	Renal tubule resbsorption (rare)	Opposite to effects of saturated renal tubular secretion.
	Biliary excretion	Decreased biliary clearance. Decreased entero-hepatic reciruculation. Renal route may become more important. AUC/dose ration increases for oral dose.

Table 7.4 The consequences of saturation on pharmacokinetic parameters. Adapted from Renwick, 1989.

The rate of elimination of a compound is subject to the law of mass action and reaction kinetics. The law of mass action states that:

"The rate at which a chemical reaction proceeds is proportional to the active masses of the reacting substances" (Curry, 1980)

The implication of this, is that a non-reversible reaction occurs at an ever-decreasing rate as the quantity of the substrate (in this case the plasma concentration of the food additive) declines. In the case of non-saturation, there is a large excess of the endogenous "reacting molecules" (this could be plasma proteins, CYP450 enzyme, p-glycoprotein etc.) for the food additive molecules to react with, and so the rate of the reaction decreases as the food additive is removed from the body. The rate of these reactions will be independent of dose, as the rate limiting step is the intrinsic rate at which the reaction occurs (R a [ ] and R = ? [ ]). The overall effect is that the slope of elimination is an exponential curve; these characteristics define the reaction as a first-order reaction. (Figure 7.1 (b).)

When saturation occurs, the substrate is in excess, and so the rate of reaction is constant over the period of time that the process is saturated (Rate = ?). The amount of time taken for the compound to be removed

then becomes dependent on the initial starting concentration (i.e. the internal dose of the food additive). The overall effect is that the slope of elimination is constant for a period of time; these characteristics define the reaction as zero order. (Figure 7.1 (a).)

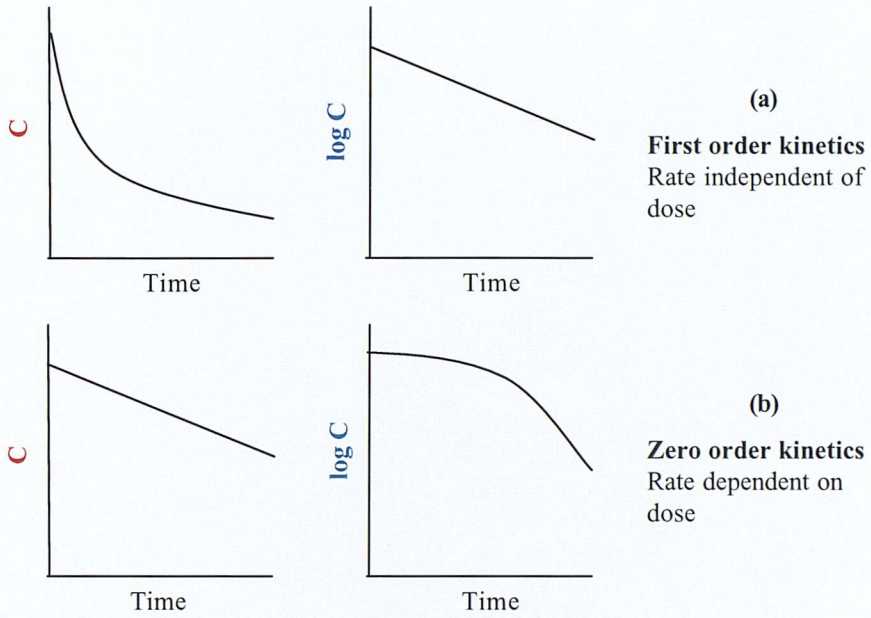


Figure 7.1 Zero-order and first order kinetics represented graphically

According to Wagner (1979, cited in Renwick, 1989) there are five tests for establishing saturation or non-linear kinetics:

1. Graphs of plasma concentration/dose plotted against time should be super-imposable for linear kinetics at different doses. Increased or decreased levels should be apparent for non-linear systems.
2. Administration of several different intravenous doses and estimation of concentration at time zero by fitting the slope of the initial elimination rate. Graphs of Cp/C0 against time should be super-impossible if linear kinetics apply.
3. Fit the concentration-time data to a linear model and derive the appropriate kinetic parameters (CL, V, k). A dose-dependent change in parameter indicates non-linearity/saturation kinetics.
4. If Michaelis-Menton kinetics apply, the percentage of drug metabolised by that pathway decreases with an increase in dose. The value of AUC/dose is not constant and plots of Cp against time curve downward.
5. Measure tissue and unbound plasma concentrations over a range of doses. A plot of tissue concentration (Ct) against unbound concentration (Cpu) should be a straight line for linear tissue extraction. Saturation would mean that the relative increases in tissue concentrations are smaller

at higher doses. The value of Ct/Cpu should be constant in a linear system. Non-linearity of tissue binding would result in this value becoming smaller at higher doses.

In the case of the data generated in this project, it is possible to compare CL/F values at the two doses in animals and in humans to determine whether or not saturation kinetics occurs (approach 3). Table 7.5 summarises the ratios between the high and low doses for both the human and animal data, highlighting where saturation kinetics are likely to have occurred.

	Human data 10 x ADI/ADI	**Animal data** NOAEL/0.1 x NOAEL
BHT	0.9	0.61
Curcumin	-	-
PG	1.2	0.9
TBZ	0.1	0.08

Table 7.5 A summary of CL/F ratios derived from the two dose studies in each species

Saturation kinetics was observed in the human and animal TBZ data sets. Non-linearity is only relevant if it occurs below the LOAEL, and affects the slope of the external-dose/internal dose curve that the NOAEL is on. Table 7.6 is a summary table of the NOAEL, LOAEL and ADI values for TBZ, and the dose range at which non-linearity was observed in the data presented.

	NOAEL	**LOAEL**	**ADI**	**Dose range in which saturation is occurring (animals)**
TBZ	10 mg/kg[a]	40 mg/kg[a]	0.1 mg/kg[b]	1-10 mg/kg

Table 7.6 A summary of the NOAEL, LOAEL and ADI values for TBZ, and the dose at which non-linearity is observed in animals.

[a] Pivotal study, Lankas & Wise, 1990 (unpublished report, cited in WHO, 1997).

[b] a 100-fold safety factor was applied to the NOAEL of 10 mg/kg, resulting in an ADI of 0.1 mg/kg/day.

Non-linear kinetics above the LOAEL could affect the slope of the dose-response curve but not alter the position of the NOAEL or the application of uncertainty factors used to derive the ADI. Non-linearity around the NOAEL or lower would significantly affect the risk assessment and should therefore be incorporated into the selection/derivation of the composite uncertainty factor (van der Heijden et al., cited in Renwick, 1999a). Quite clearly in this case, the range of non-linearity in kinetics falls around/below the NOAEL. This has implications regarding the precision of the NOAEL, and therefore the ADI. Interestingly, the non-linearity is mirrored in the human data, indicative perhaps that whilst these dose ranges result in non-linear kinetics, the dose-kinetic parameter curves may still be reasonably parallel.

Saturation of kinetics at the dose of 10 mg/kg for TBZ in rats suggests that the true no-adverse effect level or NAEL would probably be below this dose. The long-term toxicological implications of intake at saturating doses appear not to have resulted in any chronic toxicological effect in the pivotal study. It may be necessary therefore to identify the mechanism that has become saturated (this was beyond the scope of the animal studies conducted), and to make a judgement as to whether or not toxicologically significant.

If the precision of the current NOAEL is questionable, this reflects of the precision of the ADI, and so any estimates of human variability and proportions of the population covered are subject to discovering whether or not the ADI kinetics are on the linear part of the "dose-PK-effect curve". This would require a greater range of doses (less than and greater than the ADI) to be studied *in vivo* in humans.

In the case of the other food additives under study (BHT, curcumin and PG), kinetics were linear at the doses studies. The inference therefore, is that the estimate of the NOAEL is a reasonable estimate of the NAEL (the true threshold), resulting in an ADI, which as designed is likely to be protective, if not overprotective in some instances.

In conclusion, it seems that an approach that requires greater characterisation of the linearity of the external-dose/internal-dose response curve in the animal model, and preferentially in humans (where possible) would help to remove some of the uncertainty and imprecision inherent in the use of point estimates (i.e. the NOAEL/LOAEL/ADI). Areas of the dose-response would then be either linear or non-linear, and risk management decisions would have greater supporting evidence, especially when considering the consequence of intakes above the ADI.

The following text discusses possible risks for individuals with intakes above the ADI. The thoughts apply equally well to the risks to individuals whose CL/F is more than 3.16 standard deviations from the mean and who have intakes at the ADI.

7.3.6 Excursions/intakes above the ADI

Intake data relating to the compounds investigated has been reviewed in the relevant result chapters. The conclusions of these intake estimates are that generally exposure to BHT, curcumin, propyl gallate and thiabendazole is low. However intake data are derived mostly from the US and European countries, which may over-estimate "global" exposure to some compound, a possible example is BHT since developed "western" populations tend to eat more food fried in fat containing BHT. Conversely the

intake of curcumin, which is used extensively in cooking on the Indian sub-continent is likely to be underestimated based on intake data from this population.

Intake or exposure to food additives at levels exceeding the ADI, may be associated with adverse health effects. Reasons for increased intake may be due to the life-stage of the individual, the life-style of the individual or cultural practices of the individual which impacts on diet (Lawrie, 1998). For example, Lawrie (1998) reports that the PTWI (permitted tolerable weekly intake which is used for contaminants) for dibenzo-dioxins and the ADI for saccharin were exceeded by some children, as a function of the types of diet consumed. The only type of post-marketing surveillance currently undertaken for food additives is the assessment of consumption versus the numerical ADI value. Certain groups of individuals can be identified through lifestyle choices or medical conditions, e.g. tryptophan use as an antidepressant and intake above the ADI of saccharin by diabetics (Lazarus, 1996). Careful consideration during the exposure assessment and risk characterisation processes may identify possible sub-groups of the population who are likely to consume above the ADI on a regular basis.

Intakes estimates are made using a variety of methods (some of these have been described when relevant in the substrate chapters) including the use of annual production statistics, national per capita estimates, market basket surveys and food diaries. These vary in accuracy and cost, and care must be taken in interpretation of the outcome of these studies, to avoid over conservatism and to ensure that the population studied is representative of the general population.

The question of the consequences of intake/exposure to food additives at doses above the ADI has been the subject of investigation by an ILSI taskforce (WHO, 1998, Larsen & Richold, 1999). Statements regarding intakes above the ADI have been made by the SCF (Scientific Committee on Food, Europe) and the JECFA:

"There need be no undue concern when the ADIs are occasionally or slightly exceeded. These circumstances therefore do not constitute a hazard to the health of the consumer. Minor fluctuations in daily intakes of a food additive above and below the ADI are usually self-compensating if averaged out over long periods. However, if the ADI appears from estimations of intake to be exceeded for long periods, expert re-assessment of the situation may be required" (SCF, 1980).

"Because in most cases, data are extrapolated from life-time animal studies, the ADI relates to life-time use and provides a margin of safety large enough for toxicologists not to be particularly concerned about short-term use at exposure levels exceeding the ADI, providing that the average intake over longer periods of time does not exceed it" (WHO, 1987).

In the case of food additives, this seems to be a sensible approach, since this group of low molecular weight compounds generally seem to have small T ½ values and are of generally low toxicity (Renwick, 1999a). Certainly this is the case for the substrates presented in this thesis. One of the major problems in attempting to determine the possible consequences to human health of intake above the ADI, is to decide what length of exposure is likely to cause adverse effects, reflected in the following statement:

"It is impossible to make generalisations concerning the length of time during which intakes in excess of the PTWI (provisional tolerable weekly intake) would be toxicologically detrimental. Any detrimental effect would depend upon the nature of the toxicity and the biological half-lie of the chemical concerned" (WHO, 1989)

It is now generally accepted, that the length of time taken to induce the critical effect in acute toxicity studies in animals may be used as an indicator of the duration of exposure that may be incurred without adverse effect. Additionally several other factors have been identified that may be used to consider the likely impact of excursions above the ADI, including the precision of the NOAEL, the duration of exposure and the severity of the critical effect (Renwick, 1999b,c).

7.3.6.1 Precision of the NOAEL

If wide dosing intervals are used in the pivotal study, the estimated NOAEL may be considerably lower than the LOAEL, resulting in a NOAEL that may be underestimating the true NAEL. Consequently excursions above the ADI may still be in the range of exposures "covered" by the dosing interval between the NOAEL and NAEL. The intervals between the NOAEL and LOAEL for the substrates studied is 3-4 fold (see table 7.7) so that the NOAEL may slightly underestimate the NAEL. However, if the NAEL was less than the NOAEL then saturation kinetics would mean that the decrease in internal dose from NOAEL to NAEL would be greater than the decrease in external dose.

	NOAEL (mg/kg bw)	**LOAEL (mg/kg bw)**
BHT	25	100
Curcumin	220*	1520*
Propyl gallate	1910 (135)**	7455 (510)**
Thiabendazole	10	40

* Based on the dose given to male mice, not female mice, refer to chapter 4.

** Dose expressed as mg/kg in food. Figure in parentheses is the dose expressed as mg/kg bw.

Table 7.7 Dose interval between NOAEL and LOAEL for the compounds under study.

In the case of TBZ, the kinetics display non-linearity/saturation, at doses less than the NOAEL. There is a 4-fold difference between the NOAEL and LOAEL but due to saturation kinetics the increase in internal dose (AUC) would be »4. This means that the NAEL (internal dose) could be much higher than the NOAEL (internal dose). Consequently non-linear kinetics around the NOAEL will serve to increase the significance of any difference between the NOAEL and NAEL.

The life-stage used in the derivation of the NOAEL also needs to be considered in light of any excursion above the ADI. A NOAEL based on one particular life-stage (e.g. neonatal life-stage) may be over protective for other life states, (e.g. adult humans), suggesting that the ADI could be exceeded in some populations without an increase in risk. Such considerations would need to be considered on a case-by-case basis.

7.3.6.2 Duration of exposure

Excursions above the ADI are generally assumed to transient and sporadic, but the risk assessment process leading to the allocation of the ADI uses chronic data to set safe exposure limits. Intake estimates usually reflect short periods of time, and are therefore acute in nature. General toxicology tells us that chronic administration of a chemical usually results in a lower tolerable dose than acute administration. It follows then, that the most appropriate comparison to make in the consideration of excursions above the ADI, is the estimated intake vs. the acute dose that results in the critical effect. This approach is already used to consider the non-uniform residue levels of pesticides present in agricultural crops (Renwick et al., 2003). The acute reference dose (ArfD) is normally derived from the NOAEL for critical effects in short-term studies, to which the usual default uncertainty factor of 100 is applied (or chemical-specific adjustment factor if data are available).

One of the problems of the current system of risk characterisation is that neither the ADI nor the ArfD address the likely situation of exposure which is that chronic intake is likely to be low and constant, but interspersed with transient sporadic intakes above the ADI. This might be of particular importance at particular life stages, for example embryonic development, or infancy during neural development. These possible scenarios require a greater integration of the exposure and hazard assessments. The nature of exposure of the studies in this thesis is equally non-representative of the likely exposure scenario – i.e. intake of additive over the course of 12 hours for example. It is unlikely that persons would consume the ADI or 10 x ADI as a bolus dose, except in the case of accidental exposure, and so the data here represent the "worst-case scenario". This is particularly true for TBZ since saturating concentrations in the liver may not have been produced if the 10 x ADI dose was ingested slowly over a period of 12-16 hours. Mimicking non-bolus administration, for example by administering the ADI as three doses with meals, at 8am, 12.30pm and 6.30pm would been much more difficult (from the clinical and analytical perspective).

The consequences of continued intake above the ADI are less extensively discussed in the literature. Generally speaking the opinion is that the diminished margin of safety between human intake and animal NOAEL needs to be considered against the likely margin between the NOAEL and true NAEL whilst taking into account duration. This may be a case for expert judgement on a case-by case basis rather than a scenario which can be prescribed, for example, in the case of monosodium glutamate (Walker, 1999).

7.3.6.3 Reversibility/severity of critical effect

If the effect of excess intake is reversible, the consequences of excess could be minimised by subsequent intake at or below the ADI. The status of the toxicological effect may change depending on duration of exposure, i.e. reversible for short periods of time, but irreversible when exposure is chronic (Walker, 1999). The critical effects reported for the compounds under discussion are presented below in table 7.8.

Compound	Critical effect
BHT	Hepatic enzyme induction and thyroid hyperactivity
Curcumin	Reduced bodyweight and increased absolute and relative liver weights
Propyl gallate	Increased activity of phase II xenobiotic transformation enzymes (glucuronyl-transferase and glutathione-S-transferase)
Thiabendazole	Reduced bodyweight gain

Table 7.8 Reported critical effects in pivotal studies, from which the NOAEL was determined.

The severity of toxicity is a judgmental decision, but one may consider that from the effects listed in table 7.8, that none of the critical effects identified in the pivotal studies would be considered to be severely toxic. The critical effect for thiabendazole, which is the only compound where the true ADI might be being underestimated, is certainly not of grave concern.

7.3.6.4 The magnitude of exposure

Theoretically increased exposure will move sensitive individuals from a position on a no-effect dose-response to an effect dose-response. Both kinetic and dynamic aspects will affect an individual's susceptibility to a particular exposure level (Renwick, 1999d). Management of possible intakes above the ADI would depend upon the tail of the susceptibility distribution curve, whether or not sub-groups could be identified and the severity of the critical effect. The risk of any individual exceeding the 10-fold default factor for inter-individual variation, based on increased intake is thought to be small as both the toxicokinetic and toxicodynamic default factor would have to be exceeded. Based on a limited analysis of pharmaceutical data, it was estimated that only 2 persons/million would not be covered by combined factors (3.16 x 3.16) assuming a normal distribution and only 162 persons/million based on log-normal

distribution (Renwick & Lazarus, 1998).

Our data for BHT, curcumin and PG show reduced variability at the high dose, although as previously discussed this is a function of assay sensitivity. This implies that a greater percentage of the population would be covered by the 3.16-fold at a dose above the ADI. This is entirely feasible, given the conservative estimate of the ADI. However, one must be careful to characterise the human dose-response carefully before commenting on adequate protection of the population at exposure above the ADI, since the TBZ 10 x ADI data showed greater variability as the ADI data.

7.3.7 Further guidance/information on how to use data in the CSAF framework.

The IPCS have produced a guidance document (WHO, 2001) for risk assessors with two major objectives:

"...to increase common understanding and to encourage the incorporation of relevant quantitative data in a context consistent with traditional approaches to development of measures of dose/concentration response."
"...to more fully delineate appropriate avenues of research to enable more predictive estimates of risk."

In addition, articles in the literature and reported scientific debate have helped to clarify what types of data might be incorporated into the new risk assessment framework, and how these data might be generated (Meek et al., 2003, Dybing, 2003). The IPCS document contains several useful flow charts outlining the decision processes required when attempting to incorporate data into a chemical-specific risk assessment (see figure 7.4 and 7.3). The data presented in this thesis will now be discussed with reference to the standards and quality of data described within this guidance document.

7.3.7.1 Identification of chemical moiety

BHT, PG and TBZ, all undergo significant metabolism in the liver. For BHT this is the site of action (see table 7.8), and the metabolites also have the potential to contribute to any toxic effect (see introduction to chapter 3). Significantly, there was a species differences in the routes of metabolism (between rat and mouse) and so further investigation in humans to identify the metabolic products of BHT may be warranted. In the case of TBZ, a metabolite is associated with nephrotoxic effects, and so again, determination of the routes of metabolism may be necessary. PG is metabolised by non-specific esterases at multiple sites, there is little evidence in the literature to suggest that PG is more or less toxic than its metabolites, but further work (possibly *in vitro* is warranted). The internal dose of curcumin has been

shown to be very small in the data presented in this thesis and there is some evidence that hydrolysis products of curcumin may be absorbed. Whilst there is a reasonable amount of information in the literature pertaining to the pharmacological action of the metabolites, no conclusions regarding toxicity relative to the parent compound can be made. Ideally qualitative and quantitative differences in metabolism of the parent compound should be considered and determined so that species differences in toxicokinetics can be interpreted fully.

7.3.7.2 Choice of relevant pharmacokinetic parameter

All of the studies identifying the critical effect were either chronic or sub-chronic, and so a PK parameter indicative of chronic exposure is most relevant. The PK parameters presented for comparison consist of Cmax, an estimate of clearance expressed as CL/F, and AUC. The relevant kinetic parameter for comparison of the PK parameters (derived from the ADI and 10 x ADI studies) is AUC (in some cases extrapolated to infinity, in some cases calculated from observed values). When considering the possible implications to the population of intakes exceeding the ADI, one may consider all three parameters to inform the risk management processes. Cmax would be most useful for consideration in cases of sporadic, transient intakes exceeding the ADI, whilst AUC might be more useful in interpreting chronic intakes above the ADI. However, the relative change in CL/F value between the two doses (ADI vs. 10 x ADI) would be integral to interpreting likely impact of the excursion, since saturation of the clearance pathways would result in increased internal exposure.

7.3.7.3 Experimental data

In terms of the quality and relevance of the experimental data there are a number of considerations. Firstly the relevance of the population under study. In the case of the food additives studied, the entire human population is the relevant population to study, based on the critical effects observed in animal studies. The observed effects in animals occurred in adult animals, and so the relevant comparison is with adult humans, who were the subjects for the ADI and 10 x ADI data. One criticism of the study is though, that only non-smoking adults (not taking prescription medication) aged 18-65 were allowed to take part in the study. If these restrictions were not in place, one might have expected to see larger ranges in PK values, and therefore smaller proportions of the population being adequately covered by the default of 3.16-fold. Due to obvious ethical constraints (studies not permissible in infants and children), and practical constraints (older members of the population represent a problem when trying to obtain large numbers of blood samples) this was not possible. It is important to recognise therefore, the limits of the data in extrapolating values for human variability in toxicokinetic parameters.

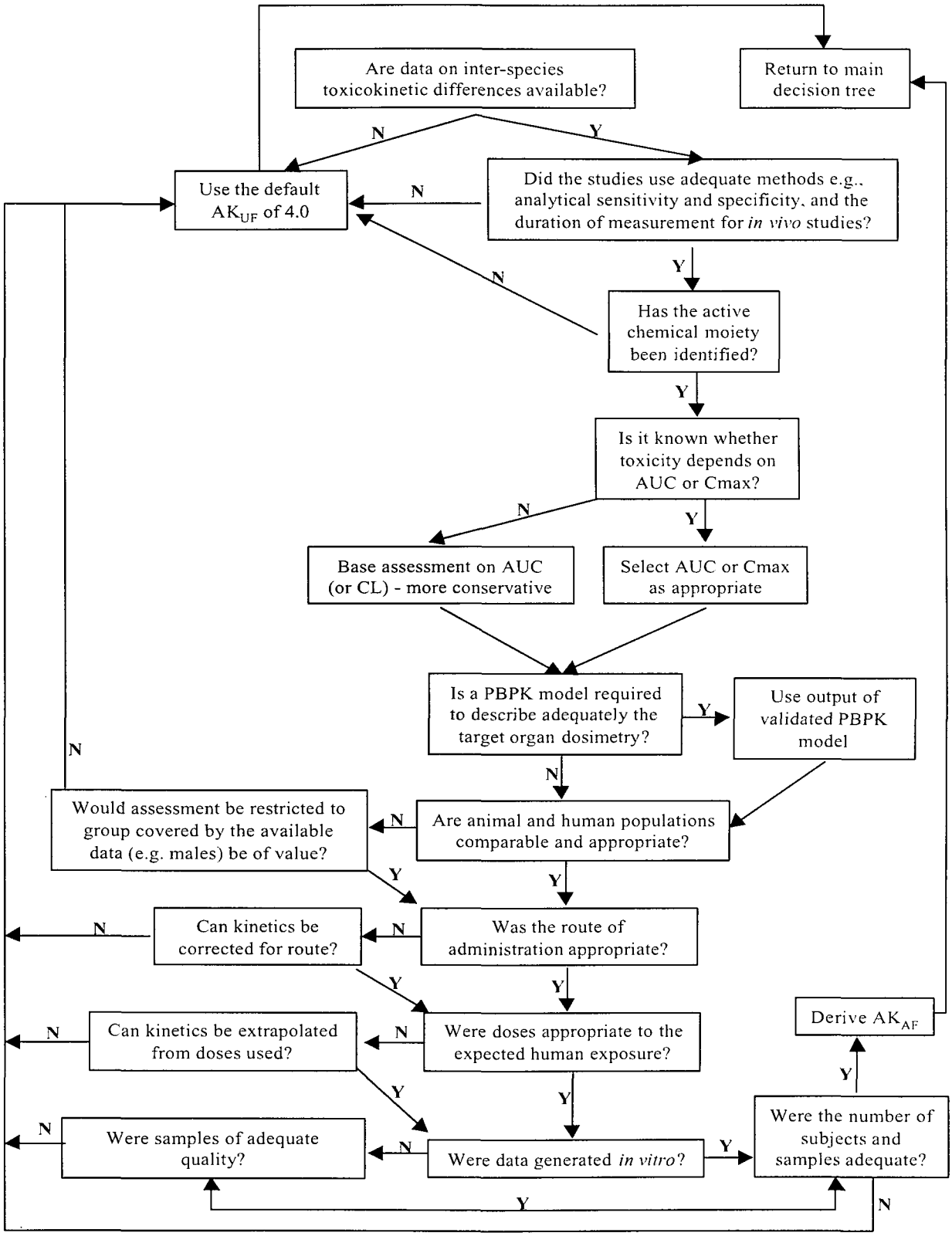


Figure 7.2 Guidance framework for the replacement of the 4.0-fold default factor for inter-species differences in kinetics with chemical specific data. Taken from WHO, 2001.

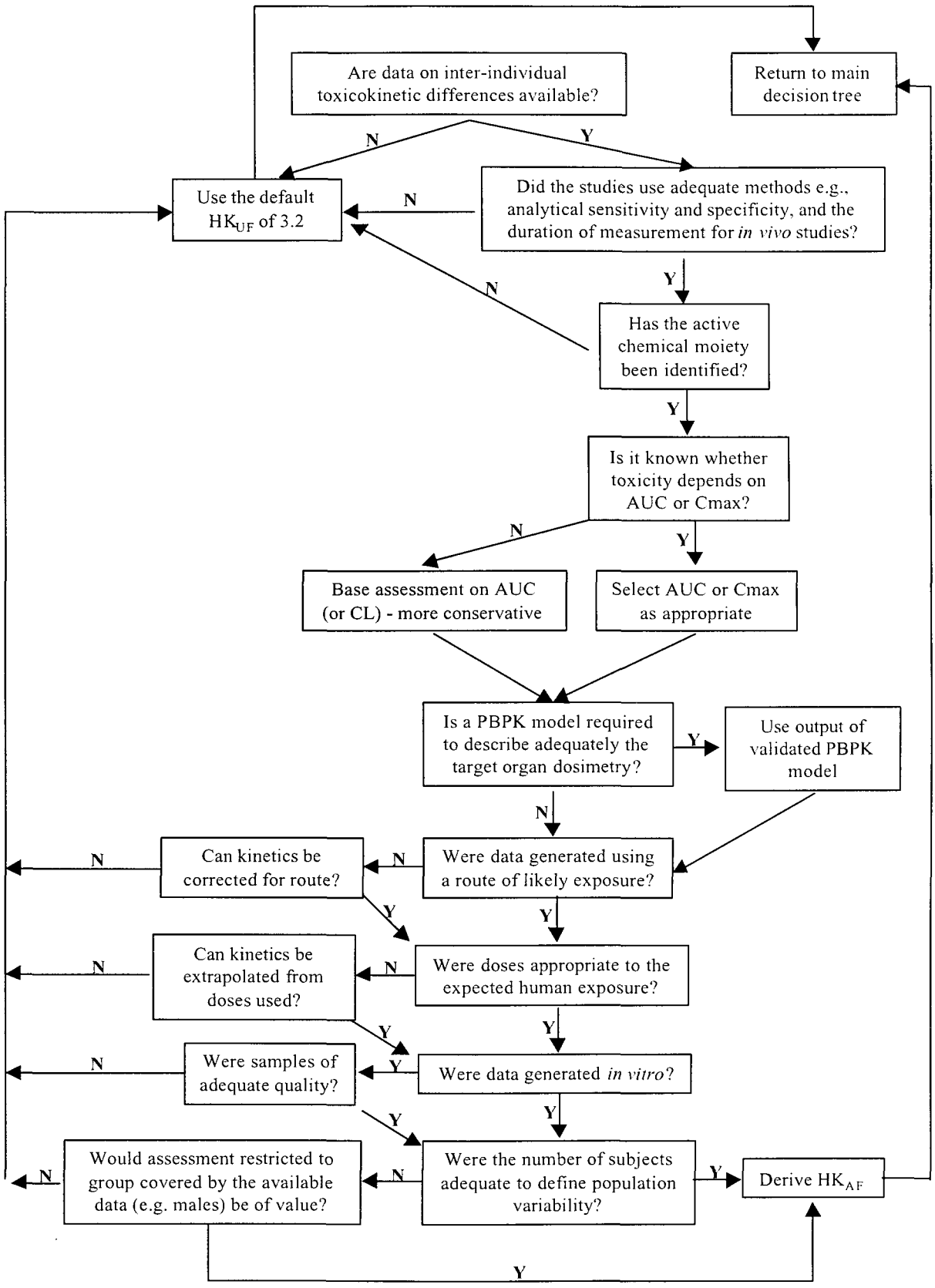


Figure 7.3 Guidance framework for the replacement of the 3.16-fold factor for variability in human kinetics with chemical-specific data. Taken from WHO, 2001.

The route of administration was certainly relevant for both species. Since food additives are consumed in food, oral dosing was deemed the most relevant route. The only possible criticism here is that animals received gavage doses in a vehicle, in the morning (i.e. after eating overnight) whilst humans received an oral bolus dose of food additive powder in a capsule with food after fasting for approximately 12 hours. This may have led to differences in absorption that would be additional to any true inter-species differences or human variability. As discussed above a bolus dose at the ADI would not represent the pattern of intake by humans or by animals if the chemical had been incorporated into the feed in the toxicity study.

The doses chosen for comparison of inter-species effect are also relevant. The possibility of saturation at the ADI in human subjects is assumed to be very small, due to the use of the NOAEL and the application of uncertainty factors. Certainly the data I have presented in this thesis support this notion. One may assume therefore, that by comparing kinetics at the NOAEL and ADI, we are comparing linear low-dose regions of the dose-response curve, at the no-effect level. The decision to conduct studies both at doses equivalent to 10 x ADI and 0.1 x NOAEL was taken, due to the very low concentrations of food additives that were detected in the plasma of subjects consuming the ADI as a bolus dose. Whilst the resulting kinetic parameters cannot be used to assess either human variability or species-differences, they do help to put the ADI studies into context, and provide useful and interesting data regarding the linearity of kinetics in humans and animals over this small dose range.

The numbers of subjects (animals and human volunteers) chosen for generation of the data set are considered to be adequate to characterise the central tendency of the PK parameters, and this central tendency, in agreement with the IPCS guidelines has been classified as the arithmetic mean of the PK parameters. Outliers in the data sets have been identified and discussed in the compound-specific chapters. The data from some compounds (BHT-ADI data for example) is much stronger than other data sets (TBZ at the ADI and PG at the ADI), however, with the 10 x ADI studies as "supporting evidence" for these data sets, some conclusions about inter-species differences can be made. The same is true for some of the 0.1 x NOAEL data, where concentrations of food additives were much lower. Clearly in the case of curcumin the sensitivity of the analytical method was not sufficient to define the concentration time profiles. Apart from the TBZ studies, the same was true of the saliva samples from the 10 x ADI studies. This meant that only limited conclusions regarding the feasibility of the use of saliva as a biomarker of internal dose were made.

The nature of the mathematical distribution of PK parameters for each compound (such as normal skewed etc.) was not determined. The numbers of volunteers studied was not enough to determine distribution adequately. Pending elucidation of the relevant metabolic pathways in humans, studies such as genotype analyses (for the relevant phase I enzymes CYP1A1, CYP1A2, BHT and TBZ respectively) or *in vitro*

experiments using whole blood (to determine the rate of hydrolysis of compounds, curcumin and PG) may have helped determine what mode of distribution was likely for the various compounds.

7.4 Future developments

This project has resulted in some very interesting data that generally support the current risk assessment framework used to derive the ADI (IPCS, 1994). Some interesting findings challenge the notion of linearity at (relatively) low doses in humans, and the usefulness of animal data to predict toxicokinetics in humans. Since studies in humans are possible, this may be perceived as a possible way of reducing uncertainty in the risk assessment of food additives. The generation of the necessary pharmacokinetic data in humans to do this retrospectively for previously assessed additives would be very costly, and almost certainly unworkable. However, the possibility of requesting *in vivo* human pharmacokinetic studies as part of the dossier of information supplied by the manufacturer prior to regulatory approval is worthy of further discussion. A recent comprehensive review by ILSI Europe (ILSI, 2003) proposed possible changes to the risk assessment process that would improve the quality and accuracy of the risk assessment process at a generic rather than chemical-specific level. Some of these latter ideas that are pertinent to subject areas covered in this thesis are presented briefly below:

"Improvement in knowledge in the shape of the dose-response curve at low doses of genotoxic and non-genotoxic carcinogens." Certainly the data presented here suggests that greater characterisation of the dose-pharmacokinetic-response curve in animals and humans may be necessary to ensure the precision of the NOAEL. Similarly the document outlined "Optimisation of study design with respect to the numbers of animals and doses used" as an aim. Together with the item previously discussed, better characterisation of the dose-response curve is a priority in toxicological studies.

"Further development of a parallelogram approach based on the comparison of *in vitro* assays utilising animal and human cells, and of biomarker- based analysis of both animal and human exposure data." The simultaneous generation of *in vivo* and *in vitro* data will allow the feasibility of such an approach to be validated. The data presented here (using plasma concentrations of the parent compound as an estimate of internal dose) was part of a wider project of research, which included the generation of *in vitro* data using microsomal assays from both species.

"Improved understanding of the biological basis for combination effects of two or more compounds." Risk assessments currently focus on compounds in isolation, however with the possible use of probalistic modelling (see chapter 1), it is possible to envisage the use of distributions of intake to predict the likely proportion of the population at risk from interactions, and revise the risk assessment as necessary.

"Development of a structured approach to the application of data on known polymorphisms in humans and animals in the risk assessment process." This is a continuing area of research, and has the potential to contribute valuable information into the risk assessment process so that adequate proportions of the population remained covered by the risk assessment. New potential sources of variability will continue to be discovered (for example human variability and inter-species differences in membrane transporters), and these will also need to be factored into relevant risk assessments

"Reassessment of the validity of the default uncertainty factors by analysis of historical data on differences between humans and experimental animals or within human sub-populations." Again this is a continuing area of research (see Dorne et al., 2002), and is of particular importance, as it avoids the use of further animals and the expense and ethical considerations of conducting human volunteers studies.

7.5 Final comments

The results in this thesis show that the default values used in the IPCS framework are generally adequate based on chronic pharmacokinetic parameters to allow for inter-species differences, and human variability. The data support the concept of chemical-specific adjustment factors to replace the default inter-species factor, since there was a large range in the values of inter-species factors calculated. This suggests that the inter-species kinetic default does not adequately reflect the kinetic differences between animals and humans. Therefore in some instances the use of animal data as the basis of an extrapolation to the likely scenario in humans will result in over- or under-estimation of the true situation, and imprecise NOAEL and subsequently ADI values. Conversely, differences between substrates in the range in proportions of the population covered by the human variability default of 3.16 were relatively small. However, further studies would be necessary to support this conclusion, since potentially sensitive sub-groups of the population were not studied. Overall these data support the breakdown of the 10-fold factors to allow for species differences and human variability in pharmacokinetics. Further research (mostly dependent on the development of suitably sensitive biomarkers of response) is required to investigate the adequacy of the dynamic defaults, and complete the validation of the IPCS framework.

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