UNIVERSITY OF SOUTHAMPTON

BIOPTERIN METABOLISM IN SENILE DEMENTIA OF THE ALZHEIMER TYPE

by

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ABSTRACT

FACULTY OF MEDICINE

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Master of Philosophy

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Senile dementia of the Alzheimer type (SDAT) is a degenerative disease of the brain leading to a loss of cognitive function for which there is currently no therapy available. It has been suggested that a deficit in the metabolism of biopterin may be implicated in the pathology of SDAT.

Tetrahydrobiopterin (BH_4) is the biologically active form of the pterin species, acting as the cofactor for the hydroxylases of phenylalanine, tyrosine and tryptophan which are involved in the biosynthesis of various neurotransmitters. The hypothesis tested was that levels of BH_4 in the plasma of SDAT patients are reduced.

The development of a high performance liquid chromatography technique with sequential electrochemical and fluorimetric detection is described. Reference ranges for pterins in plasma were determined by analysis of samples from a group of healthy younger volunteers. Plasma samples from a group of healthy, elderly volunteers were also analysed together with samples from a group of SDAT patients.

The results are presented and statistical comparisons made using non-parametric methods, as inspection showed that they were not normally distributed. A reduction in levels of plasma BH_4 was detected, in SDAT patients, but was also found in the healthy elderly volunteers as compared with the younger controls. This reflects an age related decline in the activity of dihydropteridine reductase (DHPR), an enzyme involved in the biosynthesis of BH_4, and is compatable with the findings of previous workers in the field.

Levels of BH_2, the catabolite of BH_4, are significantly raised in the healthy elderly volunteers and slightly raised in SDAT patients. This pattern of pterin concentrations suggests that an additional metabolic block occurs in SDAT, early in the pathway of BH_4 biosynthesis. The next step would be to study sepiapterin and this is discussed.

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STATEMENT

I declare that during the course of this study, with the exception below, I carried out all the work myself.

Psychiatric evaluations and blood sampling were performed by Dr. J. Gommans.

ANDREW G. POWERS

AIMS OF THE STUDY

The hydroxylase enzymes of phenylalanine,tyrosine and tryptophan, which are involved in the synthesis of various neurotransmitters, have an absolute requirement for tetrahydrobiopterin (BH_4) as a cofactor. It has been suggested that BH_4 occurs in reduced quantities in patients suffering from senile dementia of the Alzheimer type (SDAT). The aims of this study are threefold:

(1) To develop a technique, using high performance liquid chromatography, to determine levels of BH_4 together with dihydrobiopterin, biopterin and neopterin in plasma.

(2) To determine levels of the above pterins in patients suffering from SDAT together with relevant control groups, i.e. healthy younger controls and apparantly healthy elderly volunteers.

(3) In the light of the resulting findings to consider whether a supplementation of cofactor might be of value to patients suffering from SDAT.

(1) ALZHEIMER'S DISEASE

(i) The Problem of Alzheimer's Disease

Senile dementia of the Alzheimer type is the most common form of dementia, accounting for up to 50% of all cases (Kokmen 1984). In western societies it is thought to affect 10% of those over 65 years of age and 20% of those over 80 years. The morphological features to be found in the brain were first described in detail by the German scientist Alois Alzheimer in 1907 whose observations have been noted in many reviews (e.g. Bowen 1981). However, references to senile mental deficiency reach far back into history, the earliest example being attributed (in a review by Torack 1983) to the Grecian "law-giver" Solon, circa 500 B.C., who did not permit the making of wills by senile individuals.

Until recently it was common practice to refer to patients with an onset occuring before 65 years of age as pre-senile dementia. It is now realised that there is no scientific basis for making this distinction, although the terminology is still frequently used.

The clinical course of the illness may follow several patterns. There may be a loss of cognitive function commencing between 60 and 70 years, or a featureless progressive decline in intellectual cognitive skills occuring late in the seventh decade. In some patients there may be a family history where the pattern is similar to one of the two already mentioned, although familial cases are relatively rare and account for only 5-10% of the total incidence. Patients with Down's syndrome also display Alzheimer type symptoms if they survive to the third or fourth decade (Ferry 1987). It should be noted that the age of onset given above is that most frequently observed and symptoms may appear long before, or after, 60 years.

The progress of the disease may be conveniently divided into three phases, although it should be

remembered that individual cases vary and no one patient will necessarily develop all of the symptoms in the order described.

Phase 1 (Mild Dementia). At this stage the diagnosis is largely subjective, perhaps only noticed by close relatives. The patient may become apathetic, unwilling to accept change, or forget details of recent events.

Phase II (Moderate Dementia). Here there are definite signs of cognitive decline. The patient may forget recent events and become easily disorientated in both time and familiar surroundings. They may forget names of family and friends and ask repetative questions.

Phase III (Severe Dementia). At this stage the patient is severly disabled and cannot lead an independant life. Recent memories may last for only a few minutes and the patient can completely fail to recognise close relatives. Help will be required with dressing, feeding and personal hygiene. Speech may make little sense and aggressive traits may become apparent (Schneck et. al. 1982).

Due to factors such as improved health care, the proportion of the population over 65 years is increasing in western society. Consequently there is a rise in the number of individuals at risk from senile dementia of the Alzheimer type (SDAT), and this presents an enlarged workload to the geriatric health services.

The presence of a dementing person can have a large impact on the family, both in terms of workload to look after the patient and also emotionally. Such tasks as feeding, dressing and attending to the personal hygiene of the patient can be a great burden to the carer. As the patient loses the concept of time they may wake at night and the whole routine of the household can be disturbed. It is important that the carer is not neglected and ideally should have the opportunity to take holidays, pursue his/her own hobbies and interests etc. As the cognitive decline

progresses close relatives feel that they are losing a loved one and often experience feelings of grief.

It is therefore seen that SDAT is a serious and widespread problem for which no therapy is currently available.

(ii) Strutural Characteristics of Alzheimer's Disease

The main structural features of the Alzheimer's diseased brain were first described by Alois Alzheimer in 1907 (in Bowen 1981) and consist of neurofibrillary tangles and senile plaques. Although these structures can occur to a limited extent in the normal aged brain, investigations have shown an incidence of senile plaques twenty times higher in AD than control groups. No correlation has been reported between age and plaque density. At present the diagnosis of AD can only be confirmed by the identification of significant numbers of these structures at post-mortem (Etienne et. al. 1986).

Senile plaques and neurofibrillary tangles (NFT'S) are found specifically in the temporal and parietal lobes of the brain, sensory and motor areas of the cortex being largely unaffected. NFT'S occur mainly in layers III and V of the cortex while plaques occur predominantly in II and III (Ferry 1985).

NFT's occur in the cell body of the neuron and, due to their appearance, have been described as paired helical filaments. They consist of long thread-like structures, probably derived from the neurofilaments, twisted in pairs into a helix. NFT's tend to "cluster" together, unlike senile plaques which appear to be randomly distributed. Worst affected is the limbic system which controls the emotions and new memory traces. It has been speculated that aluminium may be implicated in the formation of NFT'S (Perl 1983). As long ago as 1965 aluminium salts were shown to cause NFT formation in rabbits, although the structure of induced tangles was not the paired-helical formation found in naturally occuring human examples. Some

workers have reported brain aluminium content to positively correlate with senile changes (Perl 1983).

It has also been suggested that aluminium may bind with tubulin, interfering with the formation of microtubules. Studies using electron microscopy have shown that in SDAT neurones micro-tubules are completely replaced by paired helical filaments. These studies have also shown that the filaments arise from cytomembranes which are considered to be a pathological form of the smooth endoplasmic reticulum (Gray et. al. 1987).

During the course of my studies there have been a number of publications concerned with aluminium in relation to SDAT. Aluminium, in the form of aluminosilicates, is consistantly found in the core of senile plaques. These are highly insoluble compounds and so the question is raised of how they are transported. Aluminium is known to be present in the plasma at a level of around 0.25 μmol/L and silicon, in the form of silicic acid, is a normal component of the serum at a concentration of around 21 μmol/L.

Recent investigations by Birchall and Chappel (Birchall and Chappell 1988) have demonstrated that the binding of aluminium to silicon is pH dependant under physiological conditions. Silicic acid, $Si(OH)_4$ is structurally simlar to $Al(OH)_4^-$ with which it interacts to form aluminosilicates. If the pH falls below 6.6 there is a change from silicon to phosphate binding and Birchall & Chappell have considered whether this is of significance in the move from an extra to an intracellular environment. Their investigation also showed that over pH range 5 to 9, filterable solids formed in three to five weeks. They also showed that citrate, an aluminium chelator, can modify the formation of aluminosilicates by allowing the species to form but not aggregate into filterable solids.

The binding of aluminium will determine its competition for ligands with other metal ions, such as Mg^{2+}. It is known that aluminium has a stronger binding affinity for enzymes than magnesium. This may

have significance in the pathology of SDAT and will be discussed further in section 2. Many questions remain to be answered concerning the role of aluminium in the cause and/or progression of neurological disease.

Senile plaques (SP) occur among the axons, mainly in the cortex of the brain. They appear to consist of degenerating neurons which surround a protein core, known as amyloid, which does not normally occur in the brain (Ferry 1987). SP interfere with the transmission of messages between neurons and the number in which they occur has been shown to correlate with the severity of dementia (Kokmen 1984). Amyloid contains a protein with a molecular weight of approximatly 40,000 daltons known as A4, which is also present in NFT'S. Several recent studies have concentrated on proteins in relation to Alzheimer's disease and these have been reviewed by Ferry (1986 & 1987).

Protein A4 appears to be part of a larger protein with a molecular weight of approximatly 92,000 daltons which is a normal component of the cell membrane. A recent discovery is that the precursor for the amyloid β protein contains a protease inhibitor sequence. On this basis several workers in the field have proposed the hypothesis that SP formation is due to the proteolysis of the amyloid precursor protein to give self-aggregating fragments which deposit in the brain tissues. The gene for this protein is located on chromosome 21, which is also involved with Down's syndrome. It is interesting to note that the brains of older patients with Down's syndrome degenerate in a similar manner to those of AD patients (Ferry 1987).

The gene for the very rare hereditary Familial Alzheimer's Disease (FAD) is also located on chromosome 21 (Anderton 1987). It was originally thought that the amyloid precursor and FAD genes may be the same but recent evidence (Anderton 1987) has discounted this theory. However, it is possible that the FAD gene may influence processing of the amyloid protein.

Until comparatively recently no investigation had addressed the question of whether the neurones involved

in SP were derived from one or several transmitter systems. Using non-human primate SP and antibody techniques Walker et. al. (1988) have demonstrated the involvement of multiple neuronal systems. However the question remains as to whether the initial defect occurs in one system and spreads to others or simultaneously affects several neuronal populations.

Another change which has been reported in relation to SDAT is that of granulovacuolar degeneration (e.g. Ball and Lo 1977). Granulovacules are spherical structures between 3 & 5μm in diameter with a limiting envelope. They have a central haematoxylinophylic granule 0.5 to 1.5Nm wide which appears electron dense under the electron microscope. When approximatly 20 or more of these structures are present within a neurone it appears swollen. Ball and Lo (1977) found an increase in granulovacuolar degeneration with age, although this level was exceeded in age matched SDAT subjects the incidence ranging from 2 to 100 times greater. As with NFT'S the posterior half of the hippocampus is the most susceptable and it is possible that the severity of granulovacuolar change is correlated to the incidence of NFT'S.

Other structural changes which may occur are an enlargement of the ventricles and a reduction in cerebral blood flow (Hachinski 1975).

(iii) Biochemical Changes

There have been many studies of the various biochemical changes associated with SDAT. These have been fully discussed in a number of review articles, including Davison 1981, Kokmen 1984 and Harrison 1986. The majority of reports have concentrated on abnormalities in the metabolism of the cholinergic neurotransmitter acetylcholine, although some have studied the monoamine pathway involving 5-hydroxytryptamine (5-HT or serotonin) and 5-hydroxyindoleacetic acid (5-HIAA). Normal synthesis

of the major neurotransmitters, i.e. melatonin, norepinephrine and acetylcholine, is outlined in Fig.1 which also shows the position of the pterin dependant enzymes tryptophan, tyrosine and phenylalanine hydroxylase which will be discussed later (see section 2).

Abnormalities in the concentration of neurotransmitters are closely related to the loss of neurones from the brain, leading to the suggestion that SDAT may simply be "accelerated" ageing. However, it has been demonstrated (Bowen et. al. 1979) that cell loss is selective for the neocortical neurones and that of the biochemical constituants so far measured the enzyme choline acetyl-transferase (CAT) is the most depleted. The magnitude of CAT reduction is related to the severity of dementia and also negatively correlates with the density of neurofibrillary tangles (e.g. Palmer et. al. 1987). However it is not thought to be rate limiting in acetylcholine synthesis.

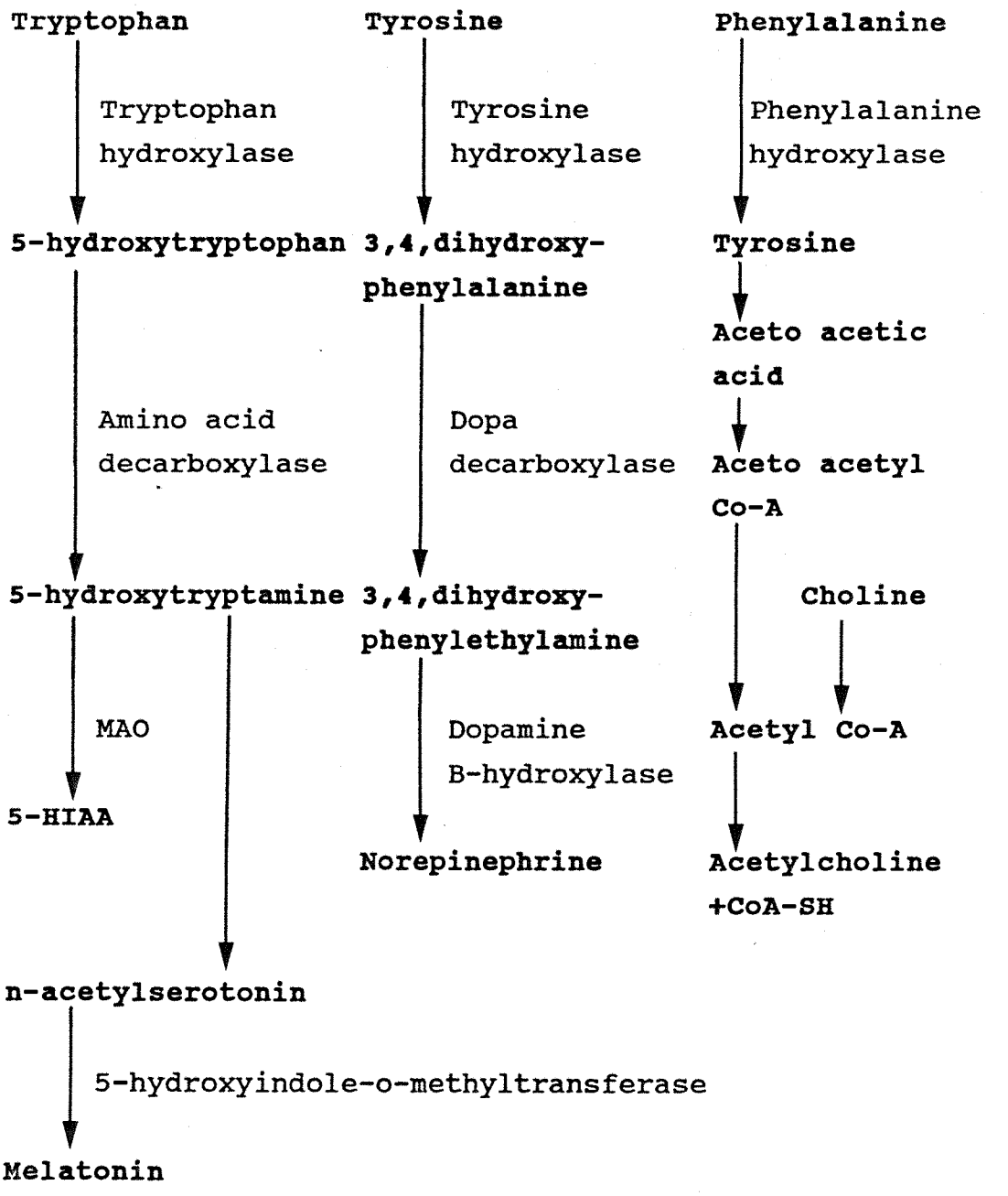
This cholinergic hypothesis, reviewed by Harrison (1986), has identified the Nucleus Basalis of Meynert (NBM) as the source of cortical innervation, an area known to degenerate in SDAT. Loss of ascending cholinergic innervation from the NBM may have parallels with Parkinsons disease. It has also been suggested that the disease may start in the hippocampus or temporal lobe before spreading along specific pathways to other areas of the brain (Hyman et.al. 1984).

Other biochemical changes include deficits in 5-HT and noradrenaline. 5-HT shows an age related decline in all areas of the brain but this only reaches significance in the gyrus cinguli. Again it has been suggested that deficits of 5-HT and noradrenaline in SDAT may be a result of nerve cell loss. A similar pattern has been observed for other biochemical factors, e.g. 5-HIAA and 3-methoxy-4-hydroxyphenylglycol (MHPG) (Gottfries et. al.1983, Palmer et. al. 1987).

Noradrenergic systems have been found to be relatively unaffected in SDAT. Modest deficits in

FIG.1.

NORMAL SYNTHESIS OF THE MAJOR NEUROTRANSMITTERS



serotonin and 5-HIAA are similar to those found in Parkinsons disease and are thought to simply reflect cell loss (D'Amato 1987).

Experimental studies have assumed that the deficits shown in the SDAT brain follow a bilateral symmetry. This question has only recently been investigated by Zubenko (Zubenko et. al. 1988) who looked at the distribution of CAT, acetylcholine esterase (AChE) and muscarinic receptors. He found a symmetrical distribution in all areas studied apart from the entorhinal cortex area of the hippocampus, an area attributed to memory loss, where CAT activity and receptor density did not show significant left/right correlation. It is interesting to note that a similar pattern was observed for tangles but not plaques, suggesting different pathologies for these lesions in SDAT (Moossy et. al. 1988).

Early investigations found that cerebral carbohydrate metabolism was altered in cases of dementia. These changes have been reviewed by Blass (Blass et. al. 1980). Features include a fall in cerebral blood flow, oxygen uptake and glucose utilisation, which are noted in both SDAT and multi-infarct dementia.

Glucose is the main substrate for cerebral oxidation and accounts for almost all of the oxygen taken up by the brain. Acetylcholine synthesis is known to be linked to carbohydrate metabolism. Very mild hypoxia will impair acetylcholine synthesis and it has been shown that anti-cholinesterase drugs (e.g. physostigmine) can delay seizures or death in cases of hypoxia. Artificially reducing inspired oxygen to 12-15% will impair short term memory, judgement and learning ability (Blass et. al. 1980).

(2) PTERINS

(i) Background

The biologically active form of the pterin species plays an essential role as cofactor for a number of enzymes involved in the synthesis of the major neurotransmitters. Their metabolism is therefore of interest in the study of pathological conditions involving a neurotransmitter deficit.

The majority of naturally occuring pterins are formed from 2-aminopteridin-4-one (or "pterin") by substitution of side chains at the 6 and/or 7 position, see figure 2. The first naturally occuring pterins to be isolated were from yellow coloured pigments in butterfly wings. Pterin derivatives are a growth factor for organisms such as Crithidia fasciculata, a protozoal parasite of the mosquito, which has been used in convenient and sensitive bioassays. It would seem unlikely that mammalian cells have a requirement for pterins as a growth factor (Nichol et. al. 1983). This is because several "normal" tumour cell lines display no detectable biopterin content or activity of guanosine triphosphate (GTP) cyclohydrolase, an enzyme involved early on in the synthetic pathway of tetrahydrobiopterin.

Tetrahydrobiopterin (BH_4)(Fig.3) is the essental cofactor for phenylalanine hydroxylase as first reported by Kaufman (Kaufman 1959) as well as for the hydroxylase for tryptophan and tyrosine. It is therefore of major importance in the formation of the relevant neurotransmitters (see Fig.1). BH_4 acts as a proton donor in the reaction, itself being oxidised to quinonoid dihydrobiopterin (q-BH_2)(Smith et. al. 1986).

FIG. 2

STRUCTURE OF PTERIN

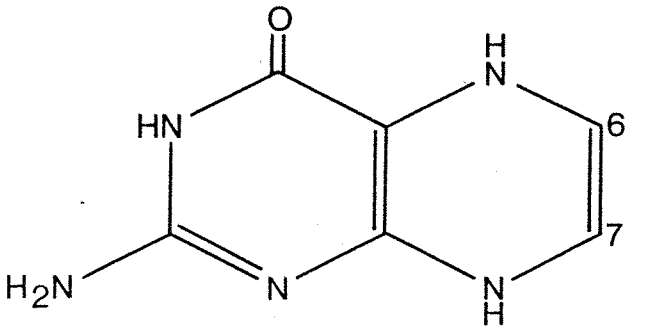
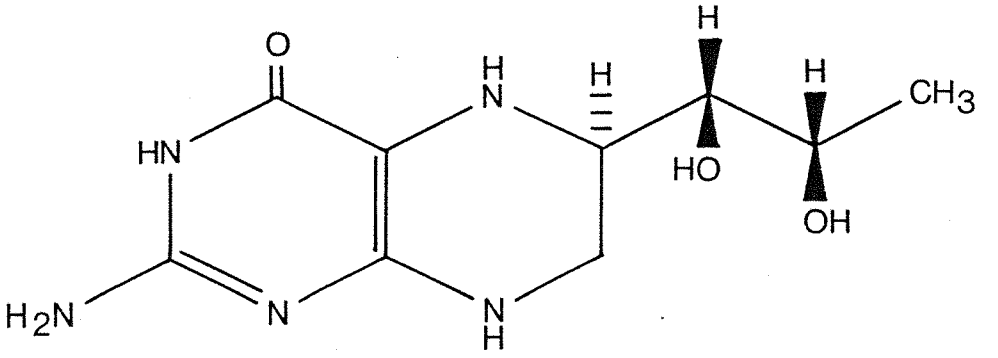


FIG. 3

TETRAHYDROBIOPTERIN (BH_4)



(ii) Biosynthesis of Tetrahydrobiopterin

The normal biosynthesis of tetrahydrobiopterin has not, as yet, been fully elucidated. It has been proposed (Nichol et. al. 1983) that two pathways are involved, (i) The conversion of guanosine triphosphate (GTP) to BH_4 and (ii) a "salvage" pathway involving the conversion of sepiapterin to BH_4. However, in mammalian cells and tissues GTP appears to be the primary precursor for the de novo synthesis of BH_4. This pathway is outlined in figure 4, from which it can be seen that sepiapterin is derived from GTP.

The quinonoid dihydrobiopterin (q-BH_2) formed by the oxidation of BH_4 in acting as cofactor for the hydroxylase enzymes is converted back to BH_4 by dihydropteridine reductase (DHPR). However q-BH_2 is unstable and will rearrange to 7,8-dihydrobiopterin (BH_2) which is not a substrate for DHPR. It is possible that some BH_2 may be converted to BH_4 by the action of dihydrofolate reductase (DHFR), but the extent to which this occurs under physiological conditions is as yet unknown.

Other workers (Heintel et. al. 1984) have indicated that under normal in vivo conditions sepiapterin may not be an intermediate in the biosynthesis of BH_4. This is because the pathway illustrated in figure 4 involves DHFR and it has been shown that BH_4 biosynthesis can still take place in the presence of DHFR inhibitors (Nichol et. al. 1983). Earlier reports (Tanaka et. al.1981) have suggested that dihydroneopterin triphosphate (NH_2P_3) is converted to sepiapterin via an intermediate compound "X". This complicates the situation still further as NADPH is considered necessary for the conversion of X to sepiapterin, although there is no change in redox potential between NH_2P_3 and sepiapterin. However NH_2P_3 is found to be converted to sepiapterin in most systems so far investigated. Heintel et. al. (1984) propose the modifications to the pathway illustrated in Fig.5 which involves the conversion of dihydroneopterin triphosphate (NH_2P_3) to BH_4 via tetrahydropterin and

FIG.4.

PROPOSED BIOSYNTHESIS OF TETRAHYDROBIOPTERIN (BH_4) IN MAN

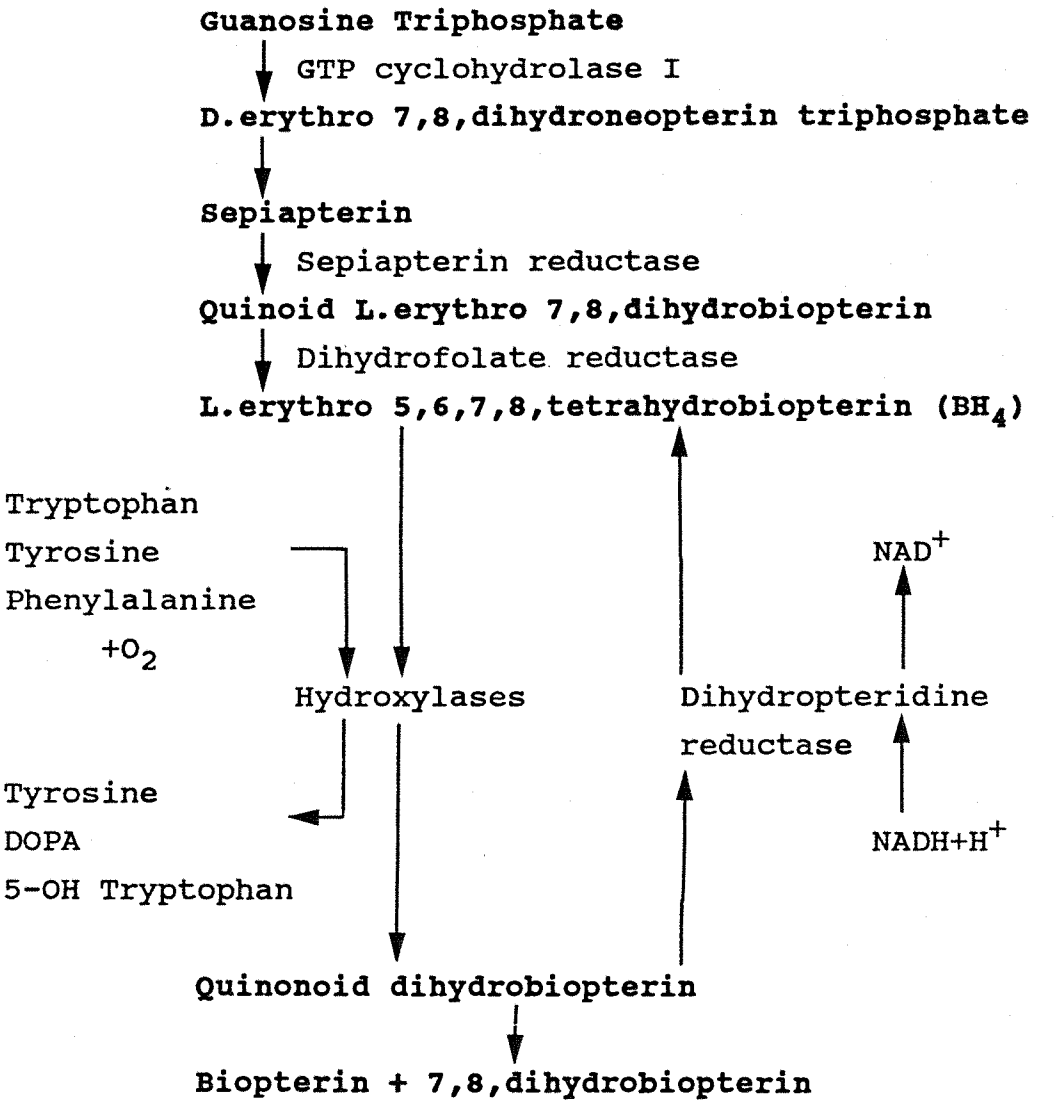
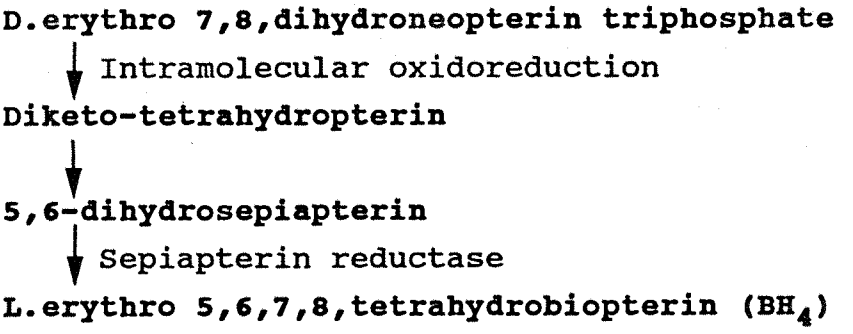


FIG.5.

ALTERNATIVE PATHWAY OF TETRAHYDROBIOPTERIN SYNTHESIS



5,6-dihydrosepiapterin intermediates. This proposal explains the above complications and is corroborated by the work of Smith (1987).

Work with primary cultures of adrenomedullary chromaffin cells (Abou-Donia et. al. 1986) has shown that the level of BH_4 present is below saturation for tyrosine hydroxylase and is therefore the rate limiting step.

(iii) Altered Metabolism of Biopterin

The metabolism of biopterin in man is altered by a number of different factors. It is important to note that urinary pterin concentrations normally display an age related change (Shintaku et. al. 1982). For example the concentration of neopterin in the urine is at its highest in neonates, whereas biopterin is highest in pre-school children (Shintaku et. al. 1982).

Lead has been found to alter the metabolism of BH_4 by the inhibition of DHPR. Eggar et. al. (1986) demonstrated a reduction of in vivo DHPR activity with long term exposure. Working with rats they found that chronic lead administration caused a rise in biopterin biosynthesis at levels of up to 1000 ppm of lead in the drinking water, but a decline at higher doses of 2000 ppm. They speculated that these results were due to a feedback mechanism attempting to maintain cellular levels of BH_4 which broke down at high doses, possibly due to an inhibition of protein biosynthesis by lead ions.

Aluminium is another element which has been associated with an interference of BH_4 synthesis. Cowburn and Blair (1987) found that adding aluminium acetate to preparations of frontal and temporal cortex from mice decreased the synthesis of BH_4. This may be due to the aluminium competing with the magnesium cofactor which is required to convert dihydroneopterin triphosphate to BH_4.

Reduced synthesis of BH_4 can be due to a deficiency of DHPR (refer Fig.4), for example in some of the so called "atypical" cases of phenylketonuria.

Patients with this type of disorder generally respond to some extent to BH_4 replacement therapy, although there is a small minority who do not (Smith et. al. 1985). Evidence (Cotton 1986) suggests that this lack of response may be due to a mutant form of the DHPR protein. The mode of action is unclear; possibilities include the binding of DHPR to qBH_2 but failure to reduce it to BH_4 or perhaps the synthesis of a reactive pterin which inactivates phenylalanine hydroxylase.

The role of folate and vitamin B12 in BH_4 biosynthesis is unknown, but both have been found to enhance synthesis in rat brains (Hamon et. al. 1986). Their results suggest that both tetrahydrofolate and vitamin B12 are required for BH_4 biosynthesis.

(iv) Biopterin Metabolism in Alzheimer's Disease

Several studies on control populations using bioassays such as that based on Crithidia fasciculata, mentioned in section 2i, have shown that the concentration of biopterin in the serum is usually maintained within a very narrow range (e.g. Aziz et. al. 1983). However other studies using more specific chromatographic methods (e.g. Young et. al. 1982) have shown that in patients suffering from AD the level of biopterin in the serum is reduced in comparison to age matched controls. Similar results have been reported for cerebrospinal fluid, again using chromatographic methodology (Kay et. al. 1986). The case for a pterin deficit is further supported by studies using the methods of specific radioimmunoassay (Sawada et. al. 1987) which have also reported biopterin deficits in the putamen and substratia nigra areas of SDAT brains. It is important to note that these deficits are not likely to be due to the cell loss associated with AD, as the authors repored either normal or slightly raised levels of neopterin, the precursor of BH_4.

The evidence would therefore suggest a deficit in the biosynthetic pathway of BH_4. Barford and co-workers (1984) find little, or no, synthesis of BH_4 in the temporal lobe of Alzheimer's patients.

Several studies have been carried out to investigate the activity of the biosynthetic enzymes. The work of Barford et. al. (1984), mentioned previously, showed that DHPR activity in the temporal lobe of the Alzheimer brain was slightly higher than in controls, but the increase did not reach significance. Young et. al. (1982) found that the DHPR activity in the plasma of AD patients was significantly lower than in controls. This contrasts with the findings of Jeeps et. al. (1986) who, working with dried blood spots, found that the DHPR activity in erythrocytes was not significantly different between SDAT patients and age matched controls. They also found no direct correlation between age and DHPR activity.

The available evidence would appear to support the hypothesis of a defect in BH_4 synthesis in Alzheimer's dementia, occuring at a point in the pathway after the production of dihydroneopterin (Q.V. figs. 4 & 5).

(v) Biopterin Metabolism and Other Neurological Disease States

(i) Hyperphenylalaninaemia including Classical and Atypical Phenylketonuria

Hyperphenylalaninaemia is a recessively inherited metabolic disorder in which the hydroxylation of phenylalanine to tyrosine is impaired. In the more severe cases this is caused by a low, or absent, activity of the enzyme phenylalanine hydroxylase. This is the so-called "classical" phenylketonuria (PKU), early diagnosis of which depends upon the detection of raised levels of phenylalanine in the blood in early life. The condition can be controlled by placing the patient on a phenylalanine controlled diet early in life, before intellectual impairment occurs.

A small proportion of patients (approximatly 1% of the total) do not respond to dietary restriction of phenylalanine alone. These patients with hyperphenylalaninaemia have a defect of pterin metabolism as opposed to a defect in phenylalanine

hydroxylase. There are at least three types which may be categorised as: a defect in the enzyme GTP cyclohydrolase I, a defect in biosynthesis after dihydroneopterin triphosphate and a defect in the enzyme DHPR (Q.V. Fig. 4). All these disorders will result in a deficit of the cofactor BH_4 and therefore diminished activity of phenylalanine hydroxylase.

Differental diagnosis of the three conditions above may be achieved by determining the ratio of biopterin to neopterin in the patient's urine, using High Performance Liquid Chromatography (HPLC)(e.g. Neiderweiser et. al. 1980, Nixon et. al. 1980, Hayakawa et. al. 1983). For example a patient with DHPR deficiency will show high biopterin and low neopterin levels while a patient with a deficit of GTP cyclohydrolase I will display low biopterin with high neopterin levels.

(ii) Parkinson's Disease

The major pathology of Parkinson's disease is a loss of nigrostriatal dopamine cells. There have been reports that biopterin in cerebrospinal fluid may be depleted by up to 50% (Moore et. al. 1987). Attempts at replacement therapy using BH_4 have only produced beneficial results with single, large (e.g. 1g) oral doses. Repeated small oral doses (200mg daily) do not appear to have a cumulative effect; this may be due to problems with small quantities of BH_4 not crossing the blood-brain barrier. However, the loss of BH_4 in Parkinson's disease is much smaller than that associated with SDAT and may simply reflect the degree of cell loss. It may therefore be possible that the ratio of cofactor to enzyme is normal and the improvements noted with large doses may be due to enzyme activity being artificially "boosted" by an excess of cofactor.

(3) CURRENT STATUS OF THERAPY IN ALZHEIMER'S DISEASE

There is currently no effective therapy for Alzheimer's disease. In caring for the patient the greatest emphasis is on providing a stable environment with well ordered daily routines which avoid confusion and stress for the sufferer.

To date the majority of attempts at a clinical treatment have centered around the cholinergic deficits already mentioned. Approaches have included the use of anticholinesterase drugs such as physostigmine. The reasoning behind this is to prevent the catabolism of acetylcholine. Recently studies have been performed using tetrahydroaminoacridine (THA) which has a longer duration of action than physostigmine. Encouraging results have been noted (Anonymous 1987) although these studies are still at an early stage and the long term effects and implications are therefore unknown. More recent work has yet to be published.

The intraventricular administration of the cholinergic agent bethanechol has been attempted in order to try and avoid the problems of blood/brain barrier penetration experienced with oral cholinergic drugs. Results reviewed by Whitehouse (Whitehouse 1988) suggest some improvement in everyday behaviour but not cognition. It is speculated that this may be due to mild cognitive toxicity making the patient passive and therefore less likely to be disruptive. Although other studies have yielded similar results it is considered that the small benefits provided by intraventricular bethanechol administration are not worth the risk posed by such a procedure.

Recent reports (Anonymous 1987) argue that it is a misconception to attempt therapy based purely on the cholinergic deficits. This is because younger SDAT patients (in the reports cited, below 79 years of age) display abnormalities in the noradrenergic and serotonergic as well as the cholinergic pathways. It is only in older subjects that the deficit is purely cholinergic. Further circumstantial evidence is

provided by the finding that although the anticholinergic drugs hyoscine and scopolamine impair learning ability the deficits produced bear a closer resemblance to Korsakoff's syndrome (a condition where the patient is confused and disorientated in time and place, most frequently noted in alcoholism) than SDAT.

(4) THE HYPOTHESIS

From the data available at the start of this study, regarding the deficits of neurotransmitters and pterins in SDAT, the hypothesis was formed that a block existed in the synthetic pathway for BH_4. From the available evidence it was not possible to say whether such a block was the primary defect, or secondary to some other factor.

The objective of this study was to develop the required methodology prior to testing the hypothesis by surveying BH_4 levels in the plasma of demented patients together with relavent control groups. If the hypothesis were to be supported it was proposed that the investigation should be extended in order to consider possible methods of replacement therapy.

(5) METHOD DEVELOPMENT

Aim of Study

The aim of this section of the study was to develop a technique using High Performance Liquid Chromatography (HPLC) for the detection and quantification of pterins in plasma. At the time of this study no published technique offered the required sensitivity and sample preparation procedures had recieved little attention. The procedures used in such a technique may be conveniently regarded in two categories: those relating to the plasma samples, i.e. (1) sample collection preparation and storage and (2) those relating to the chromatography, i.e. optimisation of the separation and sample detection. Each category was developed as described below.

(i) Samples

(a) Sample Collection

Introduction

The pterin species are highly labile and BH_4 in particular is susceptable to auto-oxidation. When collecting whole blood samples it is necessary to use an anti-oxidant as well as an anti-coagulant. Previous studies have used ascorbic acid (e.g. Hayakawa et. al. 1983) or dithioerythritol (DTE)(Howells et. al. 1986).

Ascorbic acid (Analar grade) was available from BDH Chemicals while DTE was available from two sources: Sigma Chemical Co. and Aldrich Chemical Co. These reagents were evaluated for their suitability in this study, both in terms of their interference with the HPLC analysis and their ability to protect the sample. Compatability with the HPLC system was checked by injecting solutions of the reagents for chromatographic analysis and observing the traces obtained from the detectors for noise, baseline drift etc. Performance as an anti-oxidant was assesed by following the degradation of a solution of authentic BH_4, protected by the reagent, on a spectrophotometer. In the case of DTE this was repeated with the addition of a chelating

agent (i.e. ethylenetriaminepenta acetic acid) as this had been reported to give enhanced protection of the sample (Howells et. al. 1986).

Methods

Authentic BH_4 was purchased from Dr.B.Schircks laboratories (Jona, Switzerland), this being the only commercially available source of the pure compound.

Samples were collected into foil wrapped containers in order to protect them from light and kept cool by placing on ice at collection and transfering to a refrigerator as quickly as possible. The above procedures apply to all the methodology.

(1) DTE or ascorbic acid was added to aqueous solutions of authentic BH_4 (1mg/ml) to give a final concentration of 1mg/ml and stored at $-20^{o}C$. 50μl aliquots were injected for analysis by HPLC with electrochemical and fluorimetric detectors according to the method of Howells et. al. (1986), in order to establish whether the reagent gave any interference with sample detection.

(2) In order to establish the rate of auto-oxidation under DTE protection, the degradation of authentic BH_4, dissolved in 1mg/ml DTE, was followed on a spectrophotometer at a wavelength of 267nm. This was repeated using a 50:50 combination of DTE and ethylenediaminetetra-acetic acid (EDTA)(1mg/ml of each).

Results

(1) Both compounds gave a satisfactory trace on fluorimetric detection. However, ascorbic acid was found to give severe instability problems with the electrochemical detector and DTE from Sigma Chemical Co. proved to contain impurities which again gave rise to unacceptable interference with this instrument. DTE from Aldrich Chemical Co. (Gold label grade) was found to give a satisfactory chromatographic trace on both instruments.

(2) The degradation of BH_4 in the presence of DTE alone and DTE/EDTA combined is shown in Table 1 and Fig.6.

The use of DTE alone fully protected the BH_4 samples for 75 minutes. It was found that this could be extended to 165 minutes when used in conjunction with a chelating agent such as EDTA.

Discussion

DTE is a thiol agent, preferential oxidation of -SH groups in the compound protecting the BH_4. Metal ions are known to catalyse the auto-oxidation of BH_4 (Halliwell and Gutteridge 1985). The addition of a chelating agent (i.e. EDTA) prevented this from happening and this finding was similar to that of Howells et. al. (1986). The use of a chelating agent such as EDTA had the added advantage that it acted as an anti-coagulant for the blood samples.

Conclusions

In the light of the above findings it was decided to collect blood samples into a 50:50 combination of DTE (Aldrich Chemical Co., Gold label grade) and EDTA (BDH, Analar grade), sufficient to give a final concentration of 1mg/ml of each in the whole blood.

TABLE 1

Rate of auto-oxidation of BH_4 when protected by DTE and a 50:50 DTE/EDTA combination

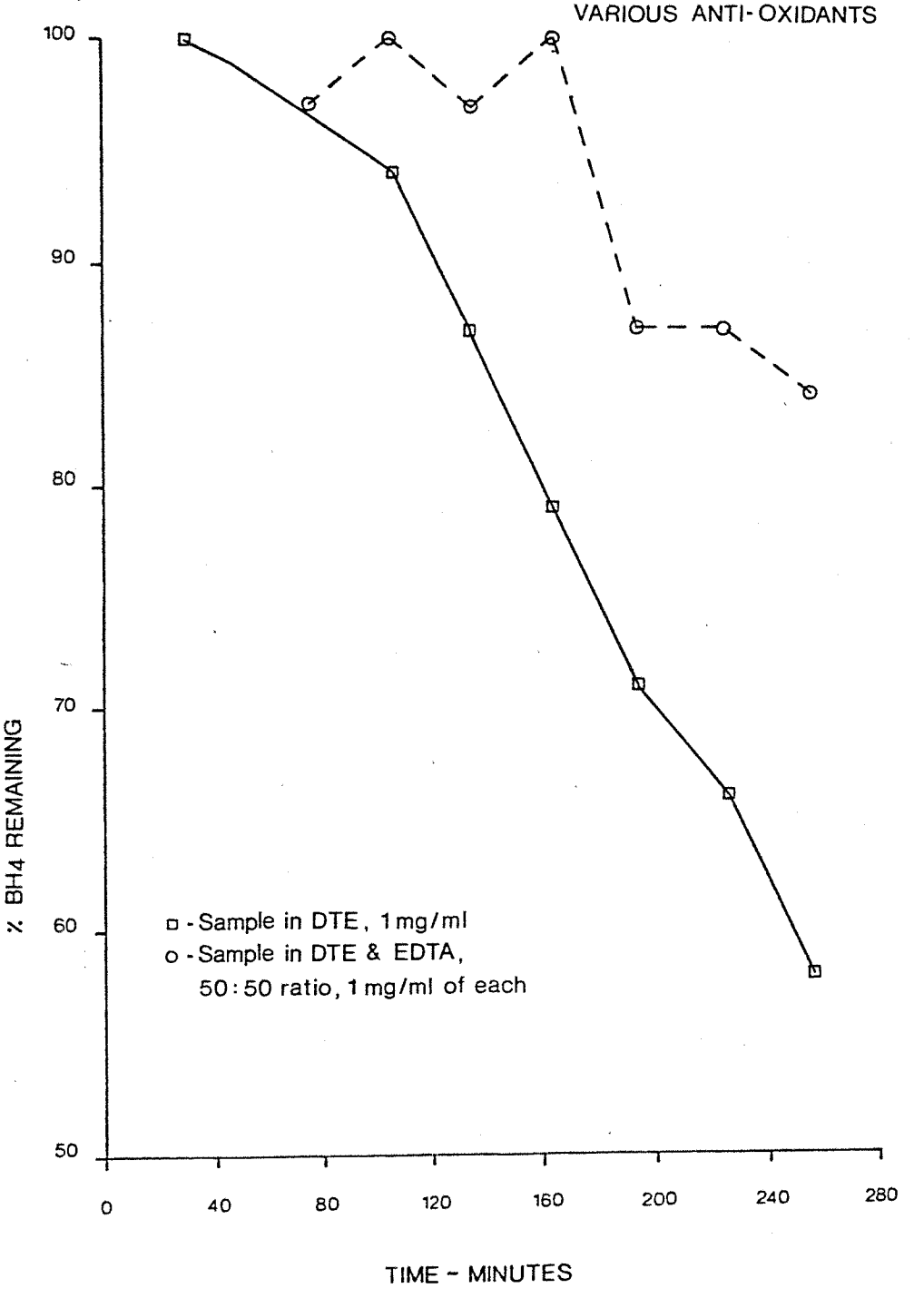
Time (minutes)	%BH_4 remaining (DTE)	%BH_4 remaining (DTE/EDTA)
0	100	100
15	100	100
30	100	-
45	99	-
75	-	97
105	94	100
135	87	97
165	79	100
195	71	87
225	66	87
255	58	84

TABLE 2

Bio-Rad Protein Assay Calibration Data and Result of Sample Analysis

[BSA]ug/ml buffer	OD_{595}
0	0.00
5	0.32
10	0.58
15	0.80
20	0.90
25	1.27
Sample	0.00

FIGURE 6
DEGRADATION OF BH_4 AT 4°C
UNDER THE PROTECTION OF
VARIOUS ANTI-OXIDANTS



(b) Sample Preparation

Introduction

In preparing samples for analysis by HPLC from whole blood it is necessary to remove the cellular fraction and larger molecules such as the plasma proteins. The cellular fraction was removed by spinning the sample in a benchtop microfuge using sample vials pre-cooled on ice and drawing off the plasma with a pasteur pipette.

In order to deproteinise the sample two different approaches appeared to offer possibilities; (i) chemical precipitation (ii) ultrafiltration. Previous techniques for the determination of total pterin content, have relied on a chemical precipitation, using a chelating agent such as trichloroacetic acid (TCA)(e.g. Dhont et. al. 1982).

For ultrafiltration the MPS-1 micropartition system produced by Amicon Ltd. appeared to offer suitable characteristics. This apparatus consisted of two chambers separated by a micropartition membrane. Plasma was loaded into the upper chamber and spun across the membrane at 2000g in a suitable centrifuge maintained at $4^{o}C$, protein free filtrate collecting in the bottom chamber. Use of a fixed angle rotor created a constant movement of plasma protein across the membrane surface, ensuring that the filter did not become blocked (Fig. 7).

The two available methodologies were evaluated as described below.

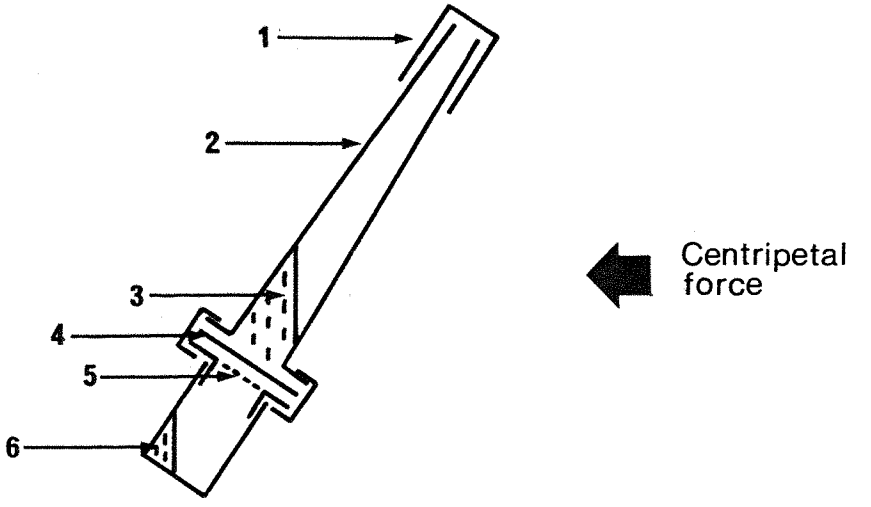
Methods

Throughout all procedures the apparatus was pre-cooled by placing on ice or using the pre-cool facility on the centrifuge rotor. Samples were shielded from light by foil wrapping the containers.

Whole blood samples were prepared as described in each of the following protocols prior to HPLC analysis by the method of Howells et. al. (1986).

FIGURE 7

MPS-1 MICROPARTITION APPARATUS



Key

1 Cap

2 Sample chamber

3 Plasma

4 Membrane

5 Support

6 Ultra-filtrate

Method 1, TCA Precipitation

(1) Collect blood into sufficient DTE & EDTA to give at least 1mg/ml whole blood of each.

(2) Spin in microfuge (3 mins.).

(3) To 0.8ml supernatant add 0.2ml of 2N TCA.

(4) Spin in microfuge (3 mins.).

(5) Filter supernatant (0.45μm).

(6) HPLC analysis.

Method 2, Ultrafiltration

(1) Collect blood into sufficient DTE & EDTA to give at least 1mg/ml whole blood of each.

(2) Spin in microfuge (3 mins.).

(3) Place supernatant in micropartition apparatus.

(4) Spin at 2000g for 45 mins.

(5) HPLC analysis.

Results

Chromatographic traces obtained from blood samples prepared by each of the above methods are shown in Fig.8. From Fig.8 it can be seen that the traces obtained were very similar for both of the techniques. However, with the TCA based method the electrochemical detector displayed a rising baseline, a problem not encountered with the micropartition system.

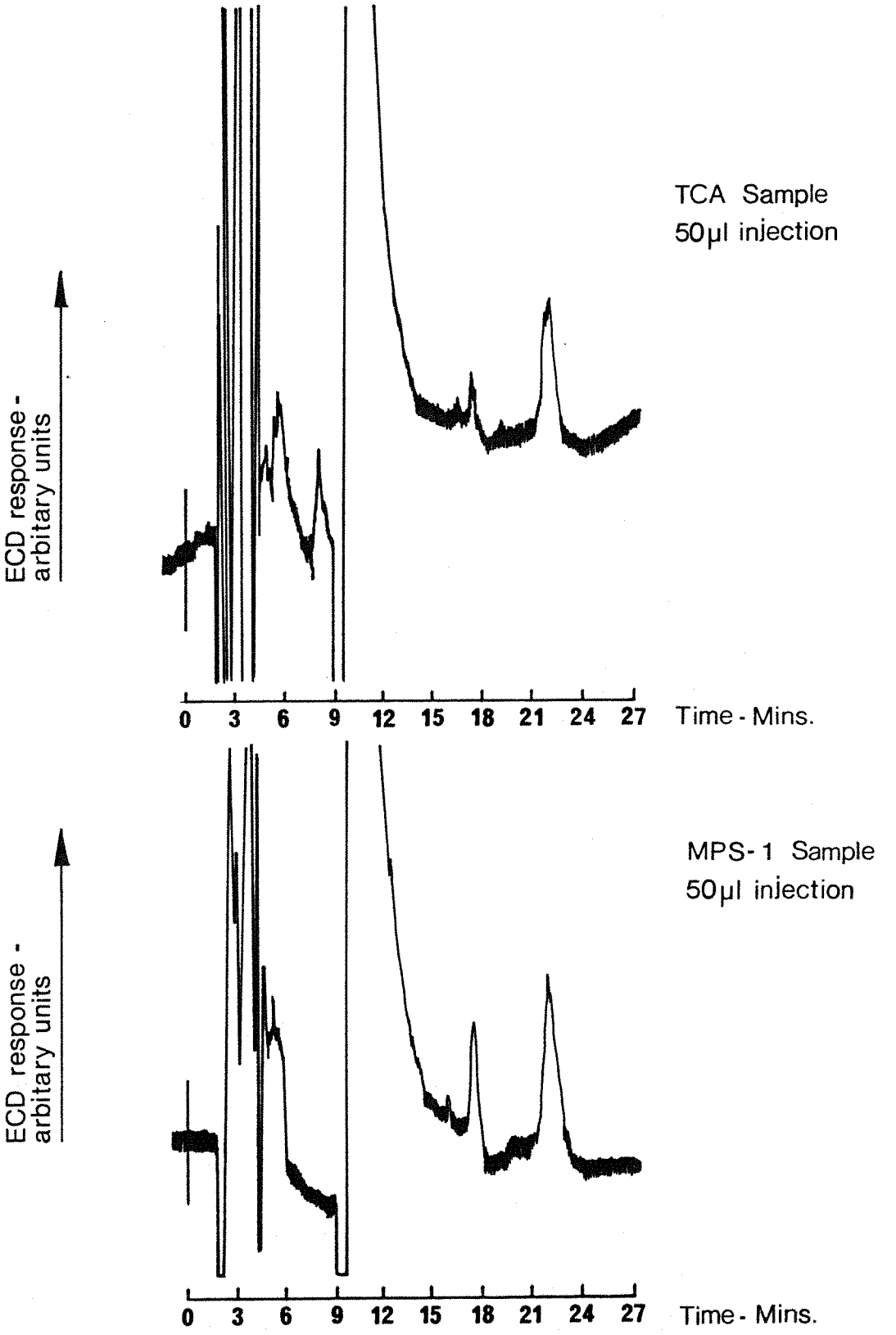
The TCA method took approximately 10 minutes to complete while the ultrafiltration technique occupied around 50 minutes.

Standard micropartition membranes, as supplied by Amicon, had a molecular weight cut-off of 30,000 dalton. These membranes gave a filtrate yield of approximately 150μl from a 500μl sample. Repeating the procedure using 100,000 dalton cut-off membranes (available only as a special order item) increased the yield to 250μl, a sufficient volume for at least two HPLC analyses.

Discussion

Both methodologies proved to be reasonably quick and convenient to use, an important feature when

FIGURE 8
COMPARISON OF CHROMATOGRAMS FROM SAMPLES PREPARED BY THE TCA & MPS-1 METHODS



handling compounds as labile as pterins. Although taking longer to perform, the filtration method offered the more convenient means of being able to pre-cool the centrifuge rotor automatically rather than relying on ice.

The rising baseline observed when analysing samples prepared by the precipitation technique was due to instability in the detector caused by the TCA. This is a highly acidic reagent and long term use may have been detrimental to the detectors electrode surface. This could have been overcome by titrating the sample to a less acidic pH, but would have necessitated the dilution of an already low pterin content.

Plasma proteins have a binding capacity for pterins, although this is largely unspecific (Rembold et. al. 1977). Reagents such as TCA have, in the methodologies of previous workers, been used to release pterins from plasma proteins during sample preparation (e.g. Zeitler and Andondonska 1986). The analysis of samples prepared by the micropartition method therfore showed the free pterin content of the blood.

After considering the above points, it was decided to use the micropartition technique, subject to the checks described in the next section, but to add a known quantity of 6-hydroxymethylpterin (6-HMP) to the sample prior to preparation to act as an internal standard for the following reasons. Quantification of this compound during the subsequent chromatographic analysis allowed correction to be made for sample recovery due to losses incurred during preparation, binding to proteins etc. It was assumed that 6-HMP had the same behaviour as the pterins of interest, with respect to sample preparation and chromatographic analysis. 6-HMP does not normally occur in the blood, thus making it a suitable candidate for use as an internal standard. To date it has only been reported to be excreted by cancer cells, both malignant and benign.

Introduction

Checks on the Ultrafiltration Technique

It was decided to use the preparative technique based on micropartition subject to a check showing that the filtrate had been sufficiently deproteinised to be suitable for analysis by HPLC. The manufacturers of the MPS-1 micropartition system claimed that the membrane retained >99.9% of the serum protein when used as described in the previous section. A protein assay was therefore carried out on the prepared filtrate.

Bio-Rad laboratories market a protein assay which is capable of measuring between 1 and 20μg protein (<25μg/ml). This assay was chosen in preference to the more traditional methods such as the Biuret or Lowry assays because it was easier to use, required only one commercially available reagent and was free from interference from contaminants. The basis of the test was the binding of an acidic Coomassie Brilliant Blue dye to the protein observed at an absorbance maximum of 595nm.

A calibration curve was constructed using a bovine serum albumin (BSA) standard which the manufacturer recommended for general use. The method is described below.

Method

Dilutions of BSA were prepared containing from 1 to 25μg/ml protein in phosphate buffer,pH 6.8.

(1) Place 0.8ml of standard (including reagent blank) or sample in test tube.

(2) Add 0.2ml Bio-Rad reagent.

(3) Mix with a vortex mixer.

(4) Between 5 mins. and 1 hour after addition of reagent measure absorbance at OD_{595} versus reagent blank.

(5) Plot OD_{595} versus standard concentration and interpolate unknown from standard curve.

Results

The results are shown in Table 2 and Fig.9. The data obtained showed that protein was not present in a detectable quantity, i.e. <1μg/ml protein. The experiment was repeated to confirm this finding, and the result was similar.

Discussion

The data obtained indicated that the protein content of plasma samples prepared by the micropartition technique was low enough for them to be suitable for analysis by HPLC.

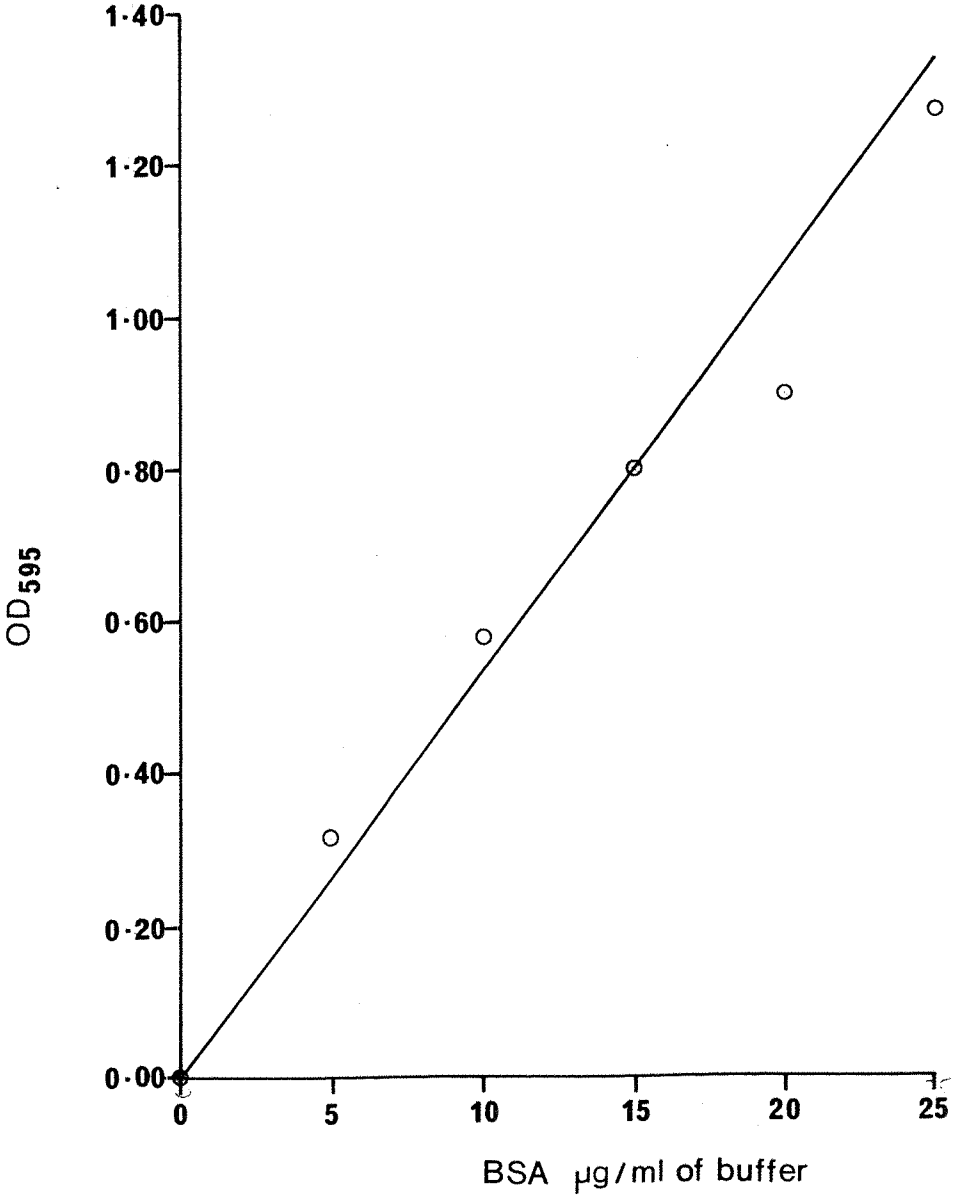
Conclusions

From the above two experiments it was concluded that the MPS-1 micropartition system offered a suitable method of sample preparation when used in conjunction with an internal standard, as described. The method was reasonably quick and convenient to use and yielded a sufficient volume of sample which was compatable with HPLC analysis.

(C) Sample Storage

After preparation the samples were stored at -70^{o}C until analysed. At this temperature and in the presence of DTE and EDTA in the quantities already specified, all the pterins under investigation remained stable and no degradation of the sample was observed over the course of these studies.

FIGURE 9
BIO- RAD CALIBRATION



(ii) Chromatography

(a) Effect of Ion Pairing Agent on Separation

Introduction

In order to analyse the prepared plasma samples it was necessary to develop a liquid chromatography method which fully separated the pterins of interest (i.e. biopterin, neopterin, BH_4 and BH_2) from each other and from other compounds in the sample. A survey of the literature showed that the method of Lunte (Lunte and Kissinger 1983) would fully resolve pterins in biological fluids.

Briefly, this method used a 250x4.6mm ODS2 reversed phase column, 5μm particle packing size, used in conjunction with a mobile phase containing phosphate buffer and an ion-pairing agent. However, the preparative procedure reported did not add an anti-oxidant, such as DTE, to the sample as found to be necessary in my study (see section 5ia) and in those of Hyland (1985) and Howells et. al. (1986).

It was therefore necessary to confirm that the pterins of interest were still fully separated. If not, the effect of altering the concentration of ion-pairing agent to re-optimise the separation needed to be investigated. In order to achieve this plasma samples, prepared as described in the previous section, were injected for HPLC analysis. Also solutions of authentic DTE and BH_4 were analysed using a mobile phase containing a variable quantity of ion-pairing agent. The methods used are described below.

Methods

(1) Mobile phase was prepared by dissolving 6.8g of potassium dihydrogen orthophosphate, 10.0mg EDTA, 20.0ml methanol and 10.0ml of 0.1M octane sulphonic acid (OSA, ion-pairing agent) in 800ml of doubly distilled water. The pH was adjusted to 2.8 by the addition of orthophosphoric acid and the final volume made up to 1 litre. Just before use 50.0mg of DTE was added and the solution filtered (0.22μm) under reduced

pressure. 75μl of prepared plasma was injected for chromatographic analysis which was performed at a flow rate of 1.3ml/minute and the mobile phase run to waste.

(2) Three batches of mobile phase were prepared as above but containing 5.0, 10.0 and 15.0ml of OSA respectively. Solutions of DTE and BH_4 were injected for analysis and the retention of the compounds noted in each case.

Results

(1) Under these conditions BH_4 was not resolved from a large chromatographic peak with a similar retention time.

(2) The retentions of DTE and BH_4 are shown in Table 3 and on Fig.10.

Discussion

From the results it can be seen that the retentions of both DTE and BH_4 could be easily altered by a change in the concentration of the ion-pairing agent when working with pure solutions. It was found that a satisfactory separation of BH_4 from other, unidentified, compounds could still not be achieved when handling plasma samples. It was therefore decided to examine a change in the column packing material as a means of improving resolution.

(b) Effect of Column Packing on Separation

Introduction

Reference to the literature of the column manufacturer (Hichrom Ltd.) indicated that the use of a column packing material with a smaller size would increase the number of theoretical plates on the column. The concept of theoretical plates gives an indication of the number of times a compound partitions between the stationary and mobile phases on a liquid chromatography column. Hence the greater the number of theoretical plates the greater the resolution of the system. A change from a 5μm to a 3μm particle size would increase the number of theoretical plates from

TABLE 3

Retention Times of Dithioerythritol (DTE) and Tetrahydrobiopterin (BH_4) under various ion-pair concentrations

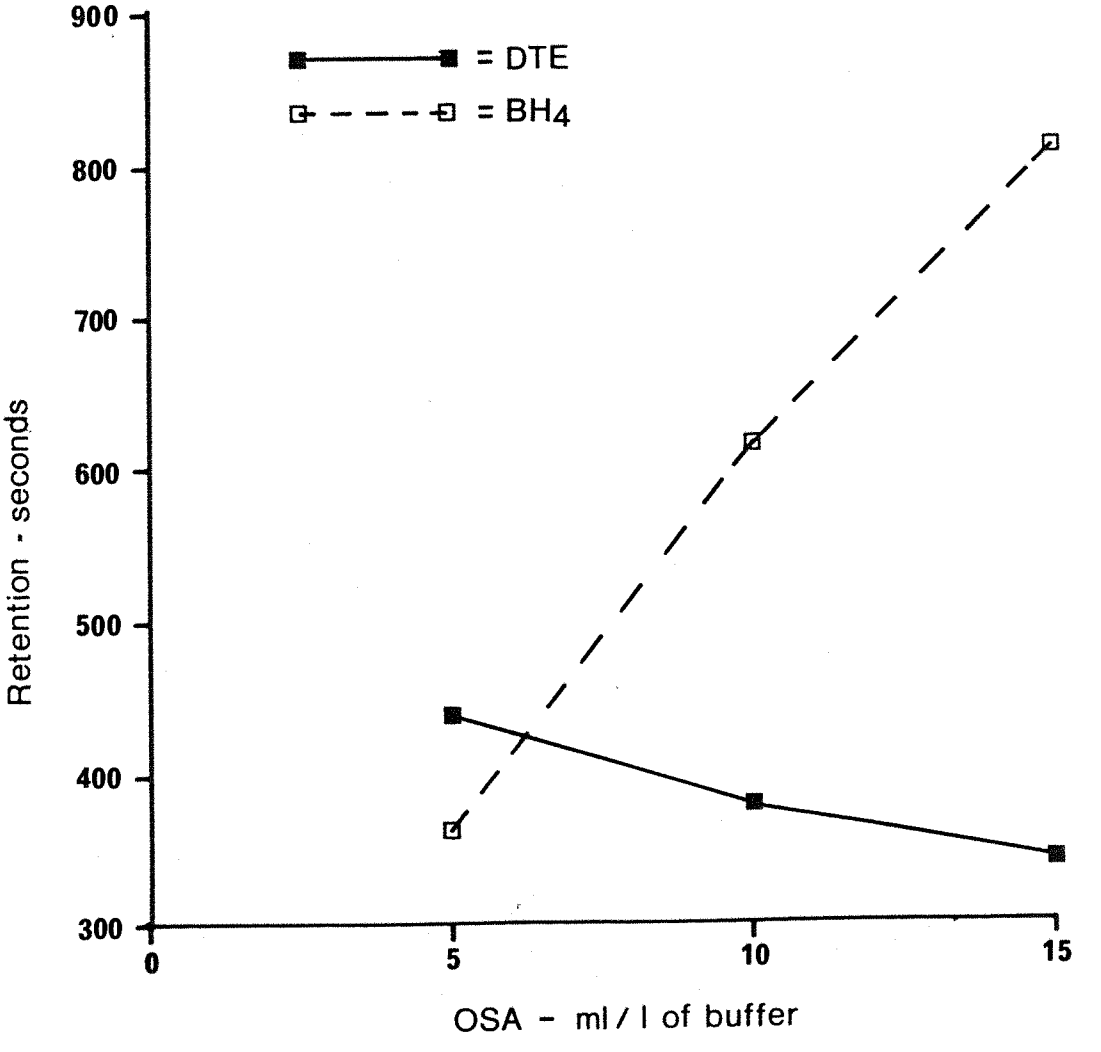
OSA (ml/litre of 0.1M soln.)	DTE retention (seconds)	BH_4 retention (seconds)
15	342	810
10	378	612
5	441	360

TABLE 4

Hydrodynamic voltammogram of BH_4

Applied potential (volts)	Detector response (arbitary units)
-0.10	-25
-0.05	-25
-0.00	+25
+0.05	+1275
+0.10	+2750
+0.15	+3000
+0.20	+3000

FIGURE 10
RETENTION TIMES OF DTE AND BH_4 UNDER VARIOUS ION-PAIR CONCENTRATIONS



60,000 to 100,000. In addition smaller packing size would cause a larger pressure drop along the length of the column. In view of the pressure limits of the pump which was available, it was decided to evaluate a 125 x 4.6mm ODS2 spherisorb reversed phase column, 3μm particle packing size.

Method

The mobile phase was as described in the previous section, and containined 10.0ml of 0.1M OSA. Chromatography was performed at a flow rate of 1.3ml/minute, 75μl of prepared plasma filtrate being injected for analysis.

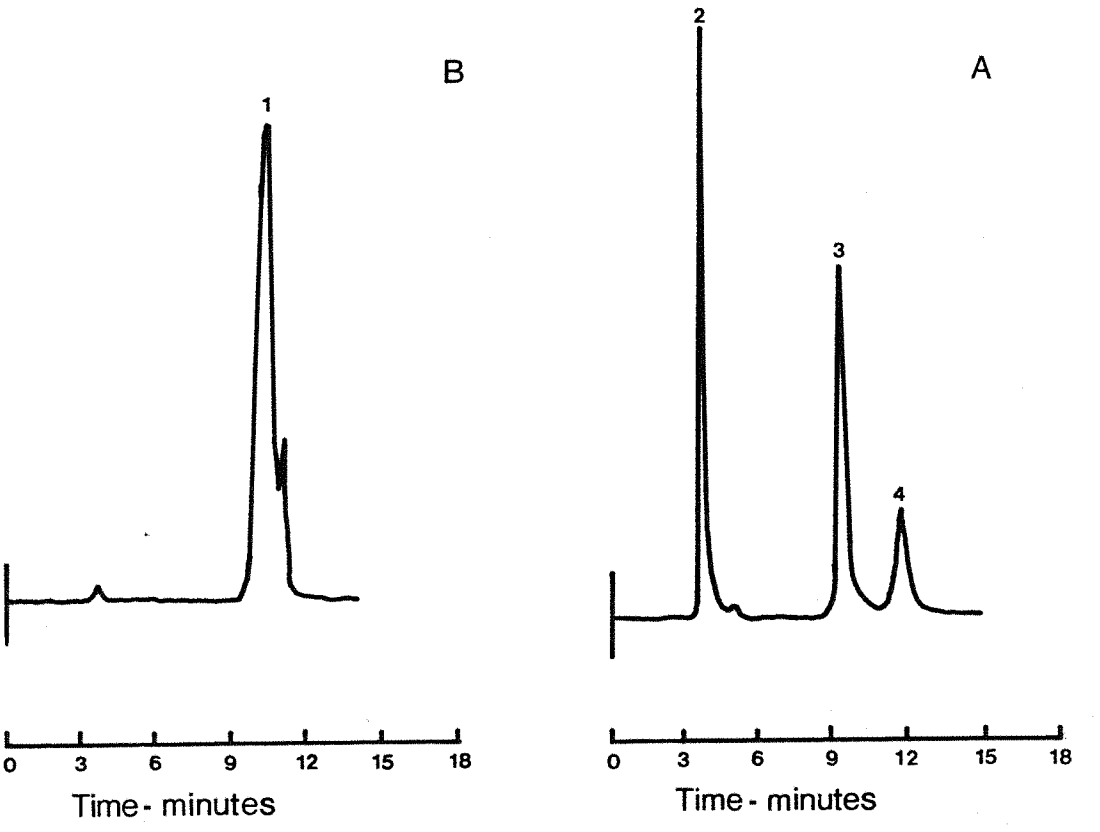
Results

A representative chromatogram from the electrochemical detector is shown in Fig.11D. From the figure it can be seen that BH_4 was well separated from other compounds in the plasma.

Discussion

Peak identification was confirmed by the methods described in section 5iib. It was therefore decided to use the 3μm packing material. The concentration of ion-pairing agent also proved to be satisfactory.

FIGURE 11
REPRESENTATIVE CHROMATOGRAMS OF AUTHENTIC COMPOUNDS AND PREPARED SAMPLES



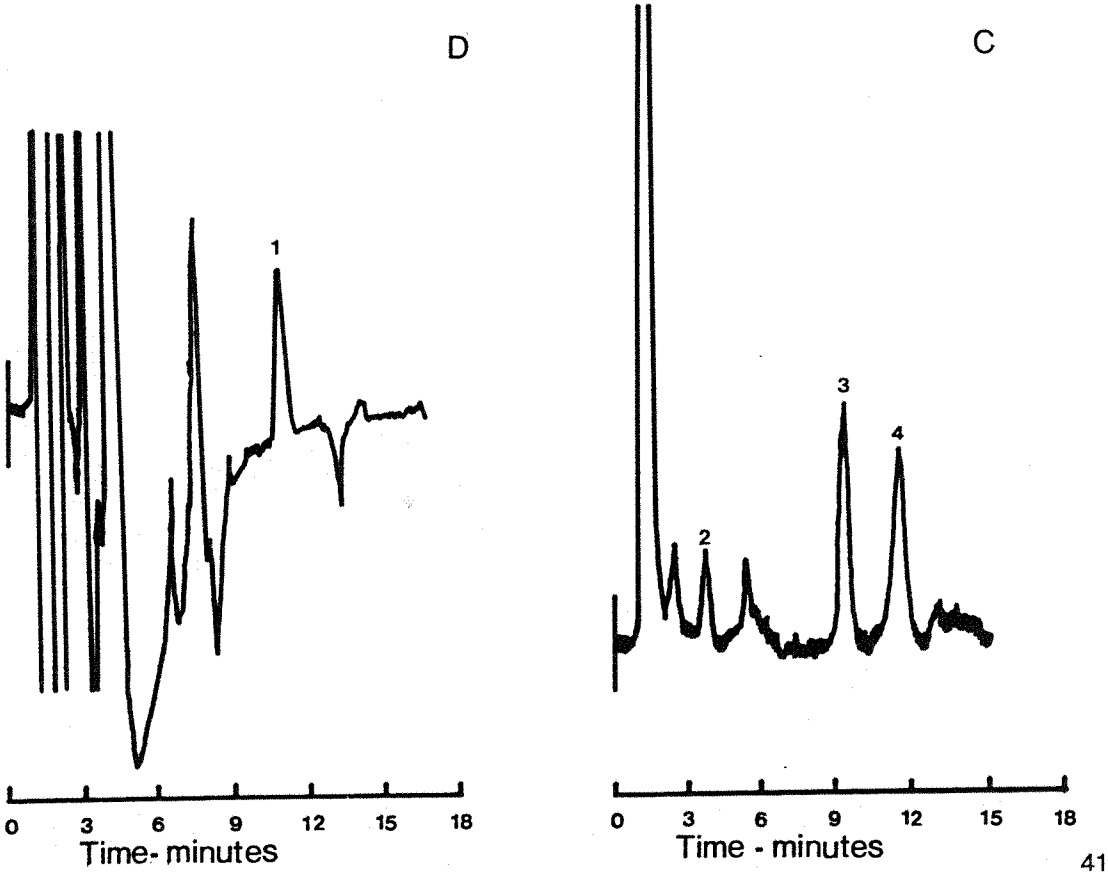


FIGURE 11

REPRESENTATIVE CHROMATOGRAMS OF AUTHENTIC COMPOUNDS AND PREPARED SAMPLES

Legend

A = Fluorescent trace of authentic compounds

B = Electrochemical trace of authentic compounds

C = Fluorescent trace of prepared sample

D = Electrochemical trace of prepared sample

Peak Identification

1 = Tetrahydrobiopterin (BH_4)

2 = Neopterin

3 = Biopterin

4 = Dihydrobiopterin (BH_2)

(c) Detection

Introduction

Detection of BH_4

Pterins exist in three oxidation states; fully oxidised biopterin and neopterin, partly oxidised BH_2 and reduced BH_4. Fully oxidised pterins are naturally fluorescent (Fukushima and Nixon 1980), a property which makes their detection and quantification relatively straightforward by the use of a fluorescence spectrometer. However, BH_2 and BH_4 are virtually non-fluorescent and so this technique is not applicable.

The majority of previous methodologies have determined the total pterin content of samples by first converting all the pterins present to the fully oxidised form. This has been achieved by a variety of chemical oxidation techniques, e.g. acid/iodine (Fukushima and Nixon 1980) or manganese dioxide (Neiderweiser et. al. 1980), but in order to estimate the pterins in the individual oxidation states alternative methodologies must be employed.

Reduced BH_4 is amenable to quantification by electrochemical detection. This is achieved by applying a known electrical potential to the compound at an electrode surface and monitoring the current associated with the resulting oxidation.

The electrochemical detector used in these studies, an ESA "Coulochem", was equipped with two analytical electrodes. A method which afforded far greater selectivity than a simple oxidation of the compound, important when handling complex samples such as plasma, was to oxidise the sample and then quantify the subsequent reduction on the second electrode. With the Coulochem detector this is known as the "redox" mode of operation.

In order to achieve the maximum possible sensitivity the voltage applied to the electrodes had to be optimised by generating an hydrodynamic voltammogram. This was done by plotting a graph of detector response (representing the current associated

with the oxidation or reduction) against the voltage applied to the electrode. The optimum setting was chosen as the lowest potential to give a maximum detector response. Generation of a hydrodynamic voltammogram is described below.

Methods

When setting up the electrochemical detector in redox mode it was necessary to generate two hydrodynamic voltammograms. The first was to optimise the applied potential for the initial oxidation of the compound on the first electrode and the second to optimise the second electrode for the subsequent reduction. Both procedures were technically identical.

A known quantity of authentic BH_4 was injected for analysis and the detector response, measured as the pen deflection on a chart recorder, noted. The electrode potential was increased and the procedure repeated.

Results

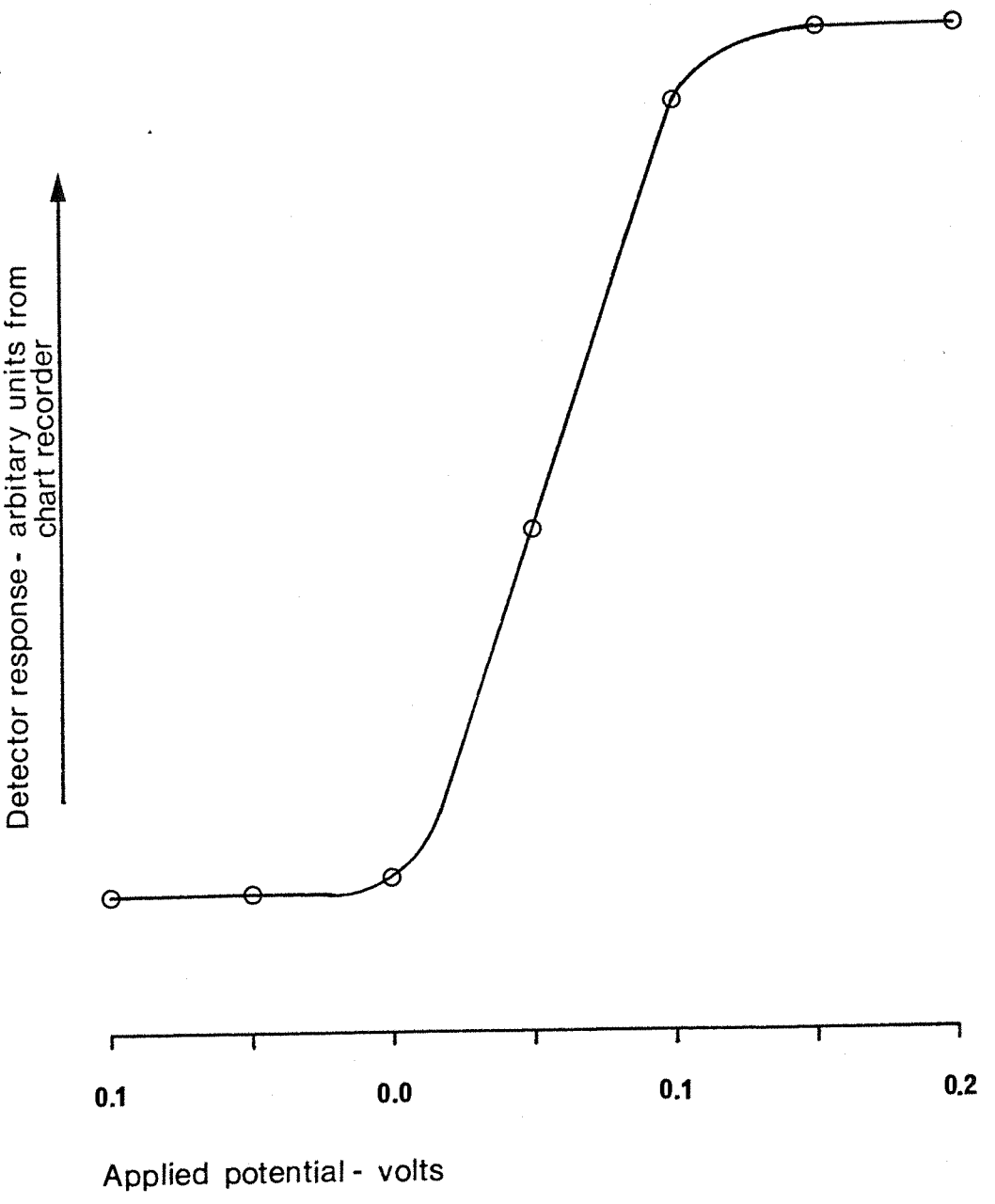
The data obtained for a typical voltammogram is displayed on Table 4 and Fig.12.

Discussion

In the case of the data presented in graph 3 the optimum electrode setting would be +0.15V. As well as being used to optimise electrode settings hydrodynamic voltammograms provide an excellent method of confirming peak identity on a chromatographic trace. If a voltammogram is generated for an unidentified compound, in the same manner as described above and is coincidental with that displayed by the authentic compound, then this is excellent evidence that the two are the same.

When setting up the electrochemical detector for redox mode it was noted that the first analytical electrode had greater sensitivity than the second. This detector was also equipped with a "conditioning cell" electrode which is normally placed in the chromatography system between pump and sample injector.

FIGURE 12
HYDRODYNAMIC VOLTAMMOGRAM OF BH_4



This was used to pre-oxidise any impurities of the mobile phase, thereby reducing interference from high background currents. As no problems were experienced with impurities it was decided to arrange the cells to carry out the initial oxidation of BH_4 on the conditioning cell electrode and the subsequent reduction on the first analytical electrode (refer Fig.13). Using this arrangement meant that quantification of BH_4 was performed on the most sensitive electrode available.

Introduction

Detection of BH_2

BH_2, like BH_4, is virtually non-fluorescent. However, using the Coulochem it was possible to convert this compound to a fully oxidised form which was fluorescent, prior to detection and quantification on a fluorescence spectrometer (Howells et. al. 1986). This oxidation took place on the second analytical electrode of the detector, which was unused in the analysis of BH_4. In order to achieve full oxidation of the compound it was necessary to optimise the electrode potential in a similar manner to that described for BH_4. In this case, as the electrode potential was increased, the response of the fluorescence detector was noted.

Methods

Generation of a "fluoresogram" is similar to the generation of a hydrodynamic voltammogram. A known quantity of authentic BH_2 was injected for analysis and the response of the fluorescence detector noted. The potential was increased and the process repeated.

Results

The data for a typical fluoresogram is shown in Table 5 and also on Fig.14.

FIGURE 13
HPLC SYSTEM

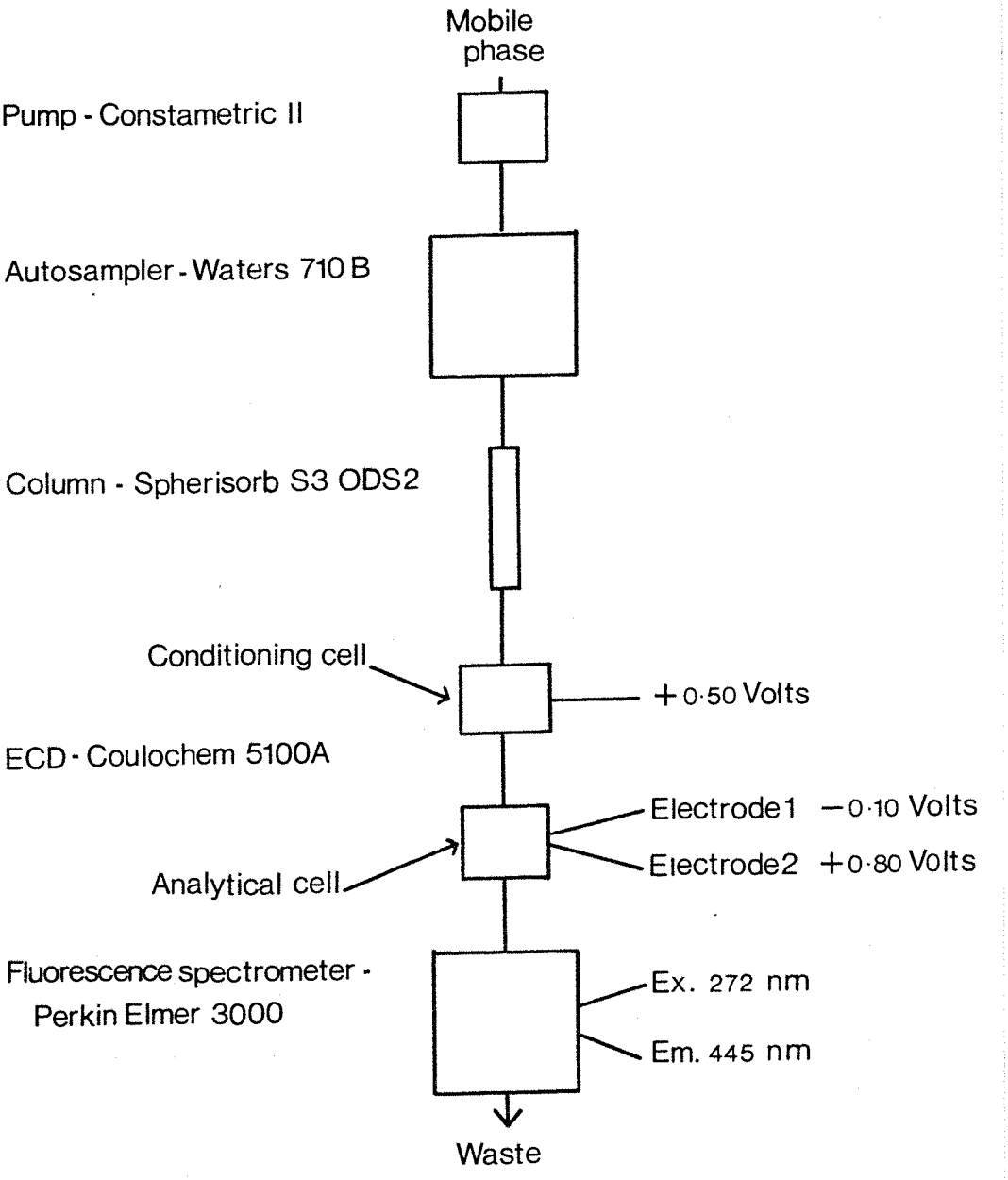


TABLE 5

Fluoresogram of BH_2

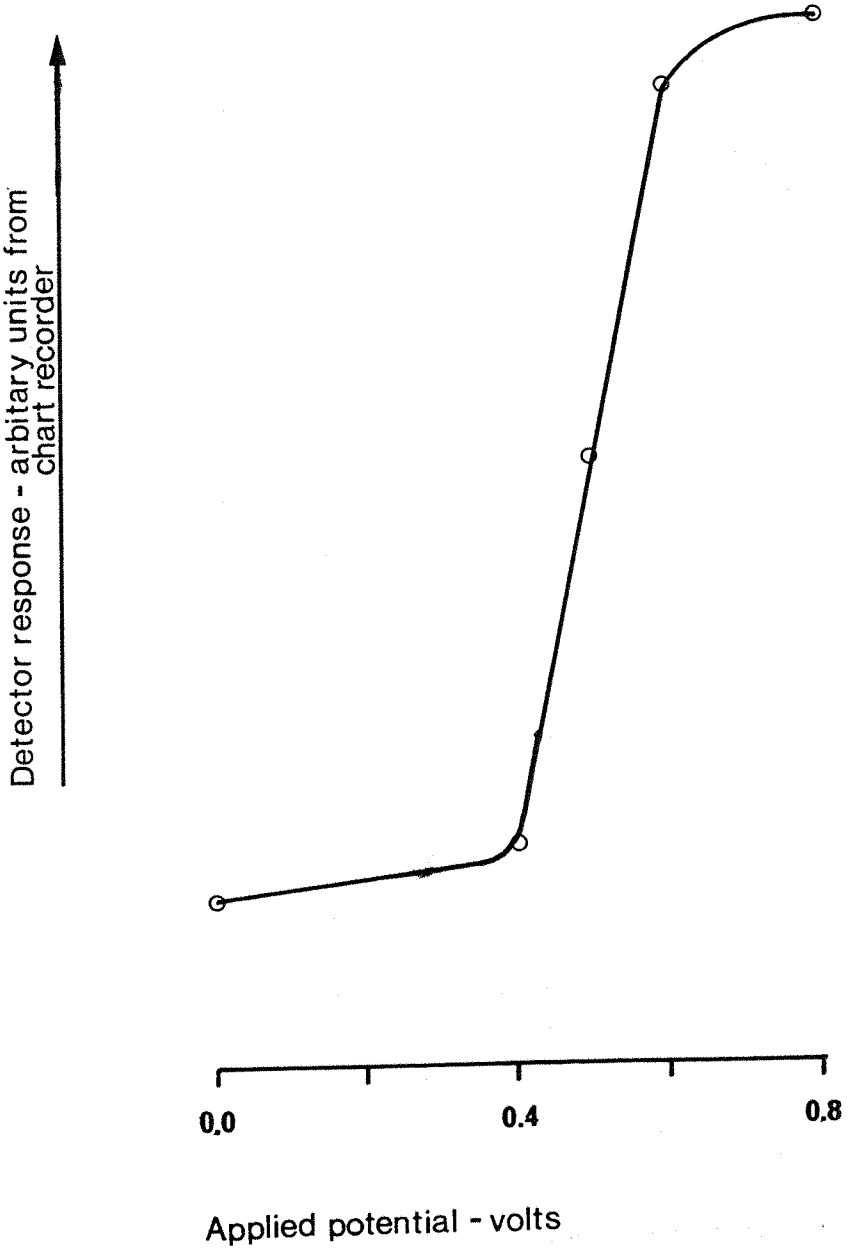
Applied potential (volts)	Detector response (arbitary units)
0.0	2.0
0.4	5.5
0.5	31.0
0.6	55.5
0.8	60.0

TABLE 6

Intra-batch coefficients of variation

Pterin	C.V.%	n
Biopterin	7.9	9
Neopterin	11.9	6
BH_2	11.2	9
BH_4	10.6	9

FIGURE 14
FLUORESOGRAM OF BH_2



Discussion

As with an hydrodynamic voltammogram the optimum potential is that which just gives maximum detector response, in this case around 0.8V. It was also necessary to set the excitation and emission wavelengths of the fluorescence spectrometer to the correct values. This is described in the next section concerned with the detection of fully oxidised pterins.

BH_2 peak identity on a chromatogram may be easily checked by switching off the electrochemical detector. This leads to the BH_2 peak on the fluorescence detector either disappearing or being significantly diminished.

Introduction

Detection of biopterin and neopterin

Fully oxidised pterins (i.e. biopterin and neopterin) are naturally fluorescent (Fukushima and Nixon 1980). Their detection and quantification may therefore be carried out fluorimetrically. It is important that the excitation and emission wavelength settings of the fluorescence spectrometer are chosen correctly. This process is described below.

Methods

The flow cell of the fluorescence spectrophotometer was first filled with a solution of biopterin. The excitation wavelength was determined by setting the emission monochromator of the instrument to zero order and then "scanning" the excitation monochromator across the range of wavelengths available, the optimum setting being that giving maximum fluorescence. Once the optimum excitation wavelength had been set the emission was determined by scanning the emission monochromator across the range of wavelengths available.

Results

The excitation and emission spectra for biopterin are shown in Fig.15A & B. Optimum wavelengths were

FIGURE 15A

EXCITATION SPECTRUM OF BIOPTERIN

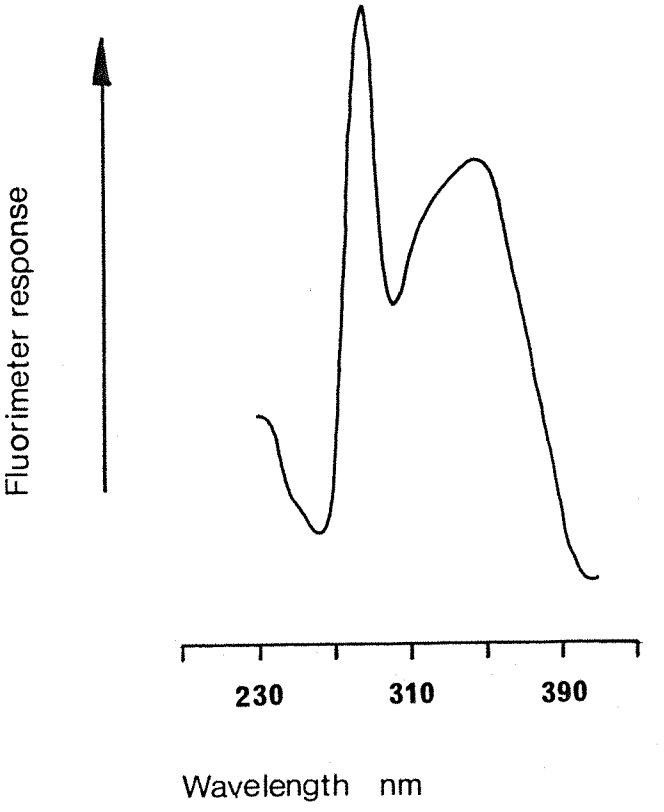
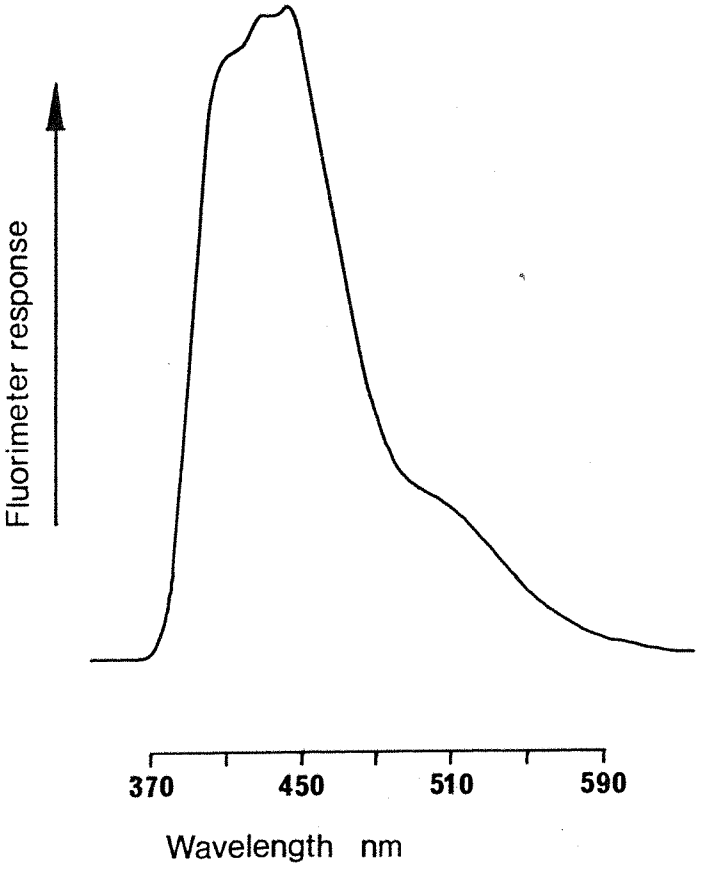


FIGURE 15B

EMISSION SPECTRUM OF BIOPTERIN



determined to be 272nm for excitation and 445nm for emission.

Discussion

The Perkin-Elmer fluorescence spectrometer used in these studies was fitted with a high intensity xenon source lamp , providing a continuum of energy over the spectrum of excitation wavelengths. The excitation wavelength given here may therefore be lower than that published for other instruments.

Confirmation of the identity of chromatographic peaks detected fluorimetrically was achieved by comparing the retention time of the sample with the authentic compound.

(iii) Final Adopted Method for Determination of Pterins in Plasma

The method used for the determination of BH_4, BH_2, biopterin and neopterin in plasma can therefore be regarded as having two stages; sample preparation and chromatographic analysis. Following method development, as described, the finalised procedures were as detailed below.

Sample preparation

(1) Collect 1ml of whole blood into a foil wrapped microfuge vial containing 1mg DTE and 1mg EDTA.

(2) Spin in benchtop microfuge for 3 minutes.

(3) Transfer 400μl of the plasma to an MPS-1 micropartition system fitted with a 100,000 dalton cut-off membrane.

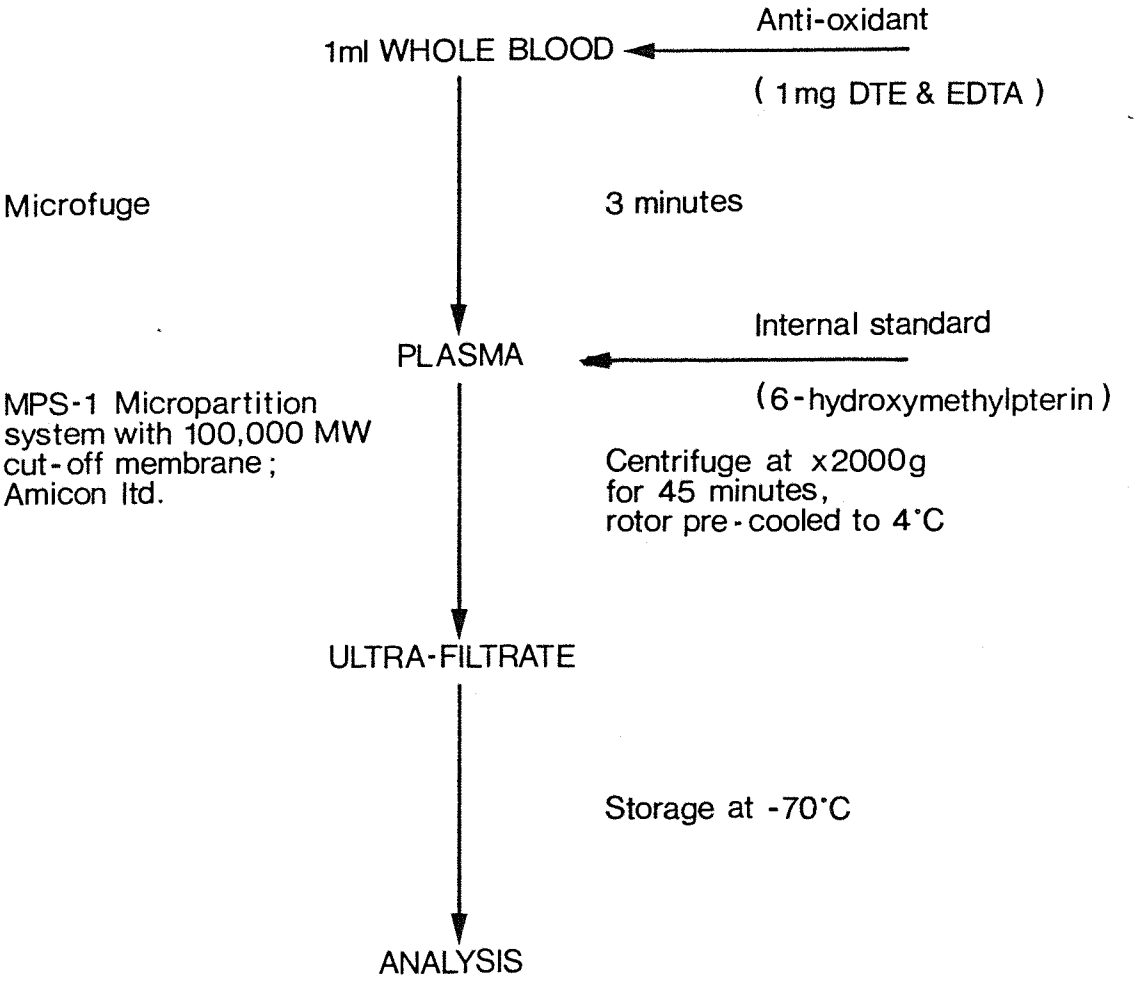
(4) Add a known quantity (e.g. 100μl of a 0.1ng solution) of 6-HMP (internal standard).

(5) Centrifuge at 2000g for 45 minutes with the rotor pre-cooled to 4^{o}C.

(6) Store ultra-filtrate at -70^{o}C until analysed.

The above is summerised in Fig.16.

FIGURE 16
SAMPLE PREPARATION PROCEDURE



NOTE – Keep sample cool and protect from light throughout procedure

Analysis

(1) Liquid chromatography was performed at a flow rate of 1.3ml/minute, mobile phase as described in section 5iia.

(2) Between 50 and 75μl of prepared plasma were injected for analysis on a 125x4.6mm S3ODS2 spherisorb reversed phase column.

(3) BH_4 was oxidised at +0.05V on the conditioning cell electrode prior to quantification by subsequent reduction at -0.10V on the first analytical electrode.

(4) BH_2 was oxidised at +0.80V on the second analytical electrode prior to fluorimetric detection.

(5) Biopterin and neopterin were quantified fluorimetrically (excitation 272nm, emission 445nm).

The chromatographic system is shown schematically in Fig.13.

(iv) Precision and limits of detection

Recovery of BH_4 from plasma "spiked" with the authentic compound had a mean of 43.9% (n=6) with an intra-batch coefficient of variation of 7.8%. Detection was linear to at least 30ng/injection with a minimum detectable quantity of 0.1ng/injection.

Sample recovery was corrected by the addition of a known quantity of 6-HMP as an internal standard prior to sample preparation. Recovery ranged from 61% to 65% (n=6) with an intra-batch coefficient of variation of 2.2% and an inter-batch coefficient of variation of 3.9% (n=6). Intra-batch coefficients of variation for the pterins of interest are shown in Table 6.

(6) STUDY GROUPS

All the following studies were carried out with the approval of the Joint Ethical Sub-Committee of the Southampton and South West Hampshire Health Authority and Southampton University Faculty of Medicine.

(i) Normal Reference Range

In order to establish a reference range for the pterins under investigation in plasma a group of volunteers aged between 21 and 46 years was recruited from the laboratory staff. Prior to taking blood samples subjects were questioned to ensure that they were free of any intercurrent infection, e.g. common cold, as previous investigations have shown neopterin levels to be raised by activation of the immune system (refer section 2iv). Blood samples were collected, prepared and analysed as previously described (section 5).

(ii) Elderly Control Group

To control for the normally occuring age related change in pterin concentrations (e.g. Shintaku et. al. 1982) a group of apparently healthy, elderly volunteers over the age of 65 years and living independantly in the community, were recruited to the study. They were selected from a register of volunteers maintained by the University Geriatric Medicine Group. Before being approached the individual's general practitioner was contacted to ensure that there was no objection to their participation in the study.

Apart from living in the community the subjects were required to be independant in active daily living, i.e. able to dress, wash and walk unassisted. Mental status was checked by administering the Folstein "Mini-Mental State" examination (Folstein et. al. 1975) which is described in Appendix IV, the criteria for inclusion being a score of at least 20/30. After ensuring that no intercurrent infection was present

blood samples were collected, prepared and analysed as previously described.

(iii) Group with Senile Dementia of the Alzheimer Type

Subjects suffering from SDAT were recruited from patients under the care of the Department of Psychogeriatrics at Moorgreen hospital. Alzheimer type subjects were differentiated from those with multi-infarct type dementia by use of an exclusion technique as described by Hachinski et. al. (1975), refer Appendix III, a score of less than 5/18 being required for inclusion in the study.

After excluding cases with multi-infarct type dementia, the patients' mental status was assesed using the Folstein examination (Appendix IV) with a score of less than 20/30 being required for inclusion in the study. Patients selected were cared for in the community as opposed to being hospital in-patients. This made the group more comparable with the elderly volunteer group, controlling for any possible effects of the hospital environment. A clinical diagnosis of SDAT was also made by a psychogeriatrician or geriatrician who also ruled out any other possible causes of dementia, e.g. drug or alcohol abuse.

After ensuring that the patient was free from intercurrent infection blood samples were collected, prepared and analysed as already described (section 5).

(7) RESULTS AND STATISTICS

(i) Study Group Data

Details of age and mental status for the three groups are detailed in Table 7. Results of the pterin assays are described in Table 8 and individual results displayed in Figure 17A-D.

From the results it is observed that the biologically active BH_4 occurs in detectable quantities in samples from only 4 healthy elderly (13.3%) and 5 patients with SDAT (15.6%). In all other instances inconsistancies between the number of samples collected and the number of analyses reported are due to contamination of the sample causing the chromatographic peaks of interest to be obscured.

TABLE 7

Age and Mental Status of Study Groups

	Control	Elderly	SDAT
Number (n)	23	30	32
Males/Females	15:8	14:16	10:22
Age (Years)			
Mean	30.2	76.8	77.1
Standard deviation	7.0	5.3	8.2
Range	21-46	88-62	96-58
Folstein Exam			
Mean Score	n/d	28.5	5.2
Standard deviation	n/d	1.4	4.7
Range	n/d	30-25	13-0
Hachinski Exam			
Mean Score	n/d	n/d	2.1
Standard deviation	n/d	n/d	1.4
Range	n/d	n/d	4-0

n/d = not done

TABLE 8

Plasma Pterin Concentrations

Assay	No. of assays above level of detection	Mean Concentration (ng/ml plasma)	95% Confidence Intervals
Neopterin			
Control	20	2.4	2.1-2.6
Elderly	29	5.7	4.6-6.8
SDAT	28	6.0	4.9-7.1
Biopterin			
Control	20	3.0	2.5-3.4
Elderly	28	1.3	0.8-1.8
SDAT	28	1.0	0.7-1.2
BH_2			
Control	20	7.2	6.0-8.3
Elderly	22	11.8	10.6-13.0
SDAT	28	9.5	8.4-10.5
BH_4			
Control	17	3.0	2.6-3.5
Elderly	4	2.4	
SDAT	5	1.6	

FIGURE 17A
PLASMA BIOPTERIN LEVELS

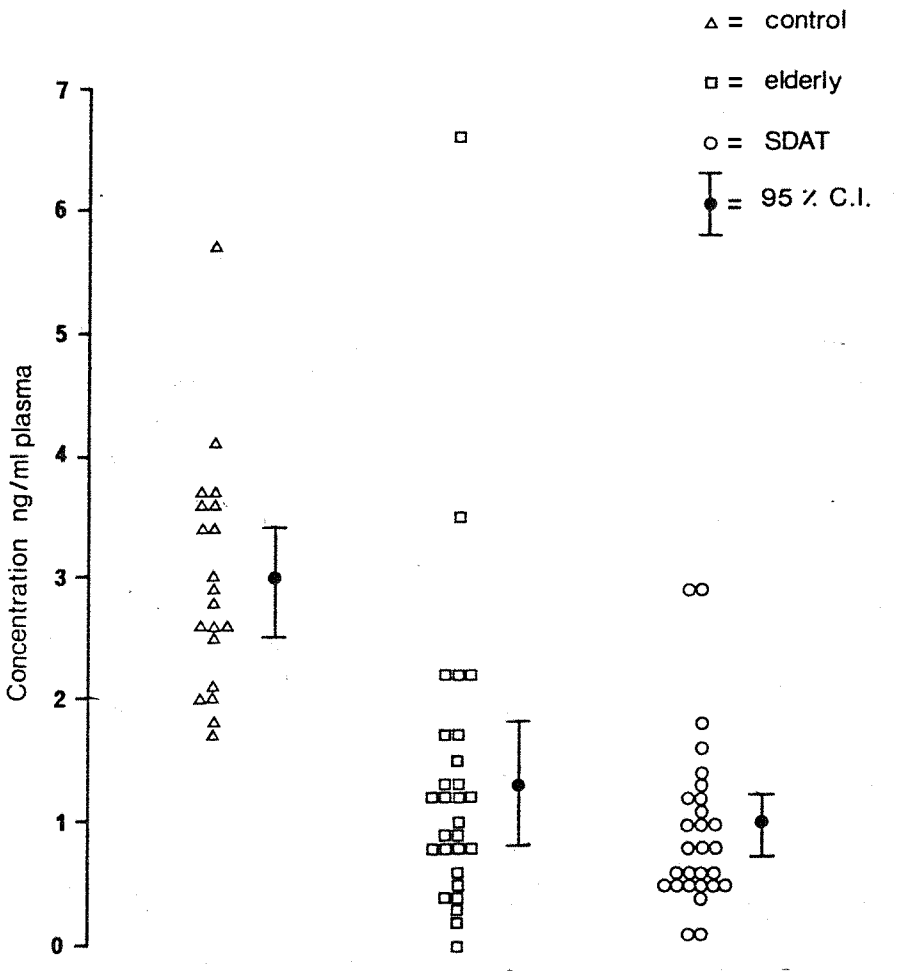


FIGURE 17B

PLASMA NEOPTERIN LEVELS

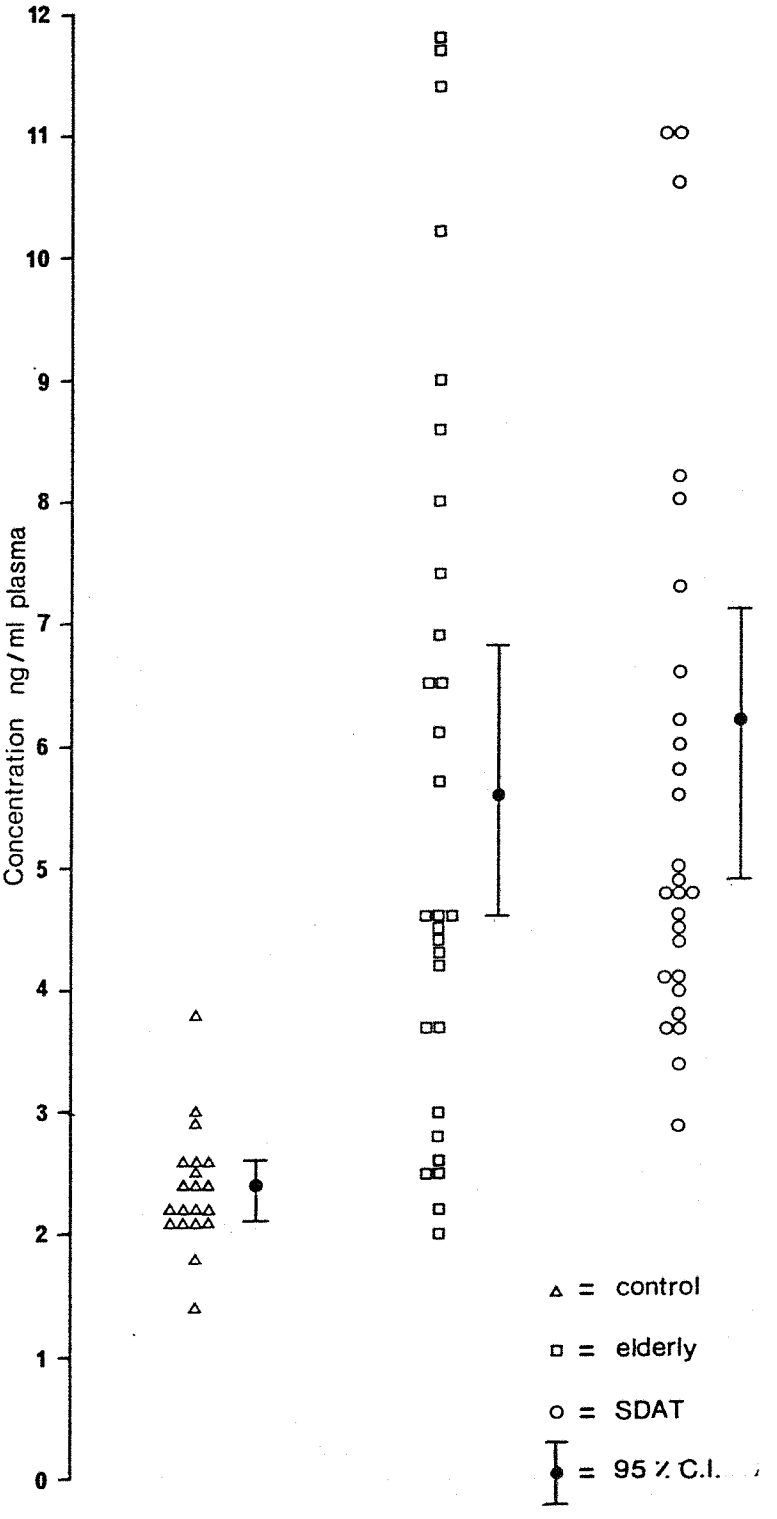


FIGURE 17C

PLASMA BH_2 LEVELS

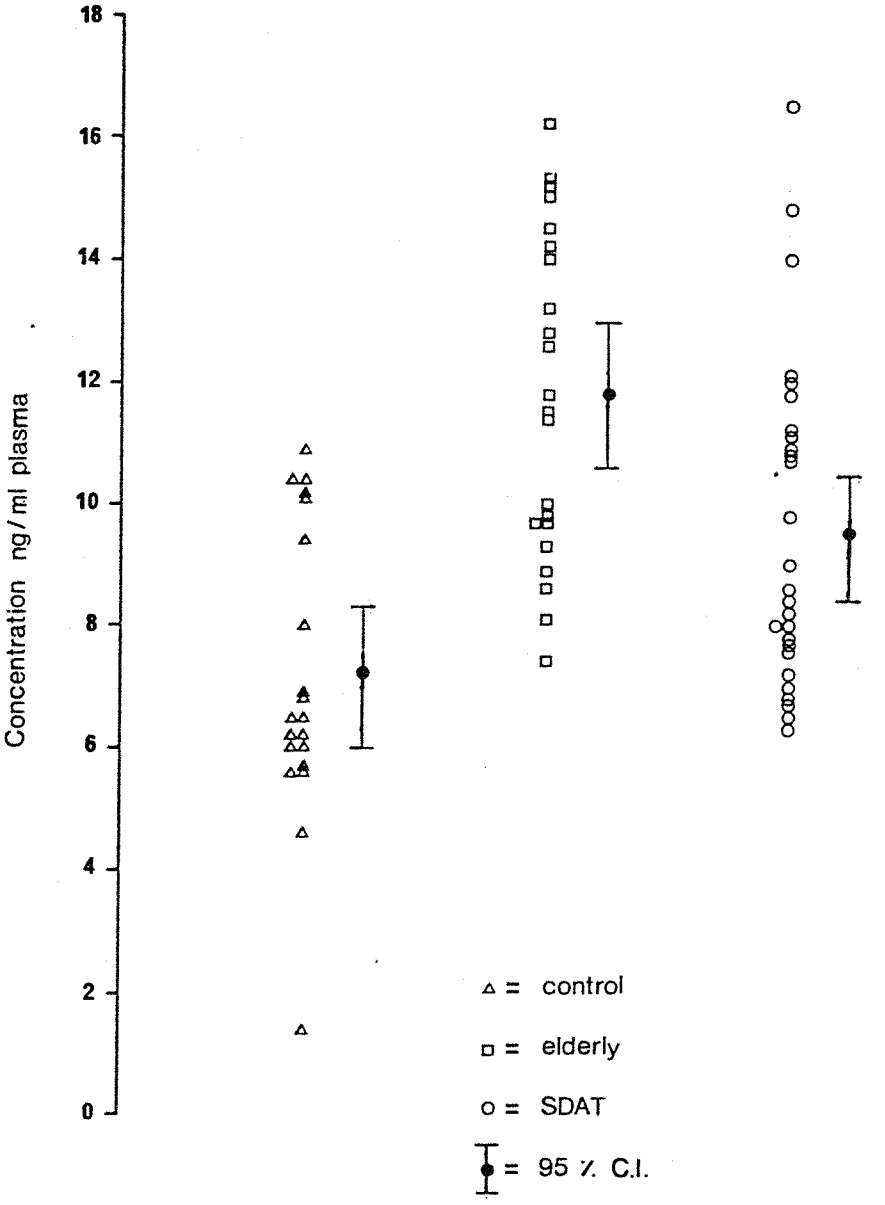
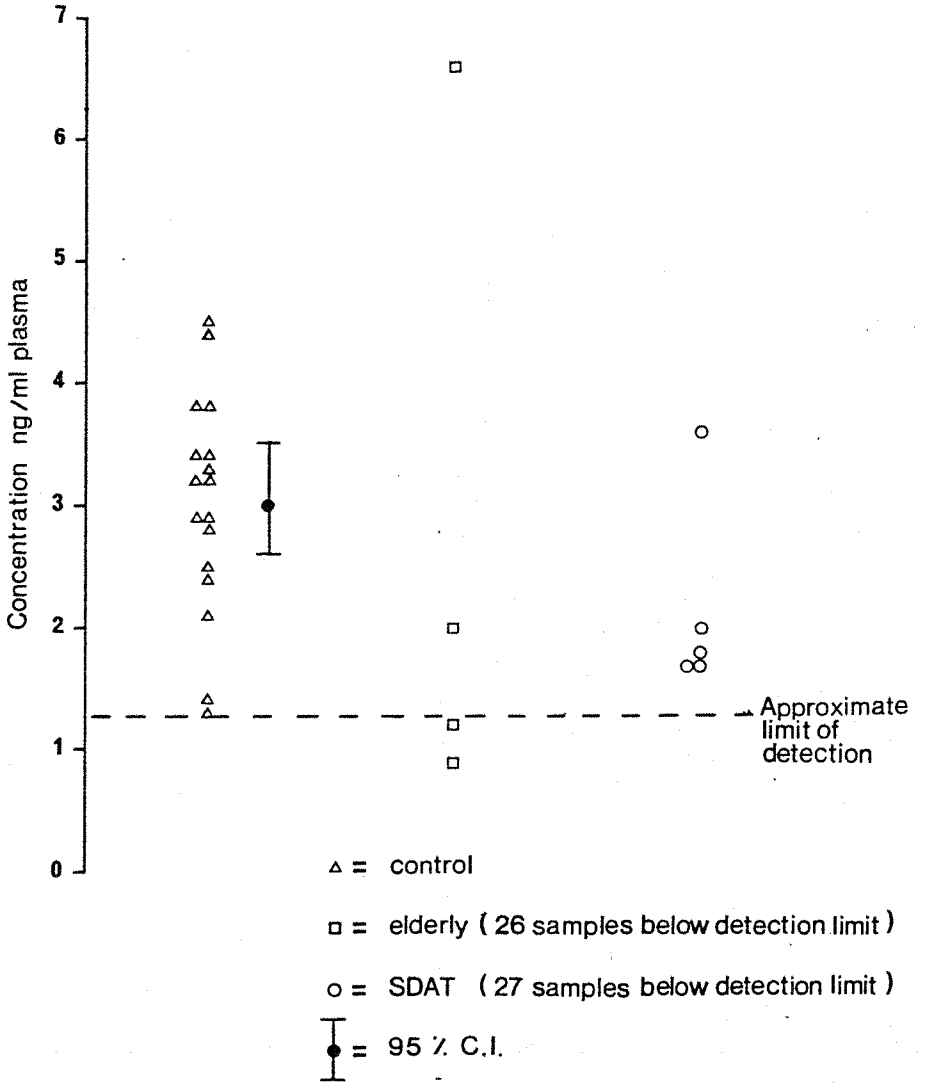


FIGURE 17D

PLASMA BH_4 LEVELS



(ii) Comparison of Data

The significance of differences between groups were calculated by the Mann-Whitney "U" test, as inspection of the results showed that the pterin concentrations were not normally distributed. This observation confirmed the findings of previous investigators (Young et. al. 1982).

The level of neopterin in the healthy elderly volunteers was significantly higher than in the younger control group ($p<0.001$) as was the level in SDAT patients ($p<0.001$). However there was little difference between the neopterin levels in healthy elderly and SDAT subjects ($p>0.05$).

Biopterin levels were decreased in healthy elderly subjects when compared with the control group ($p<0.001$) as was the case with SDAT patients ($p<0.001$). Again there was little difference between the elderly and SDAT groups ($p>0.05$).

Levels of BH_2 were significantly raised in healthy elderly subjects when compared with the younger control group ($p<0.001$). With the SDAT group levels of BH_2 lay mid-way between the younger control and elderly groups ($p<0.01$ but >0.001 when comparing the SDAT group with the control or elderly groups).

Statistical comparisons were not made for BH_4 due to the number of samples displaying a quantity of the compound below the limit of detection (approximatly 1.3ng/ml plasma).

The statistical comparisons are summerised in Table 9.

TABLE 9

Statistical Comparison of Study Groups Using the Mann-Whitney Test

Comparison	Probability
Neopterin	
Control/Elderly	<0.001
Elderly/SDAT	>0.05
Control/SDAT	<0.001
Biopterin	
Control/Elderly	<0.001
Elderly/SDAT	>0.05
Control/SDAT	<0.001
BH_2	
Control/Elderly	<0.001
Elderly/SDAT	<0.01 but >0.001
Control/SDAT	<0.01 but >0.001

(8) DISCUSSION

Visual inspection of the results obtained from the healthy elderly control group (i.e. raised neopterin and BH_2 with depleted biopterin and BH_4) would seem to show an age related decline in the enzyme dihydropteridine reductase (DHPR), which is in line with the findings of previous investigations (e.g. Young et. al. 1982). Raised levels of BH_2 show that BH_4 is being synthesized from the precursor, neopterin. However, after being oxidised to BH_2 in acting as the hydroxylase enzyme cofactor (q.v. Fig.4) it is not being recovered to the active BH_4 form, causing a build up of BH_2 levels and indicating a deficit in DHPR. This interpretation of the data is supported by the findings of Smith et. al. (1985) who demonstrated similar raised levels of BH_2 in a patient with a variant form of phenylketonuria (PKU) shown to be caused by a defect of DHPR.

The general pattern of results in the SDAT group is similar to that of the healthy elderly control group, although the elevation in levels of BH_2 is not as great. Previous work (Young et. al. 1982) showed a small age-related decline in DHPR activity in leucocytes, but a significant decrease in SDAT patients. This is clearly not consistant with the results presented here or with those reported by Jeeps et.al. (1986) who, working with dried blood spots, found DHPR activity in SDAT patients to be similar to that in healthy elderly controls. If the SDAT group had a lower level of DHPR activity than the healthy elderly then it would be expected that the accumulation of BH_2 would be higher in the healthy elderly than the dement group. One explanation of the variation in findings of Young and Jeeps is a difference in compartmentation of DHPR between leucocytes and blood spots. However, there would seem to be reasonable grounds to speculate that SDAT patients suffer from an additional metabolic block, over and above that caused by "normal" ageing, in order to explain the difference

in plasma BH_2 levels between the healthy elderly and SDAT patient groups.

It could be argued that the elevation of BH_2 is not as great in SDAT cases due to cell loss, attributable to SP or NFT, leading to an overall lower production of the compound. This would seem to be an unlikely explanation as levels of the precursor, neopterin, are elevated to a greater extent in SDAT patients than in elderly subjects, although not by a significant amount, indicating that the initial potential exists for a normal synthesis of the compound. This is again compatable with the findings of previous investigators who also discount the possibility of deficits being due to simple cell loss (e.g. Gottfries et. al. 1983, Palmer et.al. 1987).

The tentative hypothesis is therefore formed that the "additional" metabolic blockage is situated in the pathway at a point after the production of neopterin from GTP but before the synthesis of BH_4. High levels of neopterin, observed in both healthy elderly and SDAT groups, are presumably caused by an increased synthetic activity in the early stages of the pathway. This would be in response to a feedback mechanism operating from a point lying beyond the metabolic block attempting to "boost" depleted levels of biologically active BH_4. A blockage at this point in the pathway would mean that less BH_4 would be available from the initial synthetic stages and consequently levels of BH_2 in SDAT patients would be lower than in the healthy elderly group. It is also noted that the mean level of biopterin is lower in SDAT patients than in healthy elderly subjects which would again point to a lower flux through the later stages of the pathway, although the difference does not reach a level of significance.

However, the synthetic pathway leading from neopterin to BH_4 has not yet been fully elucidated. The current state of knowledge (Tanaka et. al. 1980, Heintel et. al. 1984, Smith 1987) would seem to favour a pathway which involves a sepiapterin type intermediate and the enzyme sepiapterin reductase

(Figs. 4 & 5). Three new pterins, provisionally named primapterin, anapterin and 6-oxo-primapterin, have recently been described in a patient with hyperphenylalaninaemia (Curtius et. al. 1988). Although the enzymes of BH_4 synthesis and recovery were found to be normal the patients blood phenylalanine fell in response to oral BH_4 administration. The metabolic origin of these new pterins is unknown and how they are involved with BH_4 remains unclear. Although around 50% of urinary primapterin is in the tetrahydro form it only has a cofactor activity approximatly 2.5% that of BH_4 for phenylalanine hydroxylase.

It is interesting to note that the section of the pathway under consideration is known to require a magnesium cofactor (Tanaka et. al. 1981) and it has recently been proposed that competition for enzyme binding between aluminium and magnesium may have a role to play in the pathology of SDAT (e.g. Cowburn and Blair 1987). The involvement of aluminium in SDAT is currently attracting much attention and it is therefore appropriate to give the subject some consideration.

Aluminium, in the form of aluminosilicates, is found at the core of senile plaques. Due to the highly insoluble nature of these compounds the question must be addressed of how they are transported in the brain if they are to be implicated in the pathology of SDAT. Aluminium is thought to follow the metabolic pathway of iron, at least in the extracellular environment, due to similarities in the ionic structure of the two metals (Birchall and Chappell 1988). It may be via the iron pathway of transferrin that aluminium enters the intracellular environment. Once inside the cell the slightly more acidic pH will mean that the chemistry of aluminium will differ from that of iron, probably binding to a cytosolic chelator such as citrate. The predominant aluminium species at physiological pH is $Al(OH)_4^-$, although all species will react with physiological ligands such as citrate, phosphate, silicic acid and iron binding proteins. A particularly

favourable binding site is the phosphate groups of inositol which Birchall and Chappell also suggest may be implicated in SDAT. However, it is the relative binding strengths of ligands, perhaps determined by pH differences, which will determine the *in vivo* pathway of aluminium and its competition with other ions, such as magnesium.

If abnormal pterin metabolism is implicated in the pathology of SDAT then it would appear to operate as an "all-or-nothing" phenomena. This is because inspection of individual results showed that plasma pterin concentrations did not correlate with mental status, as assessed by the Folstein "Mini-Mental State" examination (Folstein *et.al.* 1975). Also SDAT does not seem to be age dependant as there was no significant correlation between age and levels of BH_2.

In a study of this nature there is always the risk that results may be biased by artefacts of drug therapy given for treatment of other ailments, both in the healthy elderly and SDAT groups. Both patients and volunteers were recieveing a wide variety of medication including in the case of SDAT patients tricyclic anti-depressants, the most common of which was imipramine. This has infrequently been reported to cause hallucinations (Griffith 1986). However, inspection of the results showed no evidence of a related effect in either group and the number of samples should be sufficiently large to overcome any problems of bias from artefacts.

It is also important, when selecting patients for the SDAT group, that the diagnosis of AD is as accurate as possible. The inclusion of patients with multi-infarct or mixed type dementias could artificially bias the results. As SDAT cannot be confirmed until post-mortem examination has revealed significant numbers of tangles and plaques it is necessary to rely on exclusion techniques, questionaires and the experience of the medical staff.

Diagnostic methods are covered in the review by Katzman (1986) and are currently believed to have a 90%

assurance of accuracy. Modern diagnostic methods of brain imageing such as computed tomography (CT) or nuclear magnetic resonance (NMR) cannot positively identify AD but, they are useful for excluding other possible causes of dementia such as brain tumours or the damage caused by multi-infarct dementia. Questioning of relatives and close friends is of importance to determine whether the illness has a stepwise or progressive course, the former indicating multi-infarct and the latter Alzheimer's dementia. Background information is crucial, early errors in diagnosis may have been due to a failure to recognise depression as a cause of memory loss (Katzman 1986). However, in view of the data presented in table 6, I have no reason to suspect the diagnoses made in this study.

Speculation on a possible therapy for SDAT is difficult at this stage. The majority of therapies attempted so far have centered around the cholinergic deficits displayed in SDAT. Apart from a study using the potent anticholinesterase tetrahydroamionoacridine (Summers et. al. 1986) these have been largely unsuccessful. This is probably because the cholinergic deficits are only a part of a spectrum of abnormalities which may also include, for example, adrenergic defects. In the light of the results presented here I feel that insufficient grounds exist to proceed with a trial of BH_4 cofactor therapy until the pathway of synthesis from neopterin has been further elucidated. However, it is possible to speculate on some points which may have a bearing on such an approach.

Experiments have shown that although BH_4 concentration is rate limiting for tyrosine hydroxylase the peripheral administration of extra cofactor does not lead to a significant increase in enzyme activity (Kettler 1974). This is believed to be due to poor penetration of the blood-brain barrier and therefore raises questions as to the viability of BH_4 therapy and also the route of administration. Also, as already mentioned, BH_4 is a highly labile compound and so it

may be necessary to utilise a more stable, synthetic analogue. 6-Methyltetrahydropterin is a synthetic hydroxylase cofactor reported to have better blood-brain barrier penetration than BH_4 (Kapatos and Kaufman 1981). Synthetic tetrahydropterins are also believed to have a greater cofactor activity than the natural form. However, as a next step for this research an investigation of the levels of sepiapterin and/or sepiapterin reductase activity is proposed because this is currently thought to lie between neopterin and BH_4 on the synthetic pathway. Determination of sepiapterin levels in SDAT would therefore shed further light on the speculated metabolic block.

An assay of sepiapterin in plasma should not present any technical difficulties. It is a fully oxidised pterin and as a result should be naturally fluorescent, making it suitable for fluorimetric detection as described in the present study once optimum excitation and emission wavelengths have been determined. Several published methods for the detection of sepiapterin appear in the scientific literature (e.g. Woolf et. al. 1983).

In conclusion, this study has fulfilled its aim of determining cofactor levels in SDAT. Although the results do not immediatly suggest a possible replacement therapy they do support the hypothesis of a defect of pterin metabolism being implicated in SDAT. Support is given to the results of previous studies which show age-related deficits in DHPR activity and levels of biopterin. An area has been identified where further research may yield information of use in developing a therapy for SDAT.

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APPENDIX I

Reagents & Suppliers

Reagent	Grade	Supplier
Potassium dihydrogen orthophosphate	Aristar	BDH Chemicals Ltd., Poole, England
Orthophosphoric acid	Aristar	BDH Chemicals Ltd.
Ethylenediaminetetra-acetic acid (disodium salt)	Analar	BDH Chemicals Ltd.
Trichloroacetic-acid (TCA)	Analar	BDH Chemicals Ltd.
Methanol	Hipersolv	BDH Chemicals Ltd.
Ascorbic Acid	Analar	BDH Chemicals Ltd.
Dithioerythritol		Sigma Chemical Co. Poole, Dorset, England
Dithioerythritol	Gold Label	Aldrich Chemical Co. Ltd., Gillingham, Dorset, England
Octane sulphonic acid (OSA)	HPLC Grade	FSA Laboratory Supplies, Loughborough, Leics. England

Pterin standards (BH_4, BH_2, biopterin, neopterin, 6-HMP)	Dr.B.Schircks, Buechstr, Jona, Switzerland
Bio-Rad protein assay reagent	Bio-Rad laboratories Watford, Herts., England

APPENDIX II

Apparatus

Equipment	Manufacturer
ConstaMetric IIG pump	LDC/Milton Roy, Stone, Staffs., England
WISP 710B autosampler	Waters Associates Inc., Milford, Massachusets, U.S.A.
Coulochem electrochemical detector	ESA Inc., Bedford, Massachusets, U.S.A.
3000 fluorescence spectrometer	Perkin-Elmer Ltd., Beaconsfield, Bucks., England
250x4.6mm S5ODS2 spherisorb column	Hichrom Ltd., Reading,Berks., England
125x4.6mm S3ODS2 spherisorb column	Hichrom Ltd.
MPS-1 micropartition apparatus	Amicon Ltd., Stonehouse, Glos., England
Microfuge B	Beckman, Palo Alto, USA
MSE25 high speed centrifuge	Fisons, Loughborough, Leics., England

SP1800 spectrophotometer	Pye-Unicam, Cambridge, England

APPENDIX III

Hachinski Score for Multi-Infarct Dementia

Feature	Score (If present)
Abrupt onset	2
Stepwise deterioration	1
Fluctuating course	2
Nocturnal confusion	1
Relative preservation of personality	1
Depression	1
Somatic complaints	1
Emotional incontinence	1
History of hypertension	1
History of strokes	1
Evidence of associated atherscleros is	1
Focal neurological symptoms	2
Focal neurological signs	2

Interpretation:

Score 4 and below: Primary degenerative dementia

Score 7 and above: Multi-infarct dementia

Score 5 and 6: Mixed type dementia

APPENDIX IV

The "Folstein" Mini-Mental state Examination

Orientation

a) What is the; Year, season, date, day, month?

(1 point for each correct response)

b) Where are we; Country, town, district, hospital, ward?

(1 point for each correct response)

Registration

a) Name three unrelated objects, ask the patient to repeat them. (1 point for each correct response.) Repeat trial until patient has learnt all three objects or a maximum of six trials have been carried out.

Attention and calculation

a) Starting at 100, ask the patient to subtract serial 7's (i.e. 93,86,79,72,65). Stop after five subtractions. Score is the number of correct answers.

b) If the patient cannot/will not perform this task ask him to spell "WORLD" backwards. Score is the number of letters in the correct order (e.g. DLROW=5, DLORW=3).

Recall

a) Ask the patient to recall the three objects learnt above (1 point for each correct answer).

Language

a) Ask the patient to identify a wrist watch and pencil.

(1 point for each correct answer)

b) Ask the patient to repeat the sentence "No ifs ands or buts".

(Allow only one trial, score 0 or 1)

c) Ask the patient to follow these commands: Take a sheet of paper in your right hand, fold it in half, place it on the floor.

(Score 1 point for each step correctly executed)

d) From a piece of paper ask the patient to read and then obey the command "close your eyes".

(Score 1 point if he closes his eyes)

e) Ask the patient to write a sentence. It must contain a subject, verb and be sensible.

(Score 0 or 1)

f) Ask the patient to copy the intersecting pentagons design.

(Score 0 or 1)

Maximum possible score: 30

APPENDIX V

Abbreviations

AChE	Acetylcholine Esterase
BH_2	Dihydrobiopterin
BH_4	Tetrahydrobiopterin
BSA	Bovine Serum Albumin
CAT	Choline Acetyl Transferase
Co-A	Cofactor A
5-HIAA	5-Hydroxyindoleacetic acid
6-HMP	6-Hydroxymethylpterin
5-HT	5-Hydroxytryptamine
MAO	Monoamine Oxidase
MHPG	3-Methoxy-4-hydroxyphenylglycol
NFT	Neurofibrillary Tangle
PKU	Phenylketonuria
qBH_2	Quinonoid Dihydrobiopterin
SDAT	Senile Dementia of the Alzheimer Type
SP	Senile Plaque

The following published papers were included in the bound thesis. These have not been digitised due to copyright restrictions, but the links are provided.

A.G. Powers, J.H. Young, B.E. Clayton (1988) "An isocratic HPLC technique for the simultaneous detection of oxidised and reduced pterins in the plasma and other biological fluids" Annals of Clinical Biochemistry. Vol.25, pp. 235-236

A.G. Powers, J.H. Young, B.E. Clayton (1988) "Estimation of tetrahydrobiopterin and other pterins in plasma by isocratic liquid chromatography with electrochemical and fluorimetric detection." Journal of Chromatography. Vol. 432, pp. 321-328