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THE REGULATION OF HUMAN 11β-HYDROXYSTEROID DEHYDROGENASE

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ABSTRACT

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By Stephen James Donovan

Relative dysregulation of isoforms of 11β-hydroxysteroid dehydrogenase (11β-HSD) may underlie the pathogenesis of a spectrum of human diseases. Most investigations of the role of 11β-HSD activity in the aetiology of human disease have favoured analysis of the cortisol turnover quotient, plasma cortisol half-life or plasma cortisol and cortisone but have revealed no clear correlations. However, estimation of unconjugated steroid excretion may be a more incisive investigative tool since the unconjugated corticosteroid profile of urine reflects more accurately renal 11β-HSD2 activity. This thesis describes the development of three novel techniques for the sensitive estimation of unconjugated corticosteroids in biological fluids. Two utilise reversed phase HPLC with either fluorescence detection subsequent to derivatization with 7-(carboxymethoxy)-4-methylcoumarin or UV absorbance detection. The third comprises a novel radioimmunoassay for urinary free cortisone. These techniques were used to make a comparison of urinary free cortisol:cortisone ratios and total cortisol:cortisone metabolite ratios as indices of renal 11β-HSD2 activity.

Previous studies of 11β-HSD suggest that the enzyme is hormonally regulated. However, much of this research predates the discovery of 11β-HSD2 or fails to describe the mechanisms which underlie the isoform specific regulation of 11β-HSD. Extensive studies of the steroidogenic and steroid metabolising properties of the choriocarcinoma cell line, JEG-3, have established this cell line as a model of steroid metabolism and particularly 11β-HSD2 activity in trophoblastic tissue. However, few studies have attempted a systematic investigation of the hormonal regulation of 11β-HSD2 in these cells. Moreover, to date, there have been no reports of investigations of the regulation of 11β-HSD in human skeletal muscle which expresses 11β-HSD1 and is thus capable of extensive interconversion of cortisone and cortisol. Thus this thesis describes a series of novel investigations of the regulation of 11β-HSD1 in skeletal myoblasts and 11β-HSD2 in JEG-3 by steroid and peptide hormones and by pro-inflammatory and colony stimulating cytokines. Finally, this thesis describes the strong associations between glucocorticoid receptor and 11β-HSD1 expression and key features of the metabolic syndrome in human skeletal myoblasts from a cohort of men with varying degrees of insulin resistance adiposity and blood pressure. Moreover, these studies also suggest a role for 11β-HSD1 in the regulation of glucocorticoid hormone action in skeletal muscle and highlight the significance of dysregulation of isoforms of 11β-HSD in the aetiology of the metabolic syndrome.

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List of Abbreviations

11-DH	-	11-dehydrogenase
11-OR	-	11-oxoreductase
11β-HSD	-	11β-hydroxysteroid dehydrogenase
ACTH	-	adrenocorticotrophic hormone
AME	-	apparent mineralocorticoid excess
α-MSH	-	α-melanocyte stimulating hormone
AVP	-	arginine vasopressin
β-LPH	-	β-lipotrophin
BMI	-	body mass index
BSA	-	bovine serum albumin
cAMP	-	cyclic adenosine monophosphate
CAH	-	congenital adrenal hyperplasia
cDNA	-	complimentary deoxyribonucleic acid
CBG	-	cortisol binding globulin
CBX	-	carbenoxolone
CCMMC	-	7-[(chlorocarbonyl)methoxy]-4-methylcoumarin
CLIP	-	corticotrophin-like intermediate lobe peptide
CMMC	-	7-(carboxymethoxy)-4-methylcoumarin
CRH	-	corticotrophin releasing hormone
dATP	-	deoxyadenosine tri-phosphate
dCTP	-	deoxycytidine tri-phosphate
DEPC	-	diethyl pyrocarbonate
dGTP	-	deoxyguanosine tri-phosphate
DHEA	-	dehydroepiandrosterone
DMAP	-	dimethylaminopyridine
DMEM	-	Dulbecco's modified eagles medium
dNTP	-	deoxynucleoside tri-phosphate
dTTP	-	deoxythymidine tri-phosphate
DTT	-	dithiothreitol
EDC	-	1-ethyl-3-(-3-dimethylaminopropyl)-carbodiimide hydrochloride
EDTA	-	ethylenediaminetetra-acetic acid
FCS	-	foetal calf serum
GALF	-	glycyrrhetinic acid like factors
GC	-	gas chromatography
GLC	-	gas-liquid chromatography
GE	-	glycerrhetinic acid
GI	-	glycyrrizhic acid
GR	-	glucocorticoid receptor
HBSS	-	Hanks' balance salt solution
hCG	-	human chorionic gonadotrophin
HPA	-	hypothalamic-pituitary-adrenal
HPLC	-	high pressure liquid chromatography
IGF-1	-	insulin-like growth factor-1
IL-1β	-	interleukin-1β

LH	-	luteinising hormone
KRB	-	Krebs Ringer bicarbonate buffer
MOPS	-	3-[N-morpholino]propanesulphonic acid
mRNA	-	messenger ribonucleic acid
MR	-	mineralocorticoid receptor
MS	-	mass spectrometry
NAD	-	nicotinamide adenine dinucleotide
NADH	-	reduced nicotinamide adenine dinucleotide
NADP	-	nicotinamide adenine dinucleotide phosphate
NADPH	-	reduced nicotinamide adenine dinucleotide phosphate
NMR	-	nuclear magnetic resonance
ODS	-	octadecylsilane
PBS	-	phosphate buffered saline
PCR	-	polymerase chain reaction
PMSF	-	phenylmethylsulfonyl fluoride
POMC	-	pro-opiomelanocortin
RIA	-	radioimmunoassay
RT	-	reverse transcriptase
SCAD	-	short chain alcohol dehydrogenase
SDS	-	sodium dodecylsulphate
SPE	-	solid phase extraction
T3	-	3,3',5-triiodo-L-thyronine
TBST	-	tris buffered saline with tween
TCA	-	trichloroacetic acid
TE	-	tris-EDTA
TEMED	-	tetramethylethylenediame
THE	-	tetrahydrocortisone
THF	-	tetrahydrocortisol
TLC	-	thin layer chromatography
TNF-α	-	tumour necrosis factor alpha
UV	-	ultra violet
WHO	-	world health organisation

CHAPTER 1 – General Introduction

1.1 Biosynthesis and metabolism of the adrenal steroids

The adrenal cortex synthesises and secretes three main classes of steroid: the glucocorticoids, cortisol (Kendall's compound F) and corticosterone, the mineralocorticoids deoxycorticosterone and aldosterone and the adrenal androgens, dehydroepiandrosterone, androstenedione and 11β-hydroxy-androstenedione. Additionally, the adrenal cortex secretes small amounts of the precursors of these steroids into the general circulation including pregnenolone, 17α-hydroxypregnenolone, progesterone, 17α-hydroxyprogesterone, 11-deoxycortisol and relatively small amounts of the sex steroids testosterone and oestradiol. Moreover, the adrenal cortex exhibits a unique histological zonation characterised by an outer layer of cells, the zona glomerulosa, deficient in 17α-hydroxylase activity and thus incapable of producing cortisol or the adrenal androgens, which is dedicated to the synthesis of the mineralocorticoid aldosterone, whilst the two inner layers of cells, the zona fasciculata and the zona reticularis are responsible for the synthesis of the glucocorticoids and adrenal androgens respectively. A schematic representation of the adrenal steroidogenic pathway is illustrated in figure 1.1 (Page 3).

1.1.1 *Regulation of adrenal function*

The synthesis and secretion of cortisol is regulated by adrenocoricotrophic hormone (ACTH) secreted by the anterior pituitary under the influence of corticotrophin releasing hormone (CRH) which is itself a neural peptide comprising 41 amino acids secreted by the hypothalamus[1]. In contrast, aldosterone production falls predominantly under the control of the renin/angiotensin system and circulating levels of potassium[2].

ACTH (MW 4500), a straight chain peptide comprising 39 amino acids, is processed from a large precursor molecule, proopiomelanocortin (POMC) (MW 28,500), which is the product of a single messenger ribonucleic acid (mRNA) species. Subsequent post translational modification of POMC results in the production of smaller biologically active fragments which include β-Lipotropin (β-LPH), α- and β-melanocyte stimulating

hormone (α-MSH, β-MSH), β-endorphin and the N-terminal fragment of POMC (residues 1-18) which is responsible for the biological activity of ACTH. Binding of ACTH to specific receptors on the outer surface of the adrenocortical cell membrane results in the activation of adenylate cyclase with subsequent production cyclic AMP (cAMP). Increased intracellular levels of cAMP result in augmented hydrolysis of cholesterol esters, which expand the pool of free cholesterol available for steroid biosynthesis[3]. ACTH also has an effect upon the level of expression of both the steroidogenic enzymes and proteins responsible for the transport of cholesterol into the mitochondrial compartment where steroidogenesis is initiated by cleavage of the cholesterol side chain at C_{21} [3].

CRH stimulates the secretion of ACTH in a pulsatile manner and in such a way that peak levels of ACTH may be observed just prior to awakening[2]. This diurnal rhythmicity provokes a similar response in the adrenal cortex and is thus reflected in circulating levels of cortisol. Increases in ACTH secretion may be observed under conditions of stress such as pain, trauma, hypoxia, acute hypoglycaemia, exposure to cold and depression, such that they may override the normal diurnal rhythmicity[2].

Once initiated, adrenal steroidogenesis is committed to the formation of one of the three classes of adrenal steroids. This is dependent upon histological location and a cascade of enzymes which include a series of mixed function oxidases (hydroxylases), and a family of hydroxysteroid dehydrogenases. The latter are characterised by a haem moiety attached to an apoprotein found in all cytochromes but with a characteristic absorbance at 450mn, hence their classification as cytochrome P450 enzymes[4, 5]. Indeed it is considered probable that secondary to control of mobilisation of cholesterol esters to the mitochondrial compartment by ACTH, the rate limiting step for adrenal steroid biosynthesis is that of cytochrome P450 cholesterol side chain cleavage, which removes a six carbon fragment from cholesterol to produce the unsaturated steroid pregnenolone[6] (See figure 1.1, page 3)

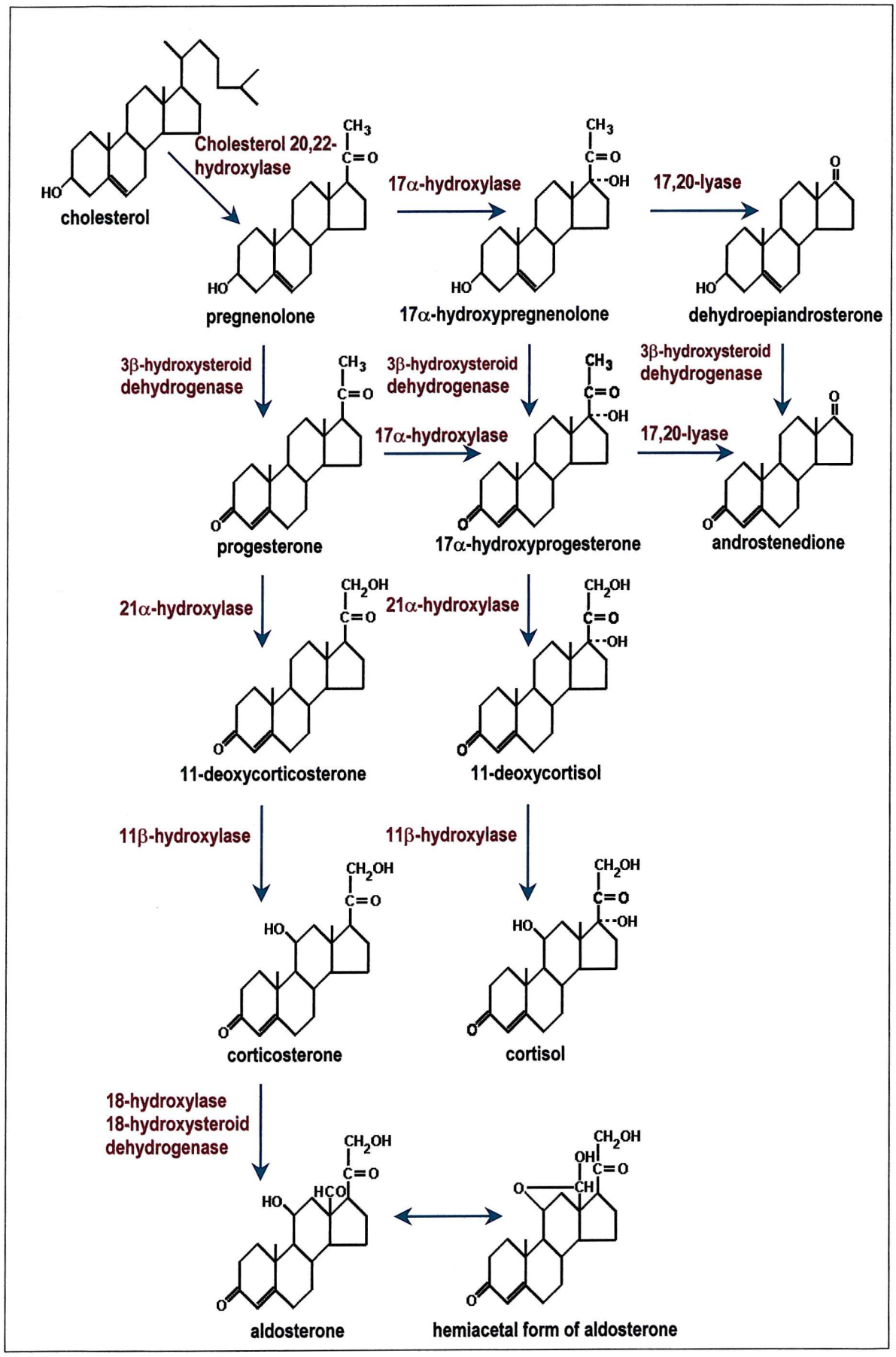


Figure 1.1. Schematic representation of adrenal steroidogensis

1.1.2 *Metabolism of cortisol*

It was not until the 1950's that paper chromatography was sufficiently developed to isolate and characterise cortisol and its metabolites from human urine[7-10]. It was these studies, however, which established cortisol as the principal adrenocorticosteroid in man, with a secretion rate of 30-80μmol/24hrs compared with a rate of 0.1-0.4μmol/24hrs for aldosterone.

Cortisol has several important physiological roles. Primarily it exerts an effect on carbohydrate metabolism by acting as an insulin antagonist, promoting a rise in blood glucose by increasing the expression and activity of the hepatic enzymes which control gluconeogenesis. Secondly, it stimulates the catabolism of proteins resulting in a negative nitrogen balance with loss of protein from both muscle and bone. Furthermore it is also known to exert a wide range of less specific physiological effects including inhibition of the inflammatory response and suppression of the reticuloendothelial system, increasing bone turnover with negative calcium balance and increasing cardiac output and vascular tone. It has also been shown to have an effect on the transport of sodium and potassium across cell membranes but the regulation of the extracellular balance of sodium and potassium is principally under the control of aldosterone[3].

In excess of 90% of circulating cortisol is bound to an α-globulin known as cortisol binding globulin (CBG) or transcortin (MW 50,000), which has a high affinity for cortisol and is synthesised in the liver. Furthermore, approximately 5% of cortisol is weakly bound to serum albumin which leaves 2-3% unbound and as such may be regarded as being biologically 'active'[3]. CBG in plasma has a cortisol binding capacity of about 550nmol/L [2] and if total plasma cortisol increases significantly above this level, the concentration of free steroid in the plasma rapidly exceeds its usual fraction of 10% of the total cortisol. Under these circumstances, significant increases in free cortisol may be observed in the urine. Urinary excretion of cortisol by the kidney is greatly hindered by its lipophylic nature. The metabolism of cortisol not only renders it biologically inactive but also increases its polarity and hence its water solubility. Indeed, less than 1% of secreted cortisol appears in the urine unchanged since most metabolic

products of cortisol are both polyhydroxylated and either sulphated or glucurono-conjugated.

The biological potency of all corticosteroids is generally considered to be dependent upon a functional hydroxyl group at carbon C_{11}. Thus dehydrogenation of the C_{11} hydroxyl to the 11-keto derivative results in a steroid with little or no biological activity[11]. Cortisol may undergo such conversion to hormonally inactive cortisone under the influence of the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD). Both cortisol and cortisone are subject to metabolism in the liver, which commences with reduction of the 4-ene-3-oxo configuration of ring A (See figure 1.2, page 7). Complete A-ring reduction results in four isomers: (1) 5β-H, 3β-OH; (2) 5β-H, 3α-OH; (3) 5α-H, 3β-OH; (4) 5α-H, 3α-OH with the 3α-hydroxy forms predominating in man (ratio of 3:1) [6]. The ratio of 5α and 5β forms is more variable and is dependent upon the steroid being reduced, thus cortisol yields a preponderance of its 5β metabolite with a ratio of about 5:1 [6]. The tetrahydro-products of this metabolism: tetrahdrocortisol (THF), 5a-tetrahydrocortisol (5α-THF), tetrahydrocortisone (THE) and 5a-tetrahydrocortisone (5α-THE) then undergo rapid glucurono-conjugation at the 3α-hydroxyl position which further increases their polarity and thus enables their excretion in urine. Approximately 30% of cortisol is excreted in the form of its tetrahydro-derivatives whilst another 30% is further reduced at C_{20} and thus excreted in the form of the cortols and cortolones. Hydroxylation of cortisol at C_6, to produce 6β-hydroxycortisol is a minor metabolic pathway for cortisol and since it is not reduced in ring A it is excreted unchanged in the urine. Whilst under normal physiological conditions only about 1% of cortisol is excreted in this form its significance rises in conditions which result in hypercortisolism and in the neonate where hepatic ring A reduction and glucurono-conjugation are not fully developed[12,13]. It is also of greater significance during pregnancy and following oestrogen treatment when the normal mechanism of cortisol metabolism through the tetrahydro derivatives appears to be impaired[14]. Small quantities of steroids, namely 11β,17,20α,21-tetrahydroxy-4-pregnen-3-one from cortisol and the corresponding trihydroxy-3,11-dione from cortisone, and 11β,17,20α,21-tetrahydroxy-5β-pregnan-3-

one which have undergone reduction at C_{20} but which have either an intact, or only a partially reduced 4-ene-3-oxo configuration may also be found in urine[6] (See figure 1.2, page 7).

The principal site of corticosteroid metabolism in man has long been thought to be the liver[15], however, an increasing body of evidence for extra hepatic metabolism of cortisol has become available over the past four decades. Several studies have clearly demonstrated that the mammalian kidney is a major site of cortisol metabolism[16,17] and that several other tissues are also capable of cortisol metabolism, including the prostate, thyroid, skeletal muscle and synovial membrane[18,19]. Following work on the metabolism of cortisol in the isolated perfused rat kidney, several studies have demonstrated that not only is the kidney an important site for the conversion of cortisol to cortisone but also that it is a site for the synthesis of the 20α- and 20β-reduced metabolites, dihydrocortisol and dihydrocortisone and for the metabolism of corticosterone to 11-dehydrocorticosterone, 20β-dihydrocorticosterone, 5α-hydroxy-4,5-dihydrocorticosterone and 20β-dihydro-1-dehydrocorticosterone[16,20-22]. Importantly, the prolonged cortisol half-life frequently observed in patients with chronic renal failure has been cited as evidence for reduced 11-deydrogenation of cortisol in the kidney[23,24]. This is thought to be due to a relative deficiency in renal 11β-HSD activity, as a consequence of advancing tubular damage, and not to inaccessibility of cortisol to renal 11β-HSD as a result of reduced glomerular filtration. This is further supported by the observation that strikingly low plasma cortisone levels are found in patients following bilateral nephrectomy, and from this data it has been suggested that the kidney is the principal source of 11-dehydrogenase activity and thus cortisone production in man[17].

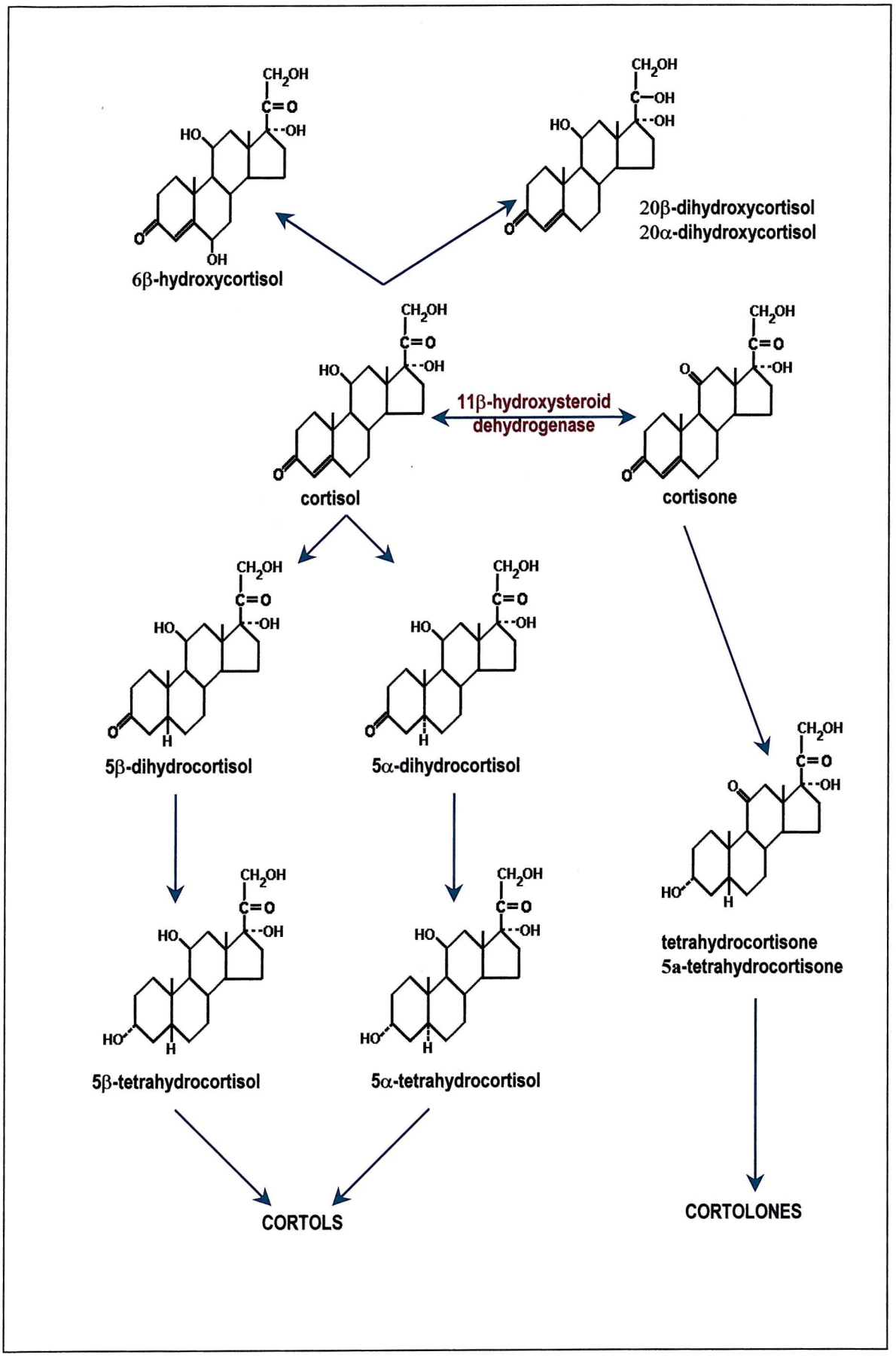


Figure 1.2. Schematic representation of adrenal steroid metabolism

1.2 Adrenal steroidogenesis and metabolism in the foeto-placental unit

Changes in the maternal pattern of circulating corticosteroids and their metabolites which result from the combined influences of foetal adrenal steroidogenesis and metabolism, and from changes in the hormonal milieu necessary for the maintenance of pregnancy are sufficiently profound to necessitate some consideration of the role of the adrenal gland and its steroidal products in the foetal and neonatal period.

By day 25 of gestation, the foetal adrenal cortex may be identified at the cranial edge of the developing mesonephros. Whilst the developing gland has been shown to express the same array of steroidogenic enzymes as that found in the adult, quantitative differences in the activities of these enzymes result in a profile of steroid secretion that is significantly different from that seen at term[25]. The most significant difference is the almost complete absence of 3β-hydroxysteroid dehydrogenase activity which results in the secretion of large quantities of Δ^5-3β-hydroxysteroids. Metabolism of the Δ^5-3β-hydroxysteroids is performed by the foetal liver and adrenal gland, which express high levels of 16α-hydroxylase and sulphotransferase[25]. Thus, the larger part of the Δ^5-3β-hydroxysteroid output of the foetal adrenal is excreted in the maternal urine in the form of 16α-hydroxylated sulphates.

By term, the human adrenal gland weighs about 4g, approximately equal to that of an adult, and secretes up to 200mg of steroid/day[26]. Over the first year of life, there is a reduction in size to about 1g in weight with extensive morphological remodelling of cellular structure (involution). Changes in function closely follow this restructuring. The bulk of steroidal material produced by the neonatal adrenal still consists of the Δ^5-3β-hydroxysteroids such as pregnenolone, 17-hydroxypregnenolone and dehydroepiandrosterone (DHEA), though secretion of both cortisol and aldosterone is significantly higher than that observed in the resting adult. Thus, the pattern of corticosteroid metabolites excreted in the urine reflects this relative difference from the adult state. As the neonatal adrenal cortex involutes during the first year of life, Δ^5-3β-hydroxysteroids gradually disappear from the circulation only to reappear again in late childhood during 'adrenarche'.

1.3 11β-hydroxysteroid dehydrogenase: an historical perspective

The interconversion of cortisol and cortisone in various mammalian tissues was first inferred from studies of the therapeutic effects of corticosteroids in the treatment of arthritis[8,27-29]. It was observed that suppression of the inflammatory response could be elicited by the oral administration of cortisone, whereas direct injection of cortisone into the affected joint produced no therapeutic response[30]. Experimental evidence also suggested that at least a proportion of administered cortisone was excreted as cortisol[31]. These observations led to the conclusion that enzymatic reduction of the 11-keto group of cortisone could result in the formation of biologically active cortisol. This was later confirmed when it was demonstrated that reversible interconversion of 11-hydroxy and 11-keto steroids occurs in the rat liver[32] due to the catalytic activity of the enzyme 11β-HSD[33]. However, it was also observed that the catalysis of 11-oxoreduction and 11β-dehydrogenation was not represented by a uniform tissue distribution since many tissues appeared to demonstrate predominantly dehydrogenase activity[16,34,35] whilst others, most significantly the liver[15,36], appeared to exhibit both oxoreductase and dehydrogenase activity.

Several mechanisms were proposed to account for the differential behaviour of 11β-HSD in various tissues: Nicholas et al[37] postulated that variability in the ratio of 11-dehydrogenase (11-DH) to 11-oxoreductase (11-OR) activity could be attributed to a parallel variability in tissue redox potential. This hypothesis was not widely accepted since several investigators demonstrated that the tissue specific ratio of catalytic activity was maintained even when pyridine nucleotide cofactors were not limiting[38,39]. A second hypothesis suggested that the net preference of catalytic direction was dependent upon local differences in tissue pH. However, pH changes within the physiological range were considered too small to affect the ratio of 11-DH to 11-OR activity to any great extent[40,41]. A third hypothesis suggested the existence of several kinetically distinct isoforms of 11β-HSD which were expressed in a tissue specific manner and which exhibited either 11-DH or 11-OR activity[10,42]. This latter suggestion was supported by evidence for the existence of separate oxidative and reductive isoforms in several enzyme systems including glyceraldehyde phosphate dehydrogenase[43]. Nevertheless, most

authors reported the results of their investigations in terms of the reversible catalytic activity of a singe enzyme which was subsequently designated as EC 1.1.1.146 11-hydroxysteroid:NADP+ 11-oxoreductase by the Nomenclature Committee of the International Union of Biochemistry[44].

The debate surrounding the physical characterisation of 11β-HSD stimulated work which resulted in its isolation and purification from the microsomal fraction of liver homogenates[41,45,46] and subsequently from other tissues including kidney[47,48] gonads[49], placenta[38,39,50] and lung[37]. Selective synthetic detergent displacement experiments suggested that hepatic 11β-HSD exists as a multi-enzyme complex in which 11-DH and 11-OR occupies different orientations and presents different degrees of accessibility to substrate within the microsomal phospholipid matrix[41]. This suggested that tissue specific conditions may alter the orientation or conformation of the enzyme complex such that it adversely affects access of substrate or inhibits enzyme activity. However, subsequent studies suggest that hepatic microsomal 11β-HSD consists of a single homogenous 34kDa protein that exhibits 11-DH but not 11-OR activity[51,52].

Following the screening and purification of positive clones from a rat liver complimentary deoxyribonucleic acid (cDNA) expression library, a 1265bp cDNA fragment containing an 861bp open reading frame was isolated[53]. The predicted amino acid sequence suggested a molecular weight of 31.7kDa, and the discrepancy with the 34kDa protein, previously identified, was thought to be due to glycosylation at Asn-X-Ser sites at positions 158-160 and 203-205. Expression of the recombinant protein in osteosarcoma, and Chinese Hamster Ovary cells in culture, revealed an enzyme which encodes both 11-DH and 11-OR activities in a manner indistinguishable from the activities in rat liver[54]. This suggests the possibility that 11-OR activity is relatively unstable under experimental conditions since activity rapidly deteriorates in cultured cells during prolonged incubation with substrate and cofactor, an observation which may explain why rat liver microsomal extracts only demonstrate 11-DH activity. Nevertheless, this work was taken as conclusive evidence that hepatic 11β-HSD exists as a single enzyme which expresses both 11-DH and 11-OR activities.

Early studies on the tissue distribution of 11β-HSD were undertaken using antisera raised in rabbits against the purified rat liver 34kDa microsomal protein[52]. Western blot analysis identified a 34kDa immunoreactive protein in liver, kidney, brain and testis and additional species of 40kDa in kidney, 26kDa in brain, 47kDa in testis and 68kDa in liver[55]. These observations inspired speculation that the additional species identified in these tissues were either the product of proteolysis, differential glycosylation or dimerisation, or that they represented the tissue specific expression of unique isoforms of 11β-HSD. This latter hypothesis was further strengthened by Northern blot analysis of various tissues using a liver 11β-HSD cDNA. These studies demonstrated that in the liver, testis, lung, heart, hippocampus and colon, mRNA for 11β-HSD consisted of a single 1700bp species whilst in the rat colon there was a 3400bp species[56,57] and in the kidney at last four species of 11β-HSD mRNA were identified[56,58].

Further direct evidence for the existence of additional isoforms of 11β-HSD came from the analysis of enzyme activity in rabbit[59,60], and rat kidney[61], and from the characterisation of 11β-HSD expression in human placenta[50], human kidney[48,62] and rectal colon[57]. By 1994 there was an overwhelming body of evidence in support of the hypothesis of the existence of several distinct isoforms of the 11β-HSD characterised in the main by: the liver isoform, which expresses predominantly 11-OR activity, and the kidney isoform, which expresses exclusively 11-DH activity[50,59,60,61,63]. These characteristics were considered sufficiently different for them to be labelled respectively as 11β-HSD type I (11β-HSD1), a low affinity, NADP/NADPH-dependent dehydrogenase/oxoreductase (apparent K_m for cortisol 2umol/L, cortisone 0.3umol/L) and 11β-HSD type II (11β-HSD2), a high affinity (apparent K_m for cortisol 50nmol/L), NAD-dependent unidirectional dehydrogenase. It was not until 1995, however, that the genes encoding 11β-HSD2 was cloned. The human 11β-HSD1 gene had already been located to chromosome one, following work performed by Tannin *et al*[64] in 1991, and in 1995 the 11β-HSD2 gene was located to chromosome 16q22 [65].

1.4 Congenital and acquired deficiency of 11β-hydroxysteroid dehydrogenase

1.4.1 *Congenital deficiency of 11β-hydroxysteroid dehydrogenase type II: The syndrome of apparent mineralocorticoid excess*

In 1974, Werder *et al* [66] described the clinical presentation of a three-year-old child with persistent, severe hypertension, low plasma renin activity, subnormal plasma aldosterone levels, antinatriuresis, hypokalaemia and metabolic alkalosis. It was also observed that blockade of the mineralocorticoid receptor with spironolactone ameliorated many of the pathological features of this condition. Three years later a similar presentation was described in a three-year-old Zuni Indian girl[67]. Extensive investigation of both plasma and urinary steroid profiles revealed that in addition to severe hypertension with low plasma renin activity, the child also exhibited a low basal plasma cortisol level and a subnormal rise in plasma cortisol in response to administration of ACTH. Moreover, urinary excretion of cortisol and 17-hydroxysteroid metabolites was significantly reduced, as was the excretion of aldosterone. However, administration of ACTH exacerbated the hypertension and hypokalaemia and suggested the existence of an unidentified ACTH dependent steroid with mineralocorticoid-like activity. Whilst the clinical and biochemical features of the syndrome shared important similarities with those of primary mineralocorticoid excess, overproduction of any known mineralocorticoid could not be detected and the epithet 'Apparent Mineralocorticoid Excess Syndrome' (AME) was adopted[68].

To date there have been fewer than thirty reported cases of AME with the classical features of low renin hypertension, hypokalaemia and metabolic alkalosis[69-77]. Whilst the more severe manifestations of untreated AME result in mortality from cerebrovascular accident or cardiovascular disease secondary to hypertension and/or hypokalaemia[70,72,78], several other, more variable, clinical features have been reported amongst patients with AME including: growth retardation[66,67], nephrocalcinosis secondary to severe hypokalaemia[73,79-81], rickets[82], muscle weakness and frank rhabdomyolysis[80,83].

There are marked similarities between AME and the presentation of several other hypertensive conditions including: Liddle's syndrome[84], a hypertensive condition caused by constitutive activation of the renal tubular sodium channel[85], and congenital adrenal hyperplasia (CAH) caused by 11β-hydroxylase deficiency or 17α-hydroxylase deficiency. However, the biochemical features of the former distinguish this syndrome from its clinically similar counterparts. AME is characterised by a reduction in the concentration of circulating mineralocorticoid and a pronounced increase in the ratio of excreted cortisol metabolites (THF, 5α-THF, the cortols and cortolic acids) compared with those for cortisone (THE, the cortolones and cortoic acids)[75,86-89]. Similarly the production of [^{3}H]-H_2O as a consequence of the metabolism of [^{3}H]-cortisol is barely detectable whilst metabolism of [^{3}H]-cortisone remains undiminished[68,73].

In the majority of reported cases of AME a second biochemical feature has been identified which is characterised by a significant increase in the ratio of urinary 5α-THF/5β-THF [68,75,88,90,91]. Indeed, this observation gave rise to the suggestion that 5α-dihydrocortisol was responsible for the mineralocorticoid effects of AME but this hypothesis was later discarded when it was demonstrated that there was no absolute increase in the plasma concentration of 5α-dihydrocortisol but that the increase in the 5α:5β metabolite ratio represented a relative deficiency of 5β-reductase activity[68,69].

Secondary changes in the metabolism of cortisol have also been observed and include a reduction in the rate of cortisol production, as measured by the sum of urinary cortisol metabolite excretion[68,90,92] which, with the exception of the case reported by New *et al*[67] is accompanied by normal serum cortisol levels. In addition, evidence that the half-life of serum cortisol is increased in patients with AME[90] suggests that the former observation is a consequence of suppression of the pituitary release of ACTH consistent with a normally functioning hypothalamic-pituitary-adrenal (HPA) feedback mechanism. This suggestion is further supported by the observation that a broad spectrum of ACTH dependent steroids including deoxycorticosterone and corticosterone are also reduced and that a normal increase in cortisol secretion follows stimulation by ACTH administration in these patients[93]. Furthermore, a relative increase in the fraction of

urinary free steroid excretion has been reported in patients with AME. Ulick *et al*[68] demonstrated that up to 43% of urinary cortisol metabolites were excreted in their unconjugated form, compared with less than 1% in normal individuals, and that the steroid components of this fraction comprised significant concentrations of 20α- and 20β-dihydrocortisol, and 6β-hydroxycortisol which represent pathways of cortisol metabolism which contribute only to a relatively small degree to the metabolism of cortisol under normal circumstances.

The link between AME and a defect in the metabolism of cortisol was ultimately drawn from empirical observations that a fall in blood pressure followed treatment of AME with dexamethasone, aminoglutethimide and metyrapone whilst administration of cortisol or ACTH caused an exacerbation of hypertension[67,72]. Furthermore, Oberfield *et al*[71] demonstrated that the effects of cortisol administration could be alleviated by the mineralocorticoid receptor (MR) antagonist, spironolactone.

In 1988 Stewart *et al*[73] reported a case of AME in which a twenty one-year-old adult male presented with hypertensive retinopathy, hypokalaemia and subnormal plasma cortisone. The profile of urinary steroid metabolites and the extended half-life of 11α-[^{3}H]-cortisol suggested that the condition was identical with those previously reported in children. The biochemical abnormalities, which were clearly indicative of a net reduction in the conversion of cortisol to cortisone, gave rise to the suggestion that AME was the consequence of a congenital deficiency of 11β-HSD but that the syndrome was characterised by a deficiency of 11β-dehydrogenation whilst 11-oxoreduction was unaffected. This hypothesis was strengthened by the observation of identical symptoms in affected siblings[71,72,90,94]. However, it was not until the identification, characterisation and the discovery of several mutations in the gene for 11β-HSD2 that AME was finally ascribed to a congenital deficiency of 11β-HSD2[62,75,76,81,95,96]. Most of these mutations have been reported to be premature stop sites or amino acid substitutions resulting in proteins with varying degrees of enzyme activity. Indeed, expression studies on the mutant cDNAs have revealed a high degree of correlation between genotype and

phenotype such that a fatal, or very severe, phenotype may be explained on the basis of specific mutations resulting in an enzyme with little or no catalytic activity[86].

A variant of AME, referred to as AME type 2 has been described[97]. The clinical presentation is similar to AME type 1 but the urinary (5α-THF+5β-THF)/THE and 5α-THF/5βTHF ratios are both normal. These observations imply that whilst cortisol still acts as a potent mineralocorticoid in the type 2 variant, there is a deficiency of both 11-OR and 11-DH activity in combination with a more generalised defect in steroid A-ring reduction[91]. Speculation that the latter defect is the primary metabolic error in the type 2 variant has yet to be proven but evidence in support of a deficiency of both 11-OR and 11-DH activity in AME type 2 has been provided by observations of the effects of carbenoxolone (CBX), a potent inhibitor of both isoforms of 11β-HSD[73,98,99]. Indeed treatment with CBX has been demonstrated to prolong the cortisol half-life but to have no effect upon the urinary THF/THE ratio[73,100].

1.4.2 *11-oxoreductase deficiency*

Clinical deficiency of 11-OR activity has been described in very few cases[101-104]. The condition is characterised by an inability to convert cortisone to cortisol, resulting in an increase in the metabolic clearance of cortisol which in turn stimulates compensatory overdrive in the HPA axis. The small number of cases so far described have all been in female patients who have presented with bilaterally enlarged adrenal glands and ACTH-driven hyperandrogenism resulting in hirsutism, dysmenorrhoea and infertility. Urinary steroid profiles in these individuals has revealed a marked increase in the excretion of cortisol and androgen metabolites and a 26 fold increase in the ratio of the tetrahydro metabolites of cortisone (THE) to cortisol (THF+5α-THF), whilst plasma and urinary free cortisol levels remain within normal limits. Nevertheless, despite biochemical features which are suggestive of 11-OR deficiency, no defects in the gene encoding 11β-HSD1 have been identified in these patients[104].

1.4.3 *Acquired deficiency of 11β-hydroxysteroid dehydrogenase*

Relatively severe states of acquired mineralocorticoid excess, the clinical features of which are not dissimilar to those of AME, have been reported in individuals who habitually consume large quantities of liquorice[105-108]. The mineralocorticoid effects of liquorice have long been recognised but were originally thought to be due to direct activation of MR by its active constituents, glycyrrhetinic acid (GE) and glycyrrizhic acid (GI)[109,110]. However, the relatively low affinity of GE and GI for the mineralocorticoid receptor and the observation that the hypertensive effects of GE in patients with Addison's disease were abolished, suggested that cortisol was an essential component in the pathogenesis of liquorice induced hypertension[109,111]. It is now recognised that both GE and GI are competitive inhibitors of 11β-HSD2 and that this is the principal mechanism underlying the mineralocorticoid properties of liquorice[112]. In support of this hypothesis, Stewart *et al*[113] demonstrated that following a 10 day regime in which volunteers ingested 200g of confectionery liquorice, the urinary cortisol/cortisone metabolite ratio and the half life of 11α-[^{3}H]-cortisol was increased and, whilst plasma cortisol levels did not change, the 24hour excretion of urinary free cortisol was markedly elevated. Moreover, the 5α/5β reduced metabolite ratio was also increased reflecting a decrease in 5β-reductase activity and resulting in a syndrome which bore a striking similarity with AME type I.

Carbenoxolone, the hemisuccinate derivative of GE, has been widely used for the successful treatment of peptic ulceration. CBX, like GE and GI, has also been reported to induce an hypertensive syndrome characterised by hypokalaemia with sodium and water retention[100]. The effects of administration of CBX are analogous with those of GE and GI in that the half-life of cortisol is prolonged and urinary free cortisol is increased, however, plasma cortisone levels are not reduced and the urinary 5α-THF/THF and (THF+5α-THF)/THE ratios remain normal. These observations are consistent with the hypothesis that ingestion of CBX promotes an acquired form of AME type 2 in which there is reduced activity of both 11-DH and 11-OR[114].

The identification of a wide range of pharmaceutical and naturally occurring inhibitors of 11β-HSD2 including, furosemide[115-117], gossypol[118] and several flavenoids such as naringenin[119], a constituent of citrus fruit has prompted several authors to propose a wider link between exogenous and endogenous inhibitors of 11β-HSD2, termed glycyrrhetinic acid like factors (GALFs) and hypertension[120-123]. Despite an inability to provide these substances with a unique identity, several studies have attempted to quantify the excretion of GALFs in sub-populations of hypertensive patients by exploring their effect upon the activity of 11β-HSD using microsomal preparations. Walker *et al*[121], using rat liver microsomes, demonstrated no correlation between GALF levels and hypertension, plasma cortisol half-life or the urinary (THF+5α-THF)/THE ratio. However, Takeda *et al*[123], using human kidney microsomes, found that GALF excretion was higher in a population of low-renin hypertensive patients compared with a group of normotensive individuals, but that the urinary (THF+5α-THF)/THE ratio was not correlated with GALF excretion. Despite obvious experimental differences and the use of two different isoforms of 11β-HSD, these contradictory data have provided no clear evidence for a relationship between GALFs, 11β-HSD and hypertension and the subject remains one of considerable controversy.

1.4.4 *11β-hydroxysteroid dehydrogenase: guardian of the mineralocorticoid receptor*

The concept that the action of steroid hormones is mediated by intracellular receptor activation was proposed over 20 years ago[124]. Research over the past two decades has identified that thyroid, steroid and related hormones exert their effects through a group of structurally related nuclear receptors which have come to constitute the 'steroid-thyroid hormone nuclear receptor superfamily' [125]. The isolation, purification and cDNA cloning of both the high-affinity, type I corticosteroid receptor termed the mineralocorticoid receptor and the low-affinity, type II corticosteroid receptor termed the glucocorticoid receptor (GR) led to the recognition of significant sequence homology at both the nucleotide and amino-acid levels for these receptors[126-128]. This is particularly apparent in the 66 amino acid C1 region within the DNA binding domain, which includes

9 totally conserved cysteine residues forming the characteristic zinc finger structures and across the entire C terminal ligand binding domain[127]. In contrast, the hypervariable amino terminal domain exhibits very low sequence homology between family members and is involved in receptor transactivation. The region that determines specificity of ligand binding for each receptor in the superfamily has been shown to be structurally and conformationaly specific, although this specificity is not absolute even at ligand concentrations within the physiological range. Indeed, *in vitro* ligand binding studies of the purified hippocampal MR and the recombinant human MR expressed in COS cells, has revealed that MR exhibits an equal affinity for both cortisol and aldosterone[127,129-133]. The degree of overlap in ligand binding between the two receptors may be accounted for by the high level of homology (57-60%) in the C-terminal, steroid binding region of the two receptors[127] while the low degree of homology (less than 15%) exhibited by the N-terminal domains may explain their specificity for target genes[134-136].

The close sequence homology exhibited by the MR and GR, specifically at the C1 region within the DNA binding domain and at the C terminal ligand binding domain, suggests that ligand specificity is not afforded by the structural uniqueness of the receptor[127,136]. These observations are further confounding in the light of evidence which confirms that the renal MR maintains aldosterone specificity despite overwhelming competition from cortisol which, under physiological conditions, circulates at about ten times the concentration of aldosterone[137]. Such observations have resulted, inevitably, in the conclusion that factors other than the receptor are responsible for aldosterone specificity in mineralocorticoid target tissue. Several hypotheses have been postulated to account for this phenomenon: Krozowski *et al*[129] proposed that aldosterone specificity could be afforded to the renal MR as a result of sequestration of circulating glucocorticoid by extravascular CBG which effectively lowers the concentration of cortisol which is 'free' to bind with MR by greater than 95%. However, in contradiction with this hypothesis, subsequent investigations[132] revealed that the specificity of renal MR was maintained in 10 day old rats despite extremely low levels of CBG and high levels of circulating glucocorticoid. A clue to the conundrum of MR specificity was finally revealed in a series of studies by Edwards *et al*[63,138] in which the *in vitro* binding of [^{3}H]-

corticosterone by adrenalectomised rat kidney sections was found to be significantly enhanced when the animals were pretreated with the 11β-HSD inhibitor, GE. These observations prompted speculation that 11β-HSD confers aldosterone specificity on the renal MR by converting active glucocorticoid to its inactive 11-keto derivative, whilst protection of the 11-hydroxyl group in aldosterone is afforded by its 11,18 hemiketal or 11,18,20 hemi-acetal configuration and thus retains its biological activity.

1.5 The molecular biology of 11β-hydroxysteroid dehydrogenase

1.5.1 *Gene and protein structures of the 11β-hydroxysteroid dehydrogenases*

The gene for human 11β-HSD1 comprises six exons spanning approximately 9kb and encodes a protein of 292 amino acids with a relative mass of 34kDa [64], whilst the human 11β-HSD2 gene comprises five exons with an approximate size of 6.2kb encoding a 41kDa protein of 405 amino acids[65] (figure 1.3, page 21).

Structural analysis has revealed that both isoforms may be considered to be members of the short chain alcohol dehydrogenase (SCAD) superfamily[53,139]. Comparison of the X-ray crystallographic structure of 11β-HSD with a wide range of SCADs from both bacterial and mammalian sources has identified a similar one-domain α/β folding pattern which is considered to be characteristic of this enzyme superfamily[139]. Similarly, alignment of 11β-HSD with 17β-hydroxysteroid dehydrogenase, 3α,20β-hydroxysteroid dehydrogenase and ribitol dehydrogenase has revealed absolute conservation of a group of structurally important sequence segments which, using the techniques of chemical modification and site directed mutagenesis, have been identified as comprising essential parts of the coenzyme binding site and catalytic centre[139-141].

11β-HSD1 and 11β-HSD2 share only about 25% amino acid sequence homology which is restricted to the structural components of the SCAD core. These include part of the nucleotide cofactor binding site (residues 85-95 in 11β-HSD1), absolutely conserved tyrosine and lysine residues that function as catalysis and a region to the N-terminal side of these residues which forms part of the steroid binding pocket in the SCAD

superfamily[142,143]. The hydrophobic N-terminal domain functions as part of the transmembrane component of both isoforms of 11β-HSD and contains a signal-anchor motif which is responsible for the translocation of the enzyme to its subcellular localisation in the endoplasmic reticulum. In 11β-HSD1 this domain is also responsible for luminal N-linked glycosylation which appears to be essential for correct protein folding and stability since mutation of glycosylation sites using rat 11β-HSD1 cDNA or inhibition of glycosyl transferase activity significantly inhibits 11-DH activity[54,144]. However, inhibition of glycosylation has no effect upon 11-OR activity which suggests that differential glycosylation of 11β-HSD1 may play a role in the tissue specific regulation of this enzyme. In contrast, post-translational modification of 11β-HSD2 has not been reported.

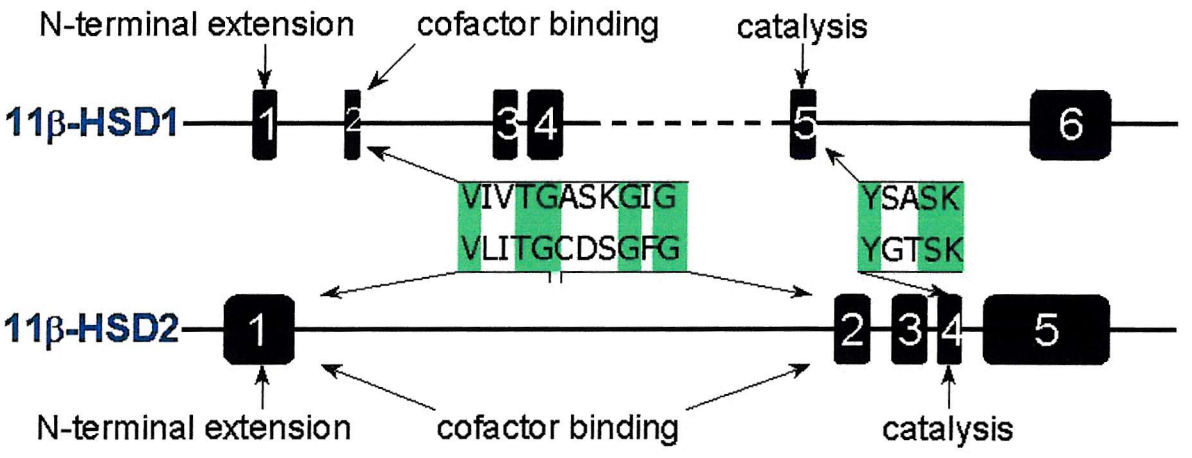


Figure 1.3 Organisational structure of the genes encoding 11β-HSD1 and 11β-HSD2.
Numbered black boxes represent exons. Highly conserved amino acid sequences are shown and identical residues within these regions are shaded in green.
Adapted from: White PC, Mune T, Fraser MR, Kayes KM, Agarwal AK. Molecular analysis of 11β-hydroxysteroid dehydrogenase and its role in the syndrome of apparent mineralocorticoid excess. Steroids 1997;62:83-8

1.5.2 *Subcellular localisation of 11β-hydroxysteroid dehydrogenase*

The subcellular localisation of 11β-HSD was first investigated in the liver[45] and subsequently in several other tissues including kidney[18], gonads[49], placenta[38] and lung[37]. These investigations identified that 11β-HSD activity was restricted to the microsomal and nuclear fractions of these tissues and that the cytosol and mitochondria were devoid of enzyme activity. Moreover, the distribution of enzyme activity between the nuclear and microsomal compartments was tissue specific, with high levels of activity in kidney nuclei and variable levels in the nuclei of cells from the rat brain[47,145]. Subsequently, investigations into the subcellular localisation of 11β-HSD2 using confocal fluorescence microscopy of Chinese Hamster Ovary (CHO) and MDCK cells transfected with a chimeric 11β-HSD2 gene fused to the coding region for the green fluorescent protein, demonstrated expression of the enzyme only in association with the endoplasmic reticulum and nuclear envelope, the outer membrane of which is considered to be part of the endoplasmic reticulum[146]. In addition, 11β-HSD2 appears to be orientated with the catalytic site of the enzyme towards the cytoplasmic surface of the endoplasmic reticulum which is in absolute contrast with 11β-HSD1 which is orientated towards the luminal side of the endoplasmic reticulum[147,148].

1.5.3 *Substrate specificity and cofactor dependence*

Analysis of the substrate specificity of 11β-HSD1, using purified rat liver microsomes, suggests that a wide variety of naturally occurring steroids with a planar A/B ring junction may serve as substrates for this enzyme[45]. In contrast, the catalytic activity of 11β-HSD1 upon steroids with either an angled A/B ring junction (i.e. 5β-reduced steroids) or an aromatic A ring configuration is significantly diminished[45,46]. Whilst modification of the substrate C_{17} side chain appears to have little effect upon the catalytic activity of 11β-HSD, removal of either the 17- or 21-hydroxyl favours oxidation over reduction at C_{11}. The introduction of halogens at C9 in the synthetic 11-hydroxysteroids, dexamethasone and 9α-fluorocortisol, appears to inhibit their oxidation by 11β-HSD, whereas reduction of the 11-keto 9α-halogenated steroid is enhanced[36,149,150]. Indeed it is thought probable that the much greater potency of 9α-fluorocortisol over the naturally

occurring non-halogenated steroids is due to its decreased rate of inactivation by 11β-HSD[151].

Several groups of structurally diverse steroidal inhibitors of 11β-HSD have been identified. Whilst comparison of the relative potency of these inhibitors demonstrates that the inclusion of 2α-CH_3, 5β-OH, 6α/β-OH, 12α-OH, 15α-OH, 16α-OH, 20α-OH, 11-oxo, 18-oxo or 16(17)-ene groups has little effect on 11-DH activity, steroids with an 11-hydroxyl group are invariably potent competitive inhibitors of 11-DH activity[38,152,153]. Fewer steroidal inhibitors of 11-OR activity have been identified but it is thought likely that inhibition of 11-OR is dependent upon the orientation of the C_{20} side chain in these steroids[38].

1.5.4 *Mutations of the 11β-hydroxysteroid dehydrogenase type II gene in AME*

To date, eleven different mutations in the 11β-HSD2 gene from studies of fifteen kindreds have been identified. Of these mutations, all affect either enzymatic activity or pre-mRNA splicing and, with the exception of a single patient who was found to be a compound heterozygote for two different mutations, all affected individuals have been found to carry homozygous mutations[80,81,95,96,154]. These data confirm AME as an autosomal recessive disease and suggest that the incidence of mutations in the 11β-HSD2 gene in the general population is relatively low. Consequently, AME is found most frequently in populations in which consanguineous marriage is common. Interestingly, six of the kindreds so far studied have been of Native American origin and whilst three of these carry the mutation; L250P, L251S, the others are each homozygous for a different mutation[96].

Of those mutations which have been identified, six have been expressed in mammalian cell culture to determine their effects upon enzyme activity. The mutation; L250P, L251S, a 3-bp substitution in exon 4 resulting in leucine-250-proline and leucine-251-serine substitution, is completely inactive whilst R337HγY338, a 3-bp deletion in exon 5 resulting in an arginine-337-histidine substitution and a deletion of tyrosine at codon 338, appears to have trace activity. R337C, a missense C-T mutation in exon 5, has in excess

of 50% of the activity of the wild type enzyme and the additional three missense C-T mutations in exon 3 have varying degrees of trace activity[86]. The remaining five mutations comprise; missense C-T mutations in exons 4 and 5, a 9-bp deletion in exon 4 resulting in tyrosine-232-serine substitution and deletion of glycine-233 and threonine-234, a 1-bp deletion in exon 5 which introduces a premature stop signal (TGA) at codon 395, a further missense mutation (R374X) in exon 5 which codes a premature stop signal resulting in a truncated protein lacking the terminal 32 amino acids and a final 11-bp deletion mutation in exon 5 which also results in a truncated protein lacking the C-terminal tail. A further non-coding mutation in intron 3 has been identified which promotes skipping of exon 4 during processing of pre-mRNA which is presumed to result in a protein which lacks a catalytic site and is therefore inactive.

Despite the small number of cases of AME, there is now a growing body of evidence to suggest that there is a significant correlation between biochemical phenotype, as determined by the precursor:product ratio, (THF+5α-THF)/THE, and genotype[86]. This is particularly true of those mutations which code a protein with partial catalytic activity such as the C-T mutations, R186C and R208C in exon 3, R213C in exon 4 and the R337C mutation in exon 5. Nevertheless, the degree of enzyme activity observed in recombinant studies may not accurately reflect those activities observed *in vivo*. Indeed, a 50% reduction in enzyme activity as a result of the R337C mutation appears to cause significant impairment of renal cortisol metabolism yet heterozygous carriers of mutations of the 11β-HSD2 gene are generally asymptomatic. Whatever the explanation for these phenomena, there is considerable speculation that further investigation, using appropriately selective and sensitive biochemical tools, may identify further mutations which result in attenuated enzyme activity and which cause relatively mild forms of AME[86].

1.6 The tissue distribution and functional role of 11β-hydroxysteroid dehydrogenase

1.6.1 *Co-localisation of 11β-hydroxysteroid dehydrogenase with the glucocorticoid and mineralocorticoid receptor*

The tissue distribution of both isoforms of 11β-HSD has been extensively investigated in a broad spectrum of mammalian species including man using both immunohistochemical and *in situ* hybridisation techniques[63,155-158]. These studies have revealed an intimate relationship between 11β-HSD1 and the GR[56,157,159,160] and between 11β-HSD2 and the MR[148,160-162]. 11β-HSD1 activity is found almost exclusively in glucocorticoid target tissues such as liver and skeletal muscle[56] whilst 11β-HSD2 activity appears to be limited to mineralocorticoid target tissue such as the distal convoluted tubule, renal collecting ducts and colon[65,163,164]. Indeed the close association between 11β-HSD1 and the GR and between 11β-HSD2 and the MR adds strength to the hypothesis that 11β-HSD2 confers aldosterone specificity upon the renal MR and has given rise to a second hypothesis that the combined effects of 11β-HSD2 and 11β-HSD1 may be responsible for regulating glucocorticoid accessibility to the GR and thus indirectly regulates tissue sensitivity to glucocorticoid[165,166]. Indeed, the relationships which exist between GR, MR and isoforms of 11β-HSD have been investigated in a variety of tissues which serve to illustrate the complexity which underpins the regulation of ligand-receptor interactions and the cascade of physiological responses which result from receptor activation.

1.6.2 *11β-hydroxysteroid dehydrogenase in the liver*

The presence of 11β-HSD1 activity in the liver was first described by Caspi *et al*[167] in 1953, and subsequently in greater detail by Hurlock and Talalay in 1959 [45]. Bush *et al*[36] later established a Michaelis constant (Km) for 11-DH activity of 20μM with NADP(H) as an efficient co-factor for the reaction in both directions. Despite an inability to characterise 11-OR activity in rat liver microsomal extracts it is known that the predominant direction of hepatic 11β-HSD1 activity in man is the conversion of cortisone to cortisol[168,169]. However, in the liver, 11-OR activity is expressed by Kupfer

cells and 11-DH activity by parenchymal cells, thus the net direction of hepatic 11β-HSD activity is a product of the sum of the enzyme activity in these two cell types[36]. In the rat, hepatic 11β-HSD1 displays a marked sexually dimorphic pattern of expression[170,171] such that hepatic 11β-HSD1 expression in male rats is some eighteen fold greater than that in females[171]. This has been explained on the basis of the sexually dimorphic pattern of growth hormone secretion in the rat[170]. However, the hormonal regulation of hepatic 11β-HSD1 expression and activity is discussed in greater detail in chapter 3.

Investigations using mice with targeted disruption of the gene encoding 11β-HSD1, have suggested that 11β-HSD1 plays a significant role in the regulation of hepatic gluconeogenesis[172]. In these animals, the expression of hepatic phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6P), enzymes that catalyse the rate limiting steps of gluconeogenesis, is markedly attenuated[172]. Moreover, earlier studies suggest that the genes for both PEPCK and G6P are regulated by glucocorticoid-inducible promoters and that in GR-deficient mice, expression of these genes is also subnormal[173]. These investigations have prompted speculation that regeneration of biologically activity glucocorticoid by hepatic 11β-HSD1 11-OR activity is a key determinant of intrahepatic gluconeogenesis in states of starvation[172]. Moreover, disruption of the gene encoding 11β-HSD1 in these mice was also found to confer resistance to the development of hyperglycaemia which was observed in wild type litter mates under conditions of stress or obesity[172].

Investigations of the effects of CBX administration in man[174] and in isolated perfused rat liver[175] have suggested that CBX inhibits hepatic 11β-HSD1 11-OR activity and that this is accompanied by an increase in whole body insulin sensitivity and glucose disposal measured by euglycaemic hyperinsulinaemic clamp[174]. However, these studies also suggested that CBX administration has no effect upon peripheral insulin sensitivity measured by forearm glucose uptake. Importantly, these observations have been explained on the basis of the inhibition of hepatic 11β-HSD1 11-OR activity by CBX resulting in a decrease in intrahepatic glucocorticoid concentrations and that this is

associated with enhanced insulin dependent down-regulation of hepatic glucose output[174].

These observations have prompted speculation that the specific inhibition of hepatic 11β-HSD1 activity may represent an important therapeutic strategy for the treatment of syndromes associated with insulin resistance[176,177].

1.6.3 *11β-hydroxysteroid dehydrogenase in the kidney*

The hypothesis that the renal MR is afforded aldosterone specificity as a result of inactivation of glucocorticoid by 11β-HSD came from observations of the clinical features of AME as discussed earlier. Immunohistochemical studies of the distribution of MR along the rat and human nephron have identified clear morphological segregation of MR immunoreactivity to the distal convoluted tubule, connecting piece and initial cortical collecting duct[178]. In contrast, however, investigations using monoclonal antisera to rat liver microsomal 11β-HSD identified significant immunostaining in the proximal convoluted tubule, medullary rays and interstitial cells of the renal papillae[155-157]. These data argued strongly against a role for 11β-HSD in maintaining renal MR specificity for aldosterone since the distance between 11β-HSD and renal MR would be too great, and the capacity for 11-DH activity expressed by the NADP(H) dependent Type I isoform of 11β-HSD too low, to afford adequate protection against physiological concentrations of glucocorticoid[48]. Nevertheless, earlier kinetic investigations of the rat and rabbit kidney had already established evidence for a gradient of 11-DH activity along the renal tubule such that conversion of cortisol to cortisone was found to be 20-30% higher in distal rather than proximal convoluted tubule[59,63,179,180]. It was not until the subsequent identification and co-localisation of 11β-HSD2, with a high capacity for 11-DH activity, with the MR in the rabbit distal collecting duct that the hypothesis that 11β-HSD confers mineralocorticoid specificity upon the renal MR was ultimately accepted[60,163]. The identification of 11β-HSD1 in the proximal segments of the renal tubule and its capacity for 11-OR activity suggests a role for this isoform in the maintenance of glucocorticoid responsiveness by the GR which exhibits a more generalised expression throughout the entire length of the nephron[181].

1.6.4 *11β-hydroxysteroid dehydrogenase in the myocardium and vascular smooth muscle*

For over 50 years it has been recognised that the maintenance of vascular tone, sensitivity of the vasculature to pressor agents and the intra- and extra-cellular levels of sodium and potassium in peripheral blood vessels are influenced by glucocorticoids[182]. In rat vascular smooth muscle these effects are known to be mediated through both MR and GR [183,184] and it is considered likely that the effects of both class of steroid proceed by independent processes in that catecholamine responsiveness is dependent upon activation of vascular GR whilst sodium/potassium transport is effected primarily through MR [185-187]. This functional specificity led Funder *et al*[188] to speculate that vascular 11β-HSD confers mineralocorticoid specificity upon the MR in a similar manner to that proposed for the kidney. The identification of both 11β-HSD1 and 11β-HSD2 in human vascular smooth muscle cells and the observation that inhibition of vascular 11β-HSD2 in these cells results in an increase in angiotensin II binding, a function attributable to activation of both MR and GR by cortisol[189], has been taken as evidence in favour of a role for 11β-HSD2 in conferring aldosterone specificity on vascular MR. However, it has also been demonstrated in both rat and human vascular smooth muscle cells that 11-OR activity predominates[182,189] and functions to regenerate active glucocorticoids from their circulating inactive 11-keto forms. Furthermore, it has subsequently been shown that in the rat, the vascular wall is itself a site of corticosteroid synthesis[190] the products of which exert paracrine or autocrine effects upon surrounding tissue. Thus, whilst the significance of changes in the ratio of vascular 11-OR and 11-DH in the pathogenesis of human hypertension remains unclear[190] there can be little doubt that the regulation of vascular tone through the action of GR, MR and both circulating and locally synthesised corticosteroid is dependent upon the combined effects of 11β-HSD1 and 11β-HSD2.

Speculation that the heart may be considered a mineralocorticoid target tissue is supported by the demonstration of MR in rat and human cardio-myocytes and fibroblasts of both the atria and ventricles[191,192]. Nevertheless, a classical role for cardiac MR has not been widely accepted and a second hypothesis which suggests a role for

corticosteroids in the maintenance of normal cardiac structure has been postulated based upon the observation that incubation of human cardiac fibroblasts with mineralocorticoid leads to increased synthesis of fibroblast collagen[193-195]. The subsequent identification of 11β-HSD1 in rat cardiac fibroblasts[196] and both 11β-HSD1 and 11β-HSD2 in human cardiac fibroblasts[197] suggest a more complex role for 11β-HSD isoforms in the myocardium and vasculature than hitherto has been generally accepted.

1.6.5 *11β-hydroxysteroid dehydrogenase in the gonads*

In male patients, Cushing's syndrome is frequently associated with hypogonadism, a phenomenon which in the male rat may be reproduced following exposure to pharmacological concentrations of glucocorticoid[198,199]. Levels of 11β-HSD activity almost as high as those found in the liver have been identified in Leydig cells which do not posses MR but do possess GR and, as a consequence, are glucocorticoid responsive. Thus, it has been postulated that 11β-HSD may function to protect the testis from excess glucocorticoid in order to preserve testosterone synthesis. This hypothesis presupposes that in the Leydig cell 11-DH activity predominates, however, there is a body of evidence to suggest that the rat testis expresses abundant 11β-HSD1 an isoform which, in the liver exhibits predominantly 11-OR activity, whilst 11β-HSD2 is undetectable[200]. Earlier studies have demonstrated that in the rat leydig cell, 11β-HSD1 expresses both 11-DH and 11-OR activity and that the relative proportion of these activities changes with pubertal maturation such that prior to puberty 11-OR activity predominates whilst in the mature Leydig cell 11-DH activity predominates[201,202]. These data have led to speculation that the ontogenic switch from 11-OR to 11-DH activity in the mature Leydig cell is essential in order to prevent glucocorticoid down regulation of testosterone biosynthesis subsequent to glucocorticoid mediated maturation of testicular function prior to puberty[201]. The mechanism underlying this switch in catalytic activity is unclear but several authors have presented evidence for the hormonal regulation of the relative 11-DH and 11-OR activity of rat testicular 11β-HSD1 *in vitro* by gluocorticoid, oestradiol, testosterone, progesterone, leutienising hormone (LH) and epidermal growth factor (EGF) [203-205]. In contrast with the rat, human testis expresses both 11β-HSD1 and

11β-HSD2 mRNA [62]. Thus the relative abundance of these isoforms, which may also be hormonally regulated, may play a significant role in the maturation and maintenance of Leydig cell function in the human.

By analogy with the role of 11β-HSD in the Leydig cell, ovarian 11β-HSD may play an equally important role. The ovary may be considered to be a glucocorticoid target tissue and several studies have demonstrated a functional role for glucocorticoid in the modulation of gonadotrophin action on granulosa cell function[206-208]. Both 11β-HSD1 and 11β-HSD2 mRNA and activity have been detected in human granulosa cells though in common with the expression of 11-OR and 11-DH activity in Leydig cells the expression of these isoforms appears to follow a developmental pattern such that 11β-HSD1 activity is abundant in the developing oocyte and leuteal body whilst 11β-HSD2 appears to be expressed at higher levels in granulosa cells during follicular recruitment and prior to ovulation[209,210]. There is evidence to suggest that the developmental regulation of 11β-HSD isoform expression in granulosa cells is gonadotrophin dependent[211]. This may represent a mechanism whereby preovulatory follicles are protected from the anti-steroidogenic effects of excess glucocorticoid whilst ovulatory and luteal follicles are afforded access to glucocorticoid which is required for the completion of oogenesis or the process of follicular rupture[209]. These observations suggest an important role for the developmental regulation of follicular glucocorticoid levels by isoforms of 11β-HSD in the maintenance of human fertility. However, whilst at least one study has demonstrated an inverse relationship between follicular 11β-HSD1 11-OR activity and successful pregnancy following *in vitro* fertilisation[212] subsequent studies have demonstrated no such correlation[213]. Whilst there is no clear explanation for the former observations at present, it has been suggested that an estimation of the ratio of 11β-HSD1 and 11β-HSD2 in follicles harvested for *in vitro* fertilisation may at least be used as a marker of follicular maturity which in turn may assist in the process of successful follicular selection[212].

1.6.6 *11β-hydroxysteroid dehydrogenase in the foeto-placental unit*

Extra-hepatic 11β-HSD activity was first described in placental tissue by Osinski[214] in1960. It is well established that passage of steroid hormones of maternal origin across the placenta is inhibited by extensive catabolism to biologically inactive compounds before entry into the foetal circulation[215]. In 1957 Migeon *et al*[216] proposed a hypothesis that suggested that one of the major roles of the placenta is the catabolism of cortisol in order to protect the foetus from the relatively high levels of steroid in the maternal circulation. This hypothesis has been given credence by work performed in late gestation in humans[216,217] which demonstrated a 28% conversion of cortisol to cortisone across the placenta near term compared with a 4% conversion in the opposite direction. At midgestation, however, in both humans and primates catalysis appears to be in the reverse direction, with significant transplacental conversion of cortisone to cortisol (35%) which is substantially greater than the rate of oxidation of cortisol to cortisone[218-221]. Based on studies in both the human and baboon it would appear that 11β-HSD2 is found predominantly in trophoblastic cells whereas 11β-HSD1 resides primarily in the decidua[222]. Importantly, the pattern of transplacental cortisol metabolism is altered during gestation from 11-oxoreduction early in pregnancy to 11-dehydrogenation near term. It is proposed that this mechanism exists because the foetal adrenal does not develop the enzymatic capacity to produce significant levels of cortisol *de novo* until late in gestation[223,224] and thus, since tightly regulated levels of cortisol are required for the normal development of the foetus, the transplacental metabolism of cortisol plays an important role, not only in protecting the foetus from high levels of maternal cortisol, but also in the regulation of foetal organ growth and development[223,224].

Significant changes in the net direction of 11β-HSD activity have also been observed in foetal tissues during human pre and perinatal development[153,225]. Throughout the second and early part of the third trimester the concentration of circulating 11-oxo-steroids greatly exceeds that of 11-hydroxy-steroids in the foetus of most species. This is also reflected in the finding that most foetal tissues express 11-DH activity in early gestation but as gestation progresses 11-OR activity develops such that the net direction of 11β-

HSD activity in most organs post-partum is oxo-reductive. This is in marked contrast with the transplacental metabolism of cortisol which is characterised by a shift from 11-OR activity to 11-DH activity as gestation progresses and suggests that foetal tissue metabolism of cortisol plays a key role in foetal maturation *per se*. Whilst it is not yet known whether gestational shifts in the ratio of 11-DH/11-OR activity in different tissues are species specific, nor when during development 11β-HSD activity first appears, it is widely accepted that these changes are intimately connected with the developmental maturation of individual organs and prepare the foetus for birth[226].

1.6.7 *11β-hydroxysteroid dehydrogenase in other tissues*

A complete discussion of the tissue specific expression and activity of isoforms of 11β-HSD is beyond the scope of this thesis and as a consequence the following paragraphs are meant to represent no more than an overview of 11β-HSD expression in tissues which are not traditionally regarded as classical mineralocorticoid or glucocorticoid target tissues.

11β-HSD1 activity and mRNA has been described in the rat cerebellum, pituitary and hypocampus[227] and, in cultured hipocampal cells, 11β-HSD1 expresses predominantly 11-OR activity[228]. Nevertheless, in the rat[229] and sheep[230] 11β-HSD1 is also believed to express 11-DH activity particularly in the hypothalamus and pituitary where it is suggested to modulate the negative glucocorticoid feedback mechanism[229,230]. Moreover, 11β-HSD2 mRNA is also expressed in the rat brain but is confined to the hypothalamus, nucleus tractus solitarus and subcommissural organ[160], however, the role of 11β-HSD2 in these regions of the brain is unclear.

Investigations of the foetal rat brain have demonstrated marked ontogenic changes in the pattern of 11β-HSD isoform expression during development which mirror developmental changes in GR and MR expression[227]. Indeed, these studies have demonstrated that 11β-HSD2 activity is expressed in all regions of the brain during midgestation, but is reduced during the third trimester, whilst 11β-HSD1 activity is not detected in the brain until the

third trimester. Furthermore, GR is expressed throughout the brain from midgestation, but MR is not detected until the last few days of gestation. Thus, it has been suggested that high levels of 11β-HSD2 at midgestation may serve to protect GR in the developing brain from activation by glucocorticoids whilst in late gestation, 11β-HSD2 expression declines and thus may allow activation of GR and MR by glucocorticoid and facilitate neuronal and glial maturation[224,231].

In the human foetal lung, the differentiation of alveolar cells[232,233], production of phosphatidyl choline, glycogen deposition and induction of the synthesis and secretion of surfactant is dependent upon the action of glucocorticoids[234,235]. It is likely that as a consequence of an increase in 11β-HSD1 11-OR activity during late gestation, reduction of cortisone to cortisol in the foetal lung brings about the final events of tissue maturation in this organ[173]. In the adult human lung, 11β-HSD2 activity and protein has been localised to the ductular cells of the tracheal and bronchial glands, type II alveolar cells and ciliated bronchial epithelial cells which are also sites known to express MR[236,237]. 11β-HSD1 activity and mRNA have also been detected in adult human lung[238] although the precise localisation of this isoform has not yet been described.

Investigations of 11β-HSD isoform expression in the adrenal gland have revealed that the midgestational human foetal adrenal gland expresses 11β-HSD2 but not 11β-HSD1[48,239,240] whilst in the adult, the adrenal gland expresses 11β-HSD1 but not 11β-HSD2[210,241-243]. Western blot analysis suggests that 11β-HSD1 is not expressed in the adrenal medulla, but is found in all three zones of the adrenal cortex, with highest levels found in the zona reticularis[210]. Whilst the role of isoforms of 11β-HSD in the human adrenal gland remains unclear it has been suggested that, in the zona reticularis, 11β-HSD1 at the cortico-medullary junction may maintain the high levels of glucocorticoid required for medullary catecholamine biosynthesis[210]. The role of 11β-HSD2 in the foetal adrenal gland is unknown.

11β-HSD2 has recently been localised to ductal and lobular epithelial cells in the human breast where it co-localises with the MR[244]. Moreover, higher levels of 11β-HSD2 expression are observed in invasive carcinoma of the breast and inhibition of 11β-HSD2 potentiates the antiproliferative effects of glucocorticoids in some breast carcinoma cell lines[245]. The expression of both 11β-HSD1 and 11β-HSD2 mRNA has been identified in normal primary human osteoblasts in culture[246]. In contrast, these same studies were unable to detect 11β-HSD1 expression in osteosracoma-derived osteoblasts but the level of GR expression in these cells was correlated with the level of 11β-HSD2 expression. Moreover, in normal osteoblasts, 11-DH activity appears to predominate[246]. These observations, whilst speculative, have led to suggestions that the expression of isoforms of 11β-HSD in human bone plays an important role in normal bone homeostasis, and may be implicated in the pathogenesis of steroid-induced osteoporosis[246].

High levels of 11β-HSD1 but not 11β-HSD2 activity and mRNA have also been identified in human adipose tissue[247,248]. Using primary cultures of adipose stromal cells, these studies suggest that 11β-HSD1 activity is higher in cells from omental rather than subcutaneous sites and that in adipose tissue 11-OR activity predominates[249]. Moreover, these studies also demonstrated that the differentiation of adipose stromal cells into adipocytes is promoted by both glucocortoids and insulin and these observations have given rise to speculation that the generation of cortisol as a consequence of 11β-HSD1 11-OR activity in omental adipose tissue may play an important role in the development of central obesity[247]. Importantly, central obesity is also strongly associated with reduced glucose tolerance, hyperinsulinaemia and other features of the metabolic syndrome[250-252]. Moreover, mice with targeted disruption of the gene encoding 11β-HSD1 do not develop the hyperglycaemia associated with obesity[172]. These observations have led to speculation that increased levels of active glucocorticoid in insulin target tissue, as a consequence of increased conversion of cortisone to cortisol by 11β-HSD1 11-OR activity, may represent a common factor

underlying the aetiology of the insulin resistance syndromes[176] and is the subject of further investigation in chapter four of this thesis.

1.7 11β-hydroxysteroid dehydrogenase in human disease

1.7.1 *The role of 11β-hydroxysteroid dehydrogenase in Cushing's syndrome*

Hypertension is a well-recognised, frequently observed, consequence of chronic glucocorticoid excess characterised by both Cushing's syndrome and exogenous administration of glucocorticoid. Approximately of 75% patients with Cushing's syndrome have bilateral adrenal hyperplasia associated with elevated plasma ACTH which is principally of pituitary origin (Cushing's disease)[253,254] or of extra-pituitary or 'ectopic' origin[255]. A smaller proportion of patients are characterised by ACTH independent Cushing's syndrome which is associated with either adrenal hyperplasia, adenoma or carcinoma[253]. Over 80% of patients with ACTH dependent Cushing's syndrome have hypertension but this increases to over 95% in patients with the ectopic-ACTH syndrome[256]. Importantly, mineralocorticoid excess characterised by hypokalaemic alkalosis is a feature of more than 95% of cases of the ectopic ACTH-syndrome which is in marked contrast with other forms of Cushing's syndrome in which fewer than 10% of patients suffer mineralocorticoid excess[257,258]. These observations have prompted speculation that the mineralocorticoid excess associated with the ectopic ACTH syndrome may be caused by ACTH dependent steroids other than cortisol such as deoxycorticosterone[259]. However, most patients with ACTH-dependent Cushing's syndrome do not have elevated levels of plasma deoxycorticosterone (Saruta T, 1986) and it is likely that the mineralocorticoid excess associated with the ectopic ACTH syndrome is due to the effects of cortisol alone[260].

The hypertension associated with exogenous glucocorticoid is dependent solely upon activation of the GR, since it is abolished by the GR antagonist, RU286, but is unaffected by MR antagonists[261,262]. However, it is likely that the hypertension associated with the ectopic ACTH syndrome is mediated through activation of both GR and MR[263] and that activation of the renal MR is a consequence of deficient inactivation of cortisol by renal 11β-HSD2[255].

Evidence in support of this hypothesis comes from the observation that an increase in the plasma cortisol/cortisone and (THF+5α-THF)/THE ratios are frequently observed in Cushing's syndrome and that this may be indicative of a decrease in 11β-HSD2 activity[264,265]. However, evidence has also been presented in which the total excretion of THE in patients with Cushing's syndrome is increased, indicative of significant 11β-HSD2 activity, despite increases in the (THF+5α-THF)/THE ratio and the urinary free cortisol:cortisone ratio[266]. These observations suggest that, despite significant renal 11β-HSD2 activity, the mineralocorticoid hypertension associated with the ectopic ACTH syndrome may be the consequence of substrate saturation of renal 11β-HSD2 as a consequence of high levels of circulating cortisol[255,265,266].

1.7.2 *The role of 11β-hydroxysteroid dehydrogenase in essential hypertension*

There is now a broad spectrum of evidence to suggest that dysregulation of cortisol metabolism may be implicated in the aetiology of essential hypertension. Several studies have demonstrated that in a significant proportion of patients with essential hypertension the plasma half life of 11α-[^{3}H]-cortisol is prolonged, though few have been able to clearly demonstrate any change in the urinary (THF+5α-THF)/THE ratio[73,123,267]. Nevertheless, there is growing speculation that 11β-HSD2 expressed in vascular smooth muscle and myocardium may play a significant role in the regulation of vascular tone through its effects upon the local metabolism of cortisol[268-271]. Moreover, several studies have demonstrated a clear correlation between vascular sensitivity to glucocorticoid and essential hypertension[271-274]. Whilst a degree of controversy still surrounds these data it is possible to speculate that increased vascular sensitivity to glucocorticoid may result from an increase in the half life of cortisol, due either to deficient inactivation of cortisol by 11β-HSD2, increased conversion of cortisone to cortisol by 11β-HSD1 or a combination of both effects[272].

1.7.3 *The foetal origins of the metabolic syndrome*

Several studies investigating possible mechanisms underlying the foetal origins of adult disease have implicated the dysregulation of transplacental cortisol metabolism in the

later development of essential hypertension in the offspring[275-278]. The observation that the administration of corticosteroids during pregnancy may lead to decreased foetal weight is well documented[279-281]. It has also been reported that there is a strong positive correlation between placental 11β-HSD activity and foetal weight at term and an inverse correlation between placental 11β-HSD activity and placental weight[282-284]. Edwards *et al*[284] hypothesised that deficiency of placental 11β-HSD at mid term could result in the transplacental passage of maternal cortisol to the foetal compartment which results in foetal growth retardation. Studies using rats treated with low-dose dexamethasone support this hypothesis since, as 9α-fluoridated corticosteroids do not undergo significant 11β-HSD catalysed dehydrogenation dexamethasone crosses the placenta largely unaltered and, in common with all glucocorticoids, may significantly retard intra-uterine growth[285]. It was also noted that animals with a low birthweight as a result of dexamethasone treatment also exhibited an increase in blood pressure in adulthood[285].

These data have prompted several authors to suggest that intrauterine exposure to high levels of cortisol represents a link between the maternal environment and foetal programming of adult disease[286]. Whilst several extensive, though retrospective, epidemiological studies have demonstrated a similar relationship between birth weight and adult hypertension in man no clear relationship between birth weight, placental weight and11β-HSD2 activity has been demonstrated and this field of research continues to be an area of active debate[282,284,287].

1.8 Overview of the thesis

It is now generally accepted that the mineralocorticoid specificity of MR and the regulation of glucocorticoid accessibility to GR occurs, predominantly as a consequence of the interconversion of cortisol and cortisone by isoforms of 11β-HSD. It is also evident, as illustrated in the preceding discussion, that the preferred direction of catalysis of 11β-HSD1 and the relative abundance of 11β-HSD1 and 11β-HSD2 may be regulated in both a maturational and tissue dependent manner. However, despite a growing body of evidence to suggest that the regulation of 11β-HSD activity may be elicited by a number

of well characterised hormones, including sex steroids, the gonadotrophins and glucocorticoid, a clear understanding of the mechanisms underlying these regulatory processes remains elusive. By analogy with the physiological, biochemical and clinical characteristics of acquired AME it has been postulated that relative dysregulation of 11β-HSD1, 11β-HSD2 or, in some instances, both isoforms of 11β-HSD may underlie the pathogenesis of a broad spectrum of diseases[73,123,264,266,267,275,276,287].

Most attempts to investigate the role of changes in 11β-HSD activity in the pathogenesis of disease have revealed no clear associations. Whilst many of these studies have favoured analysis of the cortisol turnover quotient (THF+5α-THF)/THE [288], plasma cortisol half-life[73,267] or plasma cortisol and cortisone ratios[17,112], changes in cortisol metabolism as a consequence of modest changes in 11β-HSD activity, while important , are likely to have been too small for accurate assessment by current technology. Moreover, plasma cortisol, cortisone and the ratio of their metabolites reflect the combined effect of both 11β-HSD1 and 11β-HSD2 such that changes in either 11-OR or 11-DH activity may be masked as a consequence of the opposing metabolic activity of the other isoform. Estimation of unconjugated steroid excretion which may exceed 43% of total excreted steroid in AME compared with 1% in normal individuals[69,93] may prove to be a more promising avenue of investigation since it can be argued that the unconjugated corticosteroid profile of urine reflects more accurately renal 11β-HSD2 activity. However, the analytical estimation of urinary free steroid profiles is not generally undertaken, principally because current techniques are insufficiently sensitive.

Nevertheless, there has been a growing awareness that the analysis of urinary free cortisol and cortisone may hold potential as an index of renal 11β-HSD2 activity[88,289]. Indeed, these studies have demonstrated a clear relationship between diminished renal 11β-HSD2 activity and an increase in the urinary free cortisol:cortisone ratio in AME types I and II, acquired AME (as a consequence of liquorice ingestion) and in patients with the ectopic ACTH syndrome and Cushing's disease. This index may therefore be an important diagnostic tool for the investigation of 11β-HSD2 *in vivo*. Importantly, it is

likely that improvements in analytical techniques will facilitate the sensitive, non-invasive monitoring of smaller changes in renal 11β-HSD2 activity which may, through their cumulative physiological consequences, progress to overt disease. Thus the search for direct evidence of a relationship between dysregulation of 11β-HSD and the pathogenesis of disease depends not only upon the development of a clear understanding of the mechanisms which underlie the hormonal regulation of 11β-HSD but also upon the establishment of a spectrum of analytical techniques which are sufficiently sensitive to dissect the relatively modest variations in glucocorticoid metabolism attributable to equally modest dysregulation of specific isoforms 11β-HSD.

Thus, the second chapter of this thesis describes the development and evaluation of three novel analytical techniques for the specific and sensitive estimation of unconjugated corticosteroids in biological fluids. The early pages of the chapter describe the analysis of unconjugated corticosteroids in plasma, urine and tissue culture media using solid phase extraction and reversed phase high pressure liquid chromatography (HPLC). Two novel techniques are described which employ either fluorescence detection subsequent to derivatisation with 7-(carboxymethoxy)-4-methylcoumarin in the presence of 1-ethyl-3-(-3-dimethylaminopropyl)-carbodiimide hydrochloride or UV absorbance detection. The later pages of the chapter described the development of a radioimmunoassay for the analysis of urinary free cortisone which, used in tandem with a second radioimmunoassay for the analysis of urinary free cortisol, may be used to calculate the urinary free cortisol:cortisone ratio. The remainder of the chapter describes a comparison of urinary free cortisol:cortisone ratios and total cortisol:cortisone metabolite ratios as indices of renal 11β-HSD2 activity in adults with hypopituitarism receiving growth hormone and hydrocortisone replacement therapy.

Chapter three describes a series of studies in which the analytical techniques developed in chapter two were used to characterise the differential regulation of 11β-HSD1 in human skeletal myoblasts and 11β-HSD2 in JEG-3 cells *in vitro*. Analysis of mRNA and protein is also described which demonstrates that the hormonal regulation of isoforms of 11β-HSD occurs at a pretranslational level.

Chapter four extends the regulation studies discussed in the previous chapter to an investigation of isoforms of 11β-HSD in the insulin resistance syndrome (IRS). It is well recognised that hypercortisolaemia is frequently associated with insulin resistance, hypertension, obesity and other features of the (IRS). However, there is a growing awareness that increased tissue sensitivity to glucocorticoid, regulated not only by the level of GR, but also by pre-receptor interconversion of biologically active cortisol with its hormonally inactive 11-oxo-derivative, cortisone, by isoforms of 11β-HSD, may also represent a mechanism underlying the pathogenesis of the IRS. Indeed, in support of this hypothesis it has been demonstrated that inhibition of 11β-HSD1 is accompanied by a significant increase in whole body insulin sensitivity[174]. Skeletal muscle comprises a significant proportion of total body mass and, is the principal site for the manifestation of insulin resistance and glucose intolerance. Therefore, chapter four describes the associations between GR expression and its autoregulation by glucocorticoid and the influence of 11β-HSD1 upon tissue sensitivity to glucocorticoid in primary cultures of skeletal myoblasts from a cohort of adult males with contrasting levels of insulin resistance, adiposity and blood pressure, and describes the role of these observations on current understanding of the aetiology of the IRS. This final chapter also reviews proposals for future research.

Summary of Aims

1. To develop the analytical tools necessary for the sensitive and specific quantification of unconjugated corticosteroids in biological fluids
2. To characterise the hormonal regulation of 11β-HSD1 in human skeletal myoblasts and 11β-HSD2 in JEG-3 cells, a human choriocarcinoma cell line
3. To determine the role played by relative dysregulation of 11β-HSD1 activity in human skeletal myoblasts in the pathogenesis of the insulin resistance syndrome

CHAPTER 2 – Method development

2.1 INTRODUCTION

2.1.1 *Quantitative analysis of corticosteroids: An historical perspective*

The close structural similarity of the corticosteroids and their relatively low concentrations in body fluids pose profound analytical challenges which, despite significant improvements in analytical technique, have yet to be totally overcome. The earliest methods for the analysis of steroids were based upon the use of fluorimetric or colourimetric end points, however, their principal shortcomings, namely lack of specificity, the requirement for large sample volumes and in the case of fluorimetric analysis the requirement for potentially dangerous reagents, limited their practical use in the clinical field[290,291]. By the end of the 1970's the range of analytical procedures for the measurement of specific and groups of corticosteroids had increased to include: paper chromatography[292], thin layer chromatography (TLC)[293], gas-liquid chromatography (GLC)[294], gas chromatography (GC)[295], high performance liquid chromatography (HPLC)[296-299], ultra-violet (UV) spectroscopy[300], nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS)[301]. Nevertheless, it was the development of immunoassay technology in the early 1970's that provided the tools for the relatively simple quantification of single steroids in biological fluids.

The lipophylic nature of the corticosteroids and their association with specific binding proteins in the circulation has placed considerable restrictions upon the evolution of quantitative analytical procedures for routine use in the clinical laboratory. The rapidity and potential for automation that is inherent in direct steroid analysis, requiring neither extraction nor prior chromatographic treatment, has resulted in a marked rise in the popularity of this genre of assay in the clinical field. However, the reduction in specificity and sensitivity that these techniques impose result in considerable deterioration in precision. Thus, despite their value as tools for the diagnosis of well characterised, overt disease the relatively high imprecision associated with these techniques renders them unsuitable for defining robust but smaller changes in steroid biochemistry. Importantly, an ability to detect smaller changes in steroid biochemistry

which, if persistent, may contribute to the pathogenesis of disease, would be pertinent to the development of specific therapeutic regimes thus maximising efficacy whilst minimising side effects and cost.

2.1.2 *Extraction of corticosteroids from biological fluids*

Analytical excellence, which represents the highest degree of specificity and sensitivity, demands the inclusion of three principal steps: extraction of steroid from its biological matrix; further purification of the analyte from interfering substances and quantification.

2.1.2.1 *Liquid-liquid extraction*

The metabolic products of most steroids undergo extensive hydroxylation or conjugation forming increasingly polar species which results in an array of structurally similar analytes which often share identical polar extraction characteristics. Whilst it is not recommended to rely solely upon solvent extraction for the discrimination of steroids with similar polarity, the literature is replete with examples of the efficient use of selective solvent extraction[302-304]. Furthermore, the principles of liquid-liquid extraction are sufficiently versatile to include steps which disrupt protein-binding and extract steroids from their lipoprotein matrix. The extraction of steroid from lipoprotein, however, often requires the addition of ammonium sulphate or pentylamine[305,306]. Similarly the effects of protein binding in matrices other than plasma have been investigated, several studies have demonstrated that even in saliva significant interference from protein binding may be observed in assays which do not incorporate a solvent extraction step[307].

Consideration should also be given to the analysis of steroids in urine. Thus selective extraction of unconjugated steroids may be achieved by judicious use of organic solvents such as diethyl-ether or ethyl acetate. However, the conjugated steroids usually require hydrolysis prior to analysis, and several techniques have been developed in order to overcome the difficulties associated with quantitative recovery of steroids prior to and following hydrolysis. The direct analysis of steroid conjugates following extraction from urine has been achieved by several authors and offers important alternatives to their

analysis following hydrolysis[308-310]. Nevertheless, by analogy with the analysis of steroids in plasma most authors agree that extraction of steroids from urine is obligatory for accurate, quantitative analysis[311-314].

2.1.2.2 *Solid phase extraction (SPE)*

The use of a solid phase for the quantitative extraction of steroids from biological material has gained in popularity over the past thirty years. The wide variety of solid phase media including neutral Amberlite XAD-2 [315,316] and ion exchange resins[317] have been used extensively but the development of octadecylsilane (ODS) coated microparticulate silica in syringes or cartridges has almost entirely superseded these materials[318]. The principle of the latter technology is based upon a mixture of adsorption and reverse phase partition between the liquid phase and the carbon chain which is bound to silanol groups on the surface of the microparticulate silica. The ability to vary the length of the carbon chain and the degree to which it is substituted with hydroxyl, phenyl or amino groups significantly improves the degree of selectivity which can be performed during extraction. In addition, the presence of untreated silanol groups on the silica results in a degree of adsorption which has been utilised to great effect in the separation of vitamin D metabolites[305]. The application of solid phase extraction to the quantitative analysis of corticosteroid profiles in urine has become almost universal. Techniques were initially developed for the extraction of single steroid species[313,318] but the power of this methodology for the quantitative extraction of large groups of steroids has greatly enhanced its usefulness.

2.1.3 *Purification and analysis of corticosteroid extracts*

Examples of the use of paper chromatography for the separation of steroids prior to quantification may still be seen[319,320] however, thin layer chromatography is more widely used[321,322] and careful selection of support medium and running solvent can provide highly satisfactory separations of steroid mixtures.

The development of column chromatography based upon the earlier principles of differential partition between a stationary solid phase and a liquid mobile phase resulted

in significant improvements in steroid analysis. Unfortunately, apart from a few notable exceptions there has been relatively little development of these techniques over the past three decades[56,323]. Size exclusion or 'gel filtration' column chromatography has perhaps maintained a higher degree of popularity and many examples of its use may still be found in the literature. Most of these make use of hydroxyalkoxy-Sephadex such as LH-20 or Lipidex 5000 or the combined use of ODS packed SPE cartridges and diethylaminoethyl substituted Sephadex ion exchange column chromatography which has been successfully used for the fractionation of urinary steroids into their free, glucuronide and sulphated fractions prior to analysis by GLC [324]. Applications which make use of substituted Sephadex have also been described[306].

2.1.4 *Gas chromatography (GC)*

Gas chromatography relies upon the partition of analytes between a gas mobile phase and a thermostable liquid phase which is supported upon a solid matrix. Whilst adsorption to the support medium and inconsistency in packing frequently resulted in poor chromatographic resolution, peak shape and sensitivity in earlier techniques, the advent of 0.2mm internal diameter capillary columns fabricated from fused silica has largely overcome these difficulties. High temperatures, usually in excess of 200°C, are required in order to vaporise steroids and their conjugates prior to chromatography and this is frequently accompanied by thermal degradation. Furthermore, the presence of exposed hydroxyl groups may also result in poor resolution due to adsorption during chromatography. Prechromatographic derivatisation with dimethyldichlorosilane, trimethylchlorosilane, trimethylsilylimidazole (TSIM), bistrimethylsilylacetamide (BSA) or bistrimethyletrifluoroacetamide (BSTFA) amongst others, effectively removes exposed hydroxyl groups and increases the thermal stability and volatility of hydroxysteroids and is now universally performed.

Four methods of GC detection are commonly used: Flame ionization detection (FID) is generally too insensitive for steroid analysis; the nitrogen-phosphorus detector (NPD) is more sensitive but requires the presence of nitrogen atoms, commonly introduced to steroids by formation of derivatives such as methyloximes[325]. Electron capture

detection, despite its high sensitivity is not widely used due to difficulties with detector contamination and the requirement for the formation of halogenated silyl ether derivatives[326]. Most of these detection systems have been superseded by the use of GC coupled with mass spectrometry (MS) which is frequently as sensitive as electron-capture detection but has the added advantage of improved selectivity.

The application of GC-MS to the analysis of urinary steroid profiles has changed little since it was first introduced in the 1960's [327]. Most modern procedures, with minor modifications, rely upon the formation of methyloxime trimethylsilyl ether derivatives following release of the steroids from their glucuronic acid and sulphate conjugates[93,288]. The most significant methodological development in this field has been the shift from using packed glass columns to fused silica capillary columns which has resulted in a simpler interface between the gas chromatograph and mass spectrometer and improved chromatographic resolution.

Where steroids have been shown to behave in an unpredictable manner during derivatisation, for example the 18-oxygenated steroids such as 18-hydroxycortisol; stable isotope dilution using deuterated internal standards has greatly improved precision[328]. The lack of sensitivity of these techniques has confined their application in the routine field to the analysis of urine. However, within the past decade there has been some progress in the analysis of plasma. Unfortunately, with the exception of a few procedures for the analysis of cortisol, cortisone and their metabolites[329-331], the difficulty of producing steroid extracts from a lipid rich plasma matrix has limited their application to the C_{18} and C_{19} steroids[332].

2.1.5 *High performance liquid chromatography (HPLC)*

The chromatographic analysis of steroids by HPLC has become an increasingly important area of research as reliable and sensitive equipment at relatively low cost has become available. The principles of HPLC have evolved from a background of paper, thin layer chromatography and column chromatography and much of the developmental research that has been invested in these earlier techniques can be applied with equal

success to HPLC. Analysis of steroids by HPLC has key advantages over other methods: i) high temperatures are not required and material may be recovered from the column for further analysis; ii) chromatographic resolution is superior to that of paper chromatography and TLC, and, iii) the choice of method of detection is considerably broader than those available for GC.

The selection of column packing material, length and internal diameter of the column, choice of detection system and the steroids to be analysed all have significant influences upon the outcome of their chromatography. The literature is replete with examples of the HPLC of steroids making use of adsorption, partition, ion-exchange, reverse-phase and reversed-phase ion-pair chromatography. However, reverse-phase columns packed with microparticulate silica coated with C_{18}, C_8, C_2 and phenyl materials together with polar solvent binary and tertiary mobile phases are now widely used for many applications. Careful selection of the type of stationary phase, its particle size, porosity and level of residual uncapped silanol groups, as for solid phase extraction, can greatly influence chromatographic selectivity[294]. More recently, applications which make use of immobilised cyclodextrins (macrocyclic polymers of glucose which are capable of forming inclusion complexes with steroids) have been introduced with some success into steroid chromatography[333,334]. Similarly, graphitised carbon, which has been used as an inert stationary phase for the separation of oestrogen conjugates[335] may offer advantages over silica due to its microcrystaline structure which does not contain unreacted silanol groups. Indeed, incomplete capping of silanol groups on some reverse-phase columns may have deleterious effects aside from deterioration in peak shape and chromatographic resolution. Intramolecular hydrogen bonding between uncapped silanol groups and the 18-hydroxylated steroids can lead to ring closure between C_{18} and $C_{20/21}$ or in the presence of methanol, a frequently used component of mobile phases, the formation of methyl ethyl ketals. Dimerisation and isomerisation of susceptible steroids may also be observed.

Selection of mobile phase is frequently based upon those used in equivalent TLC systems and has been reviewed by Hara *et al*[336]. Whilst isocratic chromatography has been used

for the separation of relatively simple mixtures of steroids[337] the separation of more complex mixtures is best achieved using gradient elution. The addition of pH modifiers[338], ion-pair reagents[335], the inclusion of phosphate salts[339] and the use of ternary and quaternary solvent systems[299] have each been used with some success for the analysis of steroids.

In contrast to GC, HPLC is normally performed at ambient temperatures and the requirement for derivatisation of steroids prior to chromatography in order to ensure thermal stability and volatility is therefore unnecessary. However, the lack of selectivity and structural information obtained by conventional methods of HPLC detection has restricted its uses. Techniques which interface HPLC and MS have re-established HPLC as an important and versatile implement in the evolution of steroid chromatography. Within the past decade HPLC-thermospray mass spectrometry[340,341], HPLC-electrospray mass spectrometry and HPLC-heated nebulisation mass spectrometry[342] interfaces have been developed. Sensitivities in the low picomolar range have been reported for many of these techniques and are at least equivalent with GC-MS[343,344].

2.1.6 *High-performance liquid chromatography with UV absorbance detection*

UV absorbance detection has long been a favoured technique since many of the naturally occurring steroids have some UV absorbance characteristics. The α,β-unsaturated ketone in the A-ring of the natural steroids absorbs UV light with a maximum at 240nm and with an extinction coefficient between 12,000-20,000. Those steroids which exhibit an aromatic A-ring (the oestrogens) have an absorption maximum at 280nm and isolated carbonyl groups absorb between 275-285nm. Unfortunately, UV absorbance detection is relatively insensitive with detection limits in the nanomolar range, at best, for many steroids[333]. UV absorbance detection has also been used with some success for steroids that do not naturally absorb in the UV spectrum. Agnus *et al*[334] demonstrated a detection limit of 2nM by UV absorbance for pregnanolone using a column containing immobilised cyclodextrin and with testosterone, as a component of the mobile phase, as a probe. However, those steroids which do not absorb UV light, including most of the

urinary steroid metabolites, must undergo chemical modification prior to chromatography in order to form UV-absorbing derivatives.

For most UV applications, it is accepted that the positive identification of steroid species can be made if the chromatogram demonstrates an homogenous peak with elution times identical with those of a reference compound. Nevertheless, such assumptions cannot always be made especially of chromatograms of steroids from biological fluids. This is particularly the case when complex chromatograms of extracts from urine are examined, since it may not be possible to recognise homogeneity simply by inspection. Several mechanism have been proposed in order to overcome this difficulty, such as a second chromatography on an alternative column, the use of a second solvent system or the use of the photodiode array UV absorbance detector. This instrument is capable of monitoring the column effluent at several wavelengths and may be used to provide UV-spectra which improves analytical confidence and chromatographic selectivity[339].

Alternatives to UV absorbance detection and mass spectrometry have also been explored and picomolar detection limits have been reported with the use of refractive index detection[345], electrochemical detection[346], fluorescence detection[347-349] and flow through radioactivity detectors[350]. Of these alternatives, few have found greater application to the analysis of steroids than fluorescence detection.

2.1.7 *High-performance liquid chromatography with fluorescence detection.*

Of the naturally occurring steroids, few, with the exception of the oestrogens, demonstrate native fluorescence. However, most steroids demonstrate greater or lesser fluorescence in acid solution. One of the earliest procedures which made use of fluorescence detection for the chromatographic analysis of corticosteroids was devised by Sweat in 1954 [290] and later modified for application to the analysis of free 11-hydroxycorticosteroids in human plasma by Mattingly[291]. They demonstrated that in strong sulphuric or phosphoric acid corticosteroids with an 11-hydroxyl group form fluorophores with excitation maxima and minima of 470-475nm and 520-530nm respectively. However, the development of fluorescence was highly variable and several

investigators reported the formation of multiple fluorescent products[351]. Furthermore, injection of material at low pH onto HPLC columns was found to result in deterioration in chromatographic resolution and the steroid fluorophores were found to be relatively unstable at ambient temperatures. However, modification of the technique to include extraction of the fluorophore into ethyl-acetate subsequent to acid hydrolysis produced detection limits for cortisol and corticosterone of 0.29pmol per injection, improved stability and limited the formation of multiple fluorescent products[352].

An alternative approach to fluorescence detection, which exploits the characteristics of naturally fluorescent molecules as derivatising agents for steroids, has been extensively examined. These procedures offer several advantages in that: i) there is little variation in fluorescence yield between steroid derivatives, and, ii) careful selection of both the derivatising agent and the chemical reaction involved in forming the derivative can result in a high degree of selectivity over that achieved by chromatographic separation alone.

The early history of fluorescence derivatisation is almost exclusively devoted to the development of derivatives for peptide and protein analysis using sulphonyl chlorides such as 5-dimethylaminonaphthalene-1-sulphonyl chloride (dansyl chloride)[353]. These reagents were shown to react with primary and secondary amino groups at alkaline pH and with phenols, imidazoles and alcohols at lower pH. Yet despite their successful application to the analysis of amines, amino acids, phenols, catechols and certain drugs and their metabolites in body fluids[354], they have had little application to the analysis of steroids. However, the hydrazine derivative of 5-dimethylaminonaphthaline (dansyl hydrazine) which reacts readily with aldehydes and ketones, by analogy with the formation of coloured 2,4-dinitropheylhydrazine derivatives, has been used to form fluorescent products with those corticosteroids which exhibit a keto group such as cortisol, cortisone and some of their metabolites[353].

Functional groups amongst the steroids tend to be restricted to hydroxyl, keto or phenolic groups and there is a paucity of data in the literature relating to fluorescence derivatising agents for these groups. However chlorides of carboxylic acids react freely not only with

primary and secondary amines and alcohols but also, under appropriate conditions, with tertiary amines and alcohols. Whilst they are more susceptible to hydrolysis than their sulphonyl counterparts, the rapidity with which they react at ambient temperatures makes them eminently suitable for application to the automation of pre-column derivatisation.

Of those that have found application to the analysis of steroids, carbonyl chlorides derived from 7-hydroxycoumarin demonstrate characteristics which are highly favourable. This molecule and many of its derivatives are highly fluorescent in the solid state, in solution and when adsorbed onto alumina with excitation and emission maxima of 310-320nm and 375-395nm respectively. Their steroid derivatives are also highly fluorescent, stable and exhibit excellent chromatographic characteristics. Several fluorescent acylating agents have been synthesised from 7-hydroxycoumarin but most require methylation of the 7-hydroxyl group in order to prevent self-condensation[355]. Several authors have reported highly sensitive analyses of hydroxysteroids using 7-[(chlorocarbonyl)methoxy]-4-methylcoumarin as a derivatising agent[356,357] and have demonstrated that it is at least equivalent in sensitivity with 1-anthroyl nitrile and considerably more sensitive than alternatives such as 4-dimethyamino-1-naphthoyl nitrile, 2-methyl-1,1'-binaphthalene-2'-carbonyl nitrile[358]. Indeed, the intensity of the reactions of carbonyl chlorides ensures that all primary, most secondary, except those which show significant steric hindrance, and many tertiary alcohols become derivatized which is often not the case with more gentle reactions such as those demonstrated by 1-anthoyl nitrile.

Post column derivatisation in HPLC has received relatively little attention due the restrictions imposed by the speed of reaction which must be rapid and often necessitates the use of high temperatures and post column mixing of reagents which frequently precipitates loss of sensitivity due to peak spreading. Indeed, with notable exceptions[359], few techniques have made use of post column derivatisation which has been largely superseded by precolumn derivatisation of corticosteroids.

2.1.8 *Radioimmunoassay of corticosteroids*

The introduction of radioimmunoassay in 1960 [360] and of competitive binding assay the same year[361] proved to be of profound significance in the history of steroid analysis. Steroids represent a population of molecules with little native antigenicity since their small size prohibits activation of an immune response. However, steroids may be rendered immunogenic by conjugation with carrier proteins such as bovine serum albumin, bovine thyroglobulin or keyhole limpet haemocyanin. The linkage is generally made between a free amino group on the carrier protein and the carboxyl group of an acid derivative of the steroid. The method and site of linkage, the length of the spacer arm, the number of haptens linked to the carrier and the tertiary structure of the immunogenic molecule have significant effects upon the characteristics of antisera produced in this way and offers the potential for the design of highly specific antisera capable of marked selectivity between steroids which share considerable structural similarity[362].

The close structural relationship between many of the corticosteroids and particularly between cortisol and cortisone, has greatly limited the usefulness of many antisera designed for use in radioimmunoassay. Most antisera exhibit marked cross reactivity with both cortisol and cortisone such that direct radioimmunoassay of either steroid in their biological matrix without prior selective extraction or extensive chromatographic purification frequently results in data that are potentially compromised. Indeed, the paucity of literature on the radioimmunoassay of cortisone without extensive, multistep, pre-analytical purification, highlights the problems of analytical inaccuracy imposed by poor antibody specificity[363]. Despite these drawbacks, the radioimmunoassay of urinary free cortisol subsequent to extraction with organic solvent has been used to great effect in the diagnosis of Cushing's syndrome for the past three decades and illustrates the potential of this analytical tool for the relatively rapid analysis of large numbers of specimens[364].

2.1.9 *Potential for the analysis of conjugated and unconjugated corticosteroids*

The significance of variations in biochemical phenotype and their close correlation with genotype in AME together with the characteristic changes in urinary steroids in 11-OR deficiency clearly illustrates the importance of the sensitive and specific determination of steroid profiles in biological fluids. Indeed, it has been suggested that biochemical analysis of steroid profiles in specific groups of patients may identify more modest changes in 11β-HSD activity related either to mutations which have only minor effects upon enzyme activity or to enzyme inhibition or regulation by specific agents yet to be identified[86]. Moreover, since the cloning of 11β-HSD2 and recognition of its role in the pathogenesis of AME there is general agreement that deficiency of renal 11β-HSD2 activity may be responsible for some forms of essential hypertension[73,123,267] and that an unambiguous marker of altered 11β-HSD2 activity could be an important tool for defining altered 11β-HSD2 activity with respect to the aetiology of hypertension in these cases.

The complex interplay of hepatic steroid metabolism and the interconversion of cortisol and cortisone by both isoforms of 11β-HSD requires careful dissection of the mechanisms underlying the pathogenesis of disease associated with changes in the peripheral metabolism of cortisol. This goal depends not only upon the development of analytical techniques which are sufficiently robust to detect relatively modest changes in steroid metabolism but also the establishment of *in vitro* models which enable a closer exploration of the molecular mechanisms underlying these biochemical features, unhindered by the complexity of metabolic interactions encountered *in vivo*. Many investigations have exploited the latter suggestion and examples of *in vitro* models of 11β-HSD activity are abundant in the literature[247,365-367]. However, the difficulties inherent in the quantitative analysis of corticosteroids in biological fluids are equally translated to their measurement in cell culture media *in vitro* and may have a significant impact upon the accurate analysis of enzyme kinetics. Indeed, many studies have made use of relatively crude techniques including TLC and paper chromatography[319,320] and have made the assumption that metabolism of either cortisol or cortisone *in vitro* is limited to their interconversion by 11β-HSD and that further metabolism either does not

take place or is too insignificant to affect the analytical outcome. Nevertheless, it is well recognised that several primary and tumour cell lines used for the analysis of 11β-HSD activity exhibit marked steroidogenic or steroid metabolising capabilities which are almost certain to affect the outcome of kinetic analyses and hormonal regulation of 11β-HSD.

2.1.10 *Aims of this study*

Whist the importance of GC-MS in the analysis of steroids is well established, little development in GC methodology has occurred over the past decade. In contrast, HPLC techniques which make use of UV diode-array, fluorescence and MS detection have gained in popularity as the technology has become more robust and cost effective. Nevertheless, very few applications have been published which make use of these alternatives for the investigation of dysfunction of the peripheral metabolism of cortisol, despite their potential for the production of sensitive, selective and cost effective analytical techniques. Furthermore, advances in the design and production of specific antisera have provided an opportunity to re-examine the application of radioimmunoassay technology to the analysis of cortisol and cortisone in biological fluids. This latter technology, in contrast with its chromatographic counterparts, has the added advantage of relatively large scale throughput which may be used to great effect in screening large numbers of specimens prior to selection for more rigorous analysis by chromatography. Thus, the aims of this study were to develop sensitive and specific techniques for the quantitative analysis of corticosteroids in biological fluids using:

1. HPLC with fluorescence detection for the sensitive analysis of unconjugated steroid profiles in plasma and urine;
2. HPLC with UV detection for the rapid analysis of cortisol and cortisone in tissue culture media as an aid to the study of the regulation of 11β-HSD *in vitro*;
3. radioimmunoassay techniques for the rapid analysis of unconjugated cortisol and cortisone in biological fluids for the analysis of large numbers of specimens.

2.2 Method Development I: Reversed Phase HPLC of Corticosteroids with Fluorescence Detection Using 7-[(chlorocarbonyl)methoxy]-4-methylcoumarin

2.2.1 METHODS

General chemicals and solutions were obtained from Sigma Chemical Company Ltd (Poole, Dorset, UK) and were of HPLC grade unless otherwise stated. Radiolabeled steroids were supplied by Amersham International PLC (Amersham, Bucks, UK).

2.2.1.1 *Synthesis of 7-[(chlorocarbonyl)methoxy]-4-methylcoumarin*

This was adapted from the methods of Karlsson *et al*[357] and Baker & Collis[355]. 25ml chloroform was dried by addition of 1g anhydrous sodium sulphate. 19.2ml thionyl chloride containing 1g 7-(carboxymethoxy)-4-methylcoumarin (CMMC) was heated under reflux for thirty minutes. 19.2ml dry chloroform was added and reflux heated until all solid was dissolved. Excess thionyl chloride was removed by distillation and the liquid replaced with hexane. The resulting yellow crystalline precipitate was filtered and dried under vacuum then re-crystallised from ethyl acetate containing 5% thionyl chloride. The resulting yellow crystals were freed of solvent in vacuo, 100µg was dissolved in 100ml acetonitrile and 10µl was analysed by HPLC using a Bio Rad Model 2700 solvent delivery system, AS-100T HRLC autosampler with integral Rheodyne model 7010 injection valve fitted with a 200µl injection loop (Bio Rad Laboratories Ltd. Hemel Hempstead, Hertfordshire). The column effluent was monitored using a Perkin Elmer model 3000 fluorescence spectrophotometer equipped with an 8µl flow cell (Perkin Elmer Ltd, Connecticut, USA) with excitation and emission maxima of 315nm and 380nm respectively. Chromatography was performed using a Waters Nova-Pac C_{18} 60Å 4µm 300x3.9mm I.D. analytical HPLC column (Millipore (U.K.) Ltd./Waters Chromatography Division, Watford, Hertfordshire, UK) with either 100% acetonitrile or 100% dichloromethane at a flow rate of 1.0ml/minute or with a Shodex GPC KF-806M 300x8mm I.D. analytical column (Phenomenex U.K. Ltd. Macclesfield, Cheshire, UK) with 100% tetrahydrofuran (1.0ml/minute). Infrared spectrometry was performed at the

University of Portsmouth, UK. Mass spectrometry was performed at the Department of Chemistry, Southampton University, UK.

2.2.1.2 *Corticosteroid derivatisation, solid phase extraction and HPLC*

Stock solutions of CCMMC (100mM) and 4-dimethyaminopyridine (DMAP) (100mM) were prepared using anhydrous dichloromethane as solvent. Stock solutions (200μM) of corticosterone, cortisol, cortisone, 11-deoxycorticosterone, 17α-hydroxypregnenolone, 17α-hyroxyprogesterone and pregnenolone were also prepared using acetonitrile as solvent. Using a modification of the method of Karlsson *et al*[357], 100μl of each steroid solution was evaporated under a stream of dry nitrogen in 12x100mm, glass, wide necked, screw topped tubes (L.I.P (Equipment & Services) Ltd. Shipley, West Yorkshire, UK). 15μl DMAP (100mM) and 100μl CCMMC (23mM) were added, mixed and evaporated as previously described. The contents of each tube were reconstituted with 5ml 30% acetonitrile/H_2O immediately prior to solid phase extraction. Individual Sep Pak Plus C_{18} cartridges (Millipore (U.K.) Ltd.) fitted with 20ml glass luer-lock syringes (Jencons Scientific Ltd. Leighton Buzzard, Bedfordshire, UK) were prepared by passing 5ml methanol followed by 10ml H_2O under vacuum at a flow rate of 0.5ml/minute through each cartridge. The derivatisation reaction mixture was transferred to the syringes and allowed to pass through the cartridge followed by 10ml H_2O and 10ml 30% acetonitrile/H_2O under vacuum at a flow rate of 0.5ml/minute. The steroids were recovered from the cartridge with 3ml acetonitrile and evaporated to dryness as previously described. The extracts were reconstituted in 100μl mobile phase immediately prior to use and a 10μl sample was injected onto a Waters Nova-Pac C_{18} 60Å 4μm 300x3.9mm I.D. analytical HPLC column (Millipore, UK) which was used throughout. Four mobile phases, under isocratic conditions, were examined (100% acetonitrile, 100% methanol, 70% acetonitrile/H_2O, 70% methanol/H_2O) at a flow rate of 0.7ml/minute. The HPLC column was allowed to equilibrate for 2 hours between each change of mobile phase.

2.2.1.3 *Determination of optimum reaction conditions for the derivatisation of corticosteroids*

All reactions were performed using 7μg cortisol and 7μg cortisone. Serial dilutions of CCMMC and DMAP over the concentration range 500 – 10mM were prepared using dichloromethane as solvent. Two series of reaction conditions were evaluated: (a) 100μl DMAP (100mM) with 100μl CCMMC (1-500mM); (b) 100μl CCMMC (50mM) with 100μl DMAP (1-500mM). A second series of reactions were performed using 100μl CCMMC (25mM) at temperatures of 110, 80, 50 and 37°C in sealed, screw topped, wide necked, glass tubes with incubation periods spanning 2 -120 minutes. Derivatisation and solid phase extraction were performed as previously described using 70% acetonitrile/H_2O as mobile phase (0.7ml/minute) (2.2.1.2). Peak height was recorded.

2.2.2 RESULTS

2.2.2.1 *Analysis Of 7-[(chlorocarbonyl)methoxy]-4-methylcoumarin*

The molecular mass of CCMMC was calculated to be 252.0189 ($C_{12}H_9ClO_4$) with a yield of 61.64%. Infrared absorption maxima were identified at 1795, 1700, 1610, 1385, 1265, 1160, 1137 and 915cm^{-1} and were found to be consistent with data produced by Karlsson *et al*[357] and with the structural formula of CCMMC. The molecular mass and principal ionic fragments of CCMMC was confirmed by EI-Mass spectrometry. The melting point of CCMMC was found to be 131-132°C. Homogeneity of the product was assumed from finding a single chromatographic peak which was apparent under all chromatographic conditions examined.

2.2.2.2 *Chromatography of steroid derivatives*

The instantaneous and complete derivatisation of corticosteroids using CCMMC in the presence of DMAP at room temperature has been reported by Karlsson *et al*[357]. In this study, multiple chromatographic peaks from the derivatisation of single steroids were observed, and provided evidence of incomplete derivatisation despite optimal concentrations of CCMMC and DMAP.

2.2.2.3 *Optimum reaction conditions for the derivatisation of corticosteroids with CCMMC*

Little improvement in derivatisation efficiency occurred with concentrations of CCMMC in excess of 50mM and concentrations of DMAP equimolar with CCMMC produced the highest yield (figures 2.1 and 2.2, page 58). At concentrations greater than those of CCMMC, DMAP inhibited derivatisation. Increasing reaction time at 110°C resulted in a deterioration in derivatisation efficiency (figures 2.3 and 2.4, page 59). Omission of DMAP inhibited derivatisation at temperatures below 50°C but did not limit the formation of multiple derivatives.

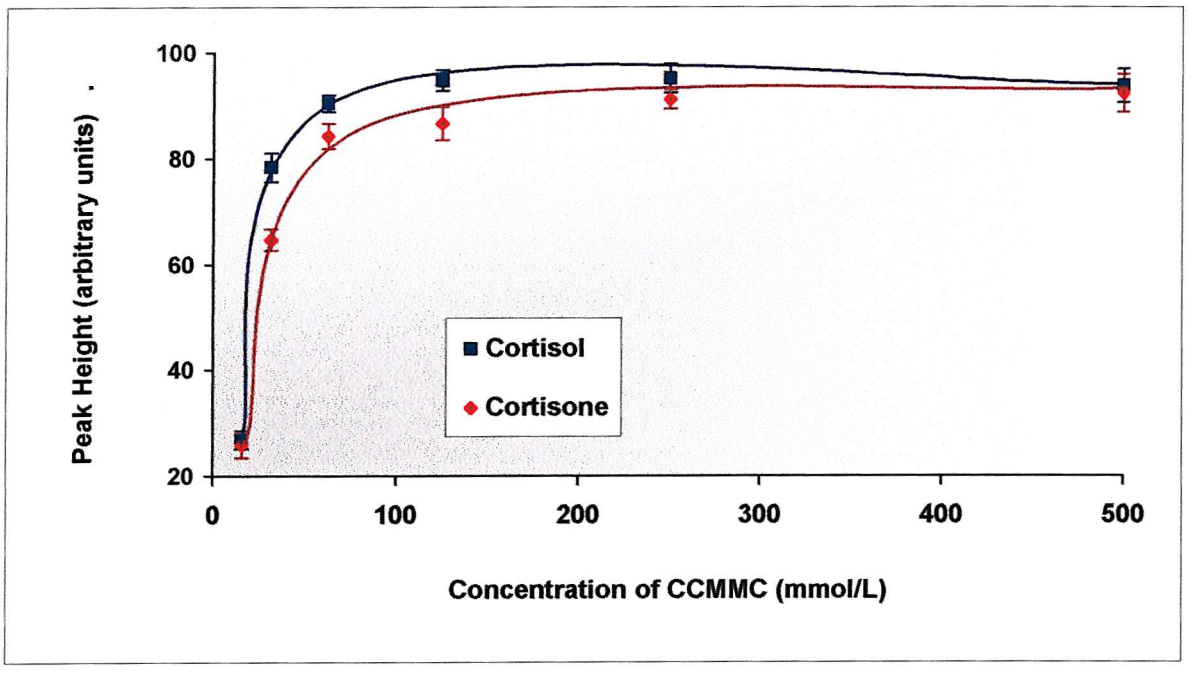


Figure 2.1 The effect of changing the concentration of CCMMC on the derivatisation of cortisol and cortisone: reactions were performed using 7µg steroid with 100µl DMAP (100mM). Derivatisation efficiency was determined from the peak height of the major steroid derivative. Each point represents the mean and standard deviation of triplicate analyses.

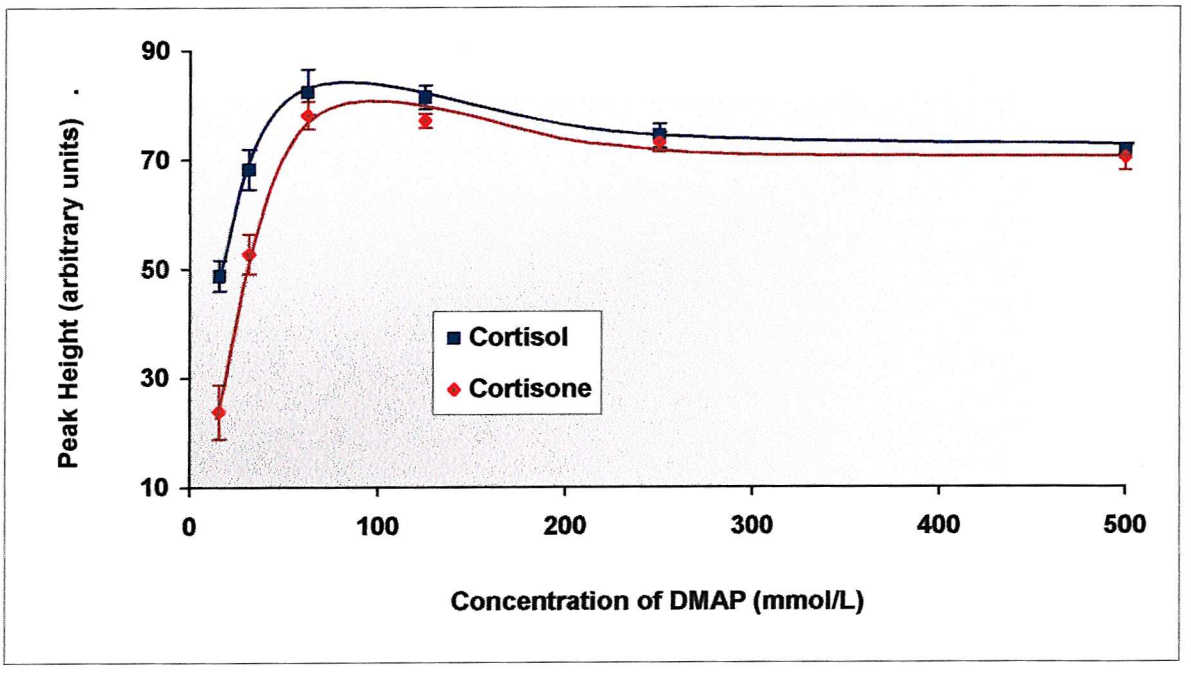


Figure 2.2 The effect of changing the concentration of DMAP on the derivatisation of cortisol cortisone with CCMMC: reactions were performed using 7µg steroid with 100µl CCMMC (50mM). Derivatisation efficiency was determined from the peak height of the major steroid derivative. Each point represents the mean and standard deviation of triplicate analyses.

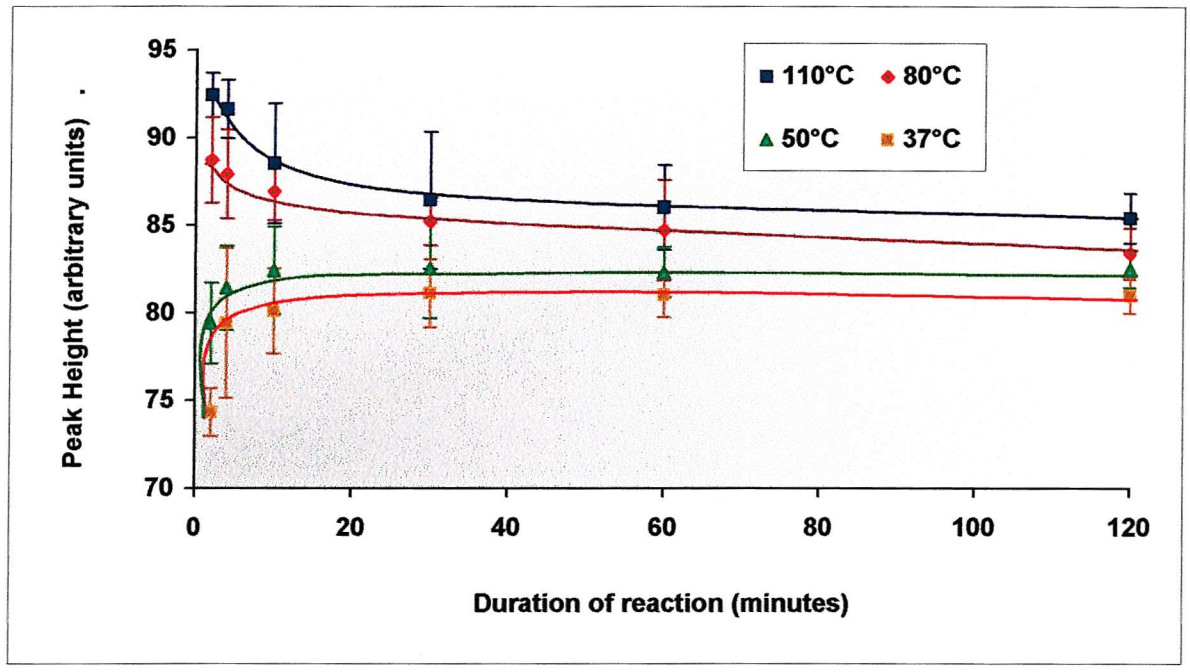


Figure 2.3 The effect of temperature on the derivatisation of cortisol with CCMMC and DMAP: reactions were performed using 7µg cortisol with 100µl DMAP (100mM) and 100µl CCMMC (25mM). Derivatisation efficiency was determined from the peak height of the major steroid derivative. Each point represents the mean and standard deviation of triplicate analyses.

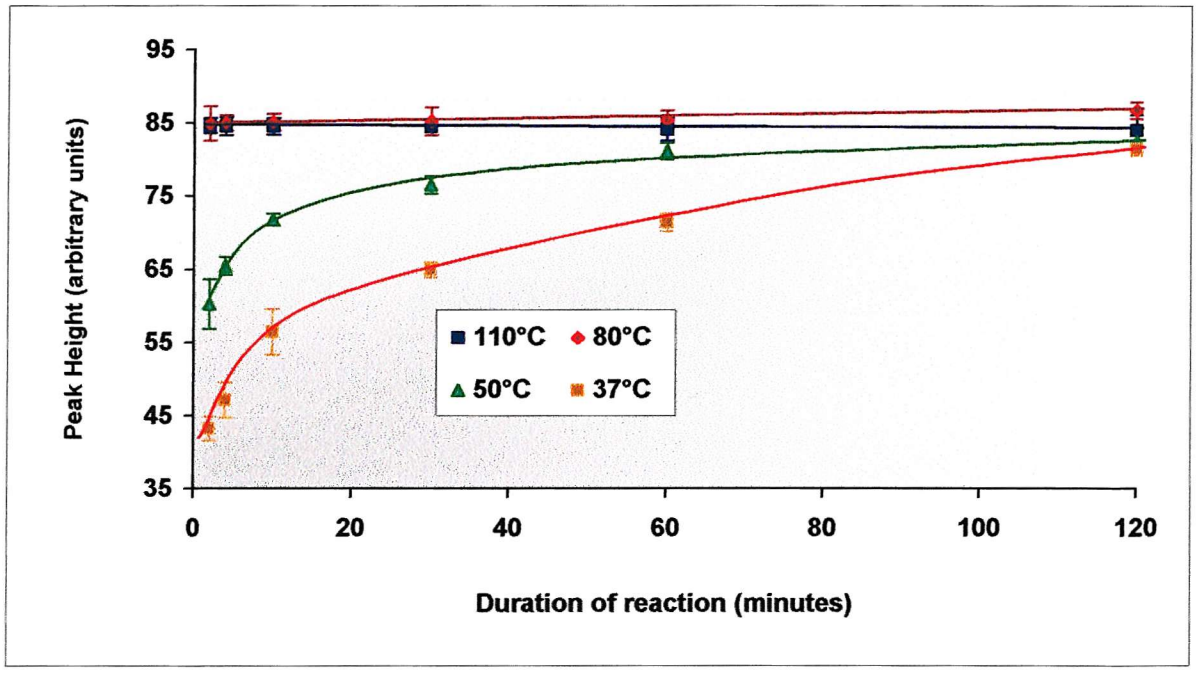


Figure 2.4 The effect of temperature on the derivatisation of cortisol with CCMMC in the absence of DMAP: reactions were performed using 7µg cortisol with 100µl CCMMC (25mM). Derivatisation efficiency was determined from the peak height of the major steroid derivative. Each point represents the mean and standard deviation of triplicate analyses.

2.2.3 SUMMARY

The formation of multiple derivatives from single analytical species is a common failing of pre-column derivatisation techniques[368]. The conformational orientation of hydroxyl moeties have important implications for their relative susceptibility to acylation and there are few exceptions to the rule that among the saturated steroids equatorial hydroxyls are more readily acylated than axial hydroxyls in the same position[300]. Moreover, primary alcohols are more susceptible to acylation than are secondary alcohols, and tertiary alcohols rarely undergo acylation. Cortisol may be considered to have only one acylable hydroxyl; this is a primary alcohol situated at C_{21}. The C_{17} hydroxyl is tertiary as is the 11β-hydroxyl which is strongly hindered by steric interactions with the two β-methyl groups at C_{10} and C_{13}, and by the β-hydrogen at C_8 and suggest that for cortisol, at least, only one product of derivatisation with CCMMC would be likely. Nevertheless, in this study at least two derivatisation products were suggested by the observation of multiple chromatographic peaks. By comparison with cortisone and 17-hydroxypregnenolone, which also yielded two derivatives, and with 17α-hydroxyprogesterone which yielded only one derivative, it is possible to speculate that in addition to derivatisation at the C_{11} hydroxyl, the tertiary alcohol at C_{17} in cortisol and cortisone was also susceptible to derivatisation, though to a lesser extent than that observed at C_{11}. These observations probably represented the products of incomplete derivatisation as a result of sub-optimal reaction conditions, particularly in the case of the tertiary alcohol at C_{17}. However, attempts to reduce the formation of multiple steroid derivatives with CCMMC by manipulation of reaction conditions and reagent concentrations had little effect. Indeed, under all reaction conditions examined, CCMMC produced multiple derivatives with cortisol, cortisone and corticosterone. Subsequent studies using the more polar, polyhydroxylated corticosteroid metabolites, including THF, 5α-THF, THE and the cortols and cortolones, also resulted in the formation of multiple derivatives. Selective solid phase extraction of the less polar corticosteroid derivatives by increasing the organic solvent content of the wash solution from 30% to 38% so that the more polar derivatives were eluted to waste resulted in little improvement. Indeed, increasing the organic component to 40% resulted in significant loss of the derivatized steroid. The improvement in chromatographic selectivity that was achieved in this exercise was not

considered to be adequate to offset the reduction in sensitivity that it imposed. Moreover, the tendency of acid chlorides to readily undergo hydrolysis to the parent carboxylic acid in the presence of water and the storage difficulties this imposed offered additional incentive to examine alternatives to derivatisation with CCMMC.

2.3 Method Development II: Reversed Phase HPLC of Corticosteroids with Fluorescence Detection Using 7-(carboxymethoxy)-4-methylcoumarin and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride

The reaction of acid chlorides with primary and secondary alcohols occurs rapidly at ambient temperatures and results in the formation of esters with the elimination of HCl. By comparison, esterification reactions which are performed under the influence of coupling reagents such as carbodiimides may be considered to be relatively gentle. These reactions exploit the phenomenon of facilitated carboxylic acid activation which may then undergo condensation esterification with alcohols with the elimination of water. The literature is replete with descriptions of the use of carbodiimides in reactions which require activation of carboxylic acids, examples of which include; the formation of antigenic conjugates of steroids, drugs and peptides for use in the production of specific antisera[362], polyethylene immobilisation of polypeptides for C-terminal sequencing[369] and as carboxyl modifying agents for protein synthesis[370] as well as for the pre-column derivatisation of 21-hydroxy-steroids with 2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole (CDB) catalysed by 1-isopropyl-3-(3-dimethylaminopropyl)carbodiimide perchlorate (IDC) prior to HPLC analysis[349]. The efficiency of derivatisation with IDC and CDB was found to be 46% for the synthetic corticosteroid, dexamethasone, with detection limits between 0.54 and 27.4 pmol/injection for dexamethasone and cortisol respectively[349]. Whilst the technique produced only one derivative for each steroid, the procedure was significantly complicated by the requirement for chemical synthesis of the fluorophore. Moreover, both CDB and IDC were found to be relatively unstable in solution at the concentrations required for reaction[349].

Thus, the parent carboxylic acid of CCMMC, CMMC, may be considered to be superior to CDB both in terms of its stability in solution at room temperature, and in that it has a

high fluorescence yield, well documented fluorescence characteristics and is available as an inexpensive, pure commercial preparation[356,357].

2.3.1 METHODS

2.3.1.1 *Derivatisation, solid phase extraction and HPLC of corticosteroids using CMMC.*

100mM stock solutions of CMMC, DMAP and 1-ethyl-3-(-3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) were prepared using anhydrous acetonitrile as solvent. 100µl of corticosterone, cortisol, cortisone, 11-deoxycorticosterone, 17α-hydroxypregnenolone, 17α-hyroxyprogesterone and pregnenolone (200µM) was evaporated to dryness (2.2.1.2). 50µl EDC (100mM), 50µl DMAP (100mM) and 100µl CMMC (100mM) were added. The reaction vessels were sealed with screw tops and placed in a heating block at 70°C for 40 minutes after which time 4ml acetonitrile/H_20 (30% v/v) was added and vortex mixed to ensure complete solvation. The steroid derivatives were recovered from solution by solid phase extraction (2.2.1.2) prior to isocratic HPLC using a Waters Nova-Pac C_{18} 60Å 4µm 300x3.9mm I.D. analytical HPLC column (Millipore, UK) with acetonitrile/H_2O (40% v/v 0.7ml/minute) as mobile phase.

2.3.1.2 *Determination of optimum concentrations of EDC, CMMC and DMAP for the derivatisation of corticosteroids with CMMC*

All reactions were performed using 7µg cortisol and 7µg cortisone. Serial dilutions of CMMC, EDC and DMAP over the concentration range 1-100mM were prepared using acetonitrile as solvent. Three series of reaction conditions were evaluated: (a) 50µl DMAP (50mM), 50µl EDC (50mM) with 100µl CMMC (1-100mM), (b) 100µl CMMC (25mM), 50µl DMAP (50mM) with 50µl EDC (1-100mM), (c) 100µl CMMC (50mM), 50µl EDC (50mM) with 50µl DMAP (1-100mM) Derivatisation, solid phase extraction and chromatography were performed as previously described (2.3.1.1 and 2.2.1.2).

2.3.1.3 *Determination of optimum temperature and duration of reaction for the derivatisation of corticosteroids with CMMC*

7µg cortisol, 100µl CMMC (25mM), 50µl DMAP (25mM) and 50µl EDC (25mM) were used throughout. Derivatisation reactions were performed at temperatures of 180, 110, 70 and 50°C in sealed, screw topped, wide necked, glass tubes with incubation periods spanning 20-240 minutes (2.3.1.1). Solid phase extraction and chromatography were performed as previously described (2.2.1.2. and 2.3.1.1).

2.3.1.4 *Derivatisation of corticosteroids with CMMC making use of alternative carbodiimide and tertiary amine catalysts*

25mM stock solutions of EDC, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluensulfonate, 1-cyclohexyl-3-(-3-dimethylaminopropyl)carbodiimide perchlorate and 50mM stock solutions of DMAP, 4-piperidinopyridine, pyridine and triethylamine were prepared using acetonitrile as solvent. All reactions were performed using 7µg cortisol. Two series of reactions were evaluated:

(a) 100µl CMMC (50mM), 50µl EDC (25mM), 50µl DMAP, 4-piperidinopyridine, pyridine or triethylamine (50mM); (b) 100µl CMMC (50mM), 50µl DMAP (50mM), 50µl EDC, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluensulfonate or 1-cyclohexyl-3-(-3-dimethylaminopropyl)carbodiimide perchlorate (25mM). Derivatisation, solid phase extraction and chromatography were performed as previously described (2.2.1.2 and 2.3.1.1).

2.3.1.5 *Chromatographic resolution: optimised for corticosteroids derivatized with CMMC*

20nmol of 5α-dihydrocortisol, 5β-dihydrocortisol, 5α-dihydrocortisone, 5β-dihydrocortisone, 6β-hydroxycortsol, 11-deoxycortisol, 11-deoxycorticosterone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, 20α-dihydrocortisol, 20β-dihydrocortisol, 20α-dihydrocortisone, 20β-dihydrocortisone, α-cortol, β-cortol, α-cortolone, β-cortolone, corticosterone, cortisol, cortisone, dehydroepiandrosterone,

fluorandrenolide, pregnenolone, progesterone, 5α-THF, THF and THE (200μM) was derivatized separately by heating at 70°C for 120 minutes in sealed glass screw-topped reaction vessels with 100μl CMMC (25mM), 50μl DMAP (50mM) and 50μl EDC (25mM). A mixture of the steroids (20nM of each) contained in separate reaction vessel were also derivatized. Chromatography was performed using a Waters Nova-Pac C_{18} 60Å 4μm 300x3.9mm I.D. analytical HPLC column (Millipore, UK) using:

(a) isocratic conditions with mobile phases comprising: (i) acetonitrile/H_2O (40% v/v 0.7ml/minute) (ii) acetonitrile/H_2O (30% v/v 0.7ml/minute)
(b) programmed gradient elution with mobile phases comprising: (i) acetonitrile/H_2O (40 –70% v/ v 0.7ml/minute) over 90 minutes, with acetonitrile/H_2O (70% v/v, 0.7ml/minute) for 20 minutes (ii) acetonitrile/(50mM KH_2PO_4,10mM acetic acid) (40 –70% v/v 0.7ml/minute) over 90 minutes, with acetonitrile/(50mM KH_2PO_4,10mM acetic acid) (70% v/v 0.7ml/minute) for 20 minutes.

2.3.1.6 *The efficiency of derivatisation of corticosteroids with CMMC*

Triplicate solutions containing 4,8,20 and 40μg cortisol and approximately 50,000dpm [1,2,6,7-^{3}H]-cortisol (Amersham International plc, Buckinghamshire, UK) (specific activity 63.0 Ci/mmol) in 200μl acetonitrile were prepared. A 50μl sample of each solution was dispensed into a glass scintillation vial (L.I.P (equipment & services) Ltd) and evaporated under a stream of dry nitrogen. The remaining solution was evaporated and derivatized (figure 2.6, page 69). The derivatized steroid was reconstituted in 110μl acetonitrile/(50mM KH_2PO_4,10mM acetic acid) (40 –70% v/v). 50μl samples were injected for HPLC (figure 2.6, page 69). Thirty minutes after injection, the column effluent was collected for 10 minutes (Model 204 Fraction Collector, Millipore UK) and evaporated under a stream of dry nitrogen. 250μl acetonitrile/H_2O (40% v/v) and 4ml Optiphase scintillation fluid (Pharmacia Wallac Ltd(U.K.), Milton Keynes, UK) was added to all vials which were capped and mixed to ensure the formation of an homogenous emulsion. Each vial was counted by liquid scintigraphy (model 1215 liquid scintilation counter (Pharmacia, UK). Quench correction was performed by recounting

each vial after the addition of 100,000dpm [^{3}H]-standards (Pharmacia, UK) (Appendix 1).

2.3.1.7 *Recovery of [1,2,6,7-^{3}H]-cortisol from urine and plasma*

Five specimens of urine were selected at random. Urinary free cortisol was measured by radio-immunoassay (RIA) (2.5.1). 10,000dpm [1,2,6,7-^{3}H]-cortisol was added to duplicate 10ml aliquots of each specimen. 1ml of each specimen was counted by liquid scintigraphy (2.3.1.6). Extraction of corticosteroids was performed using a modification of the method of Shackleton *et al*[371]. The specimens were centrifuged (3000 x g, 10minutes) and duplicate 4ml aliquots were passed through prepared Sep Pak Plus C_{18} cartridges (figure 2.6, page 69) fitted with 20ml glass luer-lock syringes (Jencons Scientific Ltd, UK), followed by 2x10ml washes with H_2O. Steroids were eluted from the cartridge with 3ml acetonitrile. The eluate was evaporated to dryness under a stream of dry nitrogen and the residue was reconstituted with 5ml ethyl acetate. The organic matrix was washed with 2ml carbonate buffer (50mM, pH8.5), centrifuged (3000 x g, 10 minutes) and the lower, aqueous layer aspirated to waste. The solvent was evaporated and the residue was reconstituted in 110µl acetonitrile/(50mM KH_2PO_4,10mM acetic acid) (40 –70% v/v). Duplicate 50µl samples were dispensed into a glass scintilation vials and counted by liquid scintigraphy (2.3.1.6).

Five plasma specimens were selected which exhibited varying degrees of jaundice, lipaemia and haemolysis. Plasma cortisol was measured by RIA at the Department of Endocrinology, Southampton General Hospital. 40,000dpm [1,2,6,7-^{3}H]-cortisol was added to triplicate 5ml aliquots of each specimen. 1ml of each specimen was counted by liquid scintigraphy (2.3.1.6). Protein was removed by the addition of 1ml acetonitrile to 1ml of plasma followed by centrifugation (3000 x g, 10minutes). The supernatant was decanted into a separate glass tube containing 5ml H_2O. Solid phase extraction and liquid scintigraphy was then performed as previously described (2.3.1.7).

2.3.1.8 *Calibration, analytical imprecision, limits of detection and recovery of corticosteroids from plasma and urine*

Three solutions containing 5α-dihydrocortisol, 5β-dihydrocortisol, 5α-dihydrocortisone, 5β-dihydrocortisone, 6β-hydroxycortsol, 11-deoxycortisol, 11-deoxycorticosterone, 17α-hydroxypregnenolone, 20α-dihydrocortisol, 20β-dihydrocortisol, 20α-dihydrocortisone, 20β-dihydrocortisone, α-cortol, β-cortol, α-cortolone, β-cortolone, corticosterone, cortisol, cortisone, dehydroepiandrosterone, pregnenolone, 5α-THF, THF and THE at concentrations of 20, 5 and 1μM were prepared using acetonitrile as solvent. 100μl of each solution containing 80μl fluorandrenolide (6μM) as internal standard was evaporated under a stream of dry nitrogen. Derivatisation and solid phase extraction was performed as described in figure 2.6, page 69. The extracts were reconstituted in 240μl acetonitrile/(50mM KH_2PO_4,10mM acetic acid) (40 –70% v/v) and a 50μl sample was injected onto the HPLC (figure 2.6, page 69). Calibration data for each steroid was produced by computer assisted linear regression analysis using peak height as the response factor (BioRad HRLC integration software V3.1, BioRad Laboratories Ltd, UK). Three urine specimens of varying concentration and composition and three pooled plasma specimens which exhibited varying degrees of lipaemia and haemolysis were obtained. 100nM and 250nM of the steroids listed above was added to a 100ml aliquot of each specimen. Duplicate 4ml and 1ml aliquots of each urine and plasma specimen respectively were extracted, derivatized and analysed by HPLC (figure 2.6, page 69).

Within-batch imprecision was estimated by the analysis of ten replicates of pooled plasma and urine specimens. Between-batch imprecision was calculated by the analysis of pooled plasma and urine specimens once in each of ten individual analyses to which 100nM of each of the above listed steroids had been added. Limits of detection were calculated by the analysis of serial dilutions of urine and plasma specimens over the range (1:1 –1:64) and were estimated to be equivalent with a signal to noise ratio of three.

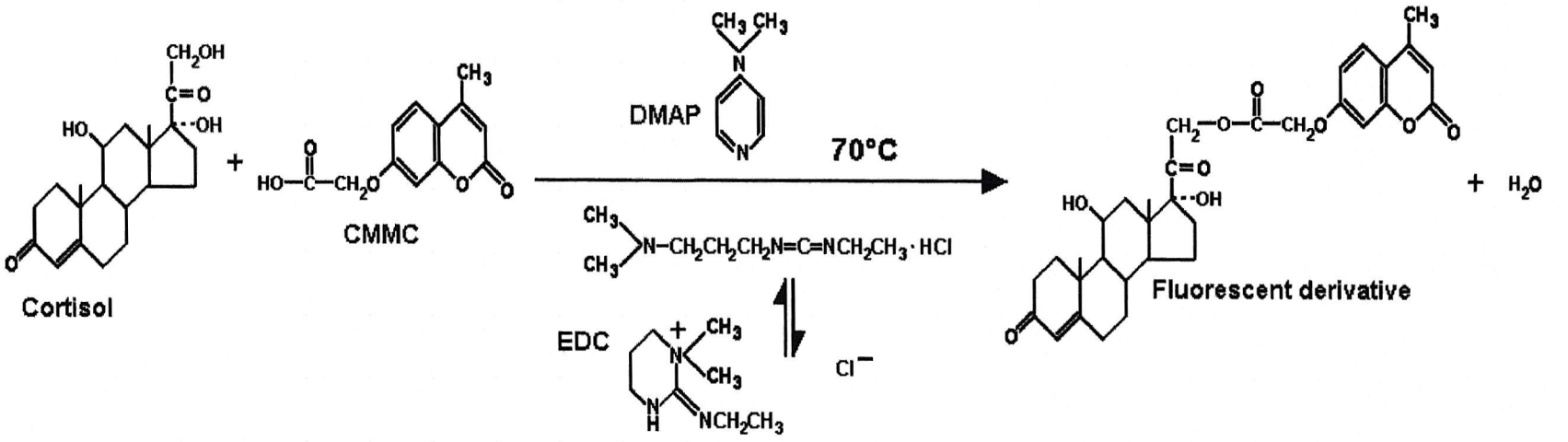
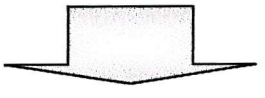


Figure2.5 Schematic representation of the derivatisation of hydroxysteroids with 7-(carboxymethoxy)-4-methylcoumarin in the presence of 1-ethyl-3-(-3-dimethylaminopropyl)-carbodiimide hydrochloride and 4-dimethylaminopyridine.

Steroids were derivatized by incubating at 70°C for 120minutes in sealed screw-capped reaction vessels using 100ml CMMC (25mM), 50ml EDC (50mM) and 50ml EDC (50mM). The reaction mixture was cooled and 5ml acetonitrile/H_2O (40% v/v) was added. The reaction mixture was vortex mixed to ensure complete solvation

Individual C_{18} Sep Pac Plus cartridges were prepared by passing 5ml methanol followed by 10ml H_2O under vacuum at a flow rate of 0.5ml/minute through each cartridge. The derivatisation reaction mixture was transferred to the syringes and allowed to pass through the cartridge followed by 10ml H_2O and 10ml 30% acetonitrile/H_2O washes under vacuum at a flow rate of 0.5ml/minute. The steroids were recovered from the cartridge with 3ml acetonitrile and evaporated to dryness under a stream of dry nitrogen.



Dried extracts were reconstituted in 100ml mobile phase immediately prior to use and a 10ml sample was injected onto a Waters Nova-Pac C_{18} 60Å 4mm 300x3.9mm I.D. analytical HPLC column (Millipore, UK). Chromatography was performed using programmed gradient elution with mobile phases comprising: acetonitrile/(50mM KH_2PO_4,10mM acetic acid) (40 –70% v/v) at 0.7ml/minute over 130 minutes, and held at acetonitrile/(50mM KH_2PO_4,10mM acetic acid) (70% v/v) at 0.7ml/minute for 20 minutes. The column was allowed to equilibrate to acetonitrile/(50mM KH_2PO_4,10mM acetic acid) (40 –70% v/v) for 20 minutes between each injection. The column effluent was monitored at excitation and emission maxima at 315nm and 380nm respectively

Figure 2.6 Flow diagram illustrating the process of derivatisation, solid phase extraction and reversed phase chromatography of corticosteroids.

2.3.2 RESULTS

2.3.2.1 *Derivatisation, solid phase extraction and HPLC of corticosteroids using CMMC*

Preliminary investigations indicated that derivatisation with CMMC, DMAP and EDC produced only one fluorescent product for each of the steroids evaluated. However, subsequent studies revealed that the more polar steroids, namely THF, THE, the cortols and cortolones frequently produced multiple derivatives when the derivatising reagents had been prepared more than 24hours prior to reaction. Isocratic chromatography was found to be inadequate to effect chromatographic resolution of all of the steroids examined in this study. Programmed gradient elution (acetonitrile/H_2O (40 –70% v/v), 0.7ml/minute over 90 minutes, with a 20 minute extension (acetonitrile/H_2O (70% v/v), 0.7ml/minute) improved chromatographic resolution but co-elution of β-cortol with α-cortolone and incomplete separation of 5α-THF and cortisone proved to be persistent failings of all chromatographic conditions investigated. Nevertheless, by careful computer aided integration it was possible to subtend an artificial baseline between 5α-THF and cortisone such that they could be accurately analysed simultaneously.

2.3.2.2 *Optimum reaction conditions for the derivatisation of corticosteroids with CMMC*

Optimal derivatisation was obtained using 100µl CMMC (25mM), 50µl DMAP (50mM), 50µl EDC (25mM) (Figures 2.7, 2.8 & 2.9, pages 71 & 72) and whilst there was little observable difference in derivatisation efficiency using carbodiimides other than EDC, there was a marked deterioration in derivatisation efficiency with all of the alternatives to DMAP evaluated in this study. Marked deterioration in derivatisation efficiency was observed when the reaction was performed at temperatures in excess of 70°C whilst at 50°C the reaction progressed too slowly for convenience (Figure 2.10, page 72). Schematic representations of the derivatisation reaction, solid phase extraction and chromatography used throughout this study are presented in figures 2.5 & 2.6, pages 68 & 69.

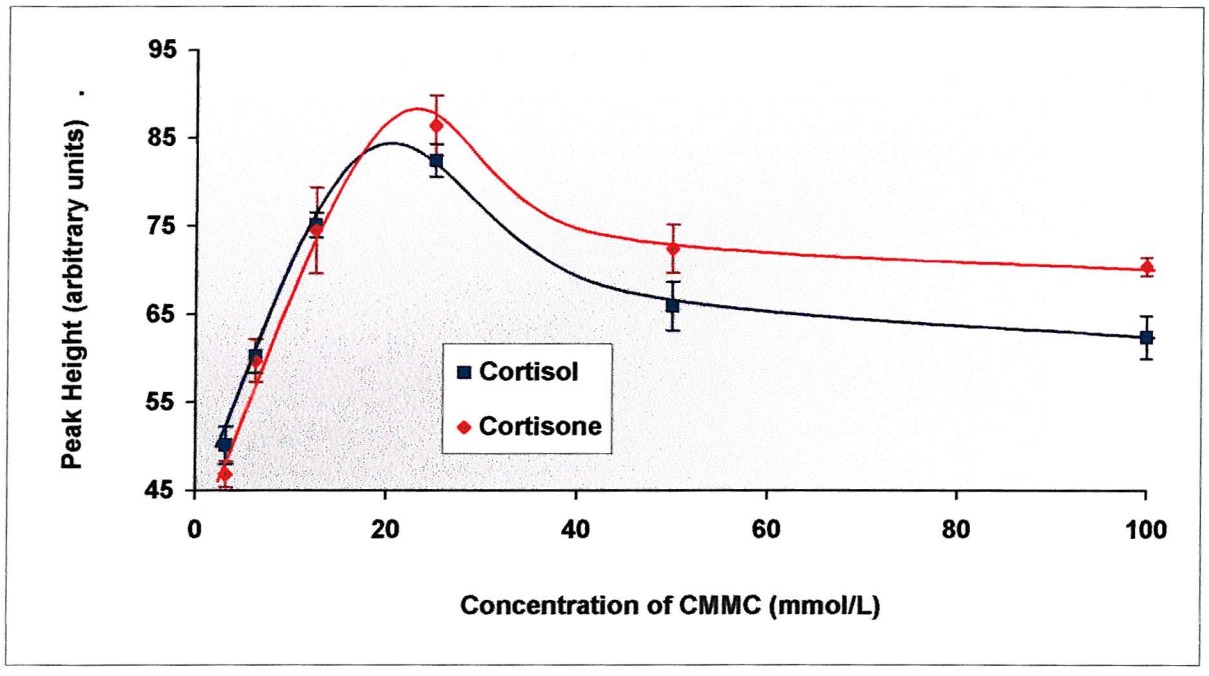


Figure 2.7 The effect of changing the concentration of CMMC on the derivatisation of cortisol and cortisone: reactions were performed using 7µg steroid with 50µl DMAP (50mM) and 50µl EDC (50mM). Derivatisation efficiency was determined from the peak height of the steroid derivative. Each point represents the mean and standard deviation of triplicate analyses.

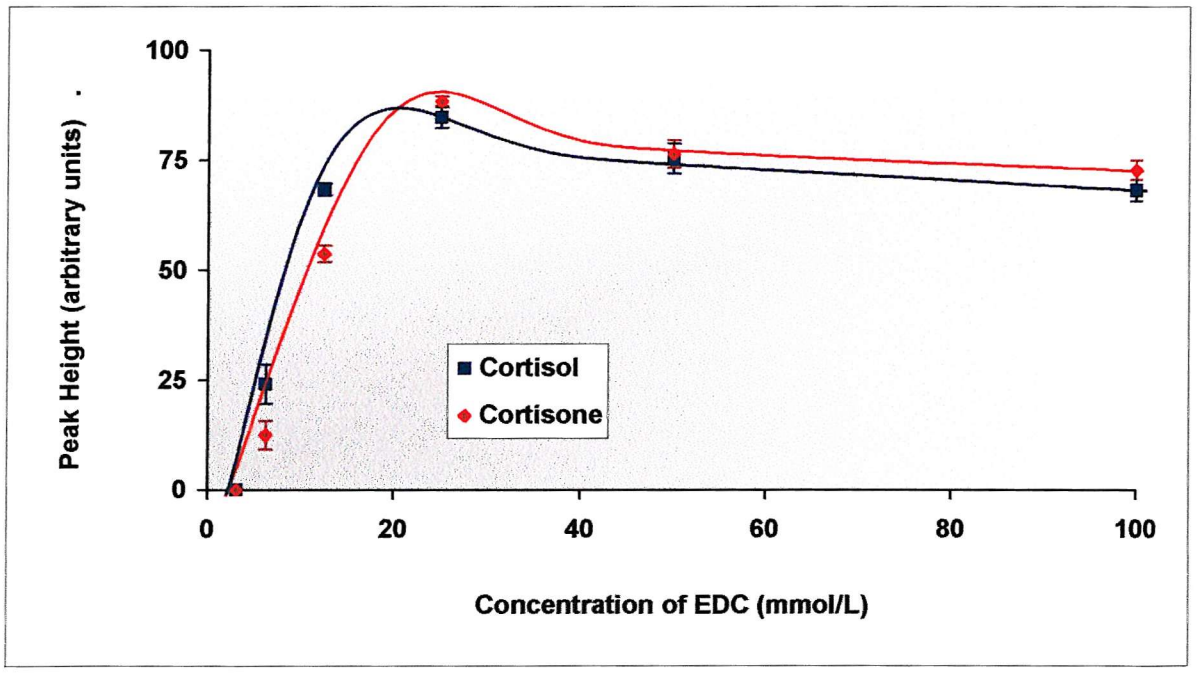


Figure 2.8 The effect of changing the concentration of EDC on the derivatisation of cortisol with CMMC: reactions were performed using 7µg steroid with 100µl CMMC (25mM) and 50µl DMAP (50mM). Derivatisation efficiency was determined from the peak height of the steroid derivative. Each point represents the mean and standard deviation of triplicate analyses.

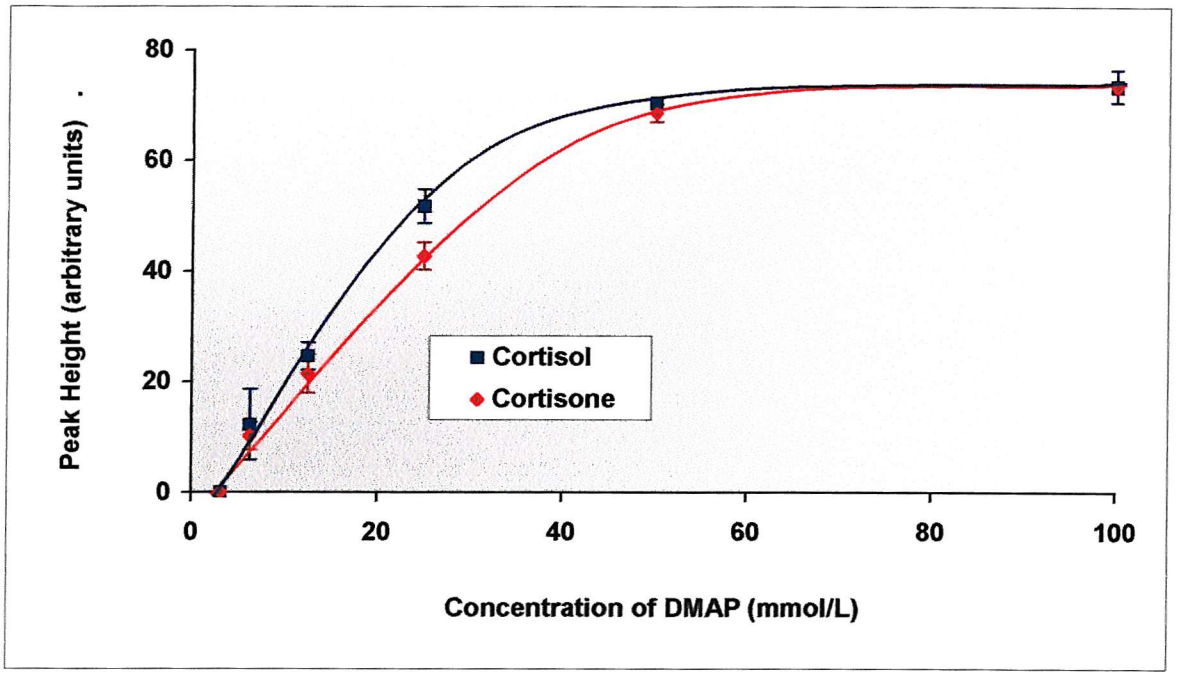


Figure 2.9 The effect of changing the concentration of DMAP on the derivatisation of cortisol with CMMC: reactions were performed using 7µg steroid with 100µl CMMC (25mM) and 50µl EDC (50mM). Derivatisation efficiency was determined from the peak height of the steroid derivative. Each point represents the mean and standard deviation of triplicate analyses.

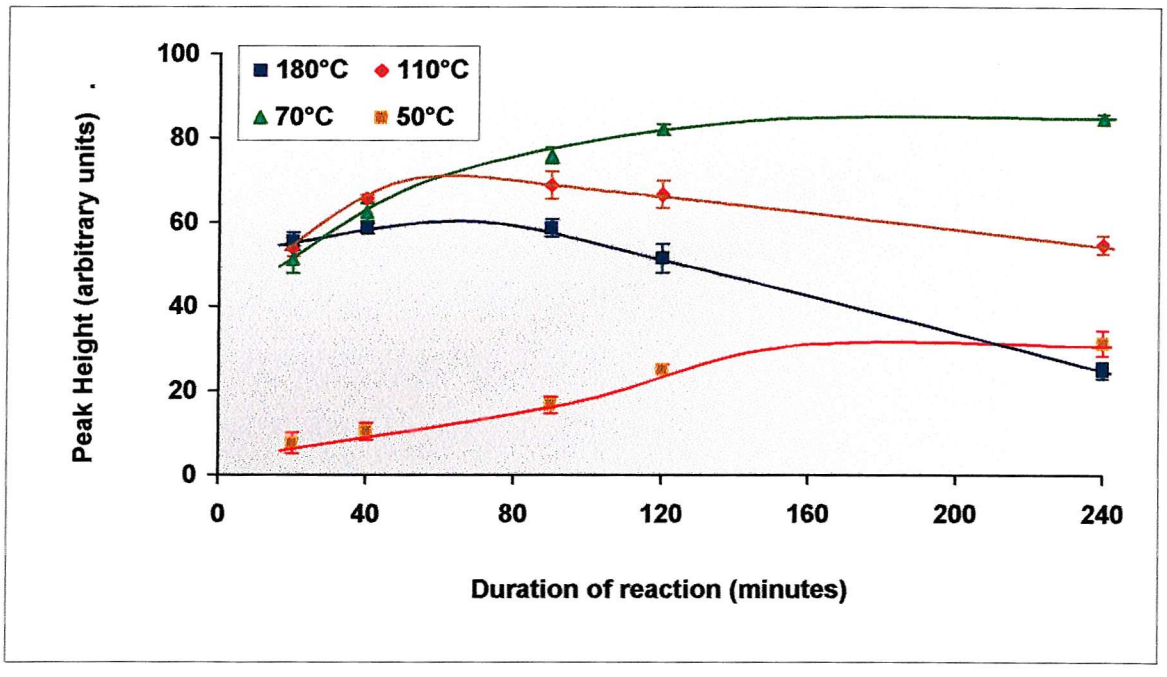


Figure 2.10 The effect of temperature on the derivatisation of cortisol with CMMC: reactions were performed using 7µg steroid with 100µl CMMC (50mM), 50µl EDC (50mM) and. Derivatisation efficiency was determined from the peak height of the steroid derivative. Each point represents the mean and standard deviation of triplicate analyses.

2.3.2.3 *Efficiency of derivatisation of corticosteroids with CMMC*

Derivatisation efficiency was calculated to be 58.9 ± 2.8% (mean ± SD), 57.2 ± 1.4%, 57.4 ± 2.1% and 59.7 ± 3.1% for 4, 8, 20 and 40μg cortisol respectively. Mean derivatisation efficiency was estimated to be 58.3 ± 1.2%.

2.3.2.4 *Recovery of [1,2,6,7-^{3}H]-cortisol from plasma and urine*

The recovery of [1,2,6,7-^{3}H]-cortisol from plasma and urine specimens with concentrations of cortisol over the range 140-990nM and <30-1380nM respectively is illustrated in table 2.1. Mean recovery of [1,2,6,7-^{3}H]-cortisol from plasma and urine was 95.3 ± 2.0% (mean ± SD) and 98.3 ± 1.6% respectively.

Table 2.1 Recovery of [1,2,6,7-^{3}H]-cortisol from plasma and urine

Specimen Cortisol (nM)	[1,2,6,7-^{3}H]-cortisol added (dpm)	[1,2,6,7-^{3}H]-cortisol recovered (dpm)	Recovery%	SD
Urine				
140	41,235	40,543	98.3	1.6
400	42,672	40,889	95.8	1.8
560	40,054	40,246	100.4	2.4
690	43,209	42,645	98.7	1.4
990	46,001	45,243	98.4	1.9
Plasma				
<30	38,452	36,124	93.9	2.4
120	38,650	37,652	97.4	1.6
230	41,126	39,100	95.1	1.7
500	39,422	38,,342	97.3	2.8
1,380	41,643	38,651	92.8	1.6

Extraction was performed in triplicate using 1ml plasma or 4ml urine containing 40000dpm or 10000dpm [1,2,6,7-^{3}H]-cortisol respectively. Mean dpm are illustrated which have been corrected for counting inefficiency, quenching and differences in sample volume.

2.3.2.5 *Calibration, linearity and limits of detection for the analysis of corticosteroids by reversed phase HPLC with fluorescence derivatisation with CMMC*

The chromatographic separation of selected corticosteroids derivatized with CMMC is illustrated in figure 2.11, page 75. Calibration curves were calculated using computer assisted linear regression using peak height as the calibration factor. Limits of detection were estimated to be 3.0 ± 2.0 nM (plasma) and 6.0 ± 2.2 nM (urine) for all steroids examined.

2.3.2.6 *Recovery and analytical imprecision*

Representative chromatograms of the derivatized extracts of plasma and urine are illustrated in figures 2.12 & 2.13 page 76. In contrast with those obtained from aqueous standard preparations, the chromatograms for both plasma and urine extracts exhibited significant increases in baseline noise and extended perturbation of the baseline at the solvent front, a phenomenon which frequently prevented the accurate quantification of 6β-hydroxycortisol, 20α- and 20β-dihydrocortisol. The presence of a significant number of unidentified peaks in the chromatogram was also characteristic of the extracts from plasma and urine. This latter phenomenon imposed a level of complexity upon the interpretation of the chromatograms that could only be successfully negotiated with the aid of electronic superimposition of chromatograms prepared from aqueous standards. Nevertheless, mean analytical recovery of corticosteroids from plasma was found to be 92.0 ± 9.9% (mean ± SD) (100nM) and 91.7 ± 7.9% (250nM) whilst recovery from urine was 90.2 ± 10.0% (100nM) and 87.7 ± 8.1% (250nM) (appendix 2). Analytical imprecision (CV%) was estimated to be 10.9 ± 4.9% (within-batch) and 12.9 ± 6.1% (between-batch) for the analysis of urine whilst analytical imprecision for plasma was 13.7 ± 9.5% (within-batch) and 15.6 ± 10.4% (between-batch) (appendix 3).

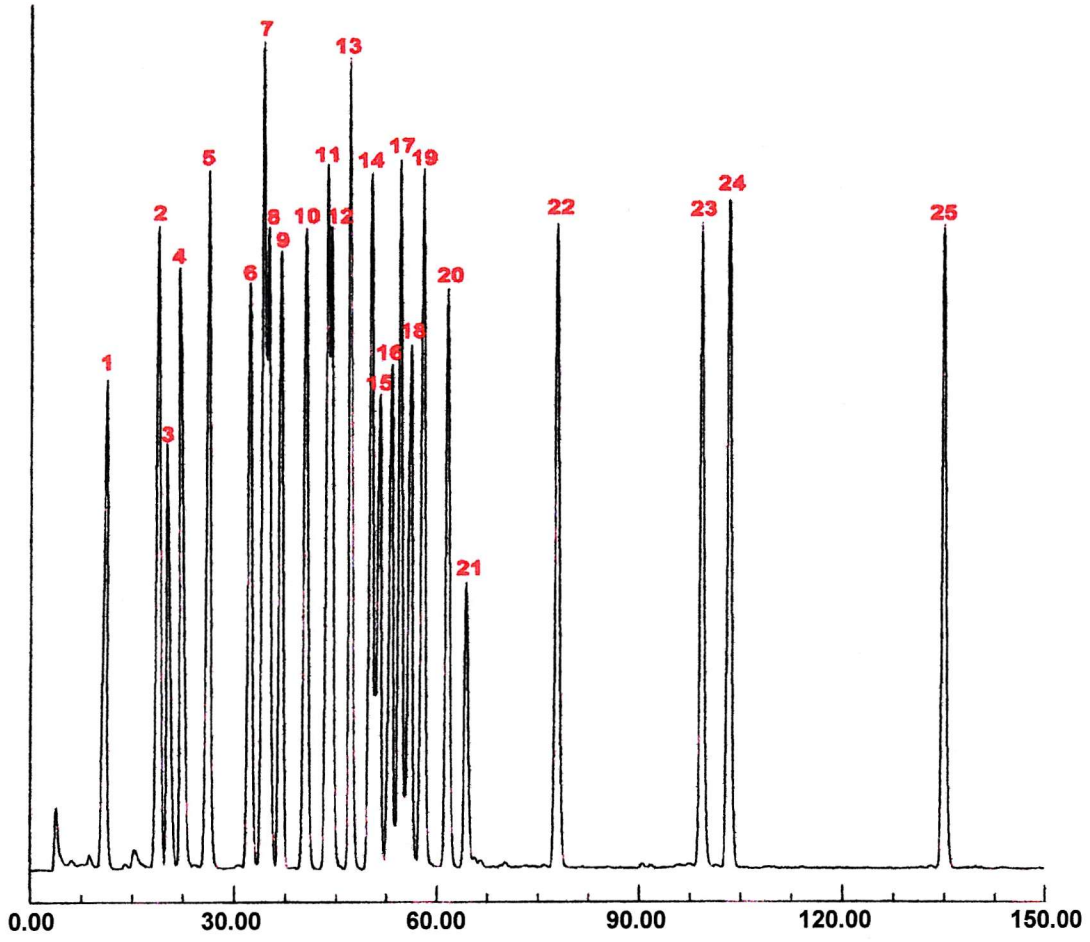


Figure 2.11 Chromatogram of selected corticosteroid/CMMC derivatives by reversed phase HPLC with fluorescence detection: The chromatogram illustrates peaks for: (1) 6β-hydroxycortisol, (2) 20α-dihydrocortisol, (3) 20α-dihydrocortisone, (4) 20β-dihydrocortisol, (5) 20β-dihydrocortisone, (6) α-cortol, (7) β-cortol, (8) α-cortolone, (9) β-cortolone, (10) cortisol, (11) cortisone, (12) 5α-THF, (13) THF, (14) THE, (15) 5α-dihydrocortisol, (16) 5β-dihydrocortisol, (17) corticosterone, (18) 5α-dihydrocortisone, (19) 5β-dihydrocortisone, (20) 11-deoxycortisol, (21) fluorandrenolide (22) 11-deoxycorticosterone, (23) 17α-hydroxypregnenolone, (24) dehydroepiandrosterone, (25) pregnenolone,

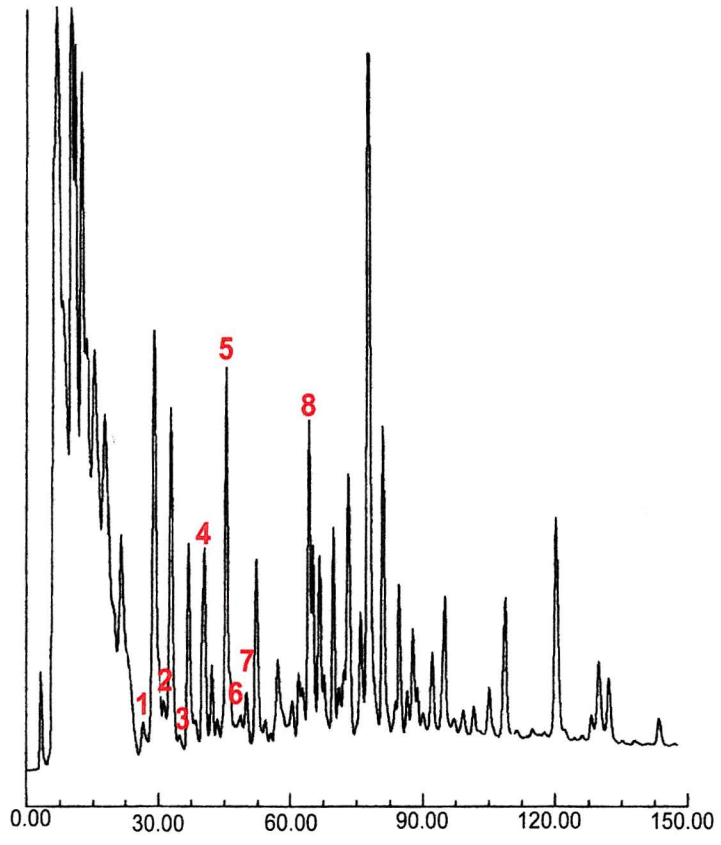


Figure 2.12 Chromatogram of urine extract corticosteroid/CMMC derivatives by reversed phase HPLC with fluorescence detection: The chromatogram represents the products of derivatisation from 4ml urine. The chromatogram illustrates peaks for:

1. α-cortol
2. β-cortol
3. β-cortolone
4. cortisol
5. cortisone
6. THF
7. THE
8. fluorandrenolide

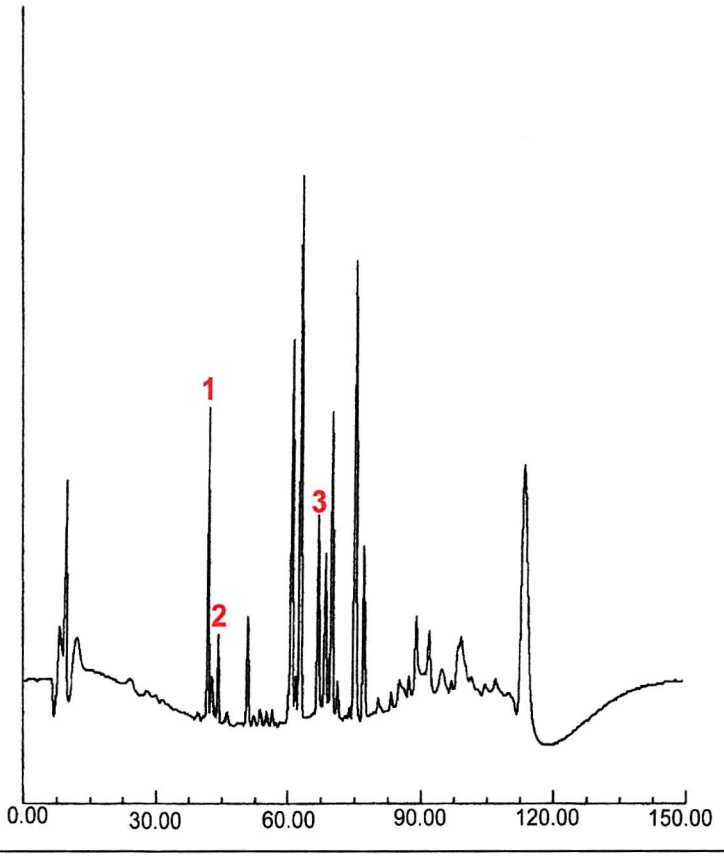


Figure 2.13 Chromatogram of plasma extract corticosteroid/CMMC derivatives by reversed phase HPLC with fluorescence detection: The chromatogram represents the products of derivatisation from 1ml plasma. The chromatogram illustrates peaks for:

1. cortisol
2. cortisone
3. fluorandrenolide

2.4 Method Development III: Reversed Phase HPLC of Corticosteroids with UV Absorbance Detection

2.4.1 METHODS

2.4.1.1 *Extraction and reversed phase HPLC of corticosteroids with UV detection*

Stock solutions (200μM) of androstendione, corticosterone, cortisol, cortisone, 11-deoxycorticosterone, 11-deoxycortisol, 20α-dihydrocortisol, 20β-dihydrocortisol, 20α-dihydrocortisone, 20β-dihydrocortisone, 6β-hydroxycortisol, 17α-hydroxyprogesterone and progesterone were prepared using anhydrous acetonitrile as solvent. Aqueous solutions of each steroid (500nM) and a mixed aqueous solution containing 500nM of each steroid were prepared. Solid phase extraction of steroids was performed using a modification of the method of Lykkesfeldt *et al*[372]. 4ml of each steroid solution containing 100μl dexamethasone (25μM) as internal standard was diluted to 10ml with H_2O and passed through prepared Sep-Pac Plus C_{18} cartidges (2.2.1.4) under vacuum at a flow rate of 0.5ml/minute. The cartridges were washed with 10ml H_2O and the steroids were recovered with 5.0ml ethyl/acetate:diethyl-ether (4:1 v/v). The eluate was washed by shaking with 2.0ml 1M NaOH saturated with Na_2SO_4, centrifuged (3000 x g, 5 minutes) and the lower, aqueous layer was aspirated to waste. The washing process was repeated using 2.0ml 1% acetic acid saturated with Na_2SO_4. The organic layer was evaporated to dryness under a stream of dry nitrogen and reconstituted in 240μl acetonitrile/(50mM KH_2PO_4,10mM acetic acid) (40 –70% v/v) and a 160μl volume was injected onto a Waters Nova-Pac C_{18} 60Å 4μm 300x3.9mm I.D. analytical HPLC column (Millipore, UK). Chromatography was effected using programmed gradient elution (table 2.2, page 79) and the column effluent was monitored for UV absorbance at 254nm using a Model 1790 programmable UV/VIS spectrophotometer fitted with an 8μl flow cell (Bio Rad, UK).

2.4.1.2 *Recovery of [1,2,6,7-^{3}H]-cortisol from tissue culture media*

Recovery of [1,2,6,7-^{3}H]-cortisol from plasma and urine was estimated as previously described (2.3.1.7 and 2.3.1.8). Recovery of [1,2,6,7-^{3}H]-cortisol from tissue culture medium (DMEM, Sigma Chemical Company, Fancy Road, Poole, Dorset, UK) containing 10% foetal calf serum (FCS) (Sigma, UK) was estimated as described for urine.

2.4.1.3 *Calibration, analytical imprecision, limits of detection and recovery of corticosteroids from plasma, urine and tissue culture media*

Aqueous solutions containing 500, 250, 100, 50 and 25nM androstendione, corticosterone, cortisol, cortisone, 11-deoxycorticosterone, 11-deoxycortisol, 20α-dihydrocortisol, 20β-dihydrocortisol, 20α-dihydrocortisone, 20β-dihydrocortisone, 6β-hydroxycortisol, 17α-hydroxyprogesterone were prepared. Urine and plasma specimens were selected as previously described (2.3.1.7 and 2.3.1.8). 100nM and 250nM of each of the steroids listed above was added to 10ml DMEM containing 10% FCS and to 10ml aliquots of each urine and plasma specimen. 1ml acetonitrile was added to duplicate 1ml aliquots of each plasma specimen containing 100μl dexamethasone (25μM) as internal standard. The protein precipitate was removed by centrifugation (3000 x g, 10 minutes). The deproteinised supernatant and 4ml aliquots of each standard, urine and DMEM specimen containing 100μl dexamethasone (25μM) as internal standard were extracted and subjected to HPLC analysis (2.4.1.1). Calibration, limits of detection and analytical imprecision was estimated as previously described (2.3.1.9)

Table 2.2 Programmed gradient elution of corticosteroids by reversed phase HPLC with UV detection

Time (Minutes)	% Mobile Phase A	% Mobile Phase B	Flow Rate (ml/minute)
0	85	15	0.8
20	18	82	0.8
30	0	100	1.3
45	85	15	0.8

Mobile phase A = (50mM KH_2PO_4, 10mM acetic acid)
Mobile Phase B = (acetonitrile/mobile phase A (65:35 v/v))

2.4.2 RESULTS

2.4.2.1 *Recovery of [1,2,6,7-^{3}H]-cortisol from plasma, urine and tissue culture media*

The recovery of [1,2,6,7-^{3}H]-cortisol from plasma and urine specimens with concentrations of cortisol over the range 140-990nM and <30-1380nM respectively is illustrated in table 2.1, page 73. Mean recovery of [1,2,6,7-^{3}H]-cortisol from plasma and urine was 98.6 ± 1.0% (mean ± SD) and 98.5 ± 0.8% respectively. The recovery of [1,2,6,7-^{3}H]-cortisol from DMEM containing 10% FCS was 98.2 ± 1.0% (mean ± SD).

2.4.2.2 *Calibration, linearity and limits of detection for the analysis of corticosteroids by reversed phase HPLC with UV detection*

The chromatographic separation of selected corticosteroids with UV detection is illustrated in figure 2.14, page 81. Calibration curves were calculated using computer assisted linear regression using peak height as the calibration factor. Limits of detection were estimated to be 10.1 ± 0.9 nM (Mean ± SD) (plasma), 16.9 ± 2.9 nM (urine) and 1.6 ± 0.8 nM (DMEM) for all steroids examined.

2.4.2.3 *Recovery and analytical imprecision*

Representative chromatograms of the extracts of plasma and urine are illustrated in figures 2.15 & 2.16, page 81. Mean analytical recovery of corticosteroids from urine was found to be 95.5 ± 3.6% (mean ± SD) (100nM) and 95.5 ± 3.6% (250nM) whilst recovery from plasma was 97.8 ± 3.2% (100nM) and 96.3 ± 4.8% (250nM) and from DMEM was 98.9 ± 2.5% (100nM) and 96.5 ± 3.0% (250nM) (appendix 4). Analytical imprecision (CV%) was estimated to be 8.0 ± 2.4% (within-batch) and 11.6 ± 2.9% (between-batch) for the analysis of urine whilst analytical imprecision for plasma was 6.5 ± 1.7% (within-batch) and 8.9 ± 1.8% (between-batch) and for DMEM was 6.1 ± 1.2% (within-batch) and 8.3 ± 2.4% (between-batch) (appendix 5).

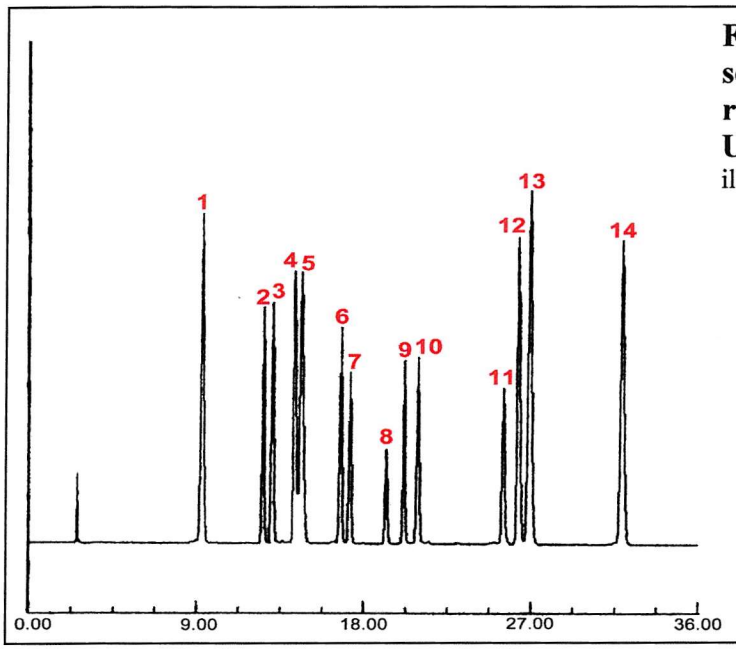


Figure 2.14 Chromatogram of selected corticosteroids by reversed phase HPLC with UV detection: The chromatogram illustrates peaks for:

1. 6β-hydroxycortsol,
2. 20α-dihydrocortisol
3. 20β-dihydrocortisol
4. 20α-dihydrocortisone
5. 20β-dihydrocortisone
6. cortisol
7. cortisone
8. dexamethasone
9. corticosterone
10. 11-deoxycortisol
11. 11-deoxycorticosterone
12. 17α-hydroxyprogesterone
13. androsteonedione
14. progesterone.

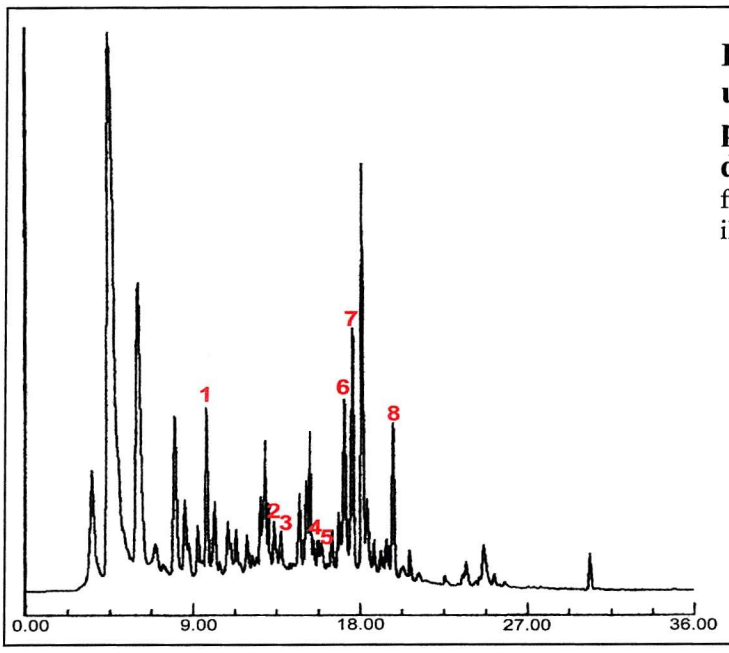


Figure 2.15 Chromatogram of urine extracts by reversed phase HPLC with UV detection: Steroids were extracted from 4ml urine. The chromatogram illustrates peaks for:

1. 6β-hydroxycortsol
2. 20α-dihydrocortisol
3. 20β-dihydrocortisol
4. 20α-dihydrocortisone
5. 20β-dihydrocortisone
6. cortisol
7. cortisone
8. dexamethasone

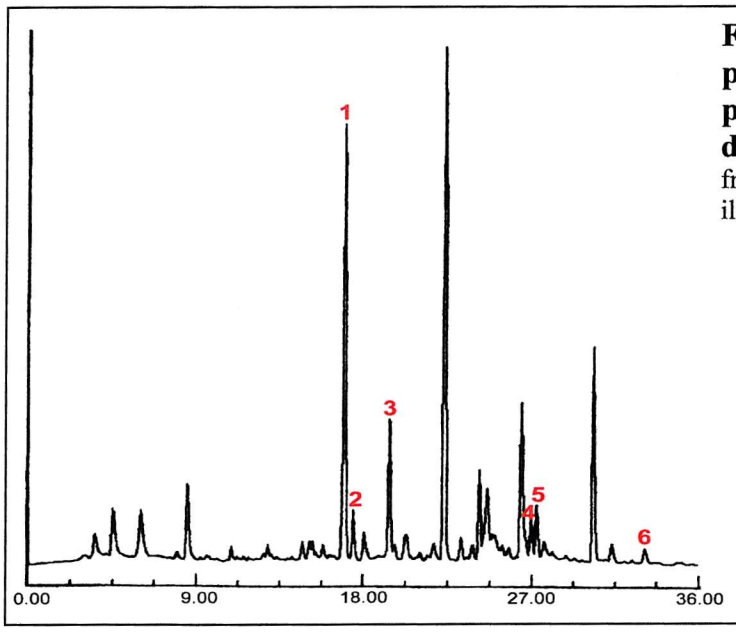


Figure 2.16 Chromatogram of plasma extracts by reversed phase HPLC with UV detection: Steroids were extracted from 4ml urine. The chromatogram illustrates peaks for:

1. cortisol
2. cortisone
3. dexamethasone
4. 17α-hydroxyprogesterone
5. androstenedione
6. progesterone

2.5 Method Development IV: Radioimmunoassay of Urinary Free Cortisone

It may be assumed that antigen/antibody interactions are governed by the law of mass action such that the equilibrium, or affinity constant (K_a), is given by the expression:-

$$Ka = \frac{k_{+1}}{k_{-1}} = \frac{[AgAb]}{[Ag]+[Ab]} \qquad (1)$$

Where k_{+1} is given as the association constant and k_{-1} is the dissociation constant, $[AgAb]$ is the molar concentration of antibody/antigen complex, $[Ag]$ is the molar concentration of free antigen and $[Ab]$ is the molar concentration of free antibody. Thus under ideal conditions, the ratio of free to bound antigen is a linear function[373]. Whilst the mathematical model of the ideal antibody/antigen interaction follows relatively simple criteria, the production of antibodies which exhibit these characteristics is considerably less straightforward.

Polyclonal antisera constitute a source of antibody that arises from a mixed population of activated B-lymphocytes and represents a source of antibody with mixed affinity constant. In contrast, monoclonal antisera are the product of a clonal cell line which expresses antibody with a uniform affinity constant. Whilst many excellent monoclonal antisera have been raised against steroids, few have demonstrated the degree of specificity and affinity of their polyclonal counterparts: a phenomenon due, at least in part, to a degree of co-operativity between antibodies in polyclonal antisera with different clonal ancestry. Therefore it may be more appropriate to discuss the binding phenomena of polyclonal antisera in terms of avidity rather than affinity, since antibody avidity may be considered to be the sum of the effects of several binding reactions with a given antigen[374]. Consequently, the development of immunoassays with the potential for the greatest sensitivity require antisera which demonstrate maximum avidity for the overall binding reaction whilst maintaining only marginal cross reactivity with structurally related antigenic species. Thus, in accordance with the law of mass action the

maximum potential sensitivity of a competitive immunoassay is given by the expression:-

$$[AbAg]/[Ag] = -K_a[AbAg](1-[AbAg])^2 \qquad (2)$$

and is achieved when the ratio of bound to free antigen is 0.3 [360].

2.5.1 METHODS

2.5.1.1 *Antibody titration and radioimmunoassay of urinary free cortisone*

Polyclonal rabbit anti-cortisone (N137) raised against 21-acetyl-cortisone-3-carboxymethyloxime-keyhole limpet haemocyanin conjugate emulsified in Freund's adjuvant was supplied by Dr. P Wood (Endocrinology Unit, Department Of Chemical Pathology, Southampton General Hospital, UK). N137 was diluted 1:50 in tris-BSA (50mM tris[hydroxymethyl]aminomethane pH 7.5 containing 0.5% (w/v) bovine serum albumin). 1ml aliquots were freeze dried and stored at –20°C. [^{125}I]-cortisone was synthesised using the chloramine-T method of Hunter and Greenwood[375] whereby 21-acetyl-cortisone-3-carboxymethyloxime was linked to [^{125}I]-histamine, purified by thin layer chromatography and stored in absolute ethanol at 4°C. A stock solution containing cortisone (1mM) in absolute ethanol was used to prepare a working standard containing cortisone (500nM) in tris-BSA. 500µl of tris-BSA or working standard were extracted by repeated inversion with 5ml dichloromethane. The phases were separated by centrifugation (3000 x g, 5 minutes) and the upper, aqueous layer was aspirated to waste. Duplicate 400µl aliquots of the remaining organic phase were dispensed into 5 x 60mm glass tubes (L.I.P (Equipment & Services) Ltd. UK) and evaporated to dryness under a stream of dry nitrogen. 300µl [^{125}I]-cortisone (10nCi/300µl) in tris-BSA, 100µl N137 (serial dilutions 1:625-1:20,000 in tris-BSA) was added to the tubes which were incubated at 37°C for two hours. Antibody bound [^{125}I]-cortisone was separated by the addition of 100µl Donkey-anti-sheep/goat SacCel (IDS Ltd, Bolden, Tyne & Wear, UK) to all tubes which were incubated at 25°C for 30 minutes. 1ml H_2O was added to all tubes which were then centrifuged (3000 x g, 10minutes) at 4°C. The supernatant was aspirated to waste and bound [^{125}I]-cortisol was estimated using a model 1260 multi-gamma counter (Pharmacia Wallac Ltd, UK).

2.5.1.2 *The effect of solvent upon liquid/liquid extraction efficiency*

500µl of cortisone (100nM and 250nM) in tris-BSA were dispensed into duplicate 12 x 100mm, glass, wide necked, screw topped tubes. 500µl tris-BSA was added to all tubes. 5ml of (i) dichloromethane, (ii) chloroform, (iii) diethyl-ether, (iv) ethyl-acetate or (v)

ethyl-acetate:diethyl-ether (4:1 v/v) was added to each tube. Extraction and radioimmunoassay was performed using N137 at a dilution of 1:5000 (2.5.1.1). The assay was standardised against serial doubling dilutions of cortisone (500 – 7.8nM) prepared using absolute ethanol as solvent. 40µl of each solution was dispensed into duplicate 5 x 60mm glass tubes, evaporated to dryness under a stream of dry nitrogen and assayed directly (without extraction) by radioimmunoassay (2.5.1.1).

2.5.1.3 *Recovery of cortisone from urine*

5nmol and 10nmol cortisone was added to duplicate 100ml aliquots from ten urine specimens of varying composition and with urinary free cortisone in the range 50-210nM (measured by HPLC with fluorescence detection) which were extracted with 5ml chloroform and analysed by radioimmunoassay using N137 at a dilution of 1:5000 (2.5.1.1). The assay was standardised against serial doubling dilutions of cortisone (500 – 7.8nM) prepared using tris-BSA as solvent. 500µl of each solution was extracted and assayed as described above.

2.5.1.4 *Evaluation of the cross reactivity of rabbit-anti-cortisone antiserum N137*

Antibody cross reactivity was estimated by Mrs C Glenn (Endocrinology Unit, Department Of Chemical Pathology, Southampton General Hospital, UK). Cross reactivity was defined as: ([*SpAg*]/[*CrAg*]) x 100; where [*SpAg*] was defined as the amount of cortisone required to produce a 50% displacement of bound [^{125}I]-cortisone and [*CrAg*] was defined as the cross reacting antigen required to reduce binding to the same degree.

2.5.1.5 *An estimation of N137 immunoreactivity attributable to urinary free cortisone*

[1,2,6,7-^{3}H]-cortisone was synthesised from [1,2,6,7-^{3}H]-cortisol (section 3.2.3). 40,000dpm/ml [1,2,6,7-^{3}H]-cortisone was added to six urine specimens of varying composition and with urinary free cortisone in the range 40-500nM (assayed by HPLC with fluorescence detection (figure 2.6, page 69)). 50µl of each specimen was placed into

20ml glass scintilation vials containing 4ml Optiphase (Pharmacia Wallac, UK) and analysed by liquid scintigraphy (2.3.1.6). 500µl of each specimen was extracted with 5ml chloroform (2.5.1.1) and assayed for immunoreactive cortisone (2.5.1.1). 800µl of the organic extract was dispensed into duplicate 5 x 60mm glass tubes and evaporated to dryness under a stream of dry nitrogen, reconstituted in 20µl chloroform and spotted onto general-purpose silica-coated TLC plates (Sigma, UK) which were developed in chloroform/ethanol (92:8 v/v) and scanned using a Bioscan 200 imager (Perkin Elmer, USA). The area corresponding with [1,2,6,7-^{3}H]-cortisone was excised from each TLC plate and eluted with 3 x 10ml absolute ethanol. The silica was removed by centrifugation (3000 x g, 5minutes). The supernatant was filtered through glass wool and evaporated to dryness under a stream of dry nitrogen. The residue was reconstituted in 1ml tris-BSA and split into two equal fractions, one of which was assayed for immunoreactive cortisone (2.5.1.1) whilst the second was analysed by liquid scintigraphy (2.3.1.6).

2.5.1.6 *Linearity, limits of detection and analytical imprecision*

Serial dilutions of three urine specimens (urinary free cortisone = 450, 385 and 260nM (estimated by HPLC with fluorescence detection (figure 2.6, page 69))) were assayed for immunoreactive cortisone (2.5.1.2). Within-batch imprecision was estimated by the analysis of ten replicates of three urine specimens. Between-batch imprecision was calculated from the mean coefficient of variation of duplicate analyses across the working range of the assay (10-500nM). The limit of detection was defined as: the measured mean plus two standard deviations divided by the initial slope of the dose response curve for 20 replicates at a cortisone concentration of 0nM [373].

2.5.1.7 *Radioimmunoassay of urinary free cortisol*

This was originally developed at the Endocrinology Unit, Department Of Chemical Pathology, Southampton General Hospital, UK[364]. 500µl urine buffered with 500µl tris-BSA was extracted with 5ml dichloromethane (2.5.1.1). 100µl [^{125}I]-cortisol (10nCi/100µl) in tris-BSA and 100µl of polyclonal sheep-anti-cortisol HP/S/631 (Guildhay, Guildford, Sussex, UK) diluted 1:2000 in tris-BSA, was added to the extracts

which were incubated at 37°C for an hour. Antibody bound [^{125}I]-cortisol was separated by the addition of 100µl Donkey-anti-sheep/goat SacCel as previously described (2.5.1.1).

2.5.2 RESULTS

2.5.2.1 *Antibody titration, extraction efficiency and recovery of cortisone from urine*

Maximum displacement of 10nCi [^{125}I]-cortisone from N137 by unlabeled cortisone (500nM) was achieved at an antibody dilution of 1:5,000 (figure 2.17, page 89). The extraction of cortisone (100nM and 250nM) was estimated to be 98.7 ± 1.3%, 94.6 ± 3.1%, 85.5 ± 1.3%, 76.2 ± 1.1% and 23.8 ± 0.5% (mean ± SD), using chloroform, dichloromethane, ethyl-acetate, ethyl-acetate:diethyl-ether (4:1 v/v) and diethyl-ether as solvent respectively. Maximum recovery was obtained using 5ml chloroform as extraction solvent and was used throughout the remainder of these studies. Recovery of 50nM and 100nM cortisone added to ten urine specimens was 94.5 ± 3.9% and 94.0 ± 4.2% respectively.

2.5.2.2 *Cross reactivity and immunoreactivity attributable to urinary free cortisone*

Specific antigenic cross reactivity with antiserum N137 was estimated to be 100% with 5α-tetrahrdocortisone, 20% with prednisolone, 18% with 5β-dihydrocortisone, 1.1% with 17α-hydroxy-11-keto-pregnanolone, 1.0% with 11-dehydrocorticosterone and 0.5% with 5β-tetrahydrocortisone. All other steroids examined in this study showed a cross reactivity of <0.1%. Immunoreactivity attributable to cortisone was estimated to be 90.7 ± 11.0% (mean ± SD) over the range 41 - 488nM (table 2.3, page 88). Serial doubling dilutions of three urine specimens (urinary free cortisone = 450, 385 and 260nM) demonstrated no appreciable deviation from linearity (r^2 = 0.998 ± 0.003, mean ± SD).

2.5.2.3 *Limits of detection and analytical imprecision*

Limits of detection for the radioimmunoassay of urinary free cortisone by radioimmunoassay with N137 were estimated to be 6nM. Within-batch imprecision

(CV%) was estimated to be 4.8, 6.7 and 8.4% (urinary free cortisone = 400, 250 and 70nM) whilst between-batch imprecision (CV%) was less than 15% across the analytical range (10-500nM) (figure 2.18, page 89).

Table 2.3 An estimation of N137 immunoreactivity attributable to urinary free cortisone.

Immunoreactive cortisone (nM)	Immunoreactivity attributable to urinary free cortisone (%)	Immunoreactivity not attributable to cortisone (nM)
41	72.8	11.2
102	86.8	13.5
125	86.9	16.4
248	98.1	4.7
301	103.8	-
488	95.7	21

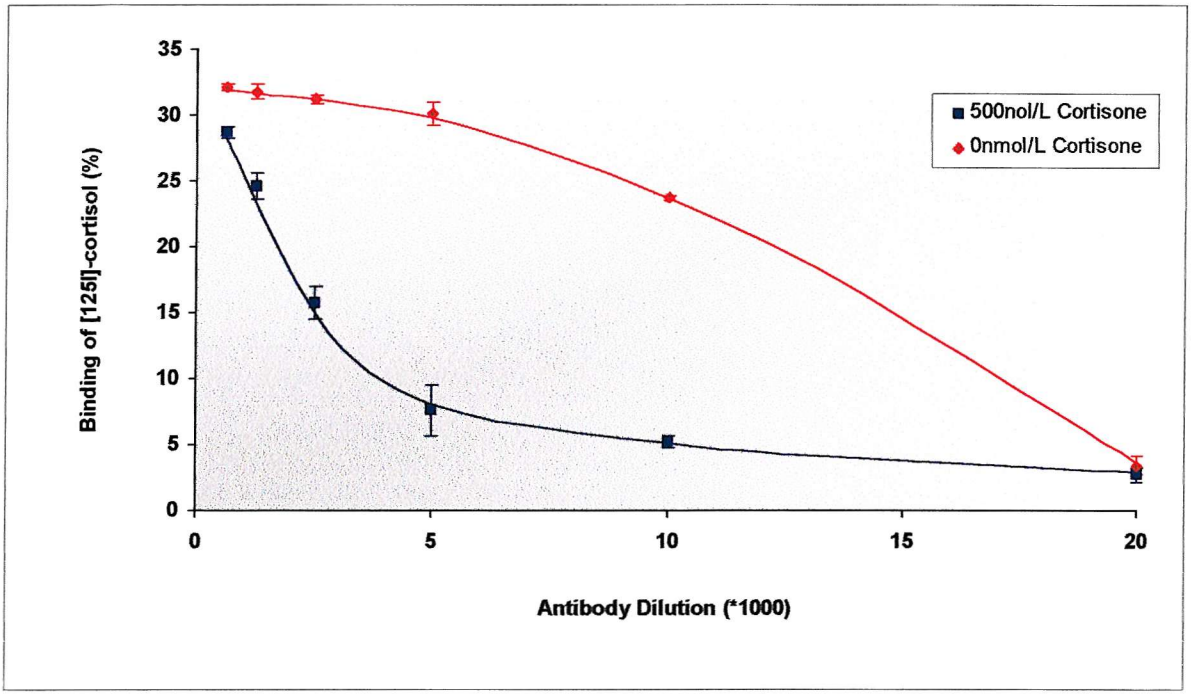


Figure 2.17 Antibody titration curve for antiserum N137: Maximum displacement of [^{125}I]-cortisol (10nCi) by 500nM cortisol was achieved at an antibody dilution of 1:5000.

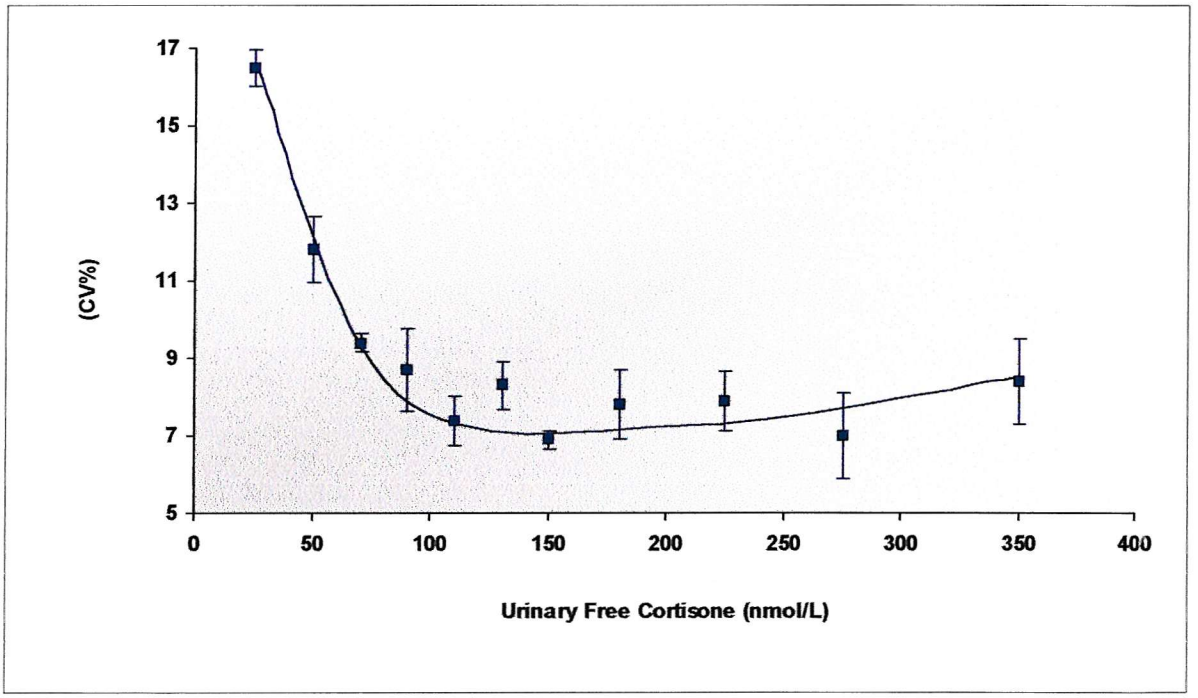


Figure 2.18 Imprecision profile for the radioimmunoassay of urinary free cortisone.

2.6 Method Development V: Methodological Comparisons

2.6.1 METHODS

2.6.1.1 *Comparison of urinary free cortisol and cortisone by HPLC and RIA*

24 hour urine collections from a cohort of 302 male and 189 female volunteers were supplied by Dr. DI Phillips, MRC Unit, Southampton General Hospital, UK. Urine samples were stored at -70°C in 5ml aliquots prior to analysis. Of these, 54 specimens were selected at random and urinary free cortisol, cortisone and cortisol:cortisone ratios were estimated by HPLC with fluorescence detection (figure 2.6, page 69), HPLC with UV detection (2.4.1.1) and RIA (2.5.1.1 & 2.5.1.7). The remaining specimens were analysed by RIA and the data were used to construct reference intervals. Statistical comparison was made using Deming linear regression[376], Spearman rank correlation coefficients, Wilcoxon signed ranks test for paired data. Difference analysis was performed using the method of Bland and Altman[377].

2.6.1.2 *Comparison of urinary free cortisol:cortisone with total cortisol:cortisone metabolite ratios*

Eight 24 hour urine collections from each of 7 adult hypopituitary patients (three male and four female age range 47-64 years) with combined growth hormone and ACTH deficiency were supplied by Dr. N Taylor, Department of Clinical Biochemistry, Kings College Hospital, UK. Prior to commencing the study, all patients were receiving hydrocortisone (5-20mg at 06:00, 12:00 and 18:00 hrs) and thyroxine replacement. Four of the patients (male:female ratio 1:1) were also receiving sex steroid replacement. All patients gave written informed consent and the study was approved by the Royal London Hospital Ethical Committee

On commencement of the study, the patients were given increasing doses of hydrocortisone (twice daily: on rising and at 18:00hrs), 10mg twice daily for the first week, 20mg in the morning and 10mg in the evening for the second week, and 40mg in

the morning and 20mg in the evening for the third week. At the end of each week, 24 hour urine specimens were collected.

Growth hormone treatment was then commenced at a dose of 0.125 units/kg/week as a single, evening, abdominal injection for 4 weeks. The growth hormone dose was increased to 0.25 units/kg/week for the remainder of the study (11 weeks).

Eight weeks after commencement of growth hormone treatment during which time the patients had received their normal maintenance hydrocortisone replacement, the dose of hydrocortisone was again adjusted at weekly intervals and 24 hour urine specimens were collected as previously described. However, changes in the hydrocortisone dose were randomised in order to obviate possible effects of prolonged growth hormone therapy. Urine samples were stored at -20°C prior to analysis.

Urinary steroid metabolite profiles were measured by gas chromatography[378]. Total 11-hydroxy-cortisol metabolites was calculated as the sum: (THF + 5α-THF + α-cortol + (β-cortol + β-cortolone)*0.5). 11-oxo-cortisol metabolites was calculated as the sum: (THE + α-cortolone + (β-cortol + β-cortolone)*0.5). Urinary free cortisol:cortisone ratios were estimated by HPLC with fluorescence detection (figure 2.6, page 69) and radioimmunoassay (2.5.1.1 and 2.5.1.6). Statistical comparison between analytical techniques was made using Deming linear regression[376], difference analysis, Spearman rank correlation coefficients and the Wilcoxon-Mann-Whitney test for two independent samples. Comparison of the effects of hydrocortisone and growth hormone replacement upon urinary free cortisol:cortisone ratios and total cortisol:cortisone metabolite ratios was made using the Wilcoxon signed rank test.

2.6.2 RESULTS

2.6.2.1 *Comparison of urinary free cortisol and cortisone by HPLC and RIA*

Urinary free cortisol measured in 54 twenty-four hour urine collections was 113.8 ± 6.4nM (mean ± SD) with a range of concentrations spanning 6.5 – 805nM. Urinary free cortisone was 110.8 ± 1.9nM with a range of 8.7 – 341nM. Close agreement (indicated by Spearman correlation) was observed between all analytical techniques ($P < 0.0001$) (table 2.4, page 93) which was confirmed by difference analysis and the Wilcoxon matched pairs signed ranks test which suggested that there were no statistically significant differences between urinary free cortisol or cortisone measured by either HPLC with fluorescence detection, HPLC with UV detection or RIA (table 2.5, page 93 & figure 2.19, page 95). Moreover, difference plots also suggested that there were no concentration dependent differences between measurements by any of the analytical techniques examined. Neither was there evidence of methodological bias (figure 2.20, page 96). However, difference plots did suggest that the greatest methodological differences were between urinary free cortisol concentrations estimated by RIA and HPLC with either UV or fluorescence detection.

Similarly, estimation of urinary free cortisol:cortisone ratios by HPLC with fluorescence detection, HPLC with UV detection or RIA showed close agreement (table 2.5, page 94) which was also confirmed by difference analysis and the Wilcoxon matched pairs signed ranks test. (table 2.6, page 94 & figure 2.21, page 97).

2.6.2.2 *Reference interval for urinary free cortisol and cortisone and the urinary free cortisol:cortisone ratio estimated by RIA*

After logarithmic transformation, the reference interval for urinary free cortisol was 102.6 (21.2 – 480.3) nM (mean and 95% confidence interval) and 63.0 (8.7 – 309.3) nM for urinary free cortisone. The reference interval for the urinary free cortisol:cortisone ratio was 0.84 (0.56 – 1.24).

Table 2.4 Correlation between urinary free cortisol and cortisone estimated by radioimmunoassay, HPLC with fluorescence detection and HPLC with UV detection

	RIA	HPLC (UV)
Urinary Free Cortisol		
HPLC (UV)	$r_s = 0.92$ (0.87 – 0.95)	*
HPLC (Fluorescence)	$r_s = 0.89$ (0.82 – 0.94)	$r_s = 0.98$ (0.97 – 0.99)
Urinary Free Cortisone		
HPLC (UV)	$r_s = 0.92$ (0.87 – 0.95)	*
HPLC (Fluorescence)	$r_s = 0.89$ (0.82 – 0.94)	$r_s = 0.98$ (0.97 – 0.99)

Data illustrates Spearman rank correlation coefficient and 95% confidence intervals for 54 paired analyses (urinary free cortisol, 6.5 – 805nM, urinary free cortisone, 8.7 – 341nM)
$P < 0.0001$ for all observations.

Table 2.5 Differences between urinary free cortisol and cortisone estimated by radioimmunoassay, HPLC with fluorescence detection and HPLC with UV detection.

	RIA	HPLC (UV)
Urinary Free Cortisol		
HPLC (UV)	5.10 (-0.75 – 12.55), $P = 0.0843$	*
HPLC (Fluorescence)	7.05 (-1.10 – 14.45), $P = 0.0923$	0.60 (-2.15 – 3.65), $P = 0.6920$
Urinary Free Cortisone		
HPLC (UV)	1.60 (-0.65 – 3.90), $P = 0.1579$	*
HPLC (Fluorescence)	1.80 (-0.45 – 3.65), $P = 0.1140$	-0.45 (-2.35 – 1.60), $P = 0.6719$

Data illustrates difference between the means and 95% confidence intervals and the statistical significance of the differences using the Wilcoxon matched pairs signed ranks test for 54 analyses (urinary free cortisol, 6.5 – 805nM, urinary free cortisone, 8.7 – 341nM)

Table 2.6 Correlation between urinary free cortisol:cortisone ratios estimated by radioimmunoassay, HPLC with fluorescence detection and HPLC with UV detection

	RIA	HPLC (UV)
Spearman correlation		
HPLC (UV)	$r_s = 0.81$ (0.76 – 0.86)	*
HPLC (Fluorescence)	$r_s = 0.78$ (0.72 – 0.84)	$r_s = 0.94$ (0.91 – 0.97)

Data illustrates Spearman rank correlation coefficient and 95% confidence intervals for 54 paired analyses.
$P < 0.0001$ for all observations.

Table 2.7 Differences between urinary free cortisol:cortisone ratios estimated by radioimmunoassay, HPLC with fluorescence detection and HPLC with UV detection.

	RIA	HPLC (UV)
Wilcoxon signed ranks		
HPLC (UV)	0.016 (-0.018 – 0.50), $P = 0.6303$	*
HPLC (Fluorescence)	0.030 (-0.011 – 0.71), $P = 0.3944$	0.014 (-0.019 – 0.47), $P = 0.4535$

Data illustrates difference between the means and 95% confidence intervals and the statistical significance of the differences using the Wilcoxon matched pairs signed ranks test for 54 analyses.

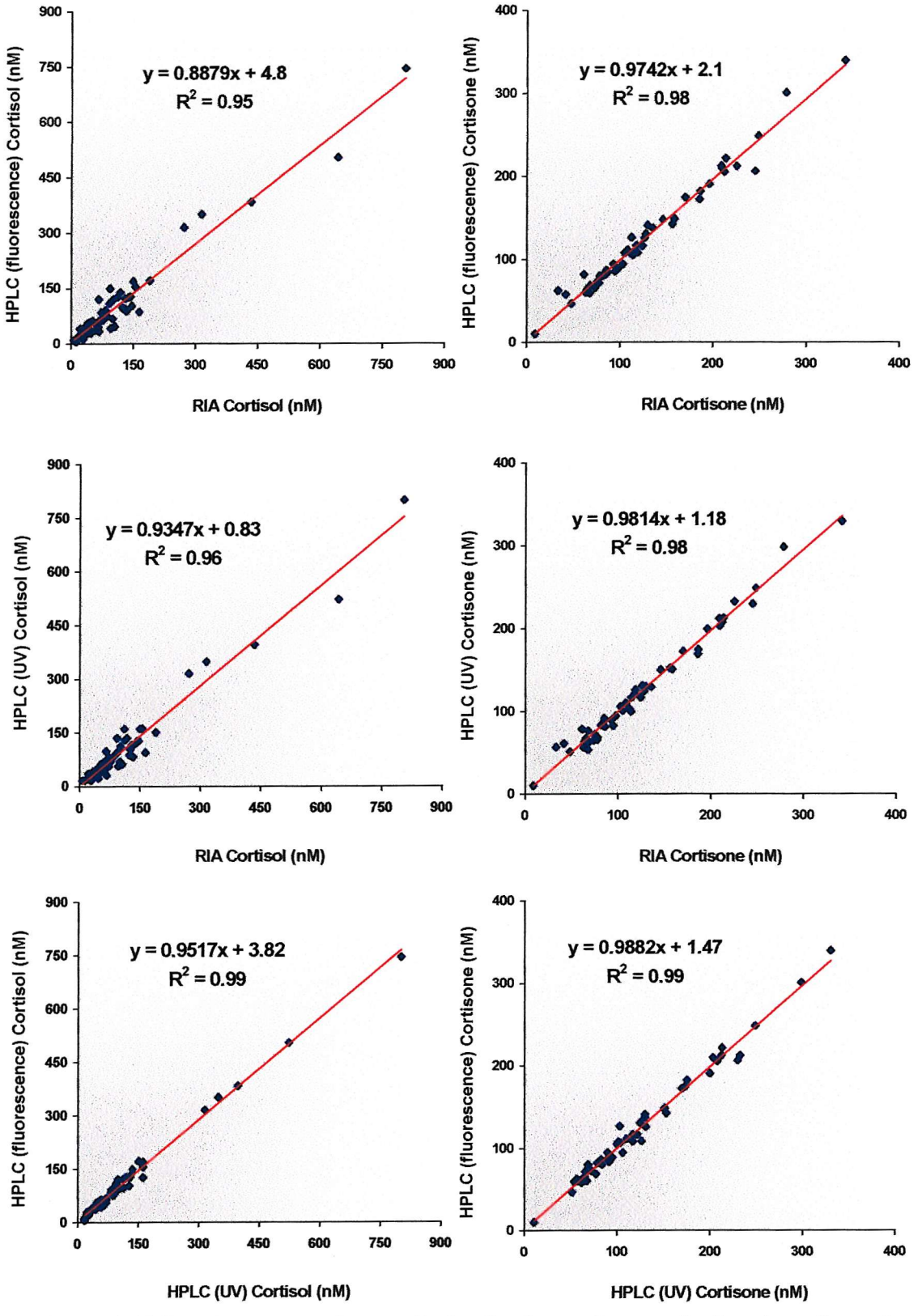


Figure 2.19 Deming regression analysis of urinary free cortisol and cortisone by HPLC with fluorescence detection, UV detection and radioimmunoassay:
Comparisons were made using 54 paired analyses (urinary free cortisol, 6.5 – 805nM, urinary free cortisone, 8.7 – 341nM).

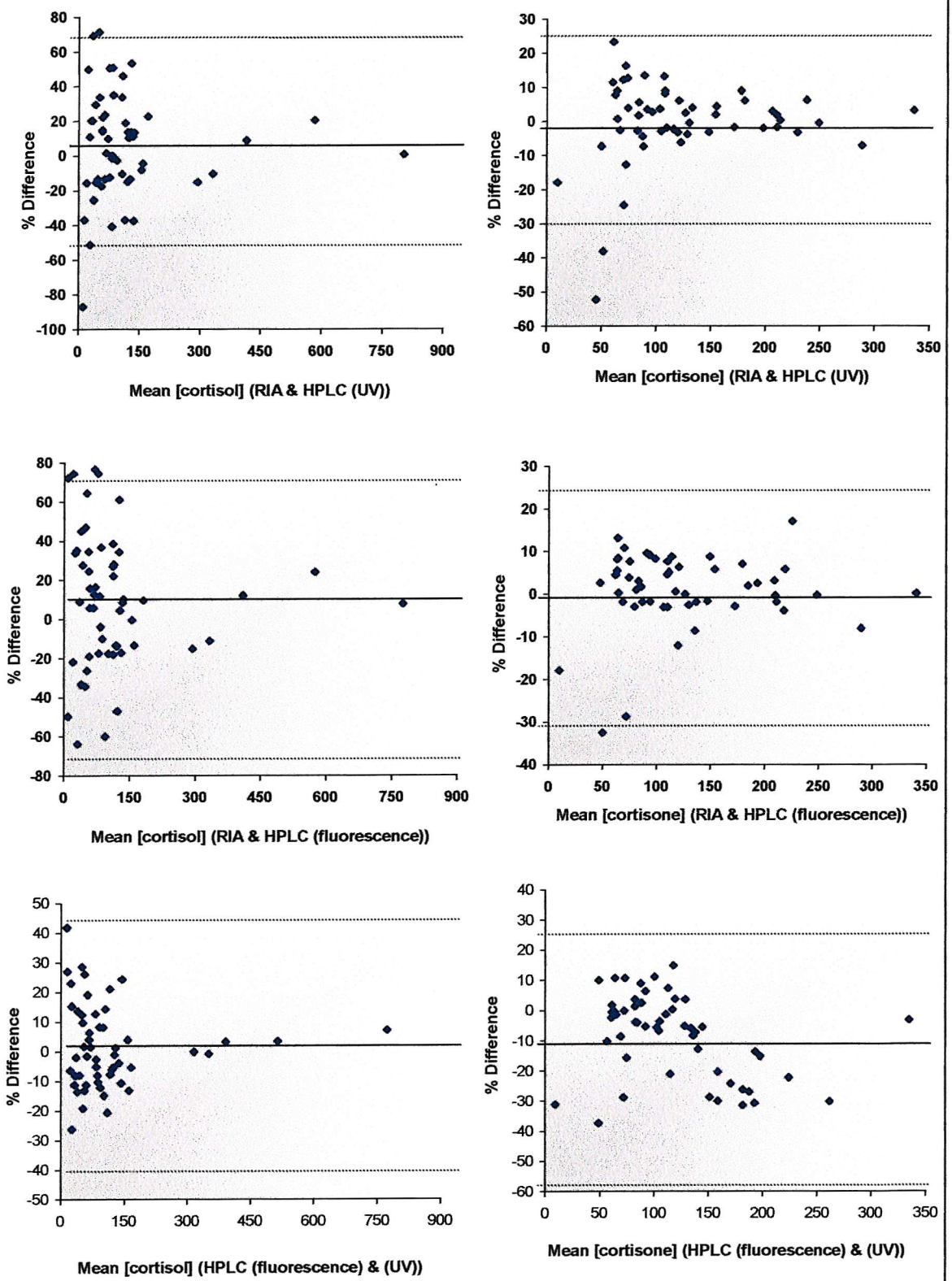


Figure 2.20 Difference analysis of urinary free cortisol and cortisone by HPLC with fluorescence detection, UV detection and radioimmunoassay: plots show the differences between 54 paired analyses measured by HPLC with UV detection, HPLC with fluorescence detection and radioimmunoassay. Solid lines represent mean difference, dashed lines represent limits of agreement.

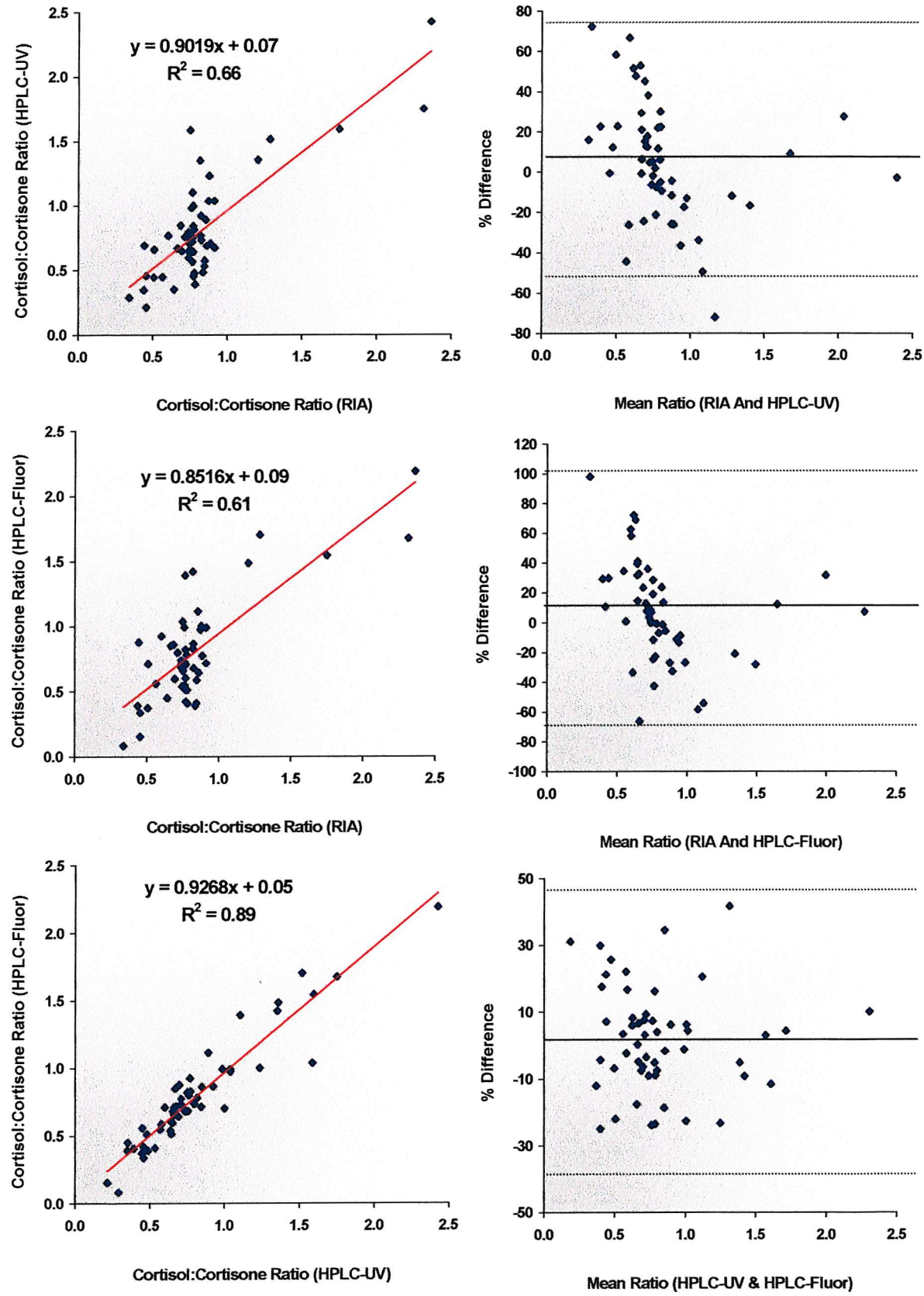


Figure 2.21 Deming regression and difference analysis of urinary free cortisol:cortisone ratios by HPLC with fluorescence detection and radioimmunoassay: plots show the Deming regression and differences between 54 paired cortisol:cortisone ratios measured by HPLC with UV detection, HPLC with fluorescence detection and radioimmunoassay. Solid lines represent mean difference, dashed lines represent limits of agreement.

2.6.2.3 *Comparison of urinary free cortisol:cortisone with total cortisol:cortisone metabolite ratios*

The Wilcoxon-Mann-Whitney test for two independent samples revealed a significant difference between the urinary free cortisol:cortisone ratio and total cortisol:cortisone metabolite ratio (difference between the medians and 95% confidence interval = 0.205 (0.054 – 0.336), $P < 0.05$). There was a weak but significant correlation between the urinary free cortisol:cortisone ratio (estimated by both RIA and HPLC with fluorescence detection) and total cortisol:cortisone metabolite ratio (table 2.8, page 100 and figure 2.22, page 101). However, this correlation was significantly weakened by the marked and unique disparity observed between the urinary free cortisol:cortisone ratio and total cortisol:cortisone metabolite ratio in the urine specimens from patient one (figure 2.23, page 102). Omission of these data from the statistical analysis improved the apparent correlation between urinary free cortisol:cortisone ratio and total cortisol:cortisone metabolite ratio (figure 2.22, page 101).

Difference plots revealed a marked difference between the urinary free cortisol:cortisone ratio and total cortisol:cortisone metabolite ratio (figure 2.22, page 101). Moreover, the magnitude of the difference between the urinary free cortisol:cortisone ratio and total cortisol:cortisone metabolite ratio increased in parallel with the total cortisol:cortisone metabolite ratio (figure 2.22, page 102). These data are consistent with the empirical observation that changes in the total cortisol:cortisone metabolite ratio were mirrored by greater changes in the urinary free cortisol:cortisone ratio (figures 2.22 & 2.23, pages 101 & 102).

Incremental doses of hydrocortisone prior to growth hormone treatment resulted in a significant increase in the total cortisol:cortisone metabolite ratio ($P < 0.001$) which was mirrored by greater increases the urinary free cortisol:cortisone ratio ($P < 0.001$) (table 2.9, page 103). Parallel changes were also observed in urinary free cortisol and urinary free cortisone (table 2.10, page 104). In contrast, incremental doses of hydrocortisone subsequent to growth hormone treatment did not result in the increase in the total cortisol:cortisone metabolite ratio or urinary free cortisol:cortisone ratio which had been

observed prior to growth hormone treatment (table 2.9, page 103). Indeed, there were no statistically significant changes in total cortisol:cortisone metabolite ratios or urinary free cortisol:cortisone ratios subsequent to growth hormone treatment despite increasing doses of hydrocortisone. Thus, statistical analysis comparing total cortisol:cortisone metabolite ratios prior to and subsequent to growth hormone treatment revealed that, with the exception of hydrocortisone dose 1, growth hormone treatment appeared to attenuate the increase in total cortisol:cortisone metabolite ratio which accompanied increases in replacement hydrocortisone prior to growth hormone treatment.

In contrast, statistical analysis comparing urinary free cortisol:cortisone ratios prior to and subsequent to growth hormone treatment revealed that growth hormone treatment appeared to significantly increase the urinary free cortisol:cortisone ratio at hydrocortisone dose 1 ($P < 0.05$) whilst growth hormone treatment appeared to have had no significant effect upon the urinary free cortisol:cortisone ratio at hydrocortisone dose 2. However, at hydrocortisone dose 3, growth hormone treatment appeared to significantly decrease in the urinary free cortisol:cortisone ratio ($P < 0.05$).

Summary:

1. increasing doses of hydrocortisone prior to growth hormone treatment resulted in a statistically significant increase in both total cortisol:cortisone metabolite ratio and urinary free cortisol:cortisone ratio
2. increasing doses of hydrocortisone subsequent to growth hormone treatment had no statistically significant effect upon either the total cortisol:cortisone metabolite ratio or the urinary free cortisol:cortisone ratio
3. growth hormone treatment attenuated the increase in total cortisol:cortisone metabolite ratio which accompanied increasing doses of hydrocortisone prior to growth hormone treatment
4. growth hormone treatment increased in the urinary free cortisol:cortisone ratio at dose 1, had no effect at dose 2 and increased the urinary free cortisol:cortisone ratio at dose 3.

Table 2.8 Correlation between urinary free cortisol:cortisone ratios and total cortisol metabolite ratios in seven hypopituitary patients receiving hydrocortisone and growth hormone therapy

	Spearman correlation
HPLC (Fluorescence)	$r_s = 0.37$ (0.11 – 0.59), $P < 0.01$
HPLC (Fluorescence) (patient 1 removed)	$r_s = 0.62$ (0.39 – 0.77), $P < 0.0001$
RIA	$r_s = 0.34$ (0.08 – 0.57), $P < 0.02$
RIA (patient 1 removed)	$r_s = 0.57$ (0.33 – 0.74), $P < 0.0001$

Data illustrates Spearman rank correlation coefficient and 95% confidence intervals $P < 0.0001$ for all observations. Marked disparity was observed between urinary free cortisol:cortisone ratios and total cortisol metabolite ratios for patient one. Thus the statistical analysis was performed both including and omitting these data.

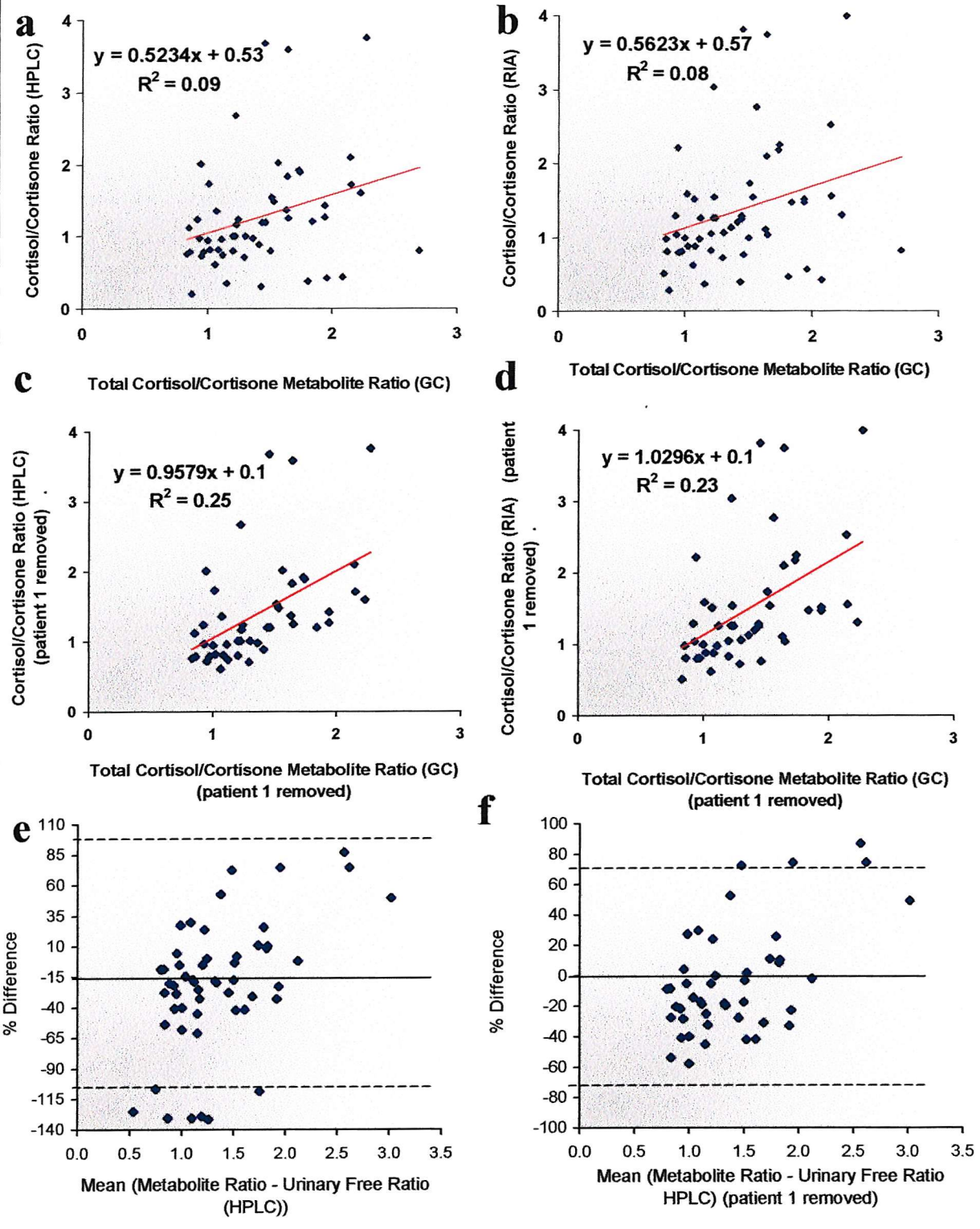


Figure 2.22 Correlation between urinary free cortisol:cortisone ratios and total cortisol:cortisone metabolite ratios in hypopituitary patients receiving combined growth hormone an hydrocortisone repalcement. Deming regression (a-d) and difference plots (e-f) for the analysis of urinary free cortisol:cortisone ratios measured by HPLC with fluorescence detection and radioimmunoassay and total cortisol:cortisone metabolite ratios. Urine specimens were from adult hypopituitary patients with combined growth hormone and ACTH deficiency prior to and after growth hormone therapy receiving varying doses of hydrocortisone replacement: Marked disparity was observed between urinary free cortisol:cortisone ratios and total cortisol metabolite ratios for patient one. Thus the statistical analysis was performed both including and omitting these data. Solid lines represent mean difference, dashed lines represent limits of agreement.

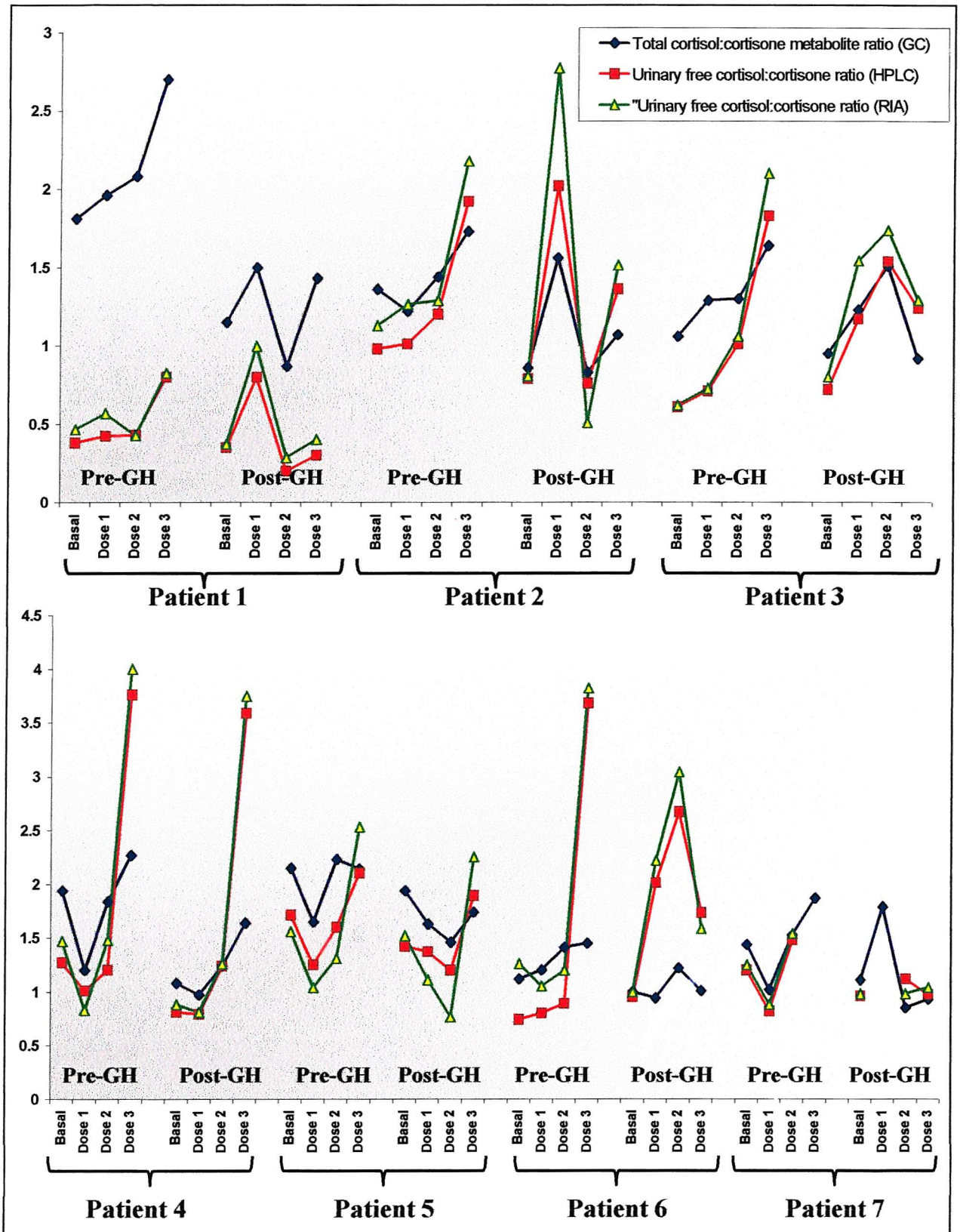


Figure 2.23 Comparison of urinary free cortisol:cortisone ratios with total cortisol:cortisone metabolite ratios. plots show urine cortisol:cortisone and total metabolite ratios from adult hypopituitary patients with combined growth hormone and ACTH deficiency before and after growth hormone therapy (GH) during varying doses of hydrocortisone replacement: dose 1 = 10mg bd; dose 2 = 20mg a.m. 10mg p.m.; dose 3 = 40mg a.m. 20mg p.m.

Table 2.9 The effect of hydrocortisone and growth hormone replacement on urinary free cortisol:cortisone ratios and total cortisol:cortisone metabolite ratios.

	Urinary Free Cortisol:Cortisone Ratio	Total Cortsiol:Cortisone Metabolite Ratio
Before Growth Hormone		
Dose 1	0.82 (0.42 –1.25)	1.22 (1.02 – 1.96)
Dose 2	1.20 (0.43 – 1.60)[a]	1.53 (1.30 – 2.23)[a]
Dose 3	2.01 (0.80 – 3.76)[bc]	1.87 (1.45 – 2.70)[bc]
After Growth Hormone		
Dose 1	1.27 (0.79 – 2.02)[g]	1.50 (0.74 – 1.79)[m]
Dose 2	1.20 (0.20 – 2.67)[dh]	1.22 (0.87 – 1.51)[jn]
Dose 3	1.36 (0.30 – 3.59)[efi]	1.07 (0.92 – 1.74)[klo]

Data illustrates median (range). Urinary free cortisol:cortisone ratios were estimated by radioimmunoassay whilst total cortisol:cortisone metabolite ratios were estimated by gas chromatography

Hydrocortisone replacement: dose 1 = 10mg bd; dose 2 = 20mg a.m. 10mg p.m.; dose 3 = 40mg a.m. 20mg p.m.

Prior to growth hormone treatment:

dose 1 vs dose 2: $^{a}P = 0.016$, dose 1 vs dose 3: $^{b}P = 0.016$, dose 2 vs dose 3: $^{c}P = 0.047$

Subsequent to growth hormone treatment:

dose 1 vs dose 2: $^{d}P = 1.000$, $^{j}P = 0.469$, dose 1 vs dose 3: $^{e}P = 1.000$, $^{k}P = 0.688$, dose 2 vs dose 3: $^{f}P = 0.688$, $^{l}P = 0.469$

Prior to growth hormone treatment vs subsequent to growth hormone treatment:

dose 1: $^{g}P = 0.047$, dose 2: $^{h}P = 0.688$ dose 3: $^{i}P = 0.031$,

dose 1: $^{m}P = 0.815$,dose 2: $^{n}P = 0.047$, dose 3: $^{o}P = 0.016$

Table 2.10 The effect of hydrocortisone and growth hormone replacement on urinary free cortisol and cortisone.

	Urinary Free Cortisol (nmol/24hrs)	Urinary Free Cortisone (nmol/24hrs)
Before Growth Hormone		
Dose 1	128.2 (71.3 – 383.0)	150.9 (41.8 – 539.4)
Dose 2	238.6 (128.4 – 601.4)[a]	237.9 (118.9 – 505.2)[a]
Dose 3	850.5 (512.7 – 3589.7)[bc]	492.1 (244.0 – 957.1)[bc]
After Growth Hormone		
Dose 1	454.9 (94.1 – 1034.9)[g]	348.0 (68.7 – 608.4)[m]
Dose 2	230.7 (61.4 – 1099.1)[dh]	303.5 (39.9 – 408.8)[jn]
Dose 3	156.6 (90.4 – 2914.6)[efi]	289.7 (90.4 – 819.6)[klo]

Data illustrates median (range). Urinary free cortisol and cortisone ratio was estimated by radioimmunoassay.

Hydrocortisone replacement: dose 1 = 10mg bd; dose 2 = 20mg a.m. 10mg p.m.; dose 3 = 40mg a.m. 20mg p.m.

Prior to growth hormone treatment:

dose 1 vs dose 2: $^{a}P = 0.016$, dose 1 vs dose 3: $^{b}P = 0.031$, dose 2 vs dose 3: $^{c}P = 0.031$

Subsequent to growth hormone treatment:

dose 1 vs dose 2: $^{d}P = 0.688$, $^{j}P = 0.438$, dose 1 vs dose 3: $^{e}P = 0.844$, $^{k}P = 0.844$, dose 2 vs dose 3: $^{f}P = 0.219$, $^{l}P = 0.469$

Prior to growth hormone treatment vs subsequent to growth hormone treatment:

dose 1: $^{g}P = 0.156$, dose 2: $^{h}P = 0.813$, dose 3: $^{i}P = 0.063$,

dose 1: $^{m}P = 0.313$, dose 2: $^{n}P = 0.688$, dose 3: $^{o}P = 0.094$

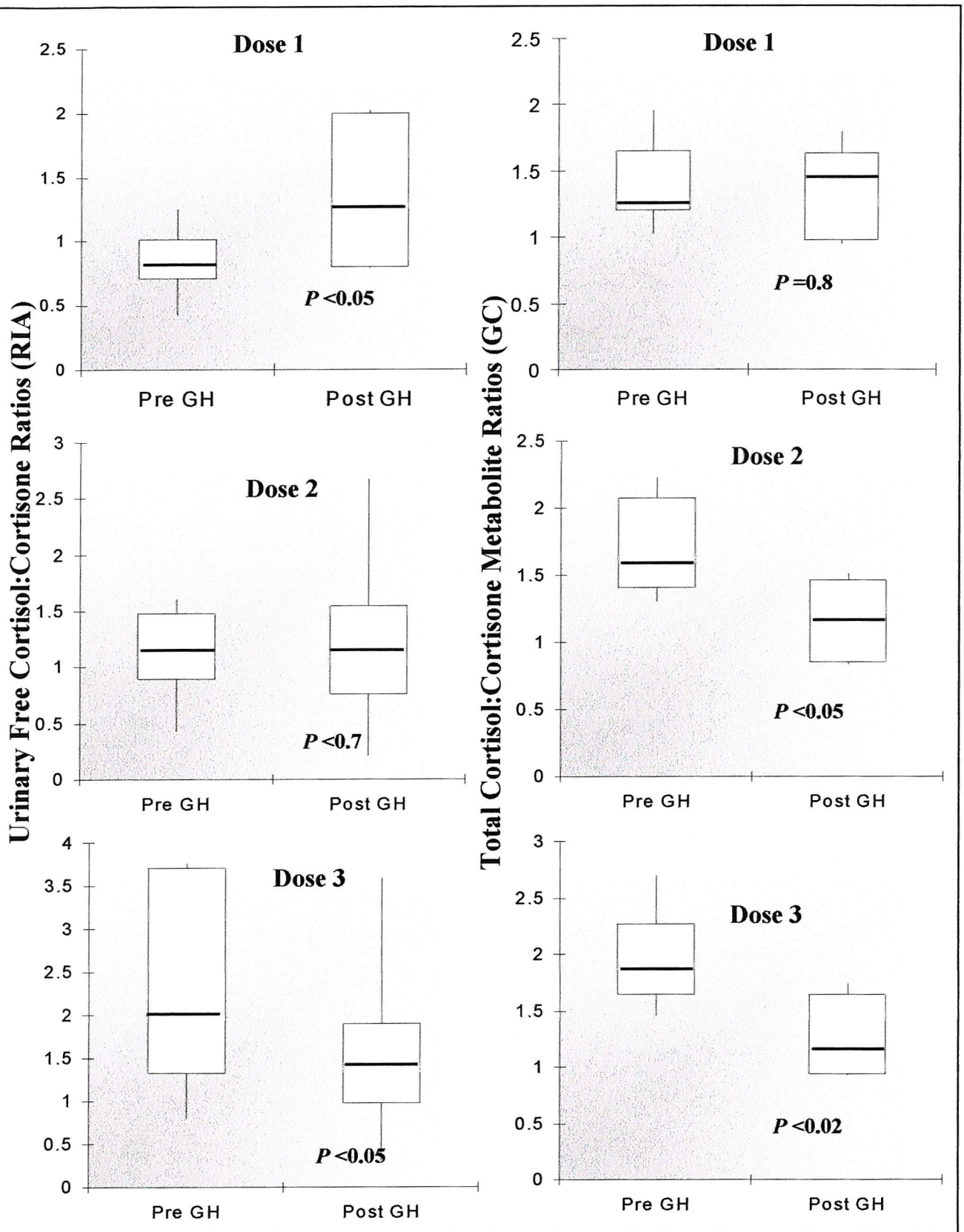


Figure 2.24 Box-whisker plot comparison of urinary free cortisol:cortisone ratios with total cortisol:cortisone metabolite ratios. Urine cortisol:cortisone and total metabolite ratios from adult hypopituitary patients with combined growth hormone and ACTH deficiency before and after growth hormone therapy (GH) during varying doses of hydrocortisone replacement: dose 1 = 10mg bd; dose 2 = 20mg a.m. 10mg p.m.; dose 3 = 40mg a.m. 20mg p.m. (The significance of the difference pre- and post growth hormone treatment is shown)

2.7 DISCUSSION

2.7.1 *Reversed phase HPLC with fluorescence detection using CMMC of corticosteroids*

One of the principal aims of this study was to develop an HPLC technique for the analysis of urinary free corticosteroid profiles as an aid to the quantification of small changes in the peripheral metabolism of cortisol. The early pages of this chapter describe the development and evaluation of a novel technique for the analysis of urinary free corticosteroids which makes use of reversed phase HPLC with fluorescence detection after derivatisation with EDC and CMMC. The satisfactory chromatographic separation and quantification of corticosteroids and their metabolites, subsequent to derivatisation with EDC and CMMC, was found to be susceptible to even modest variations in laboratory technique and reagent preparation. Additionally, fluctuations in laboratory temperature, although minimised by the use of a column heater, had very significant effects upon retention times and chromatographic selectivity, the corollary of which was co-elution of cortisone and 5α-THF and an inability to effect the resolution of the 5α- and 5β-metabolites of cortisol and cortisone.

Moreover, the analysis of both plasma and urine by this method was characterised by a marked deterioration in signal to noise ratio and by an exaggeration of baseline perturbation at the solvent front with the subsequent loss of chromatographic resolution and sensitivity. Whilst this had little impact upon the accurate analysis of corticosteroids in plasma it prevented the estimation of 6β-hydroxycortisol in urine in almost every sample and the deterioration in signal to noise ratio frequently limited analysis solely to the quantification of cortisol and cortisone. Nevertheless, when the chromatography was performed under ideal conditions, the data produced were reproducible and at least equivalent in sensitivity with alternative HPLC techniques with fluorescence detection and demonstrated marked improvements in chromatographic resolution and selectivity over most alternatives[353,356,357,359].

Urine is notable for its tendency to significant variation in composition, in the concentration of its many constituents and for it to contain natural fluorophores of dietary origin[379]. Despite rigorous pre-analytical extraction, few techniques have overcome these difficulties and background fluorescence contamination is a common failing of procedures which make use of this potentially sensitive method of detection[357] . In keeping with previous reports[355-357], the analysis of urinary free steroid profiles by HPLC with fluorescence detection in this study was frequently complicated through contamination. It is likely that this contamination originated not only from dietary fluorophores, but also with phenols, catechols and both primary and secondary amines of endogenous and dietary origin which have also been show to undergo coupling reactions with carbodiimide catalysed reactions[362,380]. Moreover, the concentration of urinary free steroids (particularly the dihydro- and tetrahydro-derivatives of cortisol and cortisone) in the patient groups investigated in this study, were found to be below the limits of detection for this technique (4.0 - 8.0nM, 8-12ng injection). Nevertheless, when the specimen appeared to be relatively free of contaminants, the procedure was found to represent a 5 to 6 fold improvement in sensitivity compared with postcolumn derivatisation techniques using dansyl hydrasine[353] and was at least equivalent to the most sensitive of precolumn derivatisation techniques[349,356,381].

The limitations imposed upon chromatographic determination of urinary steroid profiles as a consequence of the formation of multiple derivatives encountered in this study are by no means unique. This phenomenon is commonly encountered even in GC-MS applications, examples of which are typified by the formation of the syn- or anti-isomeric oxime derivatives of cortisol and 16-hydroxy-dehydroepiandrosterone and also by the formation of two products of tetrahydrocorticosterone which are separated chromatographically by approximately one minute[383]. The second of these two products co-elutes with THF necessitating an additional chromatographic step for accurate quantification[384]. In addition, marked analytical imprecision, which for some steroids can exceed 25%, is regularly reported for GC-MS techniques. This is likely to reflect the multiplicative combination of incomplete or inefficient hydrolysis of steroid conjugates and variability in recovery since internal standards are usually added prior to

derivatisation and hence do not correct for initial loss of steroids during extraction[383,385]. Thus, in this study, the addition of internal standard prior to solid phase extraction was reflected in a significant improvement in analytical imprecision (10.9 ± 6.6% for all steroids examined) despite little difference in recovery of steroid from urine when compared with analogous techniques.

In order to execute satisfactory chromatographic resolution most methodologies employ extended run times which utilise either thermal or solvent gradient elution. In order to overcome the increase in peak width observed in the later eluting peaks it is often necessary to include two or more internal standards which elute at time points flanking the chromatographic run. The linear mathematical relationship between peak height and peak width enables correction for changes in peak height throughout the chromatography but is only accurate if detector response is identical for all analytical species. This is not the case for GC-flame ionisation detectors or GC-MS since detector response is dependent upon mass and the conversion to SI-units requires additional calibration[294,383]. This disadvantage is obviated by HPLC with fluorescence detection in which there is a linear relationship between excitation, emission and concentration under conditions where quenching does not occur. In this study, there was little discernible difference in peak height between steroid conjugates, nor an appreciable change in fluorescence intensity as a result of changes in mobile phase composition. Furthermore a linear relationship between concentration and peak height was observed for all steroids investigated.

In common with the observations made of the analysis of the dihydro- and tetrahydro-derivatives of cortisol and cortisone in urine, in the patient groups investigated in this study, in plasma, unconjugated forms of these metabolites were also found to be below the limits of detection for this technique. Nevertheless, the marked deterioration in baseline noise observed during the analysis of urine extracts was less pronounced for the analysis of plasma extracts and, as a consequence, plasma cortisol and cortisone were reproducibly quantifiable in all of the specimens analysed in this study. Importantly, these observations are in marked contrast with previous studies which made use of

CCMMC (the acid chloride derivative of CMMC) and which reported prolonged baseline perturbation at the solvent front (in excess of thirty minutes in an overall chromatographic run time of 3 hours) and significant baseline noise[357]. The relatively large sample volume (1ml) required for plasma steroid analysis employed in this study is a common failing of techniques for the analysis of plasma steroids[383,386,387] and is a consequence of the relatively low levels of unconjugated steroid which are found in plasma. Moreover, the extraction of steroids from plasma is complicated by their differential protein binding. However, in the study reported herein, this was overcome by protein precipitation and as a consequence, recovery of steroids added to plasma was found to be in excess of 73%.

The application of high sensitivity chromatographic techniques for the analysis of steroid profiles has several advantages over more traditional approaches involving sequential radioimmunoassay. However, the limitations of sensitivity and the complexities of steroid extraction have prevented the successful analysis of urinary unconjugated steroids, including cortisol and cortisone, by GC-MS. However, improvements in GC and MS technology over the past five years have in part ameliorated these deficiencies through the judicious use of deuterated internal standardisation, nano-electrospray mass spectrometry and precursor ion monitoring such that the accurate determination of urinary unconjugated cortisol and cortisone by GC-MS is now a feasible though costly alternative[88,289,388]. Interestingly, within the same timeframe the interfacing of HPLC with MS has not improved to the same extent and whilst techniques for the analysis of corticosteroids which make use of HPLC coupled to particle-beam-interface and chemical-reaction-interface mass spectrometers[343,389] have been reported, they offer no advantages over GC-MS.

2.7.2 *Reversed phase HPLC with UV detection of corticosteroids: comparison with HPLC and fluorescence detection*

The difficulties encountered in the quantitative analysis of urinary free corticosteroid profiles using HPLC with fluorescence detection in this study prompted an expansion of parallel investigations using a method based upon HPLC with UV detection. This latter

study was also designed to analyse the regulation of 11β-HSD activity in cultured cells *in vitro* and will be explored in greater depth in chapters 3 and 4.

There is a considerable volume of material related to 11β-HSD activity *in vitro* and it must be noted that many investigations have made use of thin layer chromatography techniques for the analysis of interconversion of cortisol and cortisone (corticosterone and 11-dehydrocorticosterone for studies of 11β-HSD activity in the rat) which are notable for their robustness and speed of execution rather than their specificity or accuracy[48,319,320,390]. Nevertheless, within the past decade HPLC technology has been more widely used to investigate enzyme kinetics *in vitro*[146,391] and whilst most procedures have been capable of accurate evaluation of cortisol and cortisone concentrations in tissue culture media none have been reported to be sufficiently robust for the accurate determination of steroid profiles in plasma and urine.

In this study, the combined stringency of solid phase extraction and precise gradient elution comprises a novel technique which, despite a relative lack of sensitivity, by comparison with fluorescence detection, and the limited range of steroids detected by UV absorbance at 254nm is well suited to the rapid analysis of unconjugated corticosteroids in tissue culture media, urine and plasma. Indeed, the exclusion of corticosteroids without an α,β-unsaturated ketone in steroid ring A from the chromatogram reduced the number of chromatographic peaks which ensured more discriminating chromatographic resolution of 6β-hydroxycortisol and cortisone than was possible with HPLC with fluorescence detection.

In common with the observations made in section 2.7.1, many of the more polar unconjugated metabolites of cortisol and cortisone in urine were at concentrations below the UV detection limit for this technique (16.9 ± 2.9nM, 50ng/injection). However, accurate quantification of both urinary free cortisol and cortisone was possible in each of the specimens analysed in this study using either HPLC with fluorescence detection or HPLC with UV detection. This latter finding is of particular significance in the light of data from several more recent investigations which have made use of the urinary free

cortisol:cortisone ratio as an index of renal 11β-HSD2 activity[88,289] and in which this ratio has been used in combination with the total cortisol:cortisone metabolite ratio and the (THF+5α-THF)/THE ratio to characterise total body changes in 11β-HSD1 activity by excluding the contribution made by changes in 11β-HSD2 activity[392,393]. Thus, in the study reported herein, the inability to detect the more polar unconjugated corticosteroid metabolites in any of the urine specimens investigated, suggests that the analysis of urinary free corticosteroid profiles using HPLC with fluorescence detection or HPLC with UV detection is likely to be limited to conditions in which the excretion of urinary free corticosteroids is significantly raised above the normal. However, there is considerable potential for the use of either technique in the assessment of relatively small changes in the metabolism of cortisol and cortisone by isoforms of 11β-HSD which may contribute to the aetiology of many other more common diseases such as the insulin resistance syndrome[247,271,394,395].

The close correlation in the measurement of cortisol and cortisone exhibited between the two HPLC techniques in this study ($r_s = 0.98$) demonstrates the robustness of these methodologies. However, the relative complexity and expenditure in terms of time imposed upon the analysis of corticosteroids using HPLC with fluorescence detection compared with the alternative technique of HPLC with UV detection may be considered sufficient incentive to favour the former technique as a 'reference method' against which alternative techniques may be assessed. Moreover, in situations where a high rate of throughput is a limiting factor and the material is relatively simple in composition, it is clear that the technique based upon HPLC with UV detection developed in this study provides significant improvements in specificity over earlier techniques based upon TLC and paper chromatography in which monitoring of enzyme catalysed interconversion of cortisol and cortisone was monitored using tritiated tracer steroids, often at substrate concentrations considerable below the K_m of the enzyme[48,390].

By comparison with urine, the corticosteroid composition of plasma is relatively simple. However, few liquid chromatographic techniques have been reported for the analysis of corticosteroid profiles in plasma principally as a consequence of poor analytical

sensitivity and the limitations of specimen size. Despite the poorer sensitivity of HPLC with UV detection compared with HPLC with fluorescence detection described in this study, the former technique was more robust and simple to perform than the latter. Moreover, the identification of specific peaks in the chromatogram was generally more easily achieved using HPLC with UV detection compared with HPLC with fluorescence detection since the latter technique frequently resulted in a more complex chromatogram which may have been the consequence of either multiple derivative formation or the derivatisation of unknown species in plasma.

The diagnosis of inborn errors of corticosteroid biosynthesis such as congenital adrenal hyperplasia (CAH) and monitoring of therapeutic efficacy has long relied upon steroid specific radioimmunoassay or urine steroid profiling using GC-MS[383]. It is certainly true that the majority of cases of CAH are represented by mutations in the 21α-hydroxylase gene and may be identified biochemically as a consequence of a raised plasma 17α-hydroxyprogesterone which is commonly determined by radioimmunoassay. However, the rarer forms of CAH require a more extensive, time consuming and costly diagnostic program in order to identify the characteristic abnormalities in the profile of cortisol precursors and/or metabolites. Moreover, the more common, non-classical forms of CAH which include non-classical 21α-hydroxylase deficiency, non-classical 11β-hydroxyalse deficiency and non-classical 3β-hydroxysteroid dehydrogenase deficiency may also be diagnosed upon the basis of an abnormal plasma steroid profile following ACTH challenge (reviewed by New MI[396]). Nevertheless, it is likely that relatively small changes in the plasma steroid profile also characterise these conditions in the absence of ACTH challenge but their detection requires techniques which are sufficiently specific and sensitive. Whilst outside the scope of this thesis the potential for the analysis of plasma corticosteroid profiles and thus the rapid and cost effective diagnosis of CAH which is apparent in the HPLC techniques described in this chapter is clear and deserves further exploration.

2.7.3 *Radioimmunoassay of urinary free cortisone and the estimation of urinary free cortisol:cortisone ratios*

Despite the advantages of specificity which are afforded by the chromatographic determination of cortisol and cortisone in biological fluids there can be little argument that the technology is time consuming and poorly suited to studies based upon large numbers of specimens. The development of a specific radioimmunoassay for urinary free cortisone and its use in combination with an established radioimmunoassay for urinary free cortisol in this study was designed to address this inconvenience. Previous attempts to develop specific antisera to cortisone have been frustrated by extensive cross-reactivity with either cortisol or its metabolites and has necessitated extensive specimen pre-treatment in the form of chromatography using either HPLC[397-399], TLC or low pressure systems using celite or diatomaceous earth[400,401].

The marked improvements in specificity exhibited by the anti-cortisone antiserum, N137, used in this study obviated the requirement for extensive chromatographic specimen pretreatment and enabled quantification of cortisone in urine subsequent to liquid/liquid extraction using chloroform. The inclusion of a steroid extraction step prior to radioimmunoassay in this study was required in order to differentiate between unconjugated and conjugated steroid species, since cross reactivity with the relatively high concentrations of steroid conjugates in urine was anticipated and the marked differences in pH, salt and urea concentration which are characteristic of urine make a significant impact upon antibody-antigen interactions.

Despite a high degree of specificity, N137 exhibited significant specific cross reactivity with 5α-tetrahydrocortisone (100%) and 5β-dihydrocortisone (18%). However, neither steroid was at detectable concentrations in any of the urine specimens investigated in this study by HPLC with either fluorescence or UV detection. Nevertheless, it must be recognised that changes in the metabolism of cortisol or cortisone as a consequence of disease might result in significant increases in either of these cross-reacting species in urine and could potentially give erroneous results. Moreover, in a subsequent series of kinetic studies designed to analyse the regulation of 11β-HSD2 in cultured cells

(discussed in detail in chapter 3 of this thesis), N137 cross reactivity with 5β-dihydrocortisone was sufficiently significant to necessitate the use of an alternative technique.

At concentrations of urinary free cortisone in the range 40 - 500nM the fraction of N137 immunoreactivity attributable to urinary free cortisone was estimated to be greater than 90% and served to illustrate the high degree of specificity exhibited by this antiserum. This conclusion was supported by the close correlation observed between the RIA and HPLC estimations of urinary free cortisone and the absence of dose dependent differences between the two techniques. This is in marked contrast with the cross reactivity exhibited by most anti-cortisol antisera which in some instances has been estimated to give a 'true' cortisol results which reflects between 20 - 70% of the free cortisol in the specimen[364]. This latter observation may be considered to be a source of highly significant error in the estimation of urinary free cortisol by RIA and may explain the considerably greater scatter in the measured differences between the two methods compared with that observed for cortisone.

Thus, the differences observed in the estimation of urinary free cortisol:cortisone ratios by RIA compared with HPLC may be a reflection of the relatively poorer specificity exhibited by anti-cortisol antisera than to any other methodological factor. Interestingly, despite the former observations, statistical comparison of urinary free cortisol and cortisone estimated by radioimmunoassay and HPLC in this study revealed no significant differences between the methods. However empirical inspection of the data suggests a marginally positive bias in urinary free cortisol estimated by radioimmunoassay compared with HPLC which may have achieved statistical significance if the sample number had been greater. These observations serve to highlight the difficulties and importance of accurate analysis when relatively modest imprecision in measurement may have a significant effect upon the calculation of a meaningful ratio. Indeed, it is likely that the relatively poorer specificity of the urinary cortisol RIA was responsible for the minor variation in reference interval calculated in this study compared with that which has been previously reported using GC-MS[289]. Without recourse to a recognised

reference method it is difficult to speculate as to which is the more accurate though it is probable that the reproducibility of the techniques reported in this thesis renders the reference interval recorded herein no less valid than any other.

The evidence provided by this study suggests that there is little difference in the cortisol:cortisone ratio obtained by HPLC compared with that obtained by RIA, but the latter technique offers the potential for the analysis of large numbers of specimens at little cost and in relatively short periods of time compared with many of the chromatographic techniques published to date. Nevertheless, it must also be recognised that the imprecision inherent in the analytical performance of two RIA's, one for cortisol and a second for cortisone, represents the product of the imprecision of both assays and as a consequence is likely to be greater than would be expected of a single analytical step. Thus, it may be more appropriate to estimate urinary free cortisol:cortisone ratios by RIA as a preliminary analysis of large population studies in order to select sub-populations of individuals who show statistically significant variation from the reference interval for further investigation by more rigorous chromatographic techniques.

2.7.4 *Comparison of urinary free cortisol:cortisone with total cortisol:cortisone metabolite ratios*

The dynamic equilibrium which maintains appropriate circulating levels of cortisol may be considered to be regulated by a complex interplay between HPA control of cortisol secretion and the tissue specific interconversion of cortisol and cortisone by isoforms of 11β-HSD. Most authors agree that cortisol circulates at a concentration that exceeds cortisone by a factor of ten[91,112,329,401,402] yet the urinary free cortisol:cortisone ratio is most commonly reported to be between 0.5 and unity[91,289,401] and whilst this confirms the profound role of 11β-HSD2 in the renal metabolism of cortisol, it also implies significant conversion of cortisone to cortisol by 11β-HSD1 in other tissues. Moreover, it is prudent to consider the relative contribution that regulation of the dehydrogenase or oxo-reductase activity of systemic 11β-HSD1 may have upon the respective half-life of either cortisol or cortisone in the circulation and hence the fraction of cortisol to which the kidney is exposed. Therefore, analysis of plasma cortisol and cortisone or of urinary

excretion of their conjugated metabolites alone poorly reflects the contribution to cortisol metabolism effected by individual isoforms of 11β-HSD.

Speculation that the urinary free cortisol:cortisone ratio provides a more specific marker of renal metabolism of cortisol and, more significantly, of renal 11β-HSD2 activity than the total urinary cortisol:cortisone metabolite ratio has been reported by several authors[88,289]. This is founded upon the premise that the kidney is the principal source of cortisone in the human circulation[17] and that the urinary excretion of unconjugated steroid is a reflection of renal cortisol metabolism uncomplicated by metabolism attributable to 11β-HSD1 in liver and other tissues. In support of this hypothesis, investigations contemporary with this study have reported marked increases in the urinary free cortisol:cortisone ratio compared with more modest increases in the total urinary cortisol:cortisone metabolite ratio in response to treatment with carbenoxolone[289]. Whilst the mineralocorticoid like effects of GE and GI are believed to be caused solely by inhibition of renal 11β-HSD2 with consequential disturbances in the (THF + 5α-THF)/THE ratio, carbenoxolone inhibits both isoforms of 11β-HSD resulting in a prolonged cortisol half life but with relatively little effect upon the (THF + 5α-THF)/THE ratio[114,403]. Thus it may be hypothesised that, as a consequence of inhibition of both isoforms of 11β-HSD, the equilibrium of systemic interconversion of cortisol and cortisone remains unaltered and is thus reflected in a normal (THF + 5α-THF)/THE ratio whilst the rise in the urinary free cortisol:cortisone ratio may be attributable to the inhibition of renal 11β-HSD2 alone.

2.7.5 *The clinical significance of the urinary free cortisol:cortisone ratio in hypopituitary patients receiving hydrocortisone and growth hormone replacement therapy*

In this study, the relationship between the urinary cortisol:cortisone metabolite ratio and the urinary free cortisol:cortisone ratio was examined in a cohort of hypopituitary adults receiving hydrocortisone replacement and growth hormone therapy. Whilst an in depth analysis of the role of growth hormone and glucocorticoid replacement and its possible

regulation of isoforms of 11β-HSD in these patients is outside the scope of this thesis, this study illustrates the significance of the urinary free cortisol:cortisone ratio as a marker of renal 11β-HSD2 activity.

Growth hormone therapy in growth hormone deficient adults is frequently associated with sodium and water retention which has been attributed, at least in part, to activation of the renin angiotensin system[404]. However, whilst the hormonal regulation of isoforms of 11β-HSD is explored in greater detail in chapter 3 it has been suggested that the antinatriuretic properties of growth hormone may be the product of changes in the peripheral metabolism of glucocorticoid as a consequence of growth hormone dependent regulation of 11β-HSD1 [171,392,405,406,407] and glucocorticoid[210,408-410] in a tissue specific and, in the case of growth hormone, sexually dimorphic manner.

In this study a strong correlation was evident between the urinary free cortisol:cortisone ratio and the total cortisol:cortisone metabolite ratio. However, in the absence of exogenous growth hormone, whilst increasing doses of hydrocortisone were accompanied by a statistically significant rise in the urinary total cortisol:cortisone metabolite ratio there was a significantly greater rise in the urinary free cortisol:cortisone ratio. Indeed, in this study, both positive and negative changes in the total cortisol:cortisone metabolite ratio were always reflected in parallel but markedly greater changes in the urinary free cortsiol:cortisone ratio. Prior to growth hormone treatment this effect was most particularly pronounced at the highest dose of hydrocortisone replacement. While speculative, this may perhaps, reflect saturation of renal conversion of cortisol to cortisone by renal 11β-HSD2. Interestingly, there is also evidence to suggest that glucocorticoids increase 11β-HSD1 11-OR activity in human skeletal muscle (presented in chapter 3), human adipose tissue[249] and in the rat liver[168,411]. These observations suggest that increasing the dose of replacement hydrocortisone might also be accompanied by an increase in the total cortisol:cortisone metabolite ratio. Moreover, the additional cortisol generated as a consequence of glucocorticoid induction of 11β-HSD1 11-OR activity in the liver and other tissues may be presented to the

kidney alongside the administered cortisol and this too could explain the rise in urinary free cortisol:cortisone ratio observed in this study.

In common with previous observations, this study has demonstrated that in hypopituitary patients, growth hormone therapy results in an attenuation of the rise in urinary total cortisol:cortisone metabolite ratio which normally accompanies increasing doses of replacement hydrocortisone[405,412]. This observation may be explained on the basis of several possibilities: i) down-regulation of hepatic 11β-HSD1 11-OR activity in liver and other tissues resulting in a decrease in the conversion of cortisone to cortisol, ii) up-regulation of renal 11β-HSD2 11-DH activity which may result in an increase in circulating cortisone, iii) a combination of both events. Whilst down-regulation of human and rat hepatic 11β-HSD1 activity by growth hormone has been reported by several authors[171,406,412] at the time of this study, none have studied the effects of growth hormone on human renal 11β-HSD2.

When total cortisol:cortisone metabolite ratios and urinary free cortisol:cortisone ratios were compared prior to and subsequent to growth hormone treatment, small but significant differences, which were dependent upon the dose of hydrocortisone replacement, became apparent between the two indices. Thus, subsequent to growth hormone treatment, a dose of 10mg bd hydrocortisone was associated with an increase in the urinary free cortisol:cortisone ratio consistent with an apparent decline in renal 11β-HSD2 11-DH activity whilst growth hormone treatment had no significant effect upon the total cortisol:cortisone metabolite ratio. At the second dose of hydrocortisone replacement (20mg a.m.; 10mg p.m) growth hormone treatment had no significant effect upon the urinary free cortisol:cortsione ratio but resulted in a decline in the total cortisol:cortisone metabolite ratio consistent with a decline 11β-HSD1 11-OR activity in hepatic and other tissues. However, growth hormone treatment at the highest dose of hydrocortisone replacement (40mg a.m.; 20mg p.m.) was associated with a decrease in both the urinary free cortisol:cortisone ratio and the total cortisol:cortisone metabolite ratio which is consistent with an apparent increase in renal 11β-HSD2 11-DH activity or a decline in 11β-HSD1 11-OR activity in hepatic and other tissues.

These apparently paradoxical data may be explained on the basis of differential effects of growth hormone and hydrocortisone upon renal 11β-HSD2 11-DH activity. Inhibition of renal 11β-HSD2 in ACTH-deficient patients by growth hormone has recently been reported by Walker *et al*[412]. Using growth hormone doses identical with those described in this thesis and a dose of hydrocortisone replacement of 20mg a.m. and 10mg p.m., Walker *et al* noted a three fold increase in the urinary free cortisol:cortisone ratio as a consequence of growth hormone therapy. However, this compares with only a 50% increase in urinary free cortisol:cortisone ratio reported in this study.

The effects of cortisol upon renal 11β-HSD2 activity are less well recognised. However, *in vitro* studies of 11β-HSD2 activity in JEG-3 cells, a choriocarcinoma cell line, presented in chapter 3 of this thesis provides evidence to support the suggestion that 11β-HSD2 activity may be increased as a consequence of exposure to high levels (1-2μM) of cortisol. Thus, whilst speculative, the apparent inhibition of renal 11β-HSD2 activity induced by growth hormone at the lowest dose of hydrocortisone replacement in this study, may have been offset by an opposing induction of renal 11β-HSD2 activity as a consequence of higher intrarenal cortisol concentrations as the replacement dose of hydrocortisone was increased. Moreover, the decline in total cortisol:cortisone metabolite ratio effected by growth hormone treatment is consistent with a decline in 11β-HSD1 11-OR activity in hepatic and other tissues and may also have contributed to the fall in urinary free cortisol:cortisone ratio as a consequence of reduced generation of cortisol from cortisone.

It is not clear why there should have been a difference observed between the changes in urinary free cortisol:cortisone ratios in this study and those reported by Walker *et al*[412]. However, the latter study employed GC-MS analysis of urine specimens collected overnight with the results expressed as a steroid:creatinine ratio compared with 24hour urine collections used in this study.

As discussed in the introduction to this thesis and in greater detail in chapter 3, it is well recognised that 11β-HSD activity may be regulated by a number of factors including

thyroid hormones[229,413,414] and sex steroids[171]. Growth hormone is known to increase the peripheral 5'-deiodination of thyroxine (T4) to triiodotyrosine (T3) [415] and that thyroid hormones may play a role in the regulation of hepatic 11β-HSD1 activity[416] (discussed in greater detail in chapter 3). However, in the study reported herein, thyroid hormone status was monitored in each patient throughout the period of investigation and only minor changes in thyroid hormones were observed.

These observations serve to illustrate the complexity of interrelationships which contribute to the regulation of 11β-HSD as determined by either urine or plasma measurements of cortisol and cortisone and highlights the need for caution when attributing growth hormone, or indeed any other hormone, with the function of direct regulation of this enzyme. Nevertheless, the data presented in this study strongly suggest a role not only for growth hormone in the regulation of both hepatic 11β-HSD1 and renal 11β-HSD2 but also suggest that regulation of renal 11β-HSD2 by growth hormone may itself be modified by glucocorticoids. The outcome of such regulatory effects may therefore have profound implications for patients receiving combined growth hormone and hydrocortisone replacement therapy. Indeed, growth hormone therapy in humans has been reported to induce a state of glucose intolerance and insulin insensitivity[416,417]. However, this is likely to be independent of the regulation of 11β-HSD1 11-OR activity by growth hormone since inhibition of 11β-HSD1 11-OR activity by carbenoxolone increases hepatic insulin sensitivity as a consequence of a reduction in the intrahepatic conversion of cortisone to cortisol[418]. There is also growing speculation that increased generation of cortisol from cortisone by 11β-HSD1 in insulin target tissues may also contribute to the development of insulin resistance as a consequence of increased glucocorticoid hormone action[247,271,394,395]. Thus up-regulation of 11β-HSD1 11-OR activity, particularly in insulin target tissues, by exogenous glucocorticoid in patients already receiving growth hormone therapy might be expected to exacerbate the insulin resistance which accompanies growth hormone therapy.

The observations reported in the preceding paragraphs make it clear that an unambiguous analysis of the *in vivo* regulation of isoforms of 11β-HSD by growth hormone and

hydrocortisone in this study is required. It is likely, therefore, that analysis of [11α-^{3}H]-cortisol clearance in these patients prior to and subsequent to growth hormone treatment and at each of the hydrocortisone dose regimens would be required to establish putative effects of growth hormone and hydrocortisone upon renal 11β-HSD2. These data could be used as an aid to the interpretation of the value of urinary free cortisol:cortisone ratios in such a clinical context and gain insight into the possible effects of the hormonal regulation of 11β-HSD1 11-OR activity in other tissues. Importantly, the hormonal regulation of isoforms of 11β-HSD and its significance in the development of insulin resistance will be examined in chapters 3 and 4 of this thesis.

CHAPTER 3 – The hormonal regulation of 11β-hydroxysteroid dehydrogenase

3.1 INTRODUCTION

There is a significant body of evidence which supports the hypothesis that the regulation of isoforms of 11β-HSD may be mediated through the actions of a number of hormones and growth factors. However, much of this research either predates the discovery of 11β-HSD2 or fails to characterise the mechanisms which underlie the regulation of 11β-HSD in an isoform specific manner. Moreover, many of the investigations performed *in vivo* have selected to study enzyme function in animal models which, as a consequence of the considerable interspecies variation, not only in the tissue specific expression of 11β-HSD isoforms, but also in their mechanism of hormonal regulation[169], compromise the interpretation of their observations. Nevertheless, these data have served to highlight the principles underlying the hormonal regulation of isoforms of 11β-HSD and support the growing speculation that changes in enzyme activity may underlie the aetiology of a broad spectrum of diseases including essential hypertension[267,419], insulin resistance[174,176,420], glucose intolerance and central obesity[249]. Thus, a clearer understanding of the mechanisms underpinning the tissue specific pattern of expression and differential regulation of isoforms of 11β-HSD may provide important insights for the treatment of human disease.

3.1.1 *Regulation of 11β-hydroxysteroid dehydrogenase by adrenal steroids and hormones of the hypothalamic-pituitary-adrenal axis*

The tissue specific regulation of 11β-HSD activity by glucocorticoid has been explored in a number of studies[166,249,421,422]. The ectopic ACTH syndrome is characterised by high circulating levels of ACTH and plasma cortisol and by an increase in the urinary free cortisol:cortisone ratio. This latter observation has been cited as evidence for the inhibition of renal 11β-HSD2 either by ACTH or ACTH-dependent steroids. Importantly, Walker *et al*[166] using rat renal cortex and later Diederich *et al*[423] using human kidney slices demonstrated that while ACTH *per se* had no effect upon renal 11β-

HSD2 activity, the ACTH-dependent steroids: corticosterone, 18-hydroxycorticosterone and 11β-hydroxy-androstendione (which are themselves substrates for 11β-HSD2) were capable of a dose dependent inhibition of renal 11β-HSD2 activity. This is in marked contrast with the observations of Li *et al*[421] who demonstrated that administration of dexamethasone, deoxycorticosterone and 9α-fluorocortisol to adrenalectomised rats results in an increase in renal 11β-HSD2 activity of 30-50% but a decrease in 11β-HSD2 mRNA by 30-70% which may represent an increase in the rate of mRNA clearance as a consequence of translation to functional protein. However, the contradictory evidence presented by Diederich *et al*[423] and Li *et al*[421] may represent differences between experiments performed *in vivo* compared with those performed *in vitro*. Earlier studies were unable to demonstrate regulation of renal 11β-HSD2 activity by corticosterone, dexamethasone or aldosterone in rat kidney tubules[424] . However, Diederich *et al*[423] was able to demonstrate that the catalytic capacity of renal 11β-HSD2 could be saturated at high concentrations of cortisol and suggested that this may represent one mechanism whereby the plasma and urinary free cortisol:cortisone ratio may be increased in the ectopic ACTH syndrome.

Contemporary studies of the regulation of rat adrenal 11β-HSD2 activity were also unable to demonstrate a direct inhibitory effect of ACTH[425]. In contrast, later investigations demonstrated that 11β-HSD2 activity in rat adrenal cells from the zona fasciculata was decreased after incubation with ACTH; an effect that was observed even when endogenous corticosterone production was inhibited by metyrapone[426]. Importantly, the effects of metyrapone are likely to be due to inhibition of corticosterone production because it has no effect upon 11β-HSD2 activity, at least in sheep kidney microsomes[427].

In isolated rat renal collecting ducts arginine vasopressin (AVP) increases renal 11β-HSD2 activity; an effect that is reduced following adrenalectomy and restored by infusion of aldosterone but not glucocorticoid[428]. These observations are in direct conflict with an earlier report by the same author[424] but may reflect methodological

difficulties in obtaining appropriate tissue by micro-dissection. However, the latter report does suggest a role for aldosterone in the regulation of renal 11β-HSD2 activity and hence sodium and water homeostasis.

Adrenalectomey and high salt diet results in a decrease in 11β-HSD2 activity in the distal colon of weanling rats[429]. Importantly, the administration of dexamethasone to adrenalectomised rats prevents the decrease in 11β-HSD2 activity in the distal colon and administration of the potent mineralocorticoid, deoxycorticosterone acetate, to rats fed a high salt diet results in a marked increase in 11β-HSD2 activity[429]. Later studies using explant cultures of rat distal colon have confirmed the induction of 11β-HSD2 activity by glucocorticoid and aldosterone[422]. These observations suggest a role for both glucocorticoids and mineralocorticoids in the regulation of 11β-HSD in the gastrointestinal tract

Amongst the earliest evidence for the effects of glucocorticoids on 11β-HSD1 comes from studies of human skin fibroblasts *in vitro*[430]. Theses studies demonstrated that whilst 11-OR activity predominates in these cells, incubation with glucocorticoid results in a marked induction of both 11-OR and 11-DH activities; an effect that is potentiated by the removal of serum from the culture medium.

In common with 11β-HSD1 activity in the liver and skin, 11β-HSD1 activity in human adipose stromal cells is predominantly 11-OR[249]. 11β-HSD1 11-OR activity in human adipose stromal cells has been shown to increase as a consequence of exposure to glucocorticoid[249]. Since this mechanism could result in an increase in the intracellular conversion of cortisone to cortisol it has been postulated that, since 11β-HSD1 11-OR activity is higher in omental rather than subcutaneous adipose tissue, this phenomenon may play a significant role in the pathogenesis of central obesity. Similarly, hepatic 11β-HSD1 is considered to play an important role in the regulation of gluconeogenesis by regenerating active glucocorticoid from its biologically inactive 11-oxo-derivative[172]. Several studies have demonstrated induction of 11β-HSD1 11-OR activity and mRNA

expression in primary cultures of rat hepatocytes[168,411] and in 2S FAZA cells, a rat hepatic cell line, by glucocorticoids[409] whilst the latter has shown that the sequences which respond to glucocorticoid lie within the region 1800 bp of the transcription start site for 11β-HSD1.

3.1.2 *Regulation of 11β-hydroxysteroid dehydrogenase by sex hormones*

The sexually dimorphic expression of 11β-HSD1 and 11β-HSD2 has been frequently cited as evidence for the hormonal regulation of these enzymes by sex steroids. Hepatic 11β-HSD1 mRNA and 11-OR activity is significantly higher in the male rat compared with the female[170,171] and evidence for similar sexual dimorphism in 11β-HSD2 comes from studies of urinary free cortisol:cortisone ratios in man[88,431] and 11β-HSD2 mRNA expression in the mouse[432]. There is a growing body of evidence to suggest that oestradiol increases renal 11β-HSD2 11-DH activity[170] but suppresses hepatic 11β-HSD1 11-OR activity[171] and that progesterone, at least in the human, also acts to inhibit hepatic 11β-HSD1 11-OR activity but has no effect upon levels of 11β-HSD1 mRNA[210]. Moreover, administration of testosterone has been shown to increase hepatic 11β-HSD1 11-OR activity in female rats to the same levels as those seen in intact males and ablation of the pituitary eliminates the sexually dimorphic expression of hepatic 11β-HSD1 [171]. However, these observations are likely to have been affected not only by sexual differences in the secretion of growth hormone, which decreases hepatic 11β-HSD1 11-OR activity[171,406,412], but also by a relative decrease in steroidogenesis as a consequence of loss of gonadotrophin secretion.

Further evidence for the control of 11β-HSD activity by sex steroids is provided by the observation that administration of estrogens to pregnant baboons at mid-gestation results in an increase in placental 11-βHSD2 11-DH activity at term[222,433]. In support of these earlier observations, more recent evidence suggests that in term placenta, oestradiol has little or no effect upon 11β-HSD1 11-OR activity but markedly decreases 11β-HSD2 11-DH and that this effect is enhanced by progesterone[434]. Whilst the net direction of cortisol and cortisone interconversion throughout gestation may be the product of

ontogenic differences in the relative abundance of decidua and placental trophoblast which express 11β-HSD1 and 11β-HSD2 respectively, it should be recognised that regulation of enzyme activity by sex steroids may also play a role in the regulation of transplacental glucocorticoid metabolism throughout gestation.

Normal decidualisation of the endometrium is an oestrogen dependent process and is essential for successful implantation of the trophoblast and maintenance of pregnancy. High concentrations of circulating glucocorticoid, such as those seen in Cushing's syndrome or in conditions of chronic stress not only suppress pituitary secretion of LH but also render target tissues resistant to oestradiol[435]. Glucocorticoids are also thought to exert teratogenic effects on the implanting embryo and inhibit trophoblast invasion[365]. Thus, it has been suggested that the regulation of glucocorticoid hormone action in the endometrium by isoforms of 11β-HSD may play a role in trophoblastic implantation[365,366]. Importantly, the expression and activity of 11β-HSD1 and 11β-HSD2 in the endometrium appears to be regulated by sex steroids. Indeed, it has been clearly demonstrated that 11β-HSD1 activity may be enhanced by the synergistic action of oestradiol and progesterone in cultured endometrial stromal cells[365,366] and that in the rat both 11β-HSD1 and 11β-HSD2 expression is low at di-oestrus but rises markedly at pro-oetsrus, an effect which may be induced by oestradiol in ovariectomised animals[436]. These observations have given rise to the hypothesis that 11β-HSD2, expressed in endometrial stromal cells and myometrial cells, acts to protect the decidual matrix from glucocorticoid responsive proteases whilst 11β-HSD1 11-OR activity, expressed in luminal and glandular epithelial cells and in eosinophils in both the endometrial stroma and myometrium, acts to regenerate glucocorticoid which is required for the normal physiology of the epithelium[436].

The hypertensive effects of progesterone are well recognised and are thought to derive not only from direct activation of the MR but also, in part, to inhibition of isoforms of 11β-HSD[434]. It has been demonstrated that a number of endogenous substances may inhibit 11β-HSD activity including the 11α– and 11β-hydroxy metabolites of progesterone[437] and it has been hypothesised that this effect may play some role in the

development of human hypertension. In contrast, administration of dehydroepiandrosterone sulphate to spontaneously hypertensive rats results in an antihypertensive effect and a marked increase in the net conversion of corticosterone to dehydrocorticosterone in the kidney; an observation which has been interpreted as indicative of an increase in renal 11β-HSD2 activity[438].

Both oestradiol and testosterone have been shown to inhibit 11β-HSD1 11-OR activity whereas progesterone increases 11β-HSD1 activity in the rat testis[204]. It is generally accepted that the principal role of 11β-HSD in the Leydig cell is to protect androgen biosynthesis from the inhibitory effects of glucocorticoid[439]. This implies that Leydig cell 11β-HSD expresses predominantly 11-DH activity and whilst the human testis expresses both 11β-HSD1 and 11β-HSD2 [62], the rat testis expresses only 11β-HSD1, the isoform which, in the liver, is generally considered to favour 11-OR activity. Whether this anomaly may be explained on the basis of hormonal regulation of the relative abundance of 11-OR and 11-DH activities of 11β-HSD1 in the rat testis[203-205] or by the existence of a novel isoform of 11β-HSD[202] remains unclear.

3.1.3 *Regulation of 11β-hydroxysteroid dehydrogenase by thyroid hormones*

Few investigations have provided clear evidence for the regulation of isoforms of 11β-HSD by thyroid hormones. Indeed, most of these studies either predate the discovery of 11β-HSD2 or suggest that the regulation of 11β-HSD isoforms is effected indirectly[367,414,440]. Moreover, there appears to be considerable interspecies variation in the response of 11β-HSD activity to the effects of thyroid hormones and that effects measured *in vivo* are frequently not observed *in vitro*[414].

Nevertheless, indirect evidence for the regulation of 11β-HSD by thyroid hormones in man comes from the observation that hyperthyroidism is frequently accompanied by an increase in the urinary total cortisol:cortisone metabolite ratio[413] whilst hypothyroidism is accompanied by an increase in the half-life of plasma cortisol[441]. More recently, Whorwood *et al*[414] demonstrated that administration of Tri-iodothyronine (T3) to

normal adult rats results in a decline in hepatic and pituitary 11β-HSD1 11-DH activity and mRNA expression but produces no effect on 11-DH activity or gene expression in rat kidney or distal colon[414].

Evidence for interspecies variation in response to thyroid hormone *in vitro* comes from investigations which demonstrate that T3 increases hepatic 11β-HSD1 11-OR activity in rat hepatocytes but has no effect on hepatic 11β-HSD1 11-OR activity in human hepatocytes[169]. In another series of studies, Pacha *et al*[429] was unable to detect any effect of T3 on 11β-HSD2 activity either *in vivo* or in explant cultures of seven day old rat distal colon[422].

3.1.4 *Regulation of 11β-hydroxysteroid dehydrogenase by insulin*

Hypertension, central obesity and glucose intolerance are characteristics of hypercortisolaemia and may be explained upon the basis of insulin resistance as a consequence of the role of cortisol in intermediary carbohydrate metabolism as an insulin antagonist. Whilst this subject is dealt with in greater depth in chapter four of this thesis it is appropriate here to highlight the intimate relationship which exists between glucocorticoid and insulin and to postulate a possible role for insulin regulation of cortisol metabolism through regulation of isoforms of 11β-HSD.

Glucocorticoids are believed to effect their role on glucose metabolism by inhibiting peripheral insulin-dependent glucose uptake and through enhanced hepatic gluconeogenesis[176]. It has been proposed that increases in circulating levels of cortisol or changes in the level of hepatic glucocorticoid as a consequence of an increase in the conversion of cortisone to cortisol by hepatic 11β-HSD1 11-OR activity may contribute to the glucose intolerance observed in hypercortisolaemia.

Few studies have investigated the regulation of 11β-HSD by insulin and of these many are contradictory or fail to distinguish between the regulation of 11β-HSD1 11-OR and 11β-HSD1 11-DH activity. However, Hammami and Siiteri[430] demonstrated that insulin

decreases both 11β-HSD1 11-OR and 11-DH activity in skin fibroblasts and Liu *et al*[411] observed that insulin and growth hormone inhibit 11β-HSD1 11-OR activity in rat hepatocytes in culture. These observations were confirmed in another study using intact 2S-FAZA cells, a rat hepatoma cell line, which demonstrated that insulin and IGF-1 inhibit 11β-HSD1 11-DH activity[409].

11β-HSD1 11-OR activity in adipose stromal cells from omental fat is increased after exposure to glucocorticoid and insulin, a mechanism that would ensure continued exposure to glucocorticoid and thus maintain the metabolic effects of hypercortisolaemia in this tissue[247]. Similarly, 11β-HSD1 11-OR activity and mRNA expression in white adipose tissue and the adipocyte cell lines 3T3-F442A and 3T3-L1 are also increased by insulin and dexamethasone[442]. Thus, 11β-HSD1 in both hepatic and adipose tissue may represent important mechanisms for the regulation of the metabolic effects of glucocorticoid.

In streptozotocin induced insulin dependent diabetic female rats renal 11β-HSD1 11-OR activity and mRNA expression is identical with unaffected rats whilst renal 11β-HSD2 activity and mRNA expression is diminished. Administration of subcutaneous insulin to diabetic rats resulted in a return to normal levels of renal 11β-HSD2 activity and mRNA and a normalisation of blood pressure. These data have been cited as evidence for the hormonal regulation of renal 11β-HSD2 by insulin[367]. However, insulin appears to have no effect upon 11β-HSD2 activity from explant cultures of 7 day old rat distal colon[422] which suggests that the regulation of 11β-HSD2 by insulin may be tissue or cell type specific.

3.1.5 *Regulation of 11β-hydroxysteroid dehydrogenase by cytokines and growth factors*

Few investigations have examined the role of cytokines and growth factors in the regulation of isoforms of 11β-HSD. However, more recently investigations have highlighted a role for cytokines and growth factors in the regulation of 11β-HSD.

Epidermal growth factor has been shown to increase 11β-HSD1 11-OR activity in rat Leydig cells from both intact and adrenalectomised rats[205]. However, hepatocyte growth factor, epidermal growth factor, basic fibroblast growth factor and transforming growth factor beta 1 do not appear to have an effect on 11β-HSD1 11-OR activity or mRNA in primary cultures of rat hepatocytes[169].

Evagelatou *et al*[443] noted that cells from the follicular aspirates of women undergoing *in vitro* fertilisation treatment comprised a high proportion of leukocytes. Importantly, RT-PCR analysis revealed that these cells expressed 11β-HSD1 mRNA but not 11β-HSD2 mRNA but investigations of 11β-HSD activity were performed using cell homogenates by measuring 11-DH activity. Nevertheless, removal of the leukocytes resulted in a marked decline in granulosa cell 11-DH activity. Incubation with the interleukins, IL-5 and IL-6 resulted in an increase in granulosa cell 11-DH activity in the presence or absence of leukocytes whilst IL-4 and interferon-gamma (IFNγ) only induced 11-DH activity in granulosa cells which were depleted of leukocytes. IL-2 had no effect upon 11-DH activity.

The anti-inflammatory effects of glucocorticoids are well recognised and are effected through glucocorticoid receptor mediated down regulation of phospholipase-A2 transcription[444]. The pro-inflammatory cytokines, IL-1β and tumour necrosis factor alpha (TNF-α), which increase the transcription of phospholipase-A2 have also been shown to increase 11β-HSD1 11-OR activity in rat glomerular mesangial cells *in vitro*[444]. From these observations it has been hypothesised that upregulation of 11β-HSD1 activity may represent auto-regulation of the mechanism of inflammation by pro-inflammatory cytokines.

3.1.6 *Aims of this study*

A growing awareness that the activity of 11β-HSD may be altered, either as a consequence of, or indeed, as the primary cause of a number of human diseases adds significance to the preceding discussion on the regulation of 11β-HSD by a diverse range

of hormones, growth factors and cytokines. However, the available evidence suggests that the study of 11β-HSD is complicated not only by differences in regulation between cells *in vitro* compared with those *in vivo* but also by interspecies differences in both the tissue distribution of the two isoforms and in their response to the regulatory effects of a variety of hormones. Whilst it is possible to overcome the former difficulty by restricting investigations of the regulation of 11β-HSD solely to *in vivo* studies, this solution does not overcome the additional difficulty of dissecting differential regulation of 11β-HSD in an isoform specific manner. Nevertheless, *in vitro* studies offer the potential to investigate the regulation of specific isoforms of 11β-HSD in a controlled environment.

The introduction to this chapter reviews the evidence for the hormonal regulation of isoforms of 11β-HSD and, by analogy with deficiency of enzyme activity in AME and 11-oxoreductase deficiency, postulates a role for the dysregulation of 11β-HSD in the aetiology of a number of human diseases. Investigations of the regulation 11β-HSD have been reported in a broad range of human tissues and cells including those of the liver, kidney, testis, ovary, vascular smooth muscle and adipose tissue. However, to date, there have been no reports of investigations of the regulation of 11β-HSD in human skeletal muscle which represents an important glucocorticoid target tissue and which comprises as much as 40% of the total body mass in the adult male. Moreover, human skeletal muscle is known to express 11β-HSD1 [395] and is thus capable of extensive interconversion of cortisone and cortisol.

The regulation of 11β-HSD2 has been investigated in an equally broad spectrum of human tissues. Extensive studies of the steroidogenic and steroid metabolising properties of the choriocarcinoma cell line, JEG-3, have established this cell line as a model of steroid metabolism and particularly 11β-HSD2 activity in trophoblastic tissue. However, few studies have attempted a systematic investigation of the hormonal regulation of 11β-HSD2 in these cells.

Thus the aims of this study were to investigate the regulation of 11β-HSD1 in skeletal myoblasts and 11β-*HSD2* in human choriocarcinoma (*JEG*-3) cells by steroid and peptide hormones and by pro-inflammatory and colony stimulating cytokines and growth factors. These investigations served as an important pre-requisite to a clear understanding of the physiological role of 11β-HSD in the aetiology of the metabolic syndrome discussed in chapter 4.

3.2 Materials and Methods

General chemicals and solutions were obtained from Sigma Chemical Company Ltd (Poole, Dorset, UK) and were of molecular biology grade unless otherwise stated. Radiolabeled steroids were supplied by Amersham International PLC (Amersham, Bucks, UK).

3.2.1 *Preparing commonly used buffers and reagents*

3.2.1.1 *Buffers*

Phosphate Buffered Saline (PBS) was prepared by dissolving 8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4 and 0.24g KH_2PO_4 in 800ml H_2O, the pH was adjusted to pH7.4 with 10N HCl and the volume adjusted to 1L with H_2O.

3.2.1.2 *Stock solutions of steroid hormones*

Stock solutions (200μM) of 5α- and 5β-dihydrocortisol, 5α-dihydrocortisone 5α- and 5β-tetrahydrocortisol, 5α-tetrahydrocortisone, 6β-hydroxycortisol, 11-deoxycorticosterone, 11-deoxycortisol, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, 17β-oestradiol, α- and β-cortol, α- and β-cortolone, androstenedione, carbenoxolone, corticosterone, cortisol, cortisone, dehydroepiandroserone, pregnenolone, progesterone, testosterone and the glucocorticoid receptor antagonist RU38486 were prepared using absolute ethanol as solvent and stored at –20°C.

3.2.1.3 *Stock solutions of peptide hormones and growth factors*

All peptide hormones were supplied in cell culture tested lyophilised form (Sigma Chemical Company Ltd), reconstituted according to manufactures instructions and stored in 100μl aliquots at –70°C unless otherwise stated.

3,3',5-triiodo-L-thyronine (sodium salt) (T3) reconstituted with sterile PBS to provide a 150μM stock solution.

Insulin-like growth factor-1 (IGF-1) reconstituted with sterile PBS to provide a 50mg/L stock solution.

Human adrenocorticotrphic hormone (ACTH) was reconstituted with sterile PBS containing 0.1% acetic acid to provide a 10mg/L stock solution.

Human recombinant tumour necrosis factor alpha (TNF-α) (expressed in E. coli) was reconstituted with sterile H_20 to provide a 10mg/L stock solution.

Human recombinant interleukin-1β (IL-1β), human recombinant interleukin-2 (IL-2), human recombinant interleukin-6 (IL-6) and human recombinant interleukin-7 (IL-7) (all expressed in E. coli) were reconstituted with sterile H_20 to provide 10mg/L, 10mg/L, 10mg/L and 5mg/L stock solutions respectively.

Human insulin (50,000 IU), human chorionic gonadotrophin (hCG) (500,000 IU), human growth hormone (45,000 IU) and human luteinising hormone (LH) (20,000 IU) were supplied in lyophilised form in dry nitrogen (National Institute For Biological Standardisation and Control (NIBSC), Potters Bar, Hertfordshire, UK). Each peptide was reconstituted in 5ml sterile PBS containing 0.1% acetic acid. The solution was sterilised by passing it through a 0.2μm filter, aliquoted into 1ml volumes and stored at –70°C.

3.2.2 *Tissue culture*

Dulbecco's modified eagles medium (DMEM) was obtained in 500ml bottles and stored at 4°C. Foetal calf serum (FCS) was obtained in batches, filtered through a 0.45μm sterile filter (Sigma Chemical Company Ltd) and stored in aliquots (50ml) at –70°C. Non-essential and essential amino acids were obtained as 100x and 50x stock solutions and stored at –70°C. 0.5% (w/v) trypsin/ethylenediaminetetra-acetic acid (trypsion-EDTA) solution was prepared by diluting 5ml of a 10x stock solution (Sigma Chemical Co, containing 5.0g porcine trypsin and 2.0g EDTA.4Na per litre in 0.9% NaCl)) in 45ml Hanks' balance salt solution (HBSS) immediately prior to use.

3.2.1.1 *Cell lines*

All cell lines were incubated at 37°C in a humidified atmosphere of 95% air: 5% CO_2. Cells used for experiments performed in the absence of FCS were incubated for 48 hours in FCS free tissue culture medium prior to kinetic analysis.

JEG-3 Cells: A human clonal choriocarcinoma cell line. Cells were grown in $25cm^2$ flasks (Greiner labortecknik, Frickenhausen, Germany) to 80% confluence (approximately 8.2×10^6 cells/flask) in a growth medium of DMEM (without phenol red) with 0.11g/L sodium pyruvate containing 200U/ml penicillin, 50µg/ml streptomycin, 0.3mg/ml L-glutamine and 10% FCS. Culture medium was replaced at two day intervals and the cells were passaged once every 7-10 days at a ratio of 1:6. Experiments were performed in DMEM without phenol red containing either 10%FCS or in the absence of FCS.

Myoblasts: These were normal human skeletal myoblasts supplied by Dr. CB Whorwood at the Endocrine and Metabolism Unit, University Department of Medicine, University of Southampton. Primary cultures of skeletal myoblasts were established on matrix protein-coated flasks following mechanical and trypsin:EDTA disaggregation of tissue minces obtained at biopsy. The protocol was optimised to yield maximal numbers of viable satellite cells, which unlike other muscle cells retain the ability to proliferate, from an aliquot of each biopsy (15-50mg).

The establishment and proliferation of human skeletal cells, which exist as either mononuclear myoblasts or as a syncytium of fused multinuclear myotubes, was performed by modification of the methods previously described by Blau *et al*[445], Hurel *et al*[446] and Sarabia *et al*[447]. Tissue was obtained by Bergstrom needle biopsy (26-143mg) of the *vastus lateralis* from 14 Caucasian men aged 40 – 60 years. Two to three biopsies of muscle tissue was obtained from each subject during the procedure. The biopsies were immediately transferred to transport medium (Hams F10 containing 20% FCS, 1,000U/ml penicillin, 50µg/ml streptomycin and 1µg/ml amphotericine B) maintained at 4°C and micro-dissected in order to free them of fat and connective tissue. The remaining tissue was finely chopped to pieces that were smaller than $1mm^3$ with scissors. Tissue minces were washed three times with ice-cold serum free medium, re-suspended in sterile cell dispersal solution (0.05% trypsin, 0.05M EDTA in PBS) which had been pre-warmed to 37°C, transferred to sterile 50ml conical flasks and shaken at 190rpm for 60 minutes at 37°C. Optimum conditions for cell dispersal (optimised for

yield and cell viability) were determined empirically. Tissue debris was allowed to settle for one minute. The supernatant was centrifuged (550 x g, room temperature) and the cell pellet as re-suspended in cell growth medium (DMEM with 0.11g/L sodium pyruvate containing 200U/ml penicillin, 50μg/ml streptomycin, 0.3mg/ml L-glutamine, 0.25μg/ml amphotericine B and 20% FCS). The cells were washed twice with growth medium and were then plated onto gelatin/fibronectin coated 10cm petri dishes in growth medium supplemented with 1% chick embryo extract and 10-25% conditioned medium from highly proliferating myoblasts and placed in a humidified atmosphere of 95% air:5% CO_2 in a dedicated incubator (Biohit, Wolf Laboratories Ltd, UK). Culture medium was replaced twice weekly but involved removal and replacement of only 75% of the total on each occasion. The period to 90% confluence was largely dependent upon the yield of viable satellite cells but varied between 6-9 weeks.

Cells were passaged once every 21-28 days at a ratio of 1:3 and fed with 5ml culture medium at 4 day intervals. Experimental analyses were performed on 80-90% sub-confluent cells between passages 3-10 in DMEM without phenol red containing either 20%FCS or in the absence of FCS.

3.2.1.2 *Freezing, thawing and passage of cells*

Cells were stored in liquid nitrogen in 1.2ml Nalgene cryogenic vials containing up to 10^7cells. Vials of cells were gently thawed for 2-3 minutes in a 37°C water bath. The cells were transferred to a 50ml conical tube (Greiner Labortechnik, Germany) using a sterile disposable pipette and 5ml of culture medium was added one drop at a time and gently mixed to avoid cell lysis. A further 5ml tissue culture medium was added and the cells were centrifuged for 1 minute at 1000rpm. The supernatant was discarded and the cells were suspended in 10ml growth medium and pipetted into two 25cm^2 tissue culture flasks.

Cells were passaged at 80-90% confluence. The growth medium was removed and replaced with 5ml PBS wash which was gently swirled over the cells and aspirated to waste. 1ml 0.25% trypsin-EDTA solution was added to the flask, swirled over the cells

and immediately removed. The cells were incubated at 37°C in a humidified atmosphere of 95% air: 5% CO_2 for a period of 10-15 minutes in order that the cells should become detached. 2ml culture medium was added to the flask using a sterile disposable pipette and cell aggregates were dispersed by repeated gentle aspiration. Sufficient additional growth medium was added to the flasks to divide the cells into an appropriate number of clean 25cm^2 tissue culture flasks.

Cells to be stored for later use were passaged as described above and suspended in FCS containing 10% (v/v) DMSO. 0.5-1ml of cell suspension was pipetted into Nalgene cryogenic vials, which were snap frozen in a dry ice/methanol bath and placed at -70°C for 2-3 hours before finally storing in liquid nitrogen to allow gradual super freezing to -140°C.

3.2.3 *In vitro synthesis of [1,2,6,7-^{3}H]-cortisone using JEG-3 cells*

JEG-3 cells were grown to 80-90% subconfluence in a 25cm^2 tissue culture flask as previously described (3.2.2.1). The cells were grown in a serum free growth medium for a period of 24 hours. The growth medium was removed and 3ml serum free growth medium containing 250µCi [1,2,6,7-^{3}H]-cortisol (specific activity 63.0Ci/mmol) suspended in 50µl ethanol was added to the flask. The cells were incubated at 37°C in a humidified atmosphere of 95% air: 5% CO_2 for 24 hours. The growth medium was decanted into glass 50ml stoppered conical flasks and the steroids were extracted by repeated inversion with 25ml dichloromethane. The phases were separated by centrifugation (3000 x g for 5 minutes at room temperature) and the organic layer was transferred to a clean glass container. The growth medium was treated with a further 2 washes with dichloromethane followed by 3 washes with trichloromethane. The organic phases were pooled and evaporated to dryness under a stream of dry nitrogen. The extract was resuspended in 25µl dichloromethane and resolved by two way TLC using chloroform/ethanol (92:8 v/v) as the mobile phase. Separate chromatograms to which cortisol, cortisone or a mixed standard containing cortisol and cortisone were resolved simultaneously. The TLC plate containing [^{3}H]-labelled steroid was examined using a

radioimager (2.5.1.6) and the region of the TLC plate corresponding to cortisone (after comparison with the standard preparations) was removed. The silica was dispersed in 10ml trichloromethane, filtered through glass wool and evaporated to dryness under a stream of dry nitrogen. The extract was reconstituted in ethanol to give 5000dpm/µl (35.8 fmol/µl).

3.2.4 *Assaying 11β-hydroxysteroid dehydrogenase activity*

3.2.4.1 *Reagents and buffers*

HPLC mobile phases were prepared fresh for each chromatographic analysis as described in table 2.2 (page 79). Krebs Ringer Bicarbonate (KRB) buffer comprised 118mM NaCl, 3.8mM KCl, 1.19mM KH_2PO_4, 2.54mM $CaCl_2.2H_2O$, 1.19mM $MgSO_4.7H_2O$, 25mM $NaHCO_3$. The solution was charged with 95% O_2:5% CO_2 for greater than one hour followed by addition of 11.1mM glucose and 0.2% bovine serum albumin (BSA). Stock solutions of nicotinamide adenine dinucleotide phosphate (NADP), reduced nicotinamide adenine dinucleotide phosphate (NADPH), nicotinamide adenine dinucleotide (NAD) and reduced nicotinamide adenine dinucleotide (NADH) (1.25mM) were prepared by addition of sterile H_20 to pre-weighed lyophilised cofactor in glass vials.

3.2.4.2 *Kinetic analysis of 11β- hydroxysteroid dehydrogenase activity in adherent cells in culture*

JEG-3 Cells – 11β-HSD activity was measured in triplicate 25cm^2 flasks of 80-90% sub-confluent cells. Cells were incubated in DMEM (without phenol red) in both the presence and absence of 10% FCS containing a range of concentrations of cortisol (32.25 – 1000nM) for 12 hours at 37°C in an atmosphere of 95% air: 5% CO_2.

Skeletal myoblasts - 11β-HSD activity was measured in triplicate 25cm^2 flasks of 80% sub-confluent cells. Cells were incubated in DMEM (without phenol red) in both the presence and absence of 20% FCS containing a range of concentrations of cortisone (32.25 – 1000nM)) for 48 hours at 37°C in an atmosphere of 95% air: 5% CO_2.

The rate of reaction, V, was calculated from the mass of reaction product formed by a known mass of protein in one hour (pmoles of reaction product formed/mg protein/hour). Kinetic constants (apparent K_m and V_{max}) were established using Hanes plots of [S]/V versus [S], where [S] is the substrate concentration, the intercept on the abcissa is the K_m and the slope is $1/V_{max}$.

3.2.4.3 *Estimation of 11β-hydroxysteroid dehydrogenase activity in human skeletal myoblasts and JEG-3 cells subsequent to exposure to steroid and peptide hormones, growth factors and cytokines*

11β-HSD1 activity in myoblasts was estimated by incubating flasks of pre-treated cells with 5ml growth medium containing cortisone (350nM) whilst 11β-HSD2 activity in JEG-3 cells was estimated by incubating flasks of pre-treated cells with 5ml growth medium containing cortisol (350nM) such that the percent conversion of substrate to product by 11β-HSD activity was less than 50%. Thus JEG-3 cells were incubated for a period of 12 hours whilst myoblasts were incubated for periods up to 96 hours. The steroid products of this incubation were extracted and 11β-HSD activity in was estimated from the rate of conversion of substrate to product (pmol/mg protein/hour). The supernatants were then subjected to steroid analysis by solid phase extraction and HPLC with UV detection as previously described (2.4.1.1).

3.2.4.4 *Kinetic analysis of 11β- hydroxysteroid dehydrogenase and cofactor dependence in JEG-3 cell homogenates*

80% sub-confluent monolayers of JEG-3 cells in 75cm^2 flasks containing 10ml serum free DMEM (without phenol red) were dislodged using a cell scraper, transferred to 15ml conical polypropylene centrifuge tubes (Greiner Labortechnik, Germany) and centrifuged at 200 x g for 5 minutes. The supernatant was aspirated to waste and the cell pellet was resuspended in 2ml ice cold KRB containing 2mM phenylmethylsulfonyl fluoride (PMSF) and homogenised by sonication (5x 5 second pulses at 20,000Hz). The protein concentration of the homogenate was determined (3.2.5). 1mg protein was added to 4ml oxygenated KRB containing 2mM PMSF, NAD, NAHDH, NADP or NADPH (1.0 mM)

and either cortisol or cortisone at a range of concentrations (50 – 2000nM) and incubated at 37°C for 120 minutes. All assays were performed in duplicate and included control reactions containing no protein in order to define the background level of product formation. Immediately following incubation the reaction tubes were snap cooled to 4°C and centrifuged at 3000 x g to pellet cell debris. The supernatants were then subjected to steroid analysis by solid phase extraction and HPLC with UV detection as previously described (2.4.1.1).

3.2.5 *Estimating protein concentration in cell lysates and homogenates*

Based upon the method of Bradford[448] in which binding of protein to Coomassie Blue in acidic solution results in a shift in absorbance maximum from 465nm to 595nm with a linear response up to a protein concentration of 1.4mg/ml. A stock solution of bovine serum albumin containing 1.4mg/ml protein in 20ml H_2O was prepared and stored at –20°C in 500µl aliquots. Serial doubling dilutions were prepared using H_2O as solvent to cover a concentration range of 0.125 – 1.4mg/ml. Duplicate 0.1ml aliquots of each standard or specimen were pipetted into clean glass tubes, mixed with 5.0ml Bradford reagent (Sigma Chemical Company Ltd) and incubated at room temperature for 10 minutes. Absorbance at 595nm was estimated spectrophotometrically.

3.2.6 *Extraction of total RNA*

Total RNA was isolated from monolayer cultures of cells grown in 25cm^2 flasks using a single step acidified phenol/chlorofom extraction method (RNAzol B, Biogenesis, Poole, UK) as described by Chomczynski *et al*[449]. Tissue culture medium was removed and 0.5ml of RNAzol B was added to each 25cm^2 flask. The solution was swirled over the cells and left to stand at 4°C for 15 minutes. Each flask was rotated to collect the lysate as a discrete pool which was transferred into a 1.5ml eppendorf tube. 50µl chloroform:isoamyl alcohol (24:1) was added and the suspension was mixed by repeated inversion and allowed to stand at 4°C for 30 minutes. Samples were centrifuged at 10,000 x g for 20 minutes at 4°C and the aqueous layer containing total RNA was removed using a pasteur pipette and transferred to a clean 1.5ml eppendorf tube. 300µl ice cold isopropanol was added to the tube which was mixed and placed at -20°C for 2-

12 hours to precipitate the RNA. The precipitate was pelletted by centrifugation at 10,000 x g for 20 minutes. The supernatant was aspirated to waste and the pellet was washed with 500µl 70% ethanol/H_2O, centrifuged at 10,000 x g for 10 minutes. The supernatant was aspirated to waste and the pellet was allowed to dry prior to being resuspended in 40µl DEPC treated H_20.

3.2.7 *Quantification of total RNA*

Quantification of RNA and assessment of its integrity was determined spectrophotometrically at 260 and 280nm (1 absorbance unit at 260nm = 40µgRNA/ml and an absorbance ratio (260:280nm) of less than 1.0 was considered to represent 'poor quality' RNA) and by comparison with calf liver 28S and 18S ribosomal RNA markers (Pharmacia, Uppsala, Sweden) electrophoresed on a 1.0% agarose gel stained with ethidium bromide.

3.2.8 *Qualitative and semi-quantitative non-competitive RT-PCR*

First strand cDNA was synthesised from 10µg total RNA using reverse transcriptase (RT)-driven primer extension from random hexamers. 10µg RNA and 250ng random hexamers was suspended in sterile diethyl pyrocarbonate (DEPC) treated H_20 to a volume of 22µl in a sterile DEPC treated 0.2ml thin walled polypropelene polymerase chain reaction (PCR) tube (Sigma Chemical Co) which was heated to 70°C for 5 minutes and snap cooled to 4°C. 8µl 5x first strand buffer (250mM Tris-HCl (pH 8.3), 375mM KCl, 15mM $MgCl_2$), 4µl dithiothreitol (DTT) (100mM), 2µl deoxynucleoside tri-phosphate (dNTP) mix (10mM each of deoxyadenosin tri-phosphate (dATP), deoxyguanosin tri-phosphate (dGTP), deoxycytosine tri-phosphate (dCTP) and deoxythymidine tri-phosphate (dTTP)) and 10units of RNAsin (RNAse inhibitor, Promega UK, Southampton) was added, mixed and incubated at 25°C for 10 minutes and then at 42°C for 2 minutes. 200 units Superscript II (Life Technologies, GibcoBrl, Paisley, Scotland) was added and incubated at 42°C for one hour. The reaction was terminated by heating to 70°C for 15 minutes. 5-10% of this reaction served as a template for the PCR amplification. 5µl first strand cDNA was suspended in 5µl 10x

PCR buffer (100mM Tris-HCl (pH 9.0), 500mM KCl, 1% triton X-100), 3µl $MgCl_2$ (25mM), 1µl dNTP mix (10mM each), 32pmol sense and antisense primers, 5 units Taq DNA polymerase and sterile DEPC treated H_2O to a final volume of 50µl. PCR reaction conditions were 94°C for 5 minutes followed by 17-35 cycles (optimised to ensure that PCR amplification was within the logarithmic phase of the reaction which was quantified by digital camera imaging of the gel and use of Bio Image 2-D electrophoresis analysis software (Millipore UK)) of 94°C for 1 minute (denature), 56°C for 2 minutes (anneal), 74°C for 2 minutes (extend). A final 4 minute extension at 74°C was included. cDNAs including pT7/T3 hGRα cDNA fragment, pT7/T3 hGRβ cDNA fragment, pT7/T3-hGRα full length cDNA, 11β-HSD1/pcDNAI, h11β-HSD2/pGEM4Z, hMR cDNA and hNa/K ATPase αl cDNA served as positive controls for the PCR step. Negative controls included PCR of non-reverse transcribed RNA samples and omission of primers from both RT and PCR reactions.

Table 3.1 RT-PCR primer sequences and predicted product sizes

Primer		Sequence	Predicted Size
Na/K-ATPase	Sense	5'-ATATGGAACAGACTTGAGCCG-3'	652
	Antisense	5'-GGCAATTCTTCCCATCACAGT-3'	
MR	Sense	5'-AACTTGCCTCTTGAGGACCAA-3'	612
	Antisense	5'-TTTTCGGGGAGGAACCAAGG-3'	
GRβ	Sense	5'-ACTTACACCTGGATGACCAAAT-3'	585
	Antisense	5'-TCCTATAGTTGTCGATGAGCAT-3'	
11β-HSD1	Sense	5'-CTCCAGTCGGATGGCTTTTTATG-3'	571
	Antisense	5'-ACTTGCTTGCAGAATAGG-3'	
Grα	Sense	5'-ACTTACACCTGGATGACCAAAT-3'	487
	Antisense	5-TTCAATACTCATGGTCTTATCC-3'	
11β-HSD2	Sense	5'-ACCGTATTGAGTTGAACAAGC-3'	477
	Antisense	5'-TCACTGACTCTGTCTTGAAGC-3'	

3.2.9 *Northern blot analysis of 11β-hydroxysteroid dehydrogenase*

Northern blot analysis was performed with assistance from Dr. CB Whorwood at the Endocrine and Metabolism Unit, University Department of Medicine, University of Southampton.

3.2.9.1 *Reagents and buffers*

Formamide and formaldehyde were deionised by mixing with AG 501-X8 small mixed bead resin (Bio-Rad) at a ratio of 1g/10ml for 1hour at room temperature. The resin was removed by filtration through Whatmann 3M paper. Formamide was stored as 1ml and 50ml aliquots at -20°C. Gel loading dye comprised 1mM EDTA (pH8.0), 0.25% bromophenol blue, 0.25% xylene cyanol and 50% glycerol in H_2O treated with DEPC. MOPS (3-(N-morpholino)-propanesulphonic acid) electrophoresis buffer (x10) comprised 41.8g MOPS (0.2M), 16.6ml 3M DEPC treated sodium acetate (50mM) and 20ml 0.5M DEPC treated EDTA (10mM), pH adjusted to 7.0 with 6M NaOH and brought to a final volume of 1 litre. The buffer was DEPC treated, autoclaved twice and stored at room temperature in foil coated bottles. Tris-EDTA (TE) buffer comprising 10mM Tris[hydroxymethyl]aminomethane hydrochloride (Tris-HCl) and 1mM EDTA (pH8.0) was prepared from stocks of 1M Tris-HCl and 0.5M EDTA.

3.2.9.2 *Radiolabelled probes*

cDNA probes were radiolabeled with [^{32}P]-dCTP (3000Ci/mmol, Amersham International, UK) by oligonucleotide random priming of the cDNA fragment using commercially available kits (Amersham International, UK and Pharmacia, Uppsala, Sweden). cDNA was suspended to a concentration of 5-25ng/μl in TE buffer. 25-50ng of DNA was denatured by heating to 100°C for 5 minutes and snap cooled on ice. 10μl reagent mix (comprising 100mM dATP, dGTP and dTTP, random hexanucleotides in 0.5M Tris-Cl and 80mM magnesium chloride), 5μl [^{32}P]-dCTP (50μCi), sterile H_2O to a volume of 49μl and 1μl of the Klenow fragment of E. coli DNA polymerase I were added, mixed gently, microcentrifuge pulse spinned and incubated at 37°C for greater than 1 hour.

Anitsense human 11β-HSD1 cRNA probes (1.2Kb) were synthesised from the full length human 11β-HSD1 cDNA, subcloned into Bluescript KS, using T3 RNA polymerase, following linearisation with *Hind*III (methodology based upon that described by Whorwood *et al*[450]. cRNA probes were radiolabelled by incorporation of [^{32}P]UTP (3000Ci/mmol) as described by Stewart *et al*[157]. 5μl of 5 X transcription buffer (200mM Tris-HCl (pH7.5), 30mM magnesium chloride, 10mM spermidine and 50mM sodium chloride), 2.5μl DTT (100mM), 1μl RNasin (ribonuclease inhibitor, 40 units, stored at –20°C in a buffer comprising 20mM HEPES-potassium hydroxide (pH7.6), 50mM KCl, 5mM DTT and 50% glycerol), 4μl of a mixture of ATP, TTP and GTP (each at 10mM), 2.5μl UTP (100mM), 1μg of linearised template, 5μl (50Ci) [^{32}P]-UTP, 2μl RNA polymerase (20-30 units) and DEPC treated H_2O to bring the volume to 25μl were added sequentially to a sterile eppendorf tube at room temperature. The reaction mixture was incubated at 37°C for 3-4 hours. The DNA template was then digested by incubation with 1.5 units RNase free DNase I (RQ1) for 15 minutes at 37°C. The reaction was halted by addition of 1μl EDTA (200mM), the volume increased to 100μl by addition of DEPC treated H_2O. The probe was then vortex extracted with 1 volume of Tris-HCl (pH7.5) saturated phenol-chloroform-isoamylalcohol (25:24:1) followed by 1 volume of chloroform-isoamyl alcohol (24:1) and centrifuged (13,000 x g). The upper aqueous layer was transferred to another eppendorf tube containing 0.1 volumes of 3M sodium acetate (pH5.2), 2.5 volumes of ethanol and 20g yeast tRNA, vortex mixed and the probe precipitated by placing at –70°C for greater than 1 hour. Following centrifuging (13,000 x g for 10 minutes) the pellet was washed with 70% ethanol, suspended in 100μl DEPC treated H_2O and stored at -70°C.)

3.2.9.3 *Northern blot analysis*

Total RNA (30-50μg/lane) from cultured skeletal myoblasts was electrophoresed alongside RNA molecular weight markers (Amersham Pharmacia Biotech, Buckingham, UK) in a 20x20cm 1.5% agarose/ 15% formaldehyde/1x MOPS gel at a constant current of 100mA (5volts/cm), for 4-6 hours followed by transfer to Hybond N^+ membranes as described by Whorwood *et al*[56,57,414,450]. RNA samples were thawed on ice and 30-50μg

total RNA from cultured myoblasts pipetted into sterile eppendorf tubes containing 5µl 10x 3-[N-morpholino]propanesulphonic acid (MOPS) buffer (0.2M MOPS, 16.6ml 3M DEPC treated sodium acetate (50mM) and 20ml 0.5M DEPC treated EDTA (10mM), which was adjusted to pH 7.0 with 6M NaOH and brought to a final volume of 1 litre with H_2O), 9µl formaldehyde and 25µl deionised formamide. DEPC treated H_2O was added to bring the volume to 50µl. The secondary structure of the RNA was destroyed by heating to 65°C for 15 minutes and snap cooled on ice. Gel loading dye (5µl) was added, pulse centrifuged and the samples were electrophoresed alongside RNA molecular weight markers (Amersham Pharmacia Biotech, Buckingham, UK) in a 20x20cm 1.5% agarose/ 15% formaldehyde/1x MOPS gel at a constant current of 100mA (5volts/cm), for 4-6 hours. The gel was washed in transfer buffer (3.0M NaCl, 300mM tri-sodium citrate) for 20 minutes and placed on a wick of Whatmann 3M paper soaked in transfer buffer on a perspex bridge sitting in a tank of transfer buffer. Hybond N^+ nylon membrane (Amersham International) was soaked in H_2O, then in transfer buffer for up to 5 minutes and laid over the gel. Two pieces of Whatmann 3M paper soaked in transfer buffer were placed over the membrane and the parts of the wick not covered by the gel were sealed with parafilm. Paper towels were placed on top of the gel to a height of 4 cm which were covered with a perspex platform and a 500g weight to facilitate overnight capillary transfer of RNA to the membrane. The immobilised RNA was crosslinked with UV radiation (1200joules) and the membrane was stored in heat-sealed bags at 4°C.

Membranes were hybridised with either cDNA or cRNA probes for 11β-HSD1 and 18S in either 0.17M NaH_2PO_4, 0.59M Na_2HPO_4, 5mM EDTA, 7% sodium dodecylsulphate (SDS), 200µg/ml denatured salmon sperm DNA buffer (pH7.2) at 65°C for cDNA probes or 50% deionised formamide, 2xSSPE (0.36M NaCl, 0.02M NaH_2PO_4, 0.002M EDTA, pH8.0), 5x Denhardt's solution (0.02% ficoll, 0.02% polyvinylpyrrolidone, 0.1% BSA), 10% dextran sulphate, 0.1% SDS, 200µg/ml denatured salmon sperm DNA buffer at 42°C for cDNA probes or 63°C for cRNA probes. Membranes were washed in saline sodium citrate buffer (3.0M NaCl, 300mM tri-sodium citrate) containing 0.1% SDS (10 minutes at room temperature) and up to a maximum stringency of saline sodium citrate (0.15M NaCl, 15mM tri-sodium citrate):0.1% SDS (30 minutes at 68°C). Membranes

underwent phosphorimage analysis (Molecular Dynamics Model 850 Phosphorimager, Amersham Pharmacia Biotech, Buckingham, UK) and were exposed to autoradiographic film (Dupont-Cronex, GRI, UK) between intensifying screens at –70°C for 1-10 days such that the signal fell within the linear range of the film. Prior to rehybridisation, cDNA probes were removed from the membranes by washing with 1%SDS (3 hours at 70°C). cRNA probes were removed by pouring boiling 0.1%SDS onto the membrane and allowing it to cool to room temperature in a shaking waterbath.

3.2.9.4 *Northern blot hybridisation conditions*

cDNA Probes

Radiolabeled cDNA probes were denatured by heating to 95°C for 5 minutes, snap-cooled to 4°C and added to 0.17M NaH_2PO_4, 0.59M Na_2HPO_4, 5mM EDTA, 7% SDS, 200μg/ml denatured salmon sperm DNA buffer (pH7.2) at a concentration of 0.5ng/ml for 16 hours at 65°C with membranes either in heat sealed bags in polypropylene boxes in shaking water baths or in 30cm long glass bottles (Hybaid, Middlesex, UK) in convection ovens with the RNA side inner most lining the bottle. Membranes were then washed in saline sodium citrate buffer (3.0M NaCl, 300mM tri-sodium citrate) for 10 minutes at room temperature, followed by washes of increasing stringency to a maximum of saline sodium citrate (0.15M NaCl, 15mM tri-sodium citrate) containing 0.1% SDS at 65°C for 1 hour and washed in saline sodium citrate (0.15M NaCl, 15mM tri-sodium citrate) at room temperature.

cRNA Probes

Membranes were prehybridised in hybridisation buffer comprising 50% deionised formamide, saline sodium phosphate/EDTA buffer (0.36M NaCl, 0.02M NaH_2PO_4, 0.002M EDTA, pH8.0), 5x Denhardt's solution (0.02% ficoll, 0.02% polyvinylpyrrolidone, 0.1% BSA), 10% dextran sulphate, 0.1% SDS, 200μg/ml denatured salmon sperm DNA buffer at 42°C for greater than 6 hours. 15ng of probe was then heated to 65°C for 15 minutes, snap cooled on ice and pipetted into the bag or bottle containing the membrane and 10ml hybridisation buffer and incubated at precisely 63°C for 16 hours. Membranes were then washed in saline sodium citrate buffer (3.0M NaCl,

300mM tri-sodium citrate) containing 0.1% SDS for 30 minutes at room temperature followed by saline sodium citrate buffer (0.3M NaCl, 30mM tri-sodium citrate) containing 0.1% SDS at 68°C for 20 minutes.

3.2.10 *Western blot analysis of 11β-hydroxysteroid dehydrogenase*

3.2.10.1 *Reagents and buffers*

Sample loading buffer comprised 100mM Tris-HCl (pH6.8), 4% SDS, 0.2% bromophenol blue, 10% β-mercaptoethanol (added immediately prior to use) and 20% glycerol. A stock solution of 5x Tris-Glycine electrophoresis buffer comprised 125mM Tris-base, 1.25M glycine and 0.5% SDS. Tris buffered saline with Tween 20 (TBST) comprised 10mM Tris-Cl (pH8.0), 150mM NaCl and 0.05% Tween 20. Gel transfer buffer comprised 39mM glycine, 48mM Tris-base, 0.037% SDS which was prepared in 20% HPLC grade methanol. Blocking buffer comprised 20g nonfat powdered milk (Marvel; Premier Brands Ltd, Stafford, UK) in 100ml TBST. SDS molecular weight markers, comprised 8 pre-stained proteins (rabbit myosin (205kDa), β-galactosidase from E.coli (116kDa), bovine albumin (66kDa), chicken ovalbumin (45kDa), bovine carbonic anhydrase (29kDa), soybean trypsin inhibitor (20.1kDa), bovine α-lactalbumin (14.2kDa) and bovine aprotinin (6.5kDa)) in 62mM Tris-HCl (pH7.5), 2% SDS, 0.01mM EDTA, 100mM DTT, 4M urea, 0.005% bromophenol blue and 30% glycerol.

3.2.10.2 *Sample preparation*

Adherent cells were washed with ice cold PBS (x3), gently dislodged using a cell scraper and transferred to 15ml conical polypropylene centrifuge tubes (Greiner Labortechnik, Germany) in 5ml PBS containing 1mM PMSF and centrifuged at 200 x g for 5 minutes. The supernatant was aspirated to waste and the cell pellet was re-suspended in 300μl lysis buffer (PBS containing 1mM PMSF, 2% SDS, 0.5mM EDTA) at room temperature in 1.5ml Eppendorf tubes. The cells were lysed by repeated freeze thaw cycles (four times at –70°C). 100μl trichloroacetic acid (TCA) solution (100% trichloroacetic acid in H_2O w/v) was added to the lysate and vortex mixed briefly. The larger fillamentous precipitate, consisting principally of nucleic acid was allowed separate under gravity and

the supernatant, containing cellular protein suspended as a fine microparticulate precipitate, was transferred to a second 1.5ml Eppendorf tube. The lysate was centrifuged (3000 x g for 5 minutes at 4°C) to sediment the cellular protein precipitate whilst the supernatant was aspirated to waste. The cellular protein pellet was washed twice with 750µl TCA solution (2.5% w/v) and residual TCA solution was neutralised with 25µl tris-base (3M) and complete solublisation cellular proteins was achieved following addition of a further 25ml H_2O containing 1mM PMSF. The protein solution was stored at –70°C until analysis. The protein concentration of the lysate was determined using the method of Bradford (3.2.3.4).

3.2.10.3 *Preparation of human tissues for use as positive immunoreactive controls*

Approximately 250mg of human liver and kidney cortex (stored at –70°C) were homogenised separately in 2ml ice cold PBS containing 1mM PMSF. The homogenate was centrifuged and treated as for cell lysates (3.11.2)

3.2.10.4 *SDS-PAGE Electrophoresis*

10ml of 12% SDS-polyacrylamide resolving gel was prepared by mixing 4.0ml 30% acrylamide solution, 2.5ml 1.5M Tris-HCl (pH8.8), 0.1ml 10%SDS and 3.3ml H_20 which was polymerised with 100µl freshly prepared 10% ammonium persulphate and 5µl tetramethylethylenediame (TEMED). Sufficient gel mix for a Bio-Rad mini-protean II electrophoresis gel forming apparatus (BioRad, UK) was poured between glass plates to fill 60 – 80% of the volume for a gel width of 0.8mm (8cm x 10cm). Approximately 1ml isopropanol was carefully layered onto the surface of the setting gel which was allowed to set for one hour after which period the isopropanol was decanted to waste and the gel was washed with H_2O. A 5% polyacrylamide stacking gel was prepared by mixing 330µl 30% acrylamide with 250µl of 1M Tris-HCl (pH6.8), 20µl 10% SDS and 1.4ml H_2O which was polymerised with 20µl of freshly prepared 10% ammonium persulphate and 2µl TEMED. The stacking gel was poured onto the surface of the resolving gel and a teflon comb (10 lanes for a 30µl sample volume) was inserted. The gel was allowed to

polymerise for a minimum of 30 minutes after which time the teflon comb was removed and the wells were washed with H_2O. Cell lysates were diluted with an equal volume of sample loading buffer and heated to 95°C for 5 minutes to denature the proteins. Samples (10 – 100μg protein/lane) and SDS molecular weight markers were loaded onto the stacking gel and a voltage equivalent to 20 volts/cm was applied for 30 – 45 minutes.

3.2.10.5 *Electrophoretic transfer of proteins to polyvinylidene difluoride membranes*

The gel was removed from the glass plates by immersing in transfer buffer, the stacking gel was removed and the resolving gel was allowed to equilibrate in transfer buffer for 30 minutes. Six pieces of Whatmann 3M paper and one piece of immobilon P membrane (Millipore Ltd, Watford, UK) were cut to the size of the gel. The membrane was hydrated by brief immersion in methanol followed by 2 minutes in H_2O prior to equilibration for 10 - 15 minutes in transfer buffer. The Whatmann 3M paper and Biorad mini Protean II gel transblot system filter pads were also equilibrated by soaking in transfer buffer for 10 minutes. A 'transfer sandwich' consisting of a stack of 3 sheets of Whatmann 3M paper followed by the gel, the membrane and the second set of three sheets of Whatmann 3M paper were placed between the transblot system filter pads, placed into a cassette and inserted into the transblotting tank containing ice cold transfer buffer. Transfer of the proteins was effected using a current of 150 - 200mA (100 volts) for one hour. In order to confirm complete electrophoretic transfer the gel was stained with Coomassie Blue by horizontal shaking in staining solution (1g Coomassie Blue in 250ml 95% ethanol, 50ml glacial acetic acid and 200ml H_2O) for 2 hours. Gels were de-stained by washing in 25% ethanol:10% glacial acetic acid overnight.

3.2.10.6 *Immunodetection of proteins using a chemiluminescent detection system*

Following electropheretic transfer, the membrane was washed in TBST for 5 minutes. The membranes were blocked in 10ml blocking buffer at room temperature for 1 hour with gentle horizontal shaking. The membrane was rinsed twice with 25ml TBST followed by a 15 minute wash in 25ml TBST prior to overnight incubation with 10ml

primary antibody (either affinity purified sheep polyclonal antibody raised against amino acids 19-33 of human 11β-HSD1 or affinity purified sheep polyclonal antibody raised against amino acids 137-160 and 334-358 of human 11β-HSD2 obtained from The Binding Site, Birmingham, UK) diluted in TBST containing 0.1% non-fat milk (1:500 for sheep anti-human 11β-HSD1 or 1:1000 for sheep anti-human 11β-HSD2) on a horizontal shaker. The primary antibody was removed and the membrane was rinsed twice with 25ml TBST and washed with 25ml TBST (x3) for 10 minutes prior to incubation with secondary antibody (anti-sheep IgG conjugated with horse-radish peroxidase (The Binding Site, Birmingham, UK)) diluted in TBST containing 5% non-fat milk (1:40,000 for 11β-HSD1 or 1:50,000 for 11β-HSD2) for one hour at room temperature. The secondary antibody was removed and the membranes were rinsed twice with 25ml TBST and washed with 25ml TBST (x3) for 10 minutes. 11β-HSD1 and 11β-HSD2 was visualised using the supersignal enhanced chemiluminescence system (Pierce & Warriner (UK) Ltd, Chester, UK). 1ml freshly prepared chemiluminescence substrate was applied to the membrane which was subsequently sealed in clear polythene membrane and exposed to Kodak BioMax Light autoradiographic film within its linear range.

3.2.11 *Immunohistochemical analysis of human skeletal myoblasts and JEG-3 cells*

Adherent myoblasts or JEG-3 cells in $10cm^2$ petri dishes were washed with PBS, air dried and fixed with either 10% formol saline or 4% paraformaldehyde. 5-6 20mm diameter circles per dish of cells were created using a hydrophobic resin to allow multiple parallel immunostaining with different antisera. Non-specific immunostaining was diminished by incubation with 5% serum from the species from which the secondary antisera were raised (Dako A/S, Denmark) diluted in PBS for 1 hour at room temperature. The cells were washed with PBS (x3) incubated with primary antisera skeletal muscle desmin antiserum at 1:10 – 1:100, mouse monoclonal sarcomeric alpha-actinin antiserum at 1:100 – 1:1000, mouse monoclonal anti-human fibroblast surface protein antiserum at 1:500 – 1:2000, mouse monoclonal anti α-smooth muscle actin at 1:500 – 1:2000, sheep monoclonal anti-human 11β-HSD1 antiserum at 1:10 – 1:100 and

sheep monoclonal anti-human 11β-HSD2 antiserum at 1:10 – 1:100 (The Binding Site, Birmingham, UK) for either 2 hours at room temperature or overnight at 4°C in a humidified box. Antisera were prepared by dilution in PBS containing 0.05% Tween-20. Omission of primary antisera, adsorption of primary antisera with appropriate purified proteins and the use of non-immune sera (Dako A/S, Denmark) served as negative controls for specific immunostaining. The cells were washed a further three times in PBS prior to incubation with horse-radish peroxidase conjugated anti-rabbit IgG, anti-mouse IgG or anti-sheep IgG secondary antisera at 1:1000 – 1:5000 for a further 1 hour at room temperature. Immunostaining was detected following brief incubation with diaminobenzadine and visualised under light microscopy.

3.2.12 *Hormonal regulation of 11β-hydroxysteroid dehydrogenase activity in normal human skeletal myoblasts and JEG-3 cells*

Monolayer cultures of normal human skeletal myoblasts (3.2.2.1) and JEG-3 cells were grown to 90% sub-confluence in 25cm^2 flasks. Cells used for experiments performed in the absence of FCS were maintained in FCS free growth medium for a period of 48 hours prior to experimentation. Stock solutions of steroids, peptides and growth factors (table 3.2 & 3.3, page 152) were prepared as previously described (3.2.1.2 & 3.2.1.3), added to growth medium (final ethanol concentration <0.1% v/v) at concentrations and in combinations as illustrated in tables 3.2,3.3 & 3.4, page 152 & 153 and exposed to triplicate flasks of cells for up to 96 hours after which period the growth medium was removed and the cells were washed three times with 5ml hormone free growth medium. Estimation of 11β-HSD activity and the kinetic constants, K_m and V_{max}, in intact cells and homogenates of JEG-3 cells was performed as previously described (3.2.41, 3.2.4.2, 3.2.4.3 & 3.2.4.4). Assays were also performed in the presence of the glucocorticoid receptor antagonist, RU 38486 (500 – 2000mM). Statistical comparison of 11β-HSD activity (expressed as the rate of conversion of cortisone to cortisol for 11β-HSD1 or cortisol to cortisone for 11β-HSD2) was made using the unpaired 't' test after logarithmic transformation of the data or the Mann-Whitney 'U' test.

Table 3.2 Concentration of steroid hormones with which human skeletal myoblasts and JEG-3 cells were incubated prior to assay of 11β-hydroxysteroid dehydrogenase activity

Steroid	Myoblasts	JEG-3 cells
5α- & 5β-dihydrocortisol	↑	↑
5α-dihydrocortisone		
5α- & 5β-tetrahydrocortisol		
5α-tetrahydrocortisone		
6β-hydroxycortisol		
11-deoxycorticosterone		
11-deoxycortisol		
17α-hydroxypregnenolone	50 – 1000nM	50 – 2000nM
17α-hydroxyprogesterone	↓	↓
18-hydroxycorticosterone		
α- & β-cortol		
α- & β-cortolone		
Corticosterone		
Cortisol		
Cortisone		
Pregnenolone		
Carbenoxolone	50 – 200nM	
Androstenedione	5 – 200nM	50 – 1000nM
Dehydroepiandrosterone	50 – 500nM	50 – 2000nM
Testosterone	5 – 200nM	50 – 1000nM
17β-oestradiol	0.05 – 2nM	0.05 – 50nM
Progesterone	10 – 500nM	50 – 500nM

Table 3.3 Concentration of peptide hormones and growth factors with which human skeletal myoblasts and JEG-3 cells were incubated prior to assay of 11β-hydroxysteroid dehydrogenase activity

Peptide/Growth Factor	Myoblasts	JEG-3 cells
ACTH	-	5 – 500ng/L
FSH	-	50 – 200IU/L
growth hormone	0.05 – 5nM	0.05 – 5nM
LH	-	50 – 200IU/L
hCG	-	3 –30kU/L
IGF-1	50 – 500μg/L	50 – 1000μg/L
Insulin	10 – 100μU/ml	10 – 100μU/ml
T3	5 – 200pM	50 – 200pM
IL-1β	5 – 20ng/L	50 – 200ng/L
IL-2	5 – 20ng/L	50 – 200ng/L
IL-6	5 – 20ng/L	50 – 200ng/L
IL-7	5 – 20ng/L	50 – 200ng/L
TNF-α	5 – 20ng/L	50 – 200ng/L

Table 3.4 Combinations and concentration of peptides, steroids and growth factors with which human skeletal myoblasts and JEG-3 cells were incubated prior to assay of 11β-hydroxysteroid dehydrogenase activity

Myoblasts	JEG-3 cells
Insulin (10 – 100μU/ml) & cortisol (100 – 500nM)	Insulin (10 – 100μU/ml) & cortisol (0.5 – 2μM)
Insulin (10 – 100μU/ml) & cortisone (100 – 500nM)	-
Cortisol (200 – 500nM) & carbenoxolone (200nM)	Cortisol (0.5 – 2μM) & carbenoxolone (2μM)
Cortisone (200 – 500nM) & carbenoxolone (200nM)	-
-	Progesterone (500nM) & 17β-oestradiol (50nM)
Cortisol (500nM) & RU 38486 (2μM)	Cortisol (2μM) & RU 38486 (4μM)

3.3 Results

3.3.1 *Immunohistochemical analysis of human skeletal myoblasts and JEG-3 cells*

The characteristic morphology of skeletal myoblasts (figure 3.1a, page 155) and their spontaneous and serum deprivation-induced fusion to form multinuclear myotubes (figure 3.1b) in association with positive immunohistochemical staining for skeletal muscle desmin (figure 3.1c) and sarcomeric α-actinin and negative staining for human fibroblast surface antigen and α-smooth muscle actin, confirmed a yield of greater than 99% proliferating human skeletal myoblasts. Positive immunohistochemical staining for 11β-HSD1 was found to be distributed evenly throughout the cytoplasm with no staining within the nuclear compartment (figure 3.1d). Positive staining for 11β-HSD2 was not observed. Similarly in JEG-3 cells, positive immunohistochemical staining for 11β-HSD2 was found to be throughout the cytoplasm. However, there appeared to be markedly greater staining in the cytoplasm surrounding the nucleus and indeed there was also a low level of positive staining within the nuclear compartment (figure 3.2a/b, page 156).

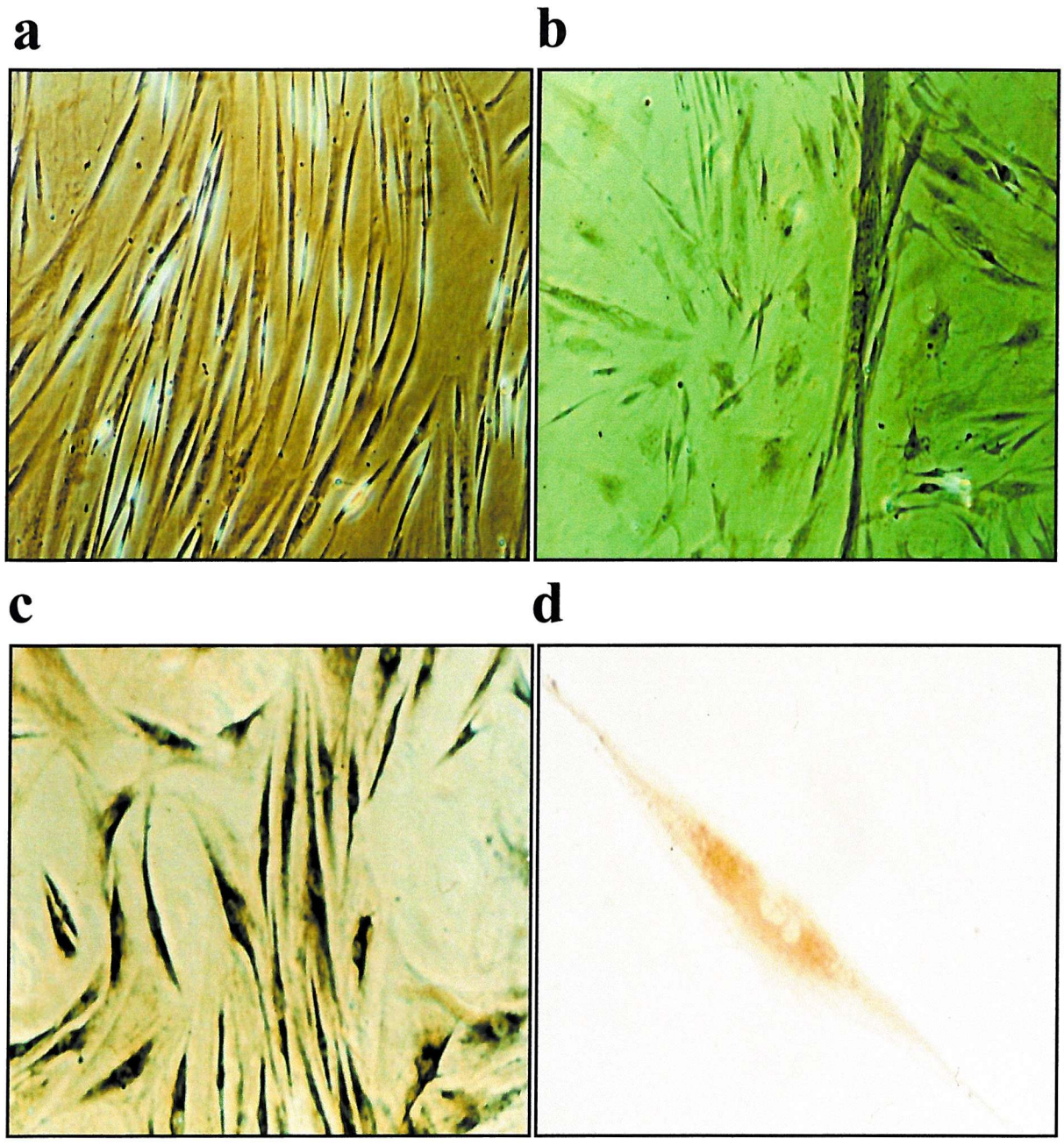


Figure 3.1 Morphological and immunohistochemical characterisation of human skeletal myoblasts. (a) morphology of human skeletal myoblasts grown in tissue culture (b) spontaneous and experimentally induced myotube formation. Immunohistochemical characterisation of skeletal myoblast phenotype was confirmed by positive staining for sarcomeric alpha-actinin (c) and negative staining for human surface fibroblast surface antigen (data not shown) (d) immunohistochemical staining for 11β-HSD1

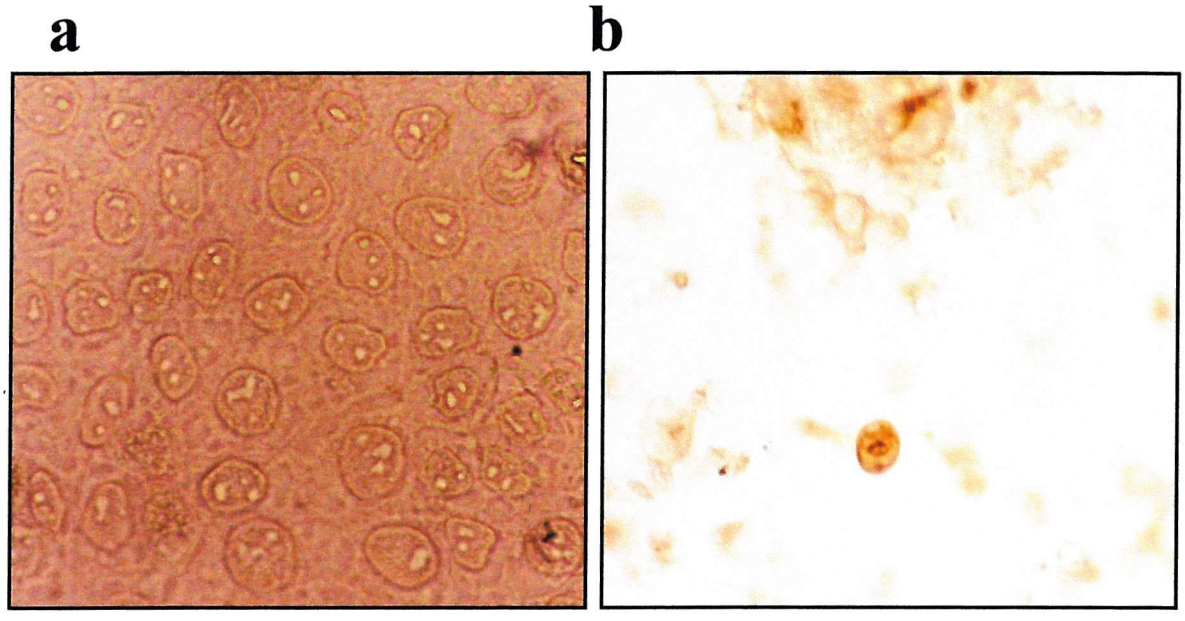


Figure 3.2 Morphological and immunohistochemical characterisation of JEG-3 cells (a) characteristic morphology of JEG-3 cells (x40) (b) positive immunostaining for 11β-HSD2 in JEG-3 cells was observed throughout the cytoplasm and within the nuclear compartment.

3.3.2 *11β-hydroxysteroid dehydrogenase in human skeletal myoblasts and JEG-3 cells*

3.3.2.1 *Human skeletal myoblasts express 11β-hydroxysteroid dehydrogenase type I mRNA, immunoreactive protein and activity*

Qualitative analysis of gene expression in myoblasts by RT-PCR using primers specific for 11β-HSD1, 11β-HSD2, and sodium/potassium-ATPase α1 subunit (Na/K-ATPase-α1) (table 3.1, page 142) revealed expression of 11β-HSD1 mRNA while 11β-HSD2 mRNA was not observed (figure 3.3b, page 158). Western blot analysis using a sheep anti-human-11β-HSD1 primary antiserum revealed a single 34kDa immunoreactive protein (figure 3.3c). In intact cells from a normal healthy subject the apparent K_m and V_{max} for the conversion of cortisone to cortisol was 476nM and 94pmol/mg protein/hour and 437nM and 46pmol/mg protein/hour in the presence and absence of 20% FCS respectively (Figure 3.3a). Conversion of cortisol to cortisone, indicative of 11-DH activity was undetectable.

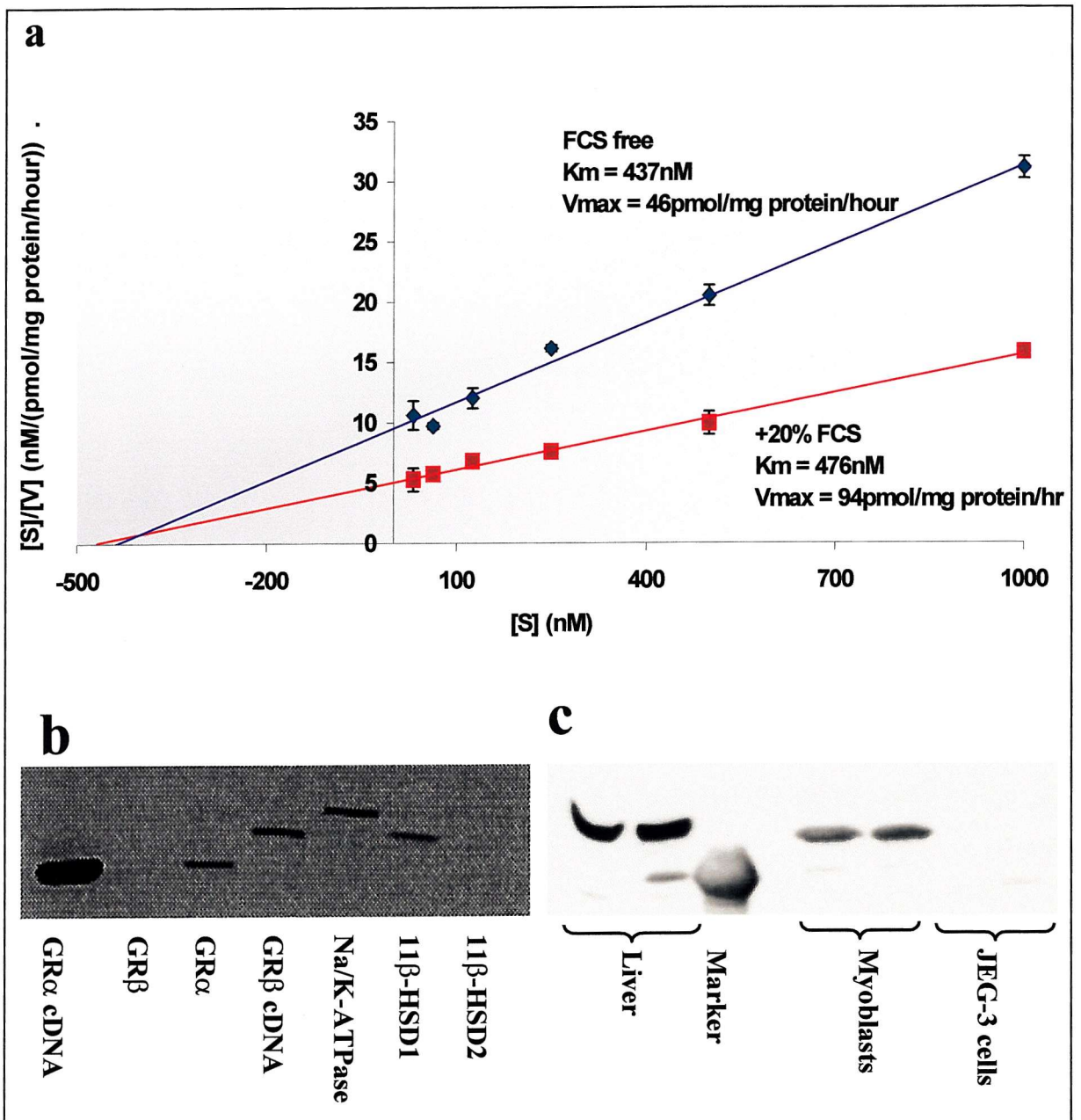


Figure 3.3 Characteristics of 11β-hydroxysteroid dehydrogenase in human skeletal myoblasts: (a) Kinetic analysis (Hanes plot) of 11β-dehydrogenase activity in intact human skeletal myoblasts in the presence and absence of 20% FCS revealed no significant difference in the affinity (K_m) of 11β-HSD1 for cortisone. However, serum removal resulted in a significant decline ($P<0.0001$) in the maximum velocity (V_{max}) of 11-oxoreductase activity. (data points represent the mean & SEM of triplicate analyses) (b) RT-PCR analysis revealed 11β-HSD1 but not 11β-HSD2 mRNA expression in human skeletal myoblasts (c) Western blot analysis of 34kDa 11β-HSD1 immunoreactive protein in skeletal myoblasts (90μg myoblast protein and 10μg liver protein loaded onto the gel). 11β-HSD1 immunoreactive protein was also detected in tissue from human liver which was used as a positive control but was not detected in JEG-3 cells.

3.3.2.2 *JEG-3 cells express 11β-hydroxysteroid dehydrogenase type II mRNA, immunoreactive protein and activity*

Qualitative analysis of gene expression in JEG-3 cells by RT-PCR using primers specific for 11β-HSD1 and 11β-HSD2 (table 3.1, page 147) revealed abundant expression of 11β-HSD2 but not 11β-HSD1 mRNA (figure 3.4c, page 160). Western blot analysis using a sheep anti-human-11β-HSD2 primary antiserum revealed a single 44kDa immunoreactive protein (figure 3.4d). The apparent K_m and V_{max} for the conversion of cortisol to cortisone in JEG-3 cell homogenates to which 1mM NAD had been added was 80nM and 50pmol/mg protein/hour respectively (figure 3.4a). In intact cells the apparent K_m and V_{max} for cortisol was 44nM and 147pmol/mg protein/hour and 53nM and 125pmol/mg protein/hour in the presence and absence of 10% FCS respectively (Figure 3.4b). Conversion of cortisone to cortisol, indicative of 11-OR activity was not observed either in intact cells or homogenates. Conversion of cortisol to cortisone or of the reverse reaction was not observed in homogenates when incubated in the presence of NADP or NADPH. This is consistent with the absence of 11β-HSD1 gene expression in these cells.

3.3.3 *Regulation of 11β-hydroxysteroid dehydrogenase by glucocorticoid*

3.3.3.1 *11β-hydroxysteroid dehydrogenase activity in human skeletal myoblasts and JEG-3 cells is unaffected by ACTH and precursors of cortisol and aldosterone biosynthesis*

11-OR activity in myoblasts and 11-DH activity in JEG-3 cells was unaffected by pre-treatment with ACTH, 5α- and 5β-dihydrocortisol, 5α-dihydrocortisone, 5α- and 5β-tetrahydrocortisol, 5α-tetrahydrocortisone, 6β-hydroxycortisol, 11-deoxycorticosterone, 11-deoxycortisol, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, α- and β-cortol, α- and β-cortolone, corticosterone or pregnenolone.

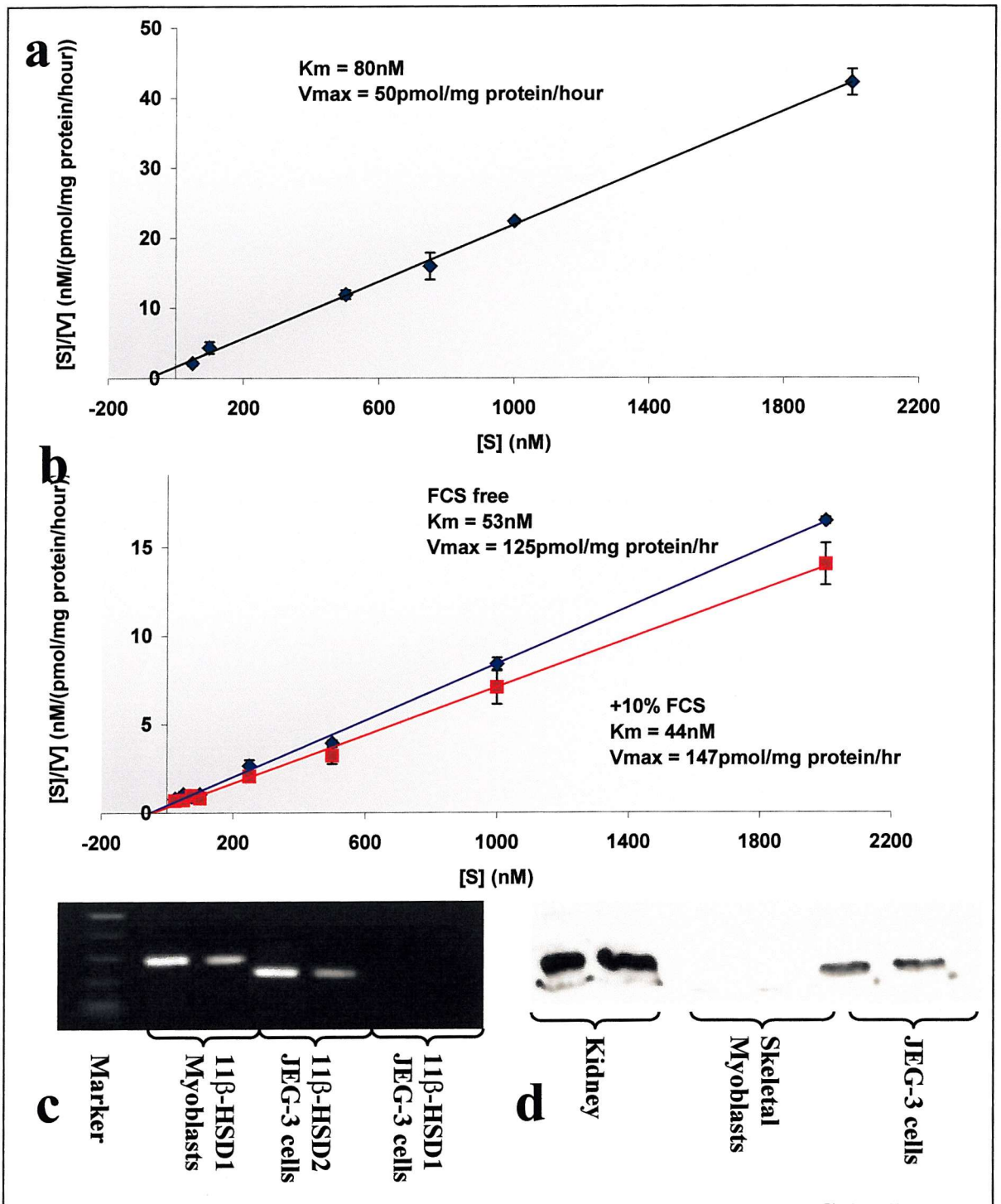


Figure 3.4 Characteristics of 11β-hydroxysteroid dehydrogenase in JEG-3 cells: Kinetic analyses (Hanes plots) of NAD dependent 11β-dehydrogenase activity in (a) JEG-3 cell homogenates (b) intact JEG-3 cells in the presence and absence of 10% FCS revealed no significant difference in the affinity (K_m) of 11β-HSD2 for cortisol. However, serum removal resulted in a significant decline ($P<0.05$) in the maximum velocity (V_{max}) of 11-dehydrogenase activity (data points represent the mean ± SEM of triplicate analyses) (c)RT-PCR analysis revealed 11β-HSD2 but not 11β-HSD1 mRNA expression in JEG-3 cells. (d) Western blot analysis of 44kDa 11β-HSD2 immunoreactive protein in JEG-3 cells (40μg total cell protein/lane loaded onto the gel). 11β-HSD2 immunoreactive protein was also detected in tissue from human kidney which was used as a positive control but was not detected in human skeletal myoblasts.

3.3.3.2 *Regulation of 11β-hydroxysteroid dehydrogenase by glucocorticoid in human skeletal myoblasts is effected at a pretranslational level and abolished by RU38486*

Pre-treatment of myoblasts with a range of concentrations of cortisol or cortisone (50 – 1000nM) for 48 hours resulted in a dose dependent increase in 11β-HSD1 mRNA, immunoreactive protein and 11-OR activity ($P < 0.05$), at concentrations of cortisol greater than 100nM (figure 3.5a, 3.6a,b,d page162 & 163). Pre-treatment with cortisol under serum free conditions enhanced 11-OR activity by a 2-3 fold greater extent than when in the presence of serum (figure 3.9, page 169). Furthermore, in keeping with the changes in 11β-HSD1 mRNA and 34kDa immunoreactive protein, multiple kinetic analyses of enzyme activity revealed that pre-treatment of myoblasts with 500nM cortisol had no significant effect on the affinity of 11β-HSD1 for cortisone but increased the maximum velocity of the reaction (V_{max}) by 40% (figure 3.5b) ($P <0.001$). Co-pre-treatment of myoblasts with cortisone and carbenoxolone abolished the induction of 11β-HSD1 mRNA expression and 11-OR activity which was observed following pre-treatment of the cells with cortisone alone (figure 3.5a, 3.6c). Pre-treatment of myoblasts with carbenoxolone alone had no effect upon 11-OR activity (figure 3.5a). Co-pre-treatment of myoblasts with cortisol (500nM) and the glucocorticoid receptor antagonist, RU38486 (2μM) completely abolished the increase in 11-OR activity which was observed following pre-treatment of myoblasts with cortisol alone (figure 3.5a).

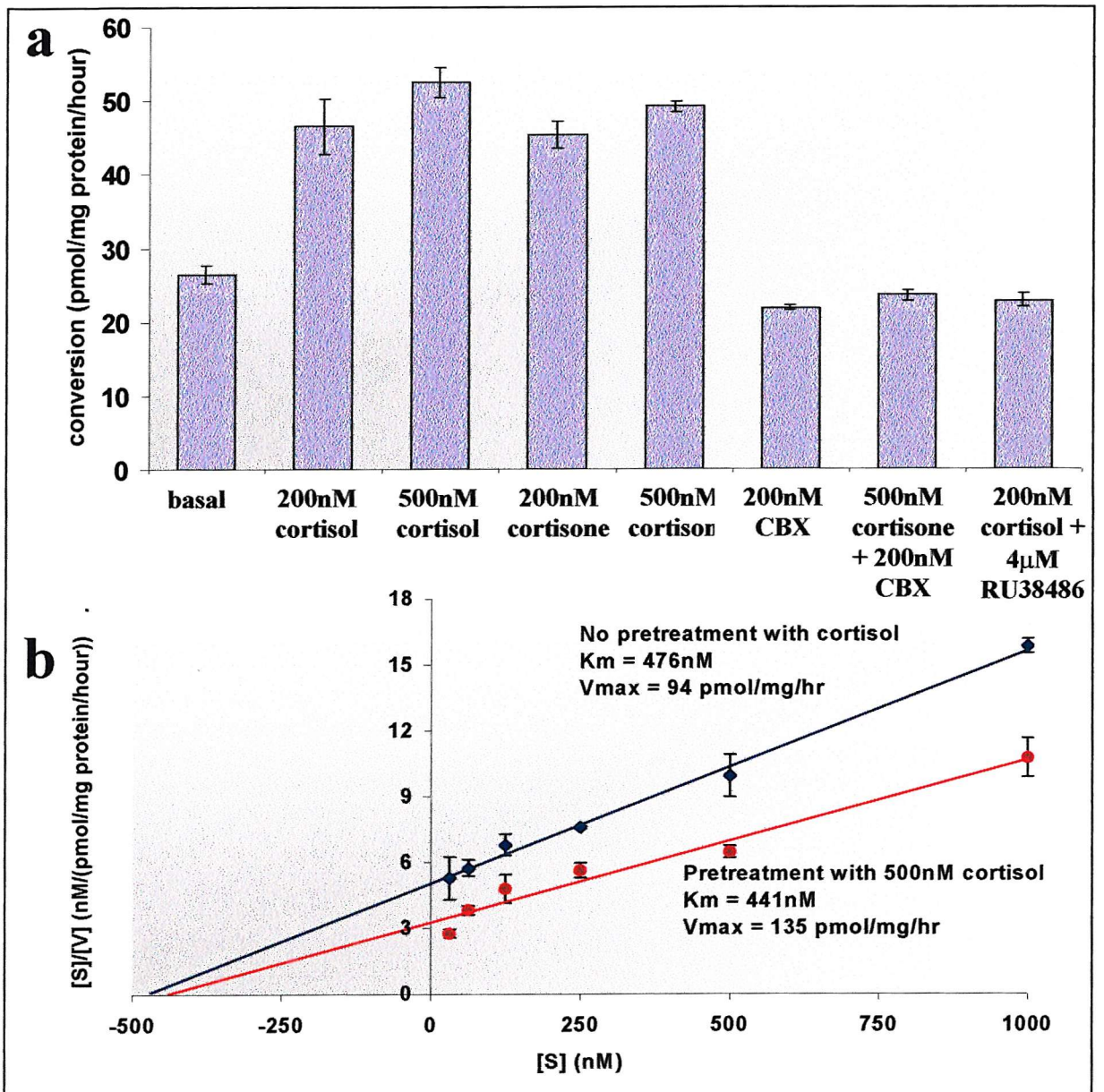


Figure 3.5 Regulation of 11β-hydroxysteroid dehydrogenase in human skeletal myoblasts by glucocorticoid: (a) analyses of 11β-HSD1 11-oxoreductase activity in skeletal myoblasts revealed a dose dependent induction of 11-oxoreductase activity following pre-treatment with cortisol and cortisone. Co-pre-treatment of skeletal myoblasts with cortisone and carbenoxolone abolished the induction of 11-oxoreductase activity which was observed following pre-treatment of the cells with cortisone alone. Co-pre-treatment of skeletal myoblasts with cortisol and the glucocorticoid receptor antagonist, RU38486, completely abolished the increase in 11-oxoreductase activity which was observed following pre-treatment of myoblasts with cortisol alone (the data are represented by the mean ± SEM of triplicate analyses) (b) Kinetic analysis of 11β-HSD1 activity in skeletal myoblasts (Hanes plot) revealed no significant change in the affinity (K_m) of 11β-HSD1 for cortisone following pre-treatment with glucocorticoid. However pre-treatment with 500nM cortisol induced a significant increase ($P<0.001$) in the maximum velocity (V_{max}) of 11-oxoreductase activity (the data points are expressed as the mean ± SEM of triplicate analyses)

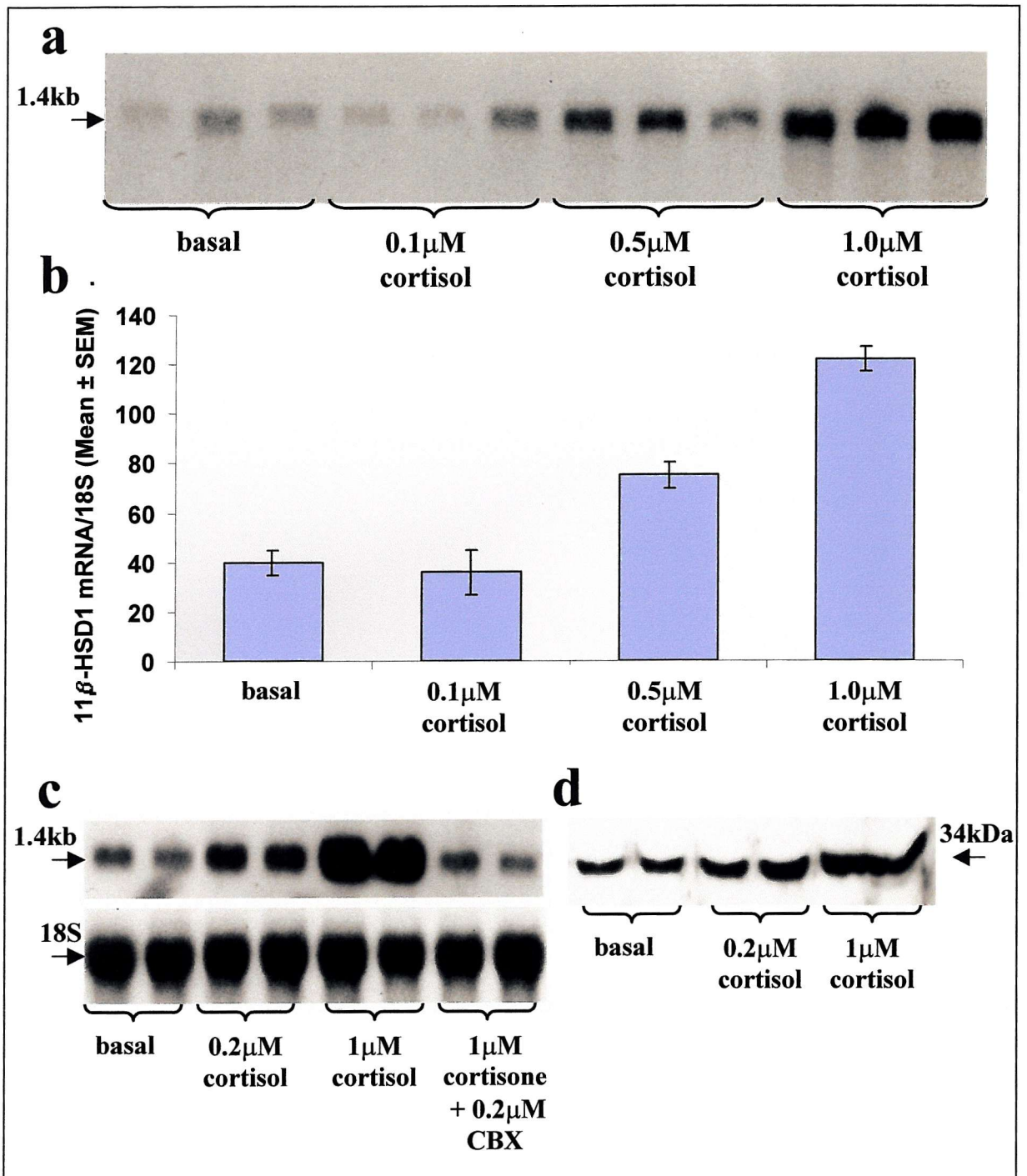


Figure 3.6 Regulation of 11β-hydroxysteroid dehydrogenase mRNA in human skeletal myoblasts by glucocorticoid: (a/b) Northern blot analysis of the glucocorticoid-induced dose dependent increase in 11β-HSD1 mRNA expression (1.4kb) in human skeletal myoblasts (data illustrates RNA from triplicate analyses and each lane represents RNA pooled from triplicate flasks). (b) Histogram of quantitative change in 11β-HSD1 mRNA/18S rRNA ratio (from (a)) (c) Northern blot analysis of the dose dependent induction of 11β-HSD1 mRNA expression in skeletal myoblasts following co-pre-treatment with cortisone & carbenoxolone (d) Western blot analysis of the dose dependent induction of 34kDa 11β-HSD1 protein in skeletal myoblasts following pre-treatment with glucocorticoid (90μg total cell protein/lane loaded onto the gel).

3.3.3.3 *Regulation of 11β-hydroxysteroid dehydrogenase by glucocorticoid in JEG-3 cells*

In common with the regulation of 11β-HSD1 11-OR activity in myoblasts, pre-treatment of JEG-3 cells with cortisol (2μM) for 24 hours also resulted in a significant increase in 44kDa 11β-HSD2 immunoreactive protein and 11-DH activity (pre-treatment with 2μM cortisol induced a 17% increase cf. basal activity, P <0.002; n = 10) (figure 3.7a/b, page 165). Multiple kinetic analyses of enzyme activity revealed that pre-treatment of JEG-3 cells with 2000nM cortisol had no significant effect on the affinity of 11β-HSD2 for cortisol but increased the V_{max} by 25% (P <0.001). However, in contrast with glucocorticoid regulation of 11-OR activity in myoblasts, the magnitude of 11-DH induction by cortisol was not increased by removal of serum from the growth medium. Nevertheless, induction of 11-DH activity as a consequence of pre-treatment with 2μM cortisol alone was was unaffected by co-pre-treatment with cortisol (2μM) and RU38486 (4μM). Pre-treatment of JEG-3 cells with RU38486 alone had no effect upon 11-DH activity. Importantly, pre-treatment of JEG-3 cells with cortisone had no effect upon 44kDa 11β-HSD2 immunoreactive protein or 11-DH activity.

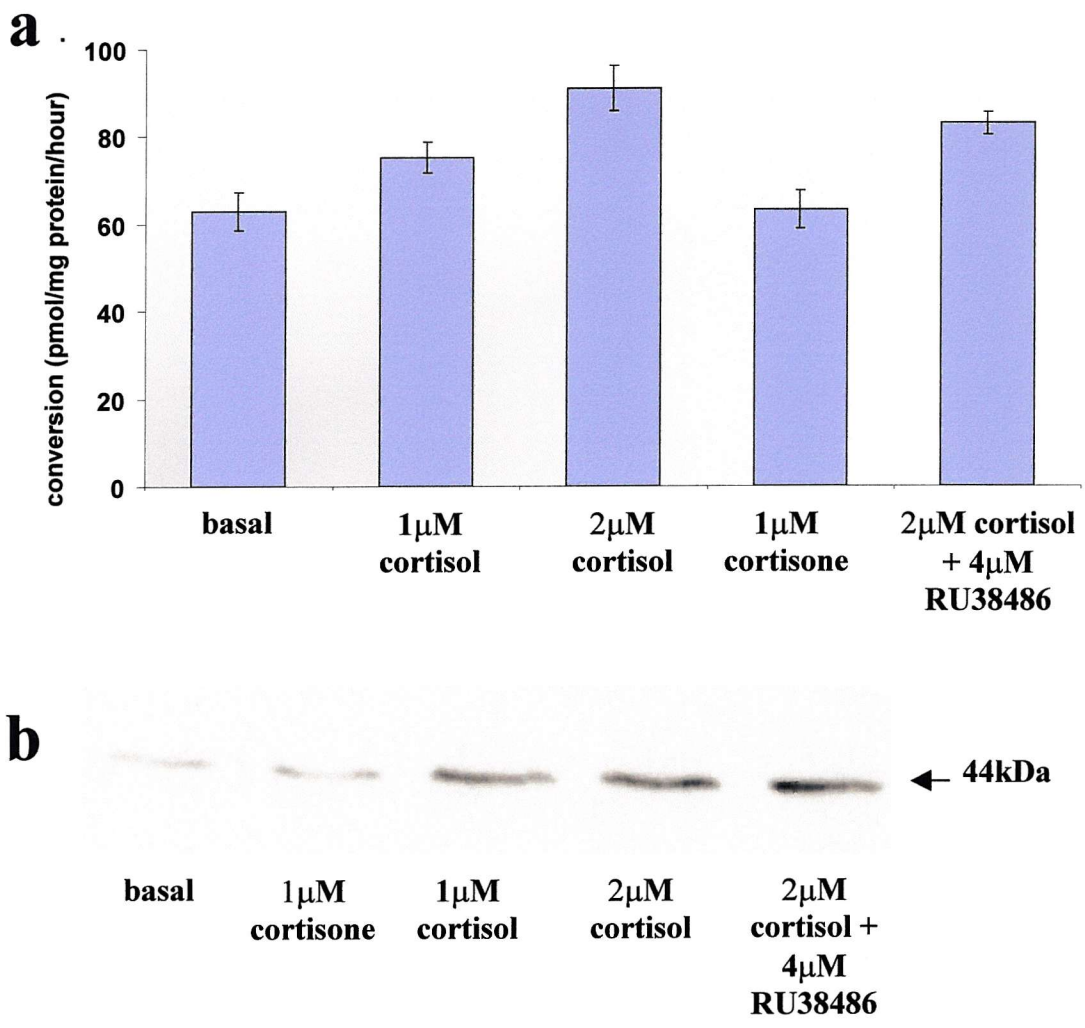


Figure 3.7 Regulation of 11β-hydroxysteroid dehydrogenase in JEG-3 cells by glucocorticoid: (a) analysis of 11β-HSD2 activity in JEG-3 cells revealed a dose dependent induction of 11-dehydrogenase activity by cortisol whilst cortisone had no detectable effect upon 11-dehydrogenase activity. Co-pre-treatment of JEG-3 cells with cortisol and the glucocorticoid receptor antagonist, RU38486, did not affect the induction of 11-dehydrogenase activity effected by cortisol alone (all data points are expressed as the mean ± SEM of triplicate analyses)
(b) Western blot analysis confirmed the induction of 44kDa 11β-HSD2 immunoreactive protein following pre-treatment of JEG-3 cells with glucocorticoid. Co-pre-treatment of JEG-3 cells with cortisol and the glucocorticoid receptor antagonist, RU38486, had no effect upon the glucocorticoid-induced increase in 44kDa 11β-HSD2 immunoreactive protein effected by cortisol alone (10μg total cell protein/lane loaded onto the gel).

3.3.4 *Regulation of 11β-hydroxysteroid dehydrogenase by insulin in human skeletal myoblasts is dependent upon serum factors*

Pre-treatment of myoblasts with insulin at a concentration of 20μU/ml in the presence of 20% FCS had no statistically significant effect upon 11β-HSD1 11-OR activity. However,in the presence of serum there was a significant reduction in 11β-HSD1 11-OR activity following pre-treatment with 40μU/ml insulin (21.8% reduction in 11β-HSD1 11-OR activity cf. basal activity, P <0.01) (figure 3.8a, page 168). Similarly, pre-treatment with 20μU/ml insulin under serum free conditions had no effect upon 11β-HSD1 11-OR activity whilst pre-treatment with 40μU/ml insulin under serum free conditions induced a significant increase in 11β-HSD1 11-OR activity (19.8% increase in 11β-HSD1 11-OR activity cf. basal activity, P <0.05) (Figure 3.8a). Western blot analysis revealed parallel, but much greater, changes in 34kDa immunoreactive protein (figure 3.8b). In common with earlier experiments, kinetic analysis of 11β-HSD1 activity revealed that pre-treatment of myoblasts with 100μU/ml insulin had no effect upon the affinity of 11β-HSD1 for cortisone (Km = 445 ± 6.1; mean ± SEM) but caused a 19% decline in V_{max} in the presence of 20% FCS (P <0.01) whilst serum removal induced a 6% increase in V_{max} which, however, failed to achieve statistical significance (P = 0.14). Pre-treatment of JEG-3 cells with insulin both in the presence and absence of 10% FCS had no detectable effect upon 11-DH activity (P = 0.2, n = 10).

3.3.5 *Regulation of 11β-hydroxysteroid dehydrogenase by insulin and glucocorticoid in combination in human skeletal myoblasts*

In the presence of 20% FCS the marked increase in 11β-HSD1 11-OR activity effected by pre-treatment with 200nM cortisol alone (1.3 fold cf. basal, P <0.05) was attenuated by co-pre-treatment with both insulin (20 - 100μU/ml) and cortisol (200nM). However, insulin had no effect on the markedly greater increase in 11β-HSD1 11-OR activity which resulted as a consequence of pre-treatment with 500nM cortisol alone (1.5 fold cf. basal, P <0.05) (figure 3.9a, page 169). In contrast, under serum free conditions, the induction of 11β-HSD1 11-OR activity effected by 200nM cortisol alone (1.7 fold cf. basal, P <0.02) was markedly increased as a consequence of co-pre-treatment with

20μU/ml insulin and 200nM cortisol (2.1 fold cf. basal, P <0.0005). However, co-pre-treatment with 100μU/ml insulin and 200nM cortisol did not further enhance 11-OR activity. The markedly greater induction of 11β-HSD1 11-OR activity following pre-treatment with 500nM cortisol under serum free conditions (2.4 fold cf. basal, P <0.0001) was also further enhanced (4 fold cf. basal, P <0.0001) as a consequence of co-pre-treatment with 100μU/ml insulin and 500nM cortisol (figure 3.9b, page 169). Northern blot analyses revealed parallel marked changes in 11β-HSD1 mRNA (figure 3.10, page 170). Co-pre-treatment of JEG-3 cells with insulin and cortisol did not further change the increase in 11-DH activity induced by pre-treatment with 2μM cortisol alone (P = 0.528; n = 10)

3.3.6 *11β-hydroxysteroid dehydrogenase activity in human skeletal myoblasts is unaffected by T3 and growth hormone but pre-treatment with IGF-1 mirrors the effects of insulin*

Pre-treatment of myoblasts with T3 (5 – 500pM) or GH (0.05 – 5nM) had no effect upon either 11β-HSD1 11-OR activity. In marked contrast however, pre-treatment of myoblasts with IGF-1 (50 – 200μg/L) in the presence of 20% FCS resulted in a significant dose dependent decline in 11-OR activity (P <0.02) whilst in the absence of serum similar pre-treatment of myoblasts resulted in a dose dependent increase in 11-OR activity (P <0.05) (figure 3.11, page 171). However, 34kDa 11β-HSD1 immunoreactive protein was unaffected by pre-treatment with IGF-1. 11β-HSD2 11-DH activity in JEG-3 cells was unaffected by pre-treatment with T3, GH and IGF-1.

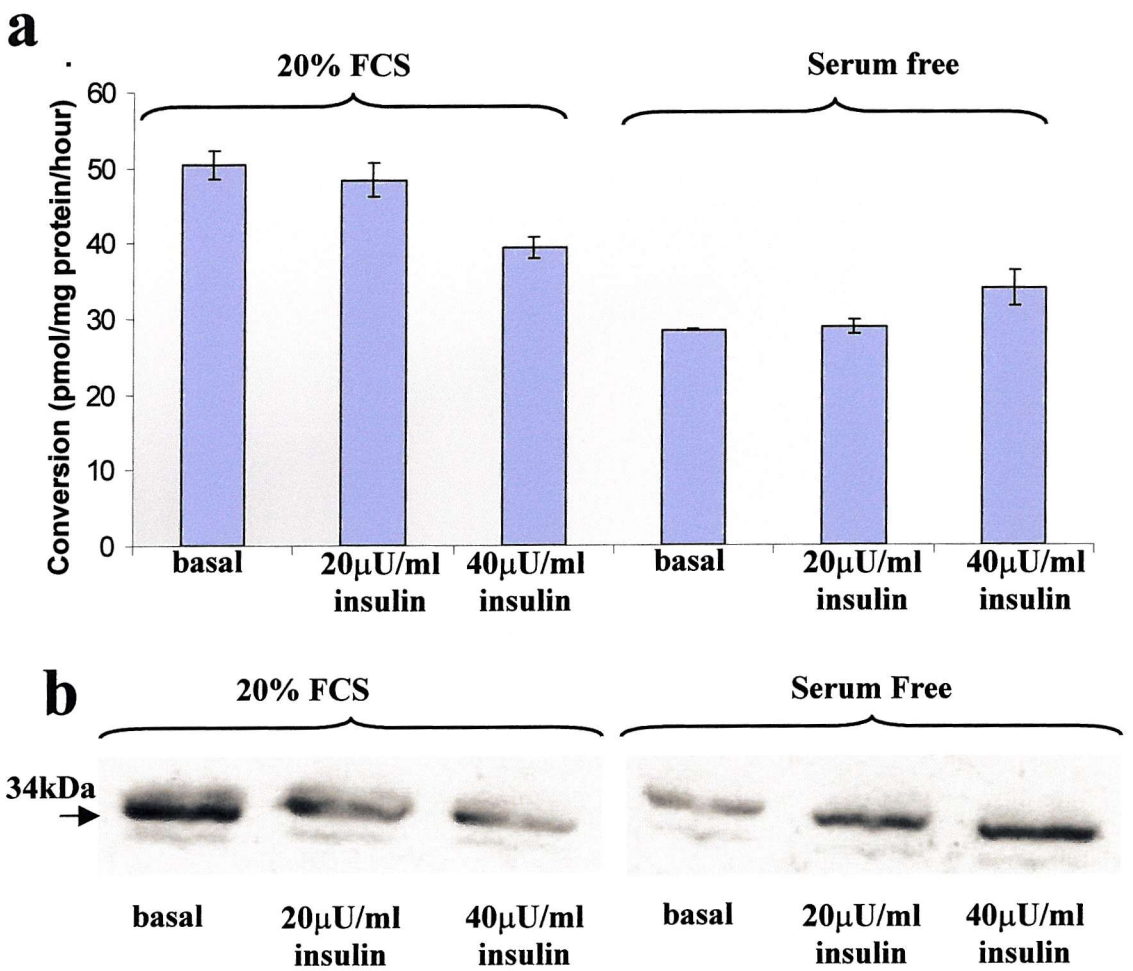


Figure 3.8 The differential regulation of 11β-hydroxysteroid dehydrogenase type I by insulin in human skeletal myoblasts is dependent upon serum factors: Kinetic and Western blot analyses of 11β-HSD 1 in skeletal myobalsts revealed a dose dependent decrease in 11-oxoreducatse activity (a) which was paralleled by a dose dependent decline in 34kDa 11β-HSD1 immunoreactive protein (b) following pre-treatment with insulin in the presence of 20% fetal calf serum (FCS). However, pre-treatment of skeletal myoblasts with insulin in the absence of FCS was accompanied by a dose dependent increase in 11-oxoreducatse activity which was paralleled by a dose dependent increase in 34kDa 11β-HSD1 immunoreactive protein (60μg total cell protein/lane loaded onto the gel) (b) (the data are expressed as the mean ± SEM of triplicate analyses)

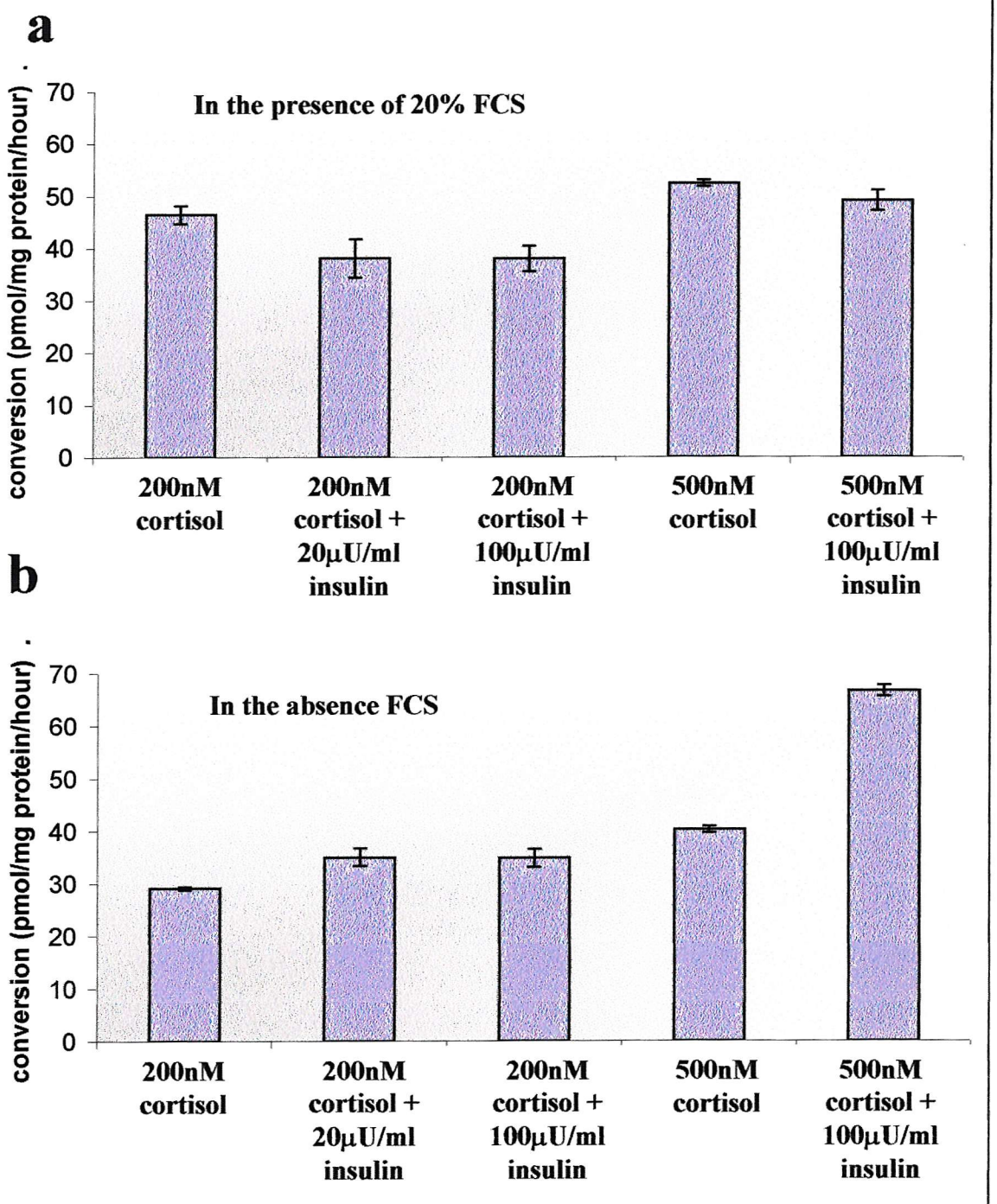


Figure 3.9 Regulation of 11β-hydroxysteroid dehydrogenase 11-oxoreductase activity in human skeletal myoblasts by glucocorticoid & insulin in combination: (a) Co-pre-treatment of skeletal myoblasts with cortisol and insulin in the presence of 20% fetal calf serum (FCS) attenuated the induction of 11β-HSD1 11-oxoreductase activity effected by cortisol alone. (b) In the absence of FCS, co-pre-treatment of skeletal myoblasts with cortisol and insulin induced a markedly greater increase in 11-oxoreductase activity than that which was effected by the same concentration of cortisol alone (the data are expressed as mean ± SEM of triplicate analyses).

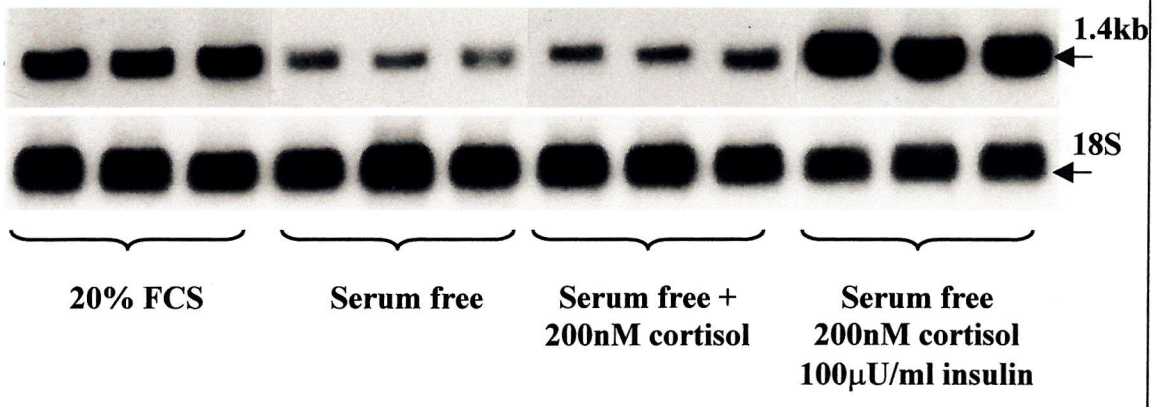


Figure 3.10 Regulation of 11β-hydroxysteroid dehydrogenase mRNA expression in human skeletal myoblasts by glucocorticoid & insulin in combination: Serum removal resulted in a marked decline in 11β-HSD1 mRNA expression. However, under serum free conditions the addition of 200nM cortisol to the growth medium resulted in a marked increase in 11β-HSD1 mRNA expression. In the absence of foetal calf serum, co-pre-treatment of skeletal myoblasts with cortisol and insulin induced a markedly greater increase in 11β-HSD1 mRNA expression than that which was effected by the same concentration of cortisol alone (the data are expressed as mean ± SEM of triplicate analyses using RNA pooled from three different flasks of human skeletal myoblasts).

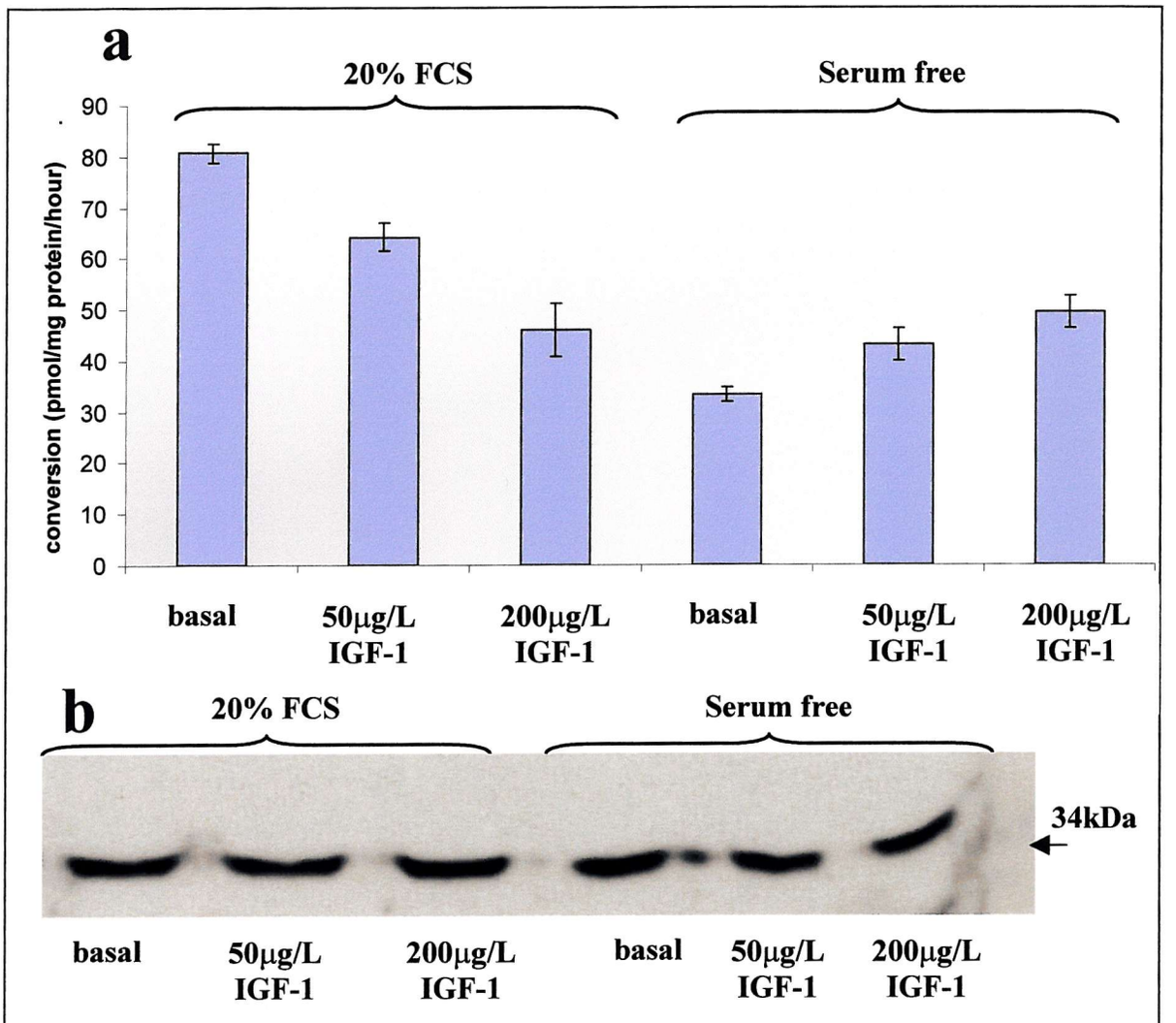


Figure 3.11 The differential regulation of 11β-hydroxysteroid dehydrogenase type I by IGF-1 in human skeletal myoblasts is dependent upon serum factors: (a) analysis of 11β-HSD1 11-oxoreducatse activity in human skeletal myobalsts revealed a dose dependent decrease in 11β-HSD1 11-oxoreducatse activity following pre-treatment with IGF-1 in the presence of 20% foetal calf serum. However, pre-treatment of skeletal myoblasts with IGF-1 in the absence of foetal calf serum was accompanied by a dose dependent increase in 11-oxoreducatse activity. (b) However, pre-treatment of human skeletal myoblasts with IGF-1 either in presence or absence of foetal calf serum had no effect on 34kDa 11β-HSD1 immunoreactive protein (90µg total cell protein/lane loaded onto the gel) (the data are expressed as the mean ± SEM of triplicate analyses)

3.3.7 *Regulation of 11β-hydroxysteroid dehydrogenase by pro-inflammatory cytokines*

3.3.7.1 *11β-hydroxysteroid dehydrogenase type 1 11-oxoreductase activity in human skeletal myoblasts is upregulated by IL1-β and TNFα*

Analyses of 11β-HSD1 activity in myoblasts subsequent to pre-treatment with IL1-β and TNFα (5-20ng/L) revealed a marked increase in 11β-HSD1 11-OR activity which, in order to maintain the percent conversion of substrate to less than 50% necessitated that analysis of 11β-HSD1 11-OR activity was estimated from the rate of conversion of cortisone to cortisol over a period of 24 hours (cf. 48 – 96 hours for previous experiments). In the presence of 20% FCS, 5ng/L IL1-β induced a 4 fold increase (P <0.0001) in 11β-HSD1 11-OR activity which was enhanced subsequent to pre-treatment with higher concentrations of IL1-β (10ng/L) (figure 3.12a, page 174). Similarly, pre-treatment of myoblasts with 5ng/L TNFα induced a 2.5 fold in increase (P <0.0001) in 11β-HSD1 11-OR activity which also increased with higher concentrations of TNFα (20ng/L). Pre-treatment of myoblasts with IL1-β and TNFα under serum free conditions was accompanied by more modest increases in 11β-HSD1 11-OR activity than those observed in the presence of 20% FCS. However, compared with basal 11β-HSD1 11-OR activity IL1-β and TNF-α induced a relative increase in 11β-HSD1 11-OR under serum free conditions such that 5ng/L IL1-β induced a 4.9 fold increase(P <0.0001) in 11β-HSD1 11-OR activity rising to a 6.2 fold increase (P <0.0001) following pre-treatment with 20ng/L IL1-β, whilst 5ng/L TNFα induced a 3.9 fold (P <0.0001) increase in 11β-HSD1 11-OR activity rising to a 4.4 fold (P <0.0001) increase following pre-treatment with 20ng/L TNFα. Multiple kinetic analyses of 11β-HSD1 11-OR activity in myoblasts subsequent to pre-treatment with 20ng/L TNFα compared with basal activity revealed a marked increase in V_{max} whilst K_m remained unaltered (apparent K_m and V_{max} under basal conditions = 476nM and 97pmol/mg protein/hour and 437nM and 46pmol/mg protein/hour in the presence and absence of 20% FCS respectively, whilst pre-treatment with 20ng/L TNFα resulted in an apparent K_m and V_{max} = 438nM and 417pmol/mg

protein/hour and 440nM and 370pmol/mg protein/hour in the presence and absence of 20% FCS). Semi-quantitative non-competitive RT-PCR and Western blot analysis revealed parallel changes in 11β-HSD1 mRNA and 34kDa immunoreactive protein (figure 3.12c/d, page 174).

3.3.7.2 *11β-hydroxysteroid dehydrogenase type 2 11-dehydrogenase activity in JEG-3 cells is downregulated by IL1-β and TNFα*

Parallel analyses of 11β-HSD2 11-DH activity in JEG-3 cells revealed a statistically significant dose dependent decline ($P < 0.05$) in 11β-HSD2 11-DH activity subsequent to pre-treatment with IL1-β and TNFα (50-200ng/L) (figure 3.13a, page 175) which was unaffected by removal of serum from the growth medium. However, Western blot analysis revealed no detectable change in 44kDa 11β-HSD2 immunoreactive protein (figure 3.13b).

3.3.7.3 *Neither 11β-hydroxysteroid dehydrogenase type 2 11-dehydrogenase in JEG-3 cells nor 11β-hydroxysteroid dehydrogenase type 1 11-oxoreductase activity in human skeletal myoblasts is affected by pre-treatment with IL-2, IL-6 or IL-7*

Pre-treatment of myoblasts and JEG-3 cells with IL-2, IL-6 and IL-7 had no detectable effect upon either 11-OR or 11-DH activity.

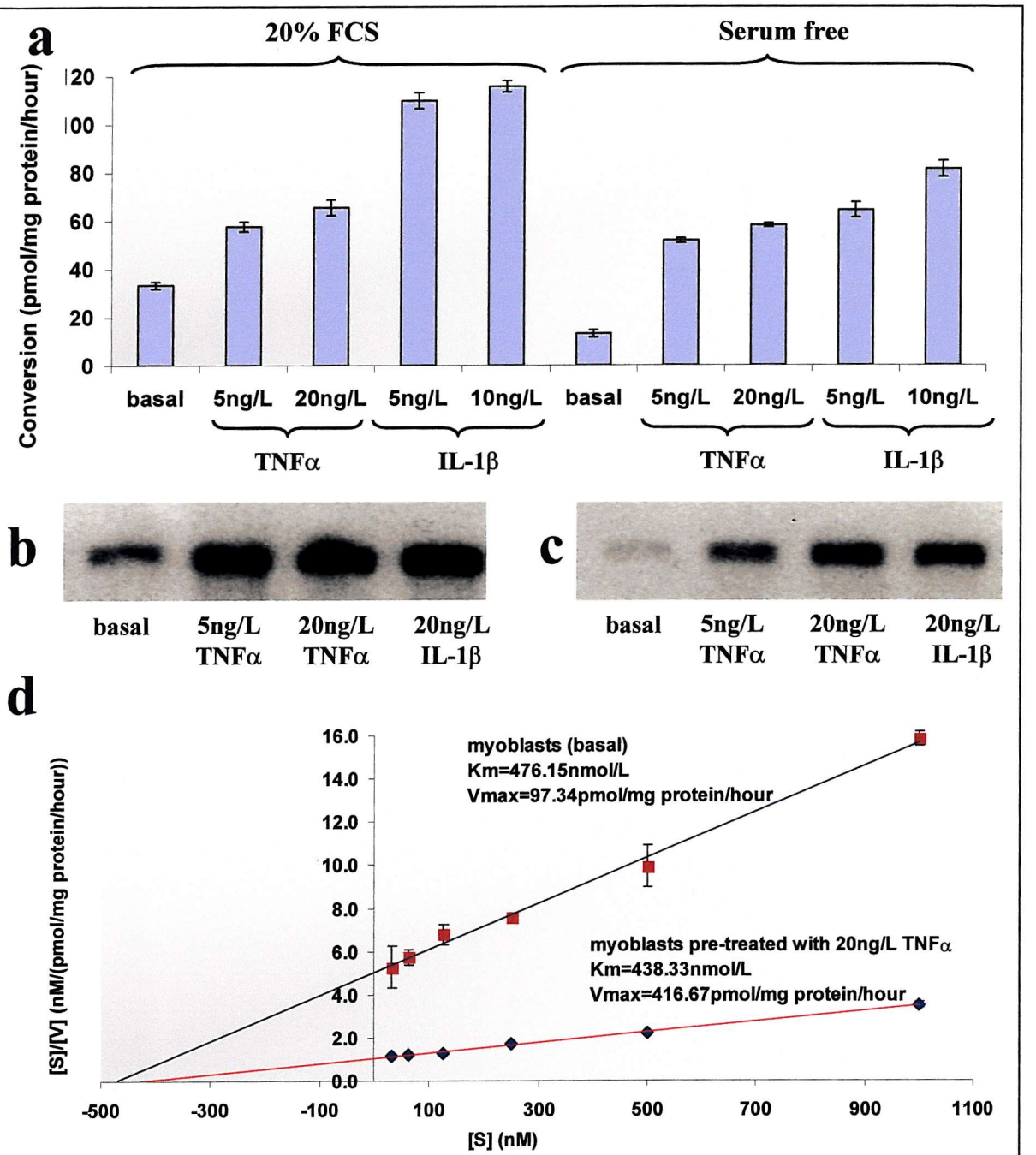


Figure 3.12 Regulation of 11β-hydroxysteroid dehydrogenase in human skeletal myoblasts by IL-1β and TNFα: (a) Analysis of 11β-HSD 1 activity in skeletal myoblasts following pre-treatment with IL-1β and TNFα in the presence and absence of 20% fetal calf serum revealed a dose dependent induction of 11-oxoreductase activity (the data are represented by the mean ± SEM of triplicate analyses) (b/c) Semi-quantitative non-competitive RT-PCR analysis of skeletal myoblasts following pre-treatment with IL-1β and TNFα confirmed parallel dose dependent increases in 11β-HSD1 mRNA (d) Kinetic analysis of 11β-HSD1 activity in skeletal myoblasts (Hanes plot) revealed no significant change in the affinity (K_m) of 11β-HSD1 for cortisol following pre-treatment with IL-1β and TNFα. However pre-treatment with 20ng/L IL-1β or TNFα induced a significant (P<0.0001) increase in the maximum velocity (V_{max}) of 11-oxoreductase activity (the data points are expressed as the mean ± SEM of triplicate analyses)

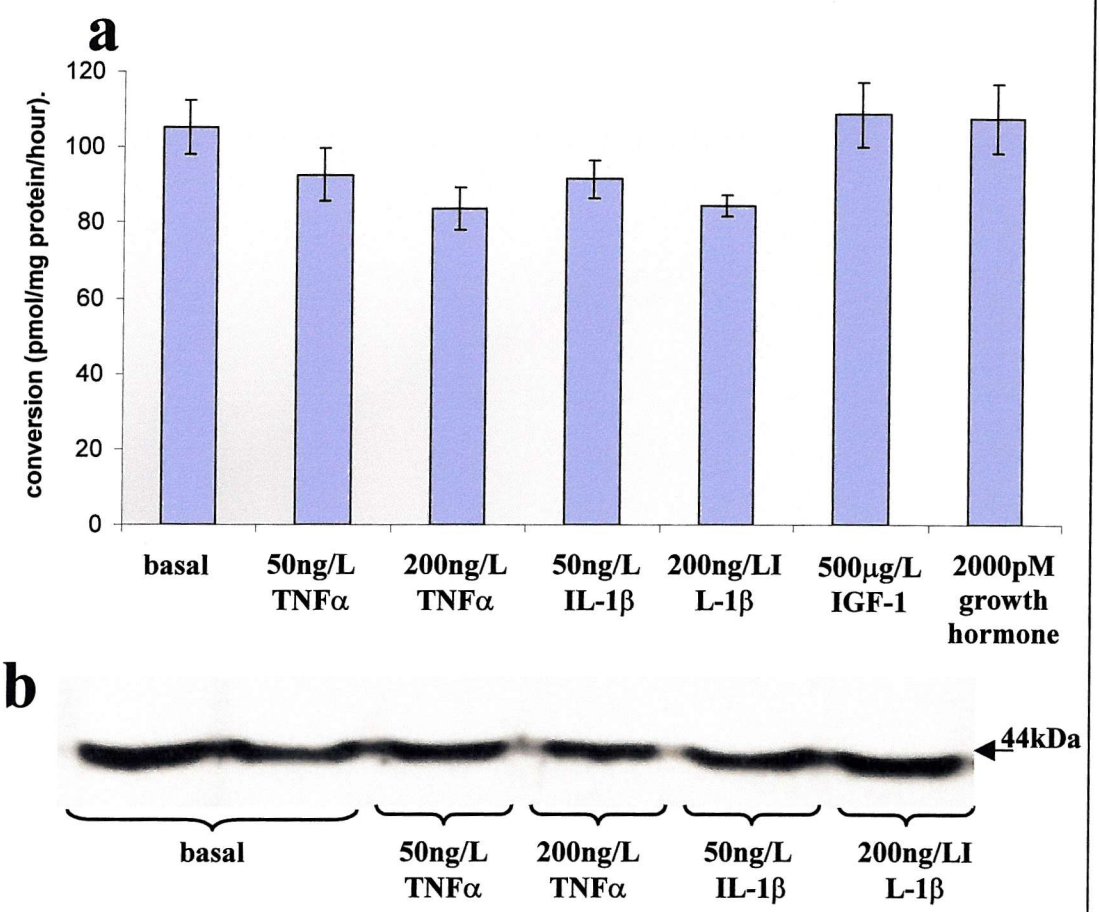


Figure 3.13 Regulation of 11β-hydroxysteroid dehydrogenase in JEG-3 cells by IL-1β and TNFα: (a) Analysis of 11β-HSD2 11-dehydrogenase activity in JEG-3 cells following pre-treatment with either IL-1β or TNFα in the presence of 10% foetal calf serum revealed a dose dependent decline in 11β-HSD2 11-dehydrogenase activity (b) However, Western blot analysis revealed that pre-treatment of JEG-3 cells with IL-1β or TNFα had no effect upon 11β-HSD2 44kDa immunoreactive protein. Removal of serum from the growth medium did not significantly alter the cytokine-induced changes in 11β-HSD2 11-dehydrogenase activity which were observed in the presence of serum. Pre-treatment with either IGF-1 or growth hormone had no detectable effect upon 11β-HSD2 11-dehydrogenase activity (20µg total cell protein/lane loaded onto

3.3.8 *Regulation of 11β-hydroxysteroid dehydrogenase by sex hormones*

3.3.8.1 *11β-hydroxysteroid dehydrogenase in human skeletal myoblasts is regulated by DHEA but not by the more potent androgens, testosterone or androstenedione*

Pre-treatment of myoblasts with DHEA (500nM) in the presence of 20% FCS was accompanied by a modest but significant decrease in 11β-HSD1 11-OR activity (14% decrease, $P < 0.05$). However, changes in 11β-HSD1 11-OR activity as a consequence of pre-treatment with DHEA at concentrations below 500nM failed to achieve statistical significance (figure 3.14a, page 177). Moreover, Western blot analysis failed to reveal a decline in 34kDa immunoreactive protein following pre-treatment of myoblasts with DHEA (figure 3.14a). Pre-treatment of myoblasts with the more potent androgens, testosterone and androstenedione at both physiological and supraphysiological concentrations (5 – 200nM), had no effect upon 11β-HSD1 11-OR activity. Removal of serum from the growth medium did not significantly alter the effects of DHEA, testosterone or androstenedione.

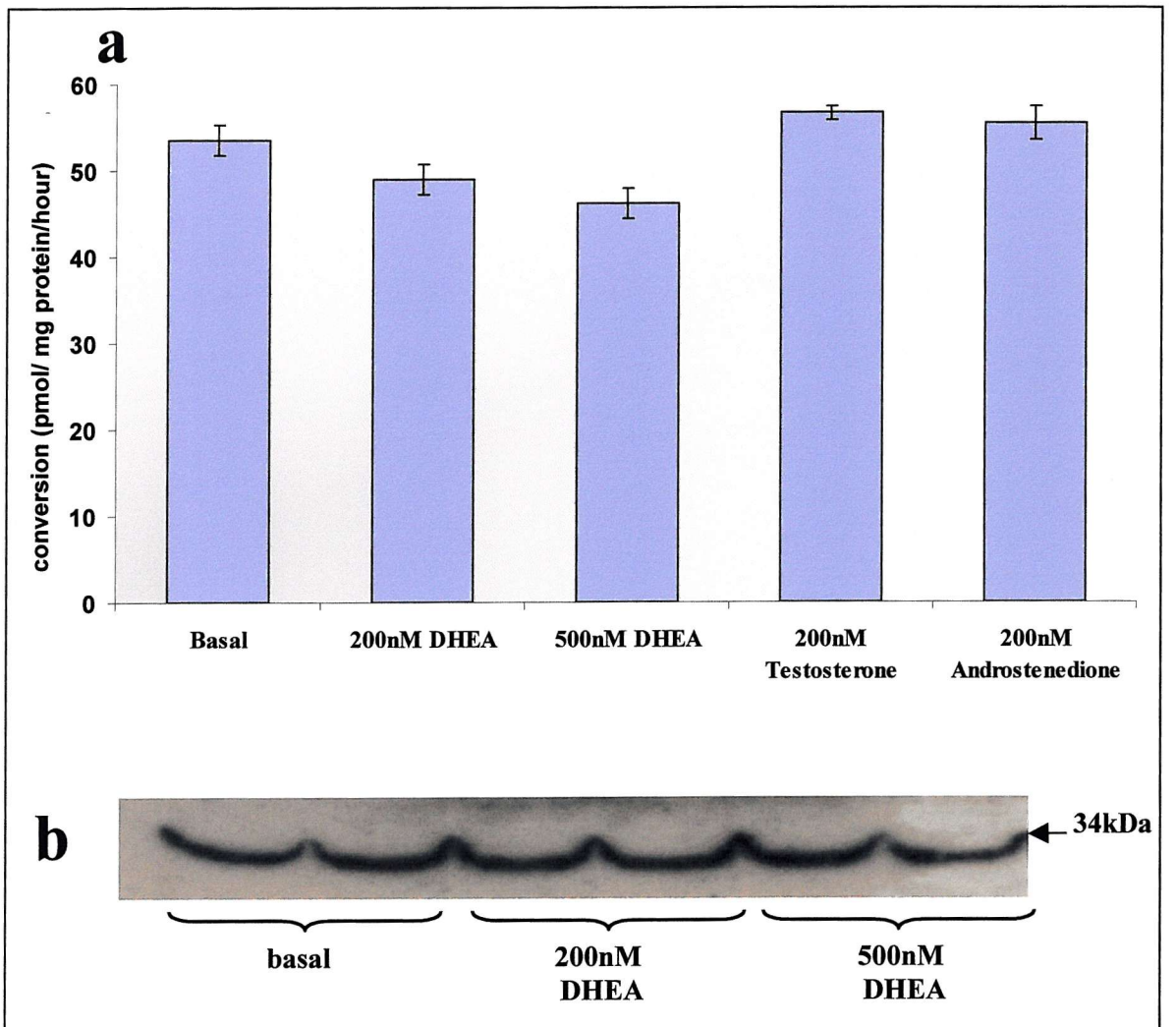


Figure 3.14 Regulation of 11β-hydroxysteroid dehydrogenase in human skeletal myoblasts by dehydroepiandrosterone, androstenedione and testosterone: (a) Analyses of 11β-HSD1 11-oxoreductase activity in human skeletal myoblasts following pre-treatment with dehydroepiandrosterone (DHEA) revealed a dose dependent decline in 11-oxoreducatse activity (b) however, Western blot analysis failed to reveal a parallel decline in 11β-HSD1 34kDa immunoreactive protein (10μg total cell protein/lane loaded onto the gel). Pre-treatment with either androstenedione or testosterone had no detectable effect upon 11β-HSD1 11-oxoreductase activity (the data are represented by the mean ± SEM of triplicate analyses).

3.3.8.2 *11β-hydroxysteroid dehydrogenase activity in JEG-3 cells is up-regulated by 17β-oestradiol but is unaffected by progesterone and the gonadotrophins*

JEG-3 cells pre-treated with progesterone (5 – 500nM) for 12 hours revealed no significant change in 11β-HSD2 11-DH activity (500nM progesterone: $P = 0.06$, n = 10) whilst similar pre-treatment with 17β-oestradiol in the presence of 10% FCS was accompanied by a statistically significant increase in 11β-HSD2 11-DH activity only when the concentration of 17β-oestradiol exceeded 50nM (pre-treatment of JEG-3 cells with 50nM 17β-oestradiol resulted in a 27% increase in 11β-HSD2 11-DH activity, $P < 0.0001$, n = 10). Lower concentrations of 17β-oestradiol also appeared to increase 11β-HSD2 11-DH activity but the change in activity failed to reach statistical significance (figure 3.15a, page 179). Parallel experiments performed using serum free growth medium revealed that pre-treatment of JEG-3 cells with 17β-oestradiol had similar effects upon 11β-HSD2 11-DH activity, such that 50nM 17β-oestradiol induced a 16% increase in 11β-HSD2 11-DH activity ($P < 0.05$) whilst changes in 11β-HSD2 11-DH activity at lower concentrations of 17β-oestradiol failed to reach significance. Co-pre-treatment of JEG-3 cells with 17β-oestradiol (50nM) and progesterone (500nM) was accompanied by an increase in 11β-HSD2 11-DH which paralleled that observed in experiments where cells has been pre-treated with 50nM 17β-oestradiol alone. Western blot analysis revealed that changes in 11β-HSD2 11-DH activity were paralleled by changes in 44kDa 11β-HSD2 immunoreactive protein (figure 3.15b, page 179). Pre-treatment with LH, FSH, hCG and with the androgens, testosterone, DHEA and androstendione had no effect upon 11β-HSD2 11-DH activity. 11β-HSD1 11-OR activity in myoblasts was unaffected by pre-treatment with either 17β-oestradiol or progesterone.

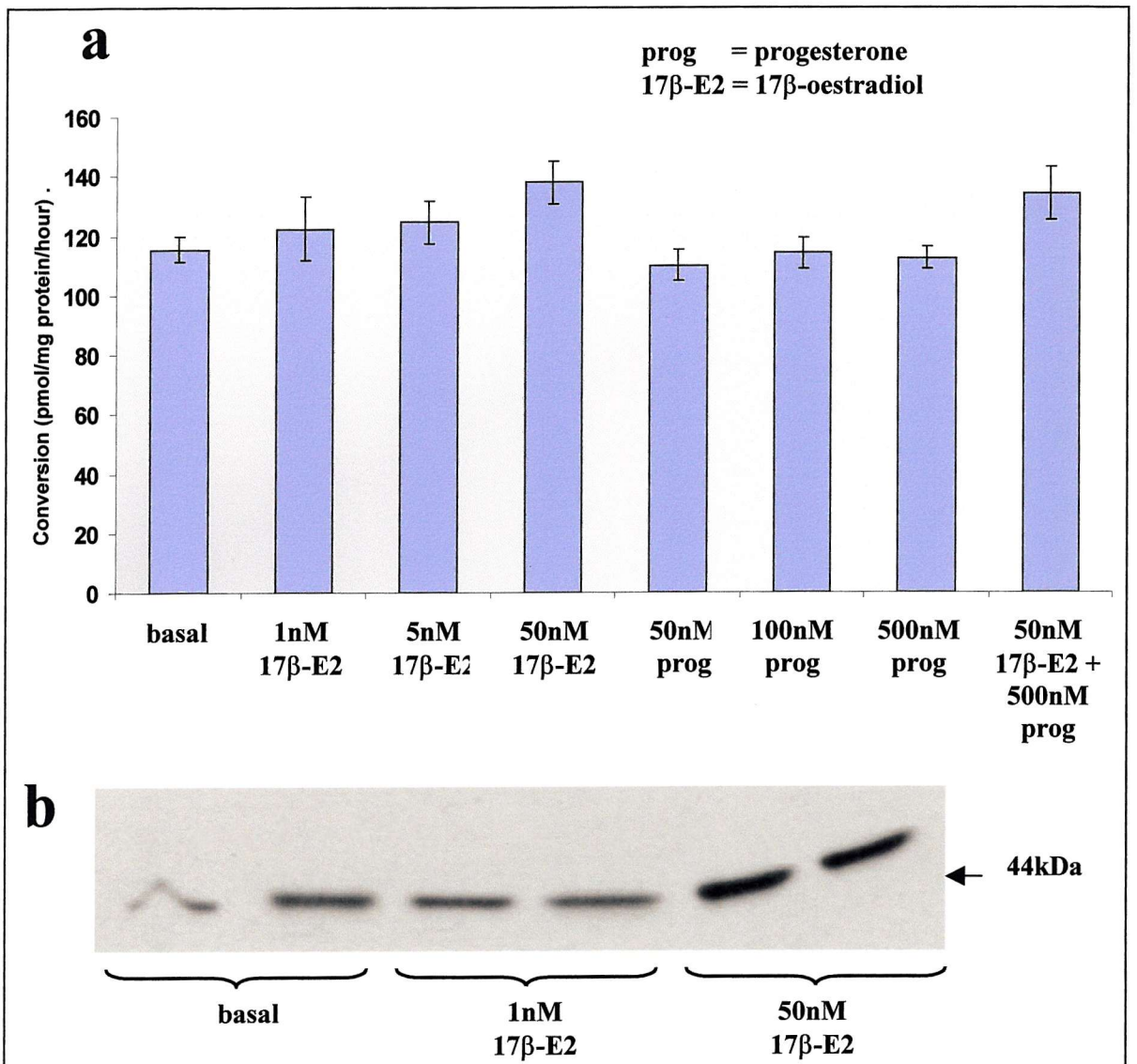


Figure 3.15 Regulation of 11β-hydroxysteroid dehydrogenase in JEG-3 cells by 17β-oestradiol and progesterone: (a) Analysis of 11β-HSD2 11-dyhydrogenase activity and Western blot analyses of 11β-HSD2 44kDa immunoreactive protein in JEG-3 cells following pre-treatment with either 17β-oestradiol revealed an small but significant ($P<0.001$) increase in 11β-HSD2 11-dehydrogenase activity when cells were pre-treated with supra-physiological concentrations of 17β-oestradiol. Pre-treatment of JEG-cells with progesterone had no effect upon 11β-HSD2 11-dehydrogenase activity (the data are represented by the mean ± SEM of triplicate analyses). Co-pre-treatment of JEG-3 cells with 17β-oestradiol and progesterone had no effect upon the induction of 11β-HSD2 11-dehydrogenase activity effected by 17β-oestradiol alone. (b) 17β-oestradiol dependent changes in 11β-HSD2 11-dehydrogenase activity were paralleled by increases in 11β-HSD2 44kDa immunoreactive protein (10μg total cell protein/lane loaded onto the gel). Removal of serum from the growth medium did not significantly alter the oestrogen-induced changes in 11β-HSD2 11-dehydrogenase activity which were observed in the presence of serum.

3.4 Discussion

3.4.1 *11β-hydroxysteroid dehydrogenase activity, mRNA and immunoreactive protein in human skeletal myoblasts*

In common with previous studies[445,451], morphological analysis of skeletal myoblasts from the vastus lateralis of human male volunteers revealed greater than 99% proliferating myoblasts which were capable of undergoing spontaneous and serum-deprivation transformation to myotubes. However, in populations of cells that are at different stages of maturation, myotube formation is rarely uniform and thus cells in culture represent an heterogenous population of myoblasts and myotubes. Therefore, in this study, levels of 11β-HSD1 expression and activity were measured exclusively in myoblasts in order to ensure uniformity of experimental conditions across a large number of tissue culture flasks. Immunohistochemical analysis of intact myoblasts revealed positive staining for 11β-HSD1 immunoreactive protein distributed throughout the cytoplasm. Importantly, this was co-localised with positive immunohistochemical staining for glucocorticoid receptor (See chapter 4). These observations lend strength to the hypothesis that 11β-HSD1 may play a role in the regulation of glucocorticoid sensitivity in skeletal muscle which represents an important glucocorticoid target tissue[56,121,165]. Neither 11β-HSD2 mRNA, immunoreactive protein nor 11β-HSD2 11-DH activity was detected in these cells and kinetic analysis of 11β-HSD1 11-OR activity in intact cells revealed an apparent K_m and V_{max} for cortisone of 476nM and 94pmol/mg protein/hour and 437nM and 46pmol/mg protein/hour in the presence and absence of 20% FCS respectively and are consistent with the cloned native 11β-HSD1 isoform. These data are in agreement with the kinetic constants for 11β-HSD1 reported in human hepatocytes (K_m for cortisone of 382nM) [168], human adipose stromal cells (K_m for cortisone of 270nM) [249] and human colonic lamina propria cells (K_m for cortisone of 500nM) [57]. Importantly, the discrepancies in kinetic constants reported for 11β-HSD1 activity in human hepatocytes and adipose stromal cells compared with those reported in the present study may be a reflection of methodological differences since the former studies calculated kinetic constants from Lineweaver/Burk transformations of the rectangular hyperbola obtained from empirical estimation of enzyme catalysed reactions,

which are intrinsically less precise, and used [^{3}H]-labelled substrate and TLC in order to estimate conversion of substrate to product. This latter methodology, whilst rapid, does not take account of metabolism of substrate by pathways other than by 11β-HSD1 and, as a consequence, the kinetic analyses reported in these studies are likely to represent only an approximation of the kinetic constants for 11β-HSD1 *in* these cells. Moreover, human hepatocytes in culture are more accurately described as hepatic stellate cells and, as a consequence, 11β-HSD1 activity in these cells may not be considered to be truly representative of hepatic 11β-HSD1 activity *in vivo*.

3.4.2 *11β-hydroxysteroid dehydrogenase activity, mRNA and immunoreactive protein in JEG-3 cells*

In this study, 11β-HSD activity in both intact JEG-3 cells and homogenates was expressed exclusively as a high affinity NAD^+ dependent 11-dehydrogenase. Kinetic analysis of 11β-HSD2 activity in intact cells revealed apparent K_m and V_{max} for cortisol of 44nM and 147pmol/mg protein/hour respectively. These data are in agreement with the kinetic constants for 11β-HSD2 reported in human kidney (K_m and V_{max}, for cortisol of 60nM and 110pmol/mg protein/hour respectively) [48] and placenta (K_m and V_{max}, for cortisol of 55nM and 300pmol/mg protein/hour respectively)[239]. Moreover, RT-PCR and Western blot analysis confirmed the presence of 11β-HSD2 but not 11β-HSD1 mRNA and immunoreactive protein in these cells. The discrepancy between apparent K_m for intact cells and homogenates (K_m = 44nM and 80nM respectively), whilst not inconsistent with previous reports[452], may be attributed to minor differences in experimental kinetic analysis since conversion of cortisol to cortisone in cell homogenates was necessarily small resulting in relatively greater imprecision in quantification. Nevertheless, the variation in 11β-HSD2 kinetic constants reported in previous studies using cell homogenates[452] may be a reflection of methodological artifact since these investigations employed relatively large concentrations of added co-factor and relied upon accurate measurement of the conversion of [^{3}H]-labelled substrate by TLC. Moreover, the concentration of [^{3}H]-labelled substrate in these studies was necessarily at concentrations which were several orders of magnitude below the K_m 11β-

HSD2 and, to date, there are no data which confirm that [^{3}H]-labelled cortisol or corticosterone undergo catalysis by isoforms of 11β-HSD in a manner analogous with native steroid.

The existence of a high affinity $NADP^+$ dependent 11-DH, termed 11β-HSD3, has been suggested by contemporary studies of cortisol metabolism in JEG-3 cell homogenates to which appropriate cofactors had been added[453]. Nevertheless, in the study reported herein, $NADP^+$ dependent conversion of cortisol to cortisone, suggestive of 11β-HSD3 activity, was not observed. Importantly, the detection of 11β-HSD3 activity has not been confirmed in these or other cells, neither has this isoform yet been cloned.

Immunohistochemical analysis of JEG-3 cells revealed 11β-HSD2 immunoreactive protein distributed predominantly in the cytoplasm immediately surrounding the nucleus with some positive staining within the nucleus itself. This confirms the findings of previous studies[453] which reported NAD^+ dependent 11β-HSD2 activity in the nuclear fraction of JEG-3 cell homogenates equivalent with that measured in the mitochondrial fraction. The role of 11β-HSD2 in the nucleus of these cells has been neglected in the literature. However, localisation of 11β-HSD2 to the nuclear compartment has been reported in other tissues including the luminal and glandular epithelia of human endometrial cells[454], human cortical collecting ducts and human colon[242,247]. In classical mineralocorticoid target tissues the mineralocorticoid receptor may be distributed, unbound to ligand, to both the nuclear and cytoplasmic compartments. Thus in these tissues, the presence of 11β-HSD2 activity in the nuclear compartment is not inconsistent with the proposed role of 11β-HSD2 as an efficient guardian of the mineralocorticoid receptor, preventing activation by cortisol[60,163]. However, JEG-3 cells, a model of trophoblastic tissue, are not generally considered to represent classical mineralocorticoid target tissue and thus the role of 11β-HSD2 in the nuclear compartment in these cells remains unclear.

3.4.3 *The hormonal regulation of 11β-hydroxysteroid dehydrogenase is effected at a pre-translational level*

Investigations of the substrate specificity of isoforms of 11β-HSD have revealed a spectrum of steroid substrates for these enzymes, some of which were capable of competitive inhibition of enzyme activity (reviewed by Monder C[455]). However, in the study reported herein, kinetic analyses were performed following the removal of growth medium containing the potent regulators of isoforms of 11β-HSD to which the cells had been previously exposed. Thus, in this study, changes in enzyme activity as a consequence of exposure to steroid and peptide hormones, was independent of possible competitive inhibition and presumably would have been dictated by transcriptional or translational regulation of enzyme expression. Indeed, throughout this study, most kinetic, Northern and Western blot analyses revealed parallel changes in 11β-HSD activity, mRNA expression and immunoreactive protein. Moreover, multiple kinetic analyses of 11β-HSD1 in skeletal myoblasts and 11β-HSD2 in JEG-3 cells revealed that increases or decreases in enzyme activity were reflected in parallel changes in the maximum velocity of the enzyme catalysed reaction (V_{max}), whilst the affinity (K_m) of the enzyme for its substrate remained unaltered. These observations are consistent with the pre-translational regulation of the quantity of functional 11β-HSD protein in these cells.

3.4.4 *Regulation of 11β-hydroxystertoid dehydrogenase in human skeletal myoblasts and JEG-3 cells by glucocorticoids*

Recent studies have provided evidence for the pre-translational regulation of 11β-HSD by glucocorticoids which is both isoform and tissue specific[166,249,421,422]. Furthermore, changes in the pattern of cortisol metabolism and excretion in conditions of cortisol excess such as the ectopic ACTH syndrome and Cushing's syndrome have given rise to speculation that elevated levels of ACTH or ACTH-dependent steroids may result in the dysregulation of isoforms of 11β-HSD[166].

In common with investigations of 11β-HSD activity in rat tissues[166] and human kidney slices[423], this study demonstrated that ACTH *per se* does not regulate 11β-HSD1 in human skeletal myoblasts or 11β-HSD2 in JEG-3 cells. Furthermore, of the steroid precursors of cortisol and aldosterone biosynthesis examined in this study, none had any detectable effect upon the activity of 11β-HSD in skeletal myoblasts or JEG-3 cells. This is in marked contrast with previous studies which demonstrated inhibition of 11β-HSD2 activity in human kidney slices by corticosterone and 18-hydroxycorticosterone[423]. However, these experiments were performed under conditions likely to reflect competitive inhibition of 11β-HSD2 activity which, by extrapolation to the kidney *in vivo*, lends support to the hypothesis that inhibition of renal 11β-HSD2 activity by ACTH-dependent steroids may underlie the changes in the pattern of urinary steroid excretion observed in patients with the ectopic ACTH syndrome[265] (discussed in the introduction to this thesis).

Previous studies of 11β-HSD1 activity have reported the glucocorticoid-dependent induction of 11β-HSD1 in a spectrum of human and animal tissues and cells in culture[166,168,249,408,409]. In this study, pre-treatment of skeletal myoblasts with physiological concentrations of glucocorticoid induced a dose dependent increase in 11β-HSD1 11-oxoreducatse activity, mRNA and immunoreactive protein. These observations provide evidence for the pre-translational induction of 11β-HSD1 gene expression by cortisol in these cells. Moreover, co-pre-treatment of skeletal myoblasts with the GR antagonist, RU38486, abolished the glucocorticoid-induced increase in 11β-HSD1 mRNA, immunoreactive protein and 11-OR activity, suggesting that the induction of 11β-HSD1 by cortisol is mediated through activation of the GR. Importantly, pre-treatment of skeletal myoblasts with cortisone, which is generally considered to be biologically inactive, also resulted in the induction of 11β-HSD1 mRNA, immunoreactive protein and 11-OR activity. However, when cells were co-pre-treated with cortisone and equimolar concentrations of carbenoxolone, an inhibitor of both isoforms of 11β-HSD, the induction of 11β-HSD1 11-OR activity effected by cortisone alone was abolished. Importantly, 11β-HSD1 11-OR activity in skeletal myoblasts was

unaffected by pre-treatment with carbenoxolone alone. These observations suggest that induction of 11β-HSD1 11-OR activity by cortisone is dependent upon the conversion of cortisone to cortisol. This represents the 'autoregulation' of 11β-HSD1 by the products of its own catalytic activity, a phenomenon which, *in vivo*, would have the effect of amplifying glucocorticoid hormone action by increasing the intracellular concentration of available cortisol. Similar observations have also been recently reported from studies of human adipose stromal cells in which induction of 11β-HSD1 11-OR activity was noted following incubation with cortisone but was abolished by co-incubation with carbenoxolone and cortisone[248]. These data support the hypothesis in favour of the existence of a mechanism of glucocorticoid receptor-mediated up-regulation of 11β-HSD1 11-OR activity which is the direct consequence of the catalytic conversion of cortisone to cortisol by 11β-HSD1 itself. Importantly the concentrations of cortisol used in this study were equivalent with those found in the general circulation and thus represent cortisol levels to which tissues may be exposed throughout the course of a normal 24 hour period.

It is widely accepted that 11β-HSD1 plays a significant role in the maintenance of tissue sensitivity to glucocorticoid by modulating the accessibility of active glucocorticoid to the glucocorticoid receptor. Thus, glucocorticoid-dependent induction of 11β-HSD1, mediated through activation of the glucocorticoid receptor, has the potential to increase the intracellular availability of active glucocorticoid which, in the absence of counter-regulatory mechanisms, would be likely to increase tissue sensitivity to glucocorticoid. The significance of this phenomenon in the aetiology of human disease is the subject of further investigations in chapter 4 of this thesis.

Removal of foetal calf serum from the medium in which skeletal myoblasts were grown induced a marked decline in 11β-HSD1 11-OR activity under basal conditions. However, pre-treatment of skeletal myoblasts with either cortisol or cortisone under conditions of serum deprivation resulted in a 2-3 fold greater induction of 11β-HSD1 11-OR activity than that which was observed following pre-treatment with cortisol or cortisone in the presence of serum. Whilst these latter observations confirm the findings of previous

investigations of 11β-HSD1 activity in skin fibroblasts[430], there is no clear evidence of a mechanism to explain the effects of serum removal upon the regulation of 11β-HSD1 by glucocorticoid. Indeed, serum removal also reduces the level of GR expression in these cells[456] which might be predicted to result in a reduction in glucocorticoid sensitivity which would inhibit the glucocorticoid-induced up-regulation of 11β-HSD1 expression. Importantly, foetal calf serum comprises a complex mixture of binding proteins, growth factors, cytokines and hormones which may serve as independent regulators of enzyme activity in cells in culture. Thus, in this study, experiments were performed both in the presence and absence of foetal calf serum in order to highlight and overcome the confounding effects of enzyme regulation by serum components of the growth medium.

Kinetic and Western blot analysis of 11β-HSD2 in JEG-3 cells following pre-treatment with cortisol at concentrations 2-3 fold those normally detected in the circulation revealed a dose dependent increase in 11β-HSD2 11-DH activity which was paralleled by changes in 11β-HSD2 immunoreactive protein. These observations are in agreement with reports of similar findings in explant cultures of rat distal colon[422]. However, following pre-treated with concentrations of cortisol equivalent with those in the general circulation, 11β-HSD2 11-DH activity in JEG-3 cells remained unchanged. Interestingly, co-pre-treatment of JEG-3 cells with cortisol and the glucocorticoid receptor antagonist, RU38486, did not attenuate the induction of 11β-HSD2 activity and immunoreactive protein effected by cortisol alone. This is in agreement with observations of glucocorticoid induction of 11β-HSD2 activity in the Ishikawa endometrial cell line[457]. Whilst speculative, these observations may be explained upon the basis that the glucocorticoid-mediated induction of 11β-HSD2 in JEG-3 cells is mediated by some unknown mechanism other than through the GR. Alternatively, these observations may be a reflection of the use of too low a concentration of RU38486. Importantly, pre-treatment of JEG-3 cells with cortisone at μM concentrations had no effect upon 11β-HSD2 11-DH activity. Together, these data support the hypothesis that induction of 11β-HSD1 expression in skeletal myoblasts by cortisone occurs as a consequence of the conversion of cortisone to cortisol by 11β-HSD1 itself.

3.4.5 *Regulation of 11β-hydroxystertoid dehydrogenase in human skeletal myoblasts and JEG-3 cells by insulin*

The observation that central obesity[458,459] and insulin resistance[176,460] are frequently associated with glucocorticoid excess has prompted considerable interest in the relationship between glucocorticoids and insulin. Moreover, evidence for the regulation of isoforms of 11β-HSD by insulin[409,411,430,442,461] has added to the growing debate surrounding the role of 11β-HSD in the development of glucocorticoid-dependent insulin resistance[176].

In this study, pre-treatment of skeletal myoblasts with insulin, in the presence of 20% foetal calf serum, induced a dose dependent decline in 11β-HSD1 11-OR activity and 11β-HSD1 34kDa immunoreactive protein. These observations are in agreement with previous reports which describe an insulin induced decline in 11β-HSD1 11-OR activity and mRNA expression in rat hepatic 2S FAZA cells[409] and primary cultures of rat hepatocytes[411]. However, in contemporary studies of 11β-HSD1 activity in human adipose stromal cells[248] and explant cultures of rat distal colon[422] insulin was observed to have no effect upon 11β-HSD1 activity. These observations suggest that the regulation of 11β-HSD1 by insulin is cell-type specific and that the evidence to date highlights a role for insulin regulation of 11β-HSD1 in tissues that are important targets for the action of both insulin and glucocorticoid.

In conditions such as non-insulin dependent diabetes melitus (NIDDM) and the metabolic syndrome the principal sites of insulin resistance are recognised to be skeletal muscle not least because it represents 25-40% of adult body mass[462,463]. Since glucocorticoids are potent antagonists of insulin action[458] and 11β-HSD1 serves to regenerate cortisol from cortisone, it is likely that the sensitive down-regulation of 11β-HSD1 by insulin in insulin target tissue represents an important regulatory mechanism underlying the maintenance of tissue insulin sensitivity. Indeed, when skeletal myoblasts were pre-treated with insulin and glucocorticoid in combination in the presence of foetal calf serum, the induction of 11β-HSD1 11-OR activity effected by glucocorticoid alone

was markedly attenuated. These observations suggest that in skeletal myoblasts, down regulation of 11β-HSD1 11-OR activity by insulin antagonises the induction of 11β-HSD1 11-OR activity by cortisol. Thus, in these cells under normal circumstances, glucocorticoid-mediated induction of 11β-HSD1 may be balanced by a counter-regulatory inhibition of 11β-HSD1 expression by insulin. In normal physiology this may be predicted to maintain levels of intracellular cortisol below those which would otherwise induce insulin resistance in these cells. It is likely, however, that these effects are tissue specific since incubation of 3T3-F442A and 3T3-L1 adipocytes[442] and human adipose stromal cells[248] with insulin and glucocorticoid alone and in combination appears to induce 11β-HSD1 activity and thus amplify glucocorticoid hormone action in these cells.

In marked contrast with the observations made when skeletal myoblasts were pre-treated with insulin in the presence of foetal calf serum, pre-treatment of these cells with insulin under serum free conditions induced a dose dependent increase in 34kDa immunoreactive protein and 11β-HSD1 11-OR activity. This is in contradiction with previous reports of a decline in 11β-HSD1 11-OR activity in skin fibroblasts following incubation with insulin under serum free conditions[430]. Moreover co-pre-treatment of skeletal myoblasts with insulin and glucocorticoid in combination, under serum free conditions, induced a marked increase in 11β-HSD1 mRNA, 34kDa immunoreactive protein and 11-OR activity which was greater than the sum of the induction observed following pre-treatment of these cells with either glucocorticoid or insulin alone. Whilst the apparently contradictory observations of 11β-HSD1 activity in skeletal myoblasts and skin fibroblasts may be explained on the basis of the tissue specific regulation of 11β-HSD1 by insulin, there is as yet no clear explanation for the opposite effect of serum removal on the insulin-dependent regulation of 11β-HSD1 in these cells.

In contrast with 11β-HSD1, there have been very few investigations of the regulation of 11β-HSD2 by insulin. In this study, insulin had no detectable effect upon 11β-HSD2 activity in JEG-3 cells. This in contrast with previous investigations of the streptozocin-

induced insulin dependent diabetic rat in which administration of insulin increases renal 11β-HSD2 activity[461]. Nevertheless, these effects may be tissue or cell-type specific and the insulin-induced up-regulation of renal 11β-HSD2 11-DH activity observed in the rat model may represent a mechanism for accelerated metabolic clearance of glucocorticoid which is paralleled by an insulin-induced decrease in 11β-HSD1 11-OR activity in insulin target tissue. Whilst the former mechanism reduces circulating levels of glucocorticoid the latter reduces intracellular levels of glucocorticoid and thus both mechanisms may serve to reduce levels of glucocorticoid hormone action and preserve insulin sensitivity in insulin target tissues. However, these observations come from pharmacologically induced insulin dependent diabetes mellitus in the rat and may not truly reflect glucocorticoid metabolism in insulin dependent diabetes mellitus man.

3.4.6 *Regulation of 11β-hydroxysteroid dehydrogenase in human skeletal myoblasts and JEG-3 cells by growth hormone and IGF-1*

The *in vivo* investigation of hepatic 11β-HSD1 has revealed evidence for the indirect down-regulation of 11β-HSD1 in this tissue by growth hormone[171,406,412,416]. To date there have been few reports of the regulation of 11β-HSD by growth hormone in tissues other than those of hepatic or renal origin[406,407,411,412]. However, growth hormone had no detectable effect upon either 11β-HSD1 in skeletal myoblasts or 11β-HSD2 in JEG-3 cells even when the cells were exposed to supraphysiological concentrations of growth hormone.

The metabolic effects of growth hormone are varied but include the mobilisation of fatty acids from triacylglycerols in adipose tissue and the stimulation of hepatic glycogenolysis. However, many of these effects are mediated by the insulin-like growth factors, IGF-1 and IGF-2 which are synthesised and secreted by the liver in response to growth hormone. In this study, pre-treatment of skeletal myoblasts with IGF-1 in the presence and absence of 20% foetal calf serum resulted in changes in 11β-HSD1 11-OR activity and 11β-HSD1 34kDa immunoreactive protein which mirrored those observed when the cells were pre-treated with insulin under similar conditions. In common with

observations made earlier in this study of the regulation of 11β-HSD by insulin, IGF-1 had no detectable effect upon 11β-HSD2 activity in JEG-3 cells.

Evidence from previous *in vivo* studies in humans and rats has supported the hypothesis that growth hormone inhibits both hepatic 11β-HSD1 and renal 11β-HSD2 activity[271,405,406]. However, the role of IGF-1 in the regulation of isoforms of 11β-HSD has not been addressed and data from this study implies that such regulation of this enzyme by growth hormone occurs indirectly and may be mediated through the action of IGF-1. Indeed, evidence in support of an indirect effect of growth hormone upon 11β-HSD activity comes from studies of gender differences in 11β-HSD activity in growth hormone deficient rats[406] and in human subjects with hypopituitarism[440]. These studies have demonstrated that in the female, *in vivo* growth hormone secretion is constant and tends to inhibit hepatic 11β-HSD1 expression whilst the male pattern of growth hormone secretion is pulsatile and has no effect upon hepatic 11β-HSD1. However, Albiston *et al*[171] showed that the regulation of 11β-HSD1 by growth hormone in the female rat may be dependent upon levels oestrogen. Indeed, a marked relationship between hepatic 11β-HSD1 activity and levels of android and gynoid fat has been reported in hypopituitary patients[440].

3.4.7 *Regulation of 11β-hydroxysteroid dehydrogenase in human skeletal myoblasts and JEG-3 cells by pro-inflammatory cytokines*

The anti-inflammatory properties of glucocorticoids are effected as a consequence of the inhibition of key mediators of the immune response. Thus, cortisol inhibits the secretion of IL-1 which reduces the stimulation of $CD4^+$ monocytes and IL-2 receptor expression. This, in turn also diminishes the synthesis and secretion of IL-2 in $CD4^+$ and $CD8^+$ cells and impairs lymphocyte proliferation[464]. This cascade of events is dependent upon the level of glucocorticoid hormone action at sites of inflammation, which is itself dependent upon intracellular levels of active glucocorticoid and levels of functional GR[465]. This has led to suggestions that local conversion of cortisone to cortisol by 11β-HSD1 is a key determinant of the extent of inflammation at sites of tissue injury[444].

Recent *in vitro* investigations of 11β-HSD1 activity have revealed evidence for the paracrine regulation of this enzyme by the pro-inflammatory cytokines, IL-1β and TNF-α, which are synthesised and secreted at sites of tissue inflammation[444]. In common with these earlier studies, pre-treatment of skeletal myoblasts with concentrations of IL-1β and TNF-α equivalent to those found in the general circulation resulted in a 5-fold increase in 11β-HSD1 11-OR activity. These observations suggest that in skeletal muscle tissue, induction of 11β-HSD1 11-OR activity by this mechanism would be predicted to be accompanied by an increase in intracellular levels of cortisol as a consequence of the local conversion of cortisone to cortisol. Moreover, in the absence of counter-regulatory mechanisms, higher intracellular levels of cortisol would result in an increase in glucocorticoid hormone action which would be accompanied by a decrease in local inflammation. Thus, while speculative, the sensitive up-regulation of 11β-HSD1 11-OR activity by IL-1β and TNF-α in skeletal muscle may represent an important mechanism underlying the tight control of local inflammation and muscle tissue repair following injury or infectious insult.

In addition to the role of promoting the intracellular regeneration of cortisol from cortisone through up-regulation of 11β-HSD1, circulating levels of IL-1β may also stimulate the release of CRH from the hypothalamus[466] and ACTH from the pituitary[467]. Indeed, during episodes of infectious insult, IL-1β is believed to excite an increase in hypothalamic-pituitary responsiveness to both endogenous and exogenous stimulation which results in an increase in glucocorticoid secretion which in turn ameliorates the metabolic stress induced by infectious challenge[466].

In this study IL-1β and TNF-α induced a small, but significant decline in 11β-HSD2 11-DH activity in JEG-3 cells. Whilst the concentration of IL-1β and TNF-α required to induce the down-regulation of 11β-HSD2 11-DH activity in these cells was an order of magnitude greater than that required to up-regulate 11β-HSD1 11-OR activity in skeletal myoblasts it must be remembered that the degree of regulation of 11β-HSD is isoform specific and is likely to be dependent upon the expression of appropriate signalling

pathways. Thus, if renal 11β-HSD2 activity is regulated by IL-1β and TNF-α in a similar manner to that observed in JEG-3 cells, this may represent a mechanism which results in a reduction in the metabolic clearance of cortisol. The combined evidence for increased hypothalamic-pituitary-adrenal activity, sensitive up-regulation of 11β-HSD1 and down-regulation of renal 11β-HSD2 as a consequence of exposure to IL-1β lend support to the hypothesis that these phenomena represent an auto-regulatory mechanism in order to limit the extent of the inflammatory response.

Recent investigations have reported a marked increase in circulating levels of TNF-α in obese patients and in patients with NIDDM. Moreover, these studies have also revealed that serum levels of TNFα correlate with hyperinsulinaemia and decreased insulin sensitivity[468,469] and that in obesity, TNFα is over-expressed not only by adipose tissue but also by skeletal muscle tissue[470,471]. Furthermore, serum levels of TNFα fall and insulin sensitivity increases as a consequence of dietary and pharmacological treatment of obesity[468,470-472].

The mechanism of TNFα induced insulin resistance is complex but is thought to involve down regulation of GLUT-4 gene expression thereby decreasing insulin-stimulated glucose transport[473]. TNFα also exerts direct effects upon insulin receptor signalling by inhibition of insulin receptor tyrosine kinase through increased phsophorylation of insulin-receptor substrate-1 [474]. From the observations made in this study, it is possible to speculate that the paracrine, or indeed intracrine, induction of 11β-HSD1 in muscle tissue by TNFα may contribute to the insulin resistance of obesity as a consequence of increased intracellular levels of glucocorticoid and thus increased levels of glucocorticoid hormone action. Furthermore, by analogy with AME, down-regulation of renal 11β-HSD2 activity by TNFα in obesity derived insulin resistance may account for the hypertension which frequently accompanies obesity, NIDDM and the metabolic syndrome.

3.4.8 *Regulation of 11β-hydroxysteroid dehydrogenase in human skeletal myoblasts by dehydroepiandrosterone*

Indirect evidence for the regulation of 11β-HSD by androgens has come from studies of the sexually dimorphic expression of isoforms of 11β-HSD in the rat[170,171], in man[88,431] and in the mouse[432]. Most of these studies imply that *in vivo* regulation of 11β-HSD occurs as a consequence of the potent androgenic effects of testosterone. In the present study, pre-treatment of skeletal myoblasts and JEG-3 cells with a range of concentrations of the potent androgens, androstenedione and testosterone, had no detectable effect upon 11β-HSD1 11-OR activity or 11β-HSD2 11-DH activity. However, exposure to DHEA, a relatively week androgen, significantly attenuated 11β-HSD1 11-OR activity in skeletal myoblasts but had no detectable effect upon 11β-HSD2 11-DH activity in JEG-3 cells. These observations confirm previous reports of a DHEA-dependent decline in hepatic 11β-HSD1 in spontaneously hypertensive rats following treatment with DHEA-sulphate[438]. However, DHEA-sulphate treated rats showed a marked increase in renal 11β-HSD2 activity. Nevertheless, the difference between the observations made by Homma *et al*[438] and those made of 11β-HSD2 activity in JEG-3 cells in the study reported in this thesis may reflect differences in species and cell-type specific regulation of 11β-HSD2 by DHEA. Furthermore, the observations of Homma *et al*[438] suggest that the activity of hepatic steroid sulphatases may also play a significant role in mediating the effects of administered DHEA-sulphate on 11β-HSD1 activity. This leads to the intriguing possibility that despite the relatively low levels of DHEA in the general circulation, enzymatic de-sulphation of DHEA-sulphate, which circulates at μmolar concentrations, by steroid-sulphatase activity may play a role in the tissue specific regulation of isoforms of 11β-HSD activity in a variety of tissues. Moreover, recent reports have suggested that the age related decline in DHEA-sulphate levels is associated with an increased risk of cardiovascular disease and insulin resistance[475-477]. However, to date, there is no evidence for age related changes in 11β-HSD1 activity in man.

Importantly in this study, the failure of androstenedione and testosterone to effect the regulation of 11β-HSD1 11-OR activity in skeletal myoblasts suggests that regulation of

11β-HSD1 by DHEA is not mediated by the androgen receptor and the mechanism underlying the regulation of 11β-HSD1 by DHEA awaits explanation. Interestingly, in contrast with the DHEA-dependent decline in 11β-HSD1 11-OR activity, Western blot analysis failed to detect a decline in 11β-HSD1 34kDa immunoreactive protein. These observations suggest that down-regulation of 11β-HSD1 activity following exposure to DHEA in skeletal myoblasts may be dependent upon post-translational modification of 11β-HSD1 protein. Indeed, peptide sequence studies have revealed two possible N-glycosylation sites in the cloned rat 11β-HSD1 at N203 and N158 [144] and inhibition of glycosylation using tunicamycin in a vaccinia virus expression system results in partial inhibition of 11β-HSD1 11-DH activity but does not affect 11β-HSD1 11-OR activity[54]. Moreover site directed mutagenesis of the two glycosylation sites in Chinese hamster ovary cells have shown that modification of the first site decreases 11β-HSD1 11-DH and 11-OR activity by 25% and 50% respectively whilst mutation of the second site results in complete abolition of catalytic activity[144]. These data suggest that N-glycosylation is required for 11β-HSD1 activity in the rat. However, there is no evidence that N-glycosylation is required for human 11β-HSD1 activity. In contrast, human 11β-HSD2 contains only one possible N-glycosylation site. However, Western blot analyses have revealed contradictory evidence that human 11β-HSD2 is N-glycosylated *in vivo*[242,454,478].

3.4.9 *Regulation of 11β-hydroxysteroid dehydrogenase in JEG-3 cells by oestradiol and progesterone*

In common with previous investigations of the regulation of 11β-HSD2 in the rat[170] and baboon[433], in this study, pre-treatment of JEG-3 cells with 17β-oestradiol resulted in an increase in 11β-HSD2 activity. However, recent studies of 11β-HSD2 11-DH activity in term placenta, indicate that 17β-oestradiol decreases the expression of 11β-HSD2 11-DH activity and that this effect was enhanced by progesterone in a dose dependent manner[434]. In contrast, in the present study, pre-treatment of JEG-3 cells with progesterone had no detectable effect on 11β-HSD2 activity whilst co-pre-treatment of JEG-3 cells with 17β-oestradiol. Moreover, co-pre-treatment of JEG-3 cells with 17β-

oestradiol and progesterone in combination did not alter the induction of 11β-HSD2 effected by 17β-oestradiol alone.

11β-HSD1 11-OR activity in skeletal myoblasts was unaffected by pre-treatment with either 17β-oestradiol or progesterone. This is in marked contrast with reports that describe the inhibition of hepatic 11β-HSD1 by progesterone[210]. However, Ricketts *et al*[210] reported inhibition of 11β-HSD1 11-OR activity but no effect upon 11β-HSD1 mRNA expression in rat and human hepatocyte cultures. Thus the decline in 11β-HSD1 11-OR activity observed in the study reported by Ricketts *et al*[210] may be a consequence of competitive inhibition of 11β-HSD1 11-OR activity by progesterone rather than pre-translational regulation of 11β-HSD1 gene expression.

Earlier *in vitro* and *in vivo* investigations of 11β-HSD activity in JEG-3 cells and in the rat[122,479] have provided evidence for the competitive inhibition of both 11β-HSD1 and 11β-HSD2 by 11-hydroxy-metabolites of progesterone. Analysis of foetal blood has revealed high levels of the 11-hydroxy-metabolites of progesterone at term[225] and competitive inhibition of placental 11β-HSD2 may explain, at least in part, the net change in the direction of cortisol:cortisone interconversion throughout the period of gestation. Moreover, these observations suggest that, in addition to the pre-translational regulation of 11β-HSD2 by 17β-oestradiol, competitive inhibition of isoforms of 11β-HSD *in vivo* either by progesterone itself or by its metabolites may contribute to the sexual dimorphism of 11β-HSD activity[437].

3.4.10 *Concluding remarks*

This study has revealed for the first time, the constitutive expression and pre-translational regulation of 11β-HSD1 in skeletal myoblasts by a broad spectrum of steroid and peptide hormones. Similarly, this study has also demonstrated the pre-translational regulation of 11β-HSD2 in JEG-3 cells, a model of trophoblastic tissue. However, many of the observations made in this chapter have also served to highlight the intimate relationship between the tissue-specific hormonal regulation of isoforms of 11β-HSD and the

homeostatic regulation of the intermediate metabolism of carbohydrate through the regulation of tissue sensitivity to glucocorticoid. These data support hypothesis that glucocorticoid antagonism of insulin action may represent a common aetiology underlying the metabolic syndrome and the insulin resistance of NIDDM.

Skeletal muscle represents one of the principal target tissues for insulin stimulated glucose uptake and disposal and may comprise as much as 40% of total body mass in the adult human male. Thus, chapter 4 of this thesis describes the analysis of *in vitro* 11β-HSD1 and glucocorticoid receptor expression in human skeletal myoblasts, and their relationship to the regulation of tissue sensitivity to glucocorticoid and *in vivo* insulin resistance, in a cohort of male volunteers with contrasting levels of insulin resistance and adiposity.

CHAPTER 4 – dysregulation of 11β-hydroxysteroid dehydrogenase: its role in the metabolic syndrome

4.1 INTRODUCTION

4.1.1 *Glucocorticoids and insulin resistance*

The metabolic, or insulin resistance syndrome, characterised by glucose intolerance, hyperinsulinaemia, dyslipidaemia and hypertension, constitutes a spectrum of clinical features which are frequently associated with type II diabetes[480,481]. Obesity may also be accompanied by a similar clinical phenotype which represents a significant adverse risk factor for the development of premature atherosclerosis and cardiovascular disease[250-252].

Cushing's syndrome has long been recognised as a model of obesity and decreased insulin sensitivity is a common consequence of exposure to supraphysiological concentrations of cortisol[458,482,483]. The effects of glucocorticoids on glucose and insulin metabolism are complex but include the promotion of gluconeogenesis and glycogen synthesis[484,485], inhibition of glycogenolysis and reduction of glucose disposal to the intracellular compartment as a consequence of inhibition of the translocation of the glucose transporter, GLUT-4, to the cell membrane[486,487,488]. Moreover, glucocorticoids promote the differentiation of pre-adipocytes into mature fat cells[489], diminishes glucose uptake and stimulates lipoprotein lipase activity in adipose tissue which results in an increase in lipid mobilisation and triglyceride sequestration in visceral fat depots[487,488]. Additionally, glucocorticoids inhibit the activity of lipoprotein lipase in skeletal muscle and diminishes uptake of circulating triglyceride which contributes to the clinical and atherogenic features of dislipidaemia associated with the insulin resistance syndrome[490]. Importantly, insulin resistance, impaired glucose tolerance and dyslipidaemia comprise the principal features of the metabolic syndrome. This has led to the hypothesis that increased levels of glucocorticoid hormone action may represent a common aetiology underpinning these cardiovascular risk factors.

4.1.2 *The regulation of tissue sensitivity to glucocorticoid*

Cross-sectional studies have revealed strong positive associations between circulating levels of cortisol, blood-pressure, glucose intolerance and hypertriglyceridaemia and have led to suggestions that chronic activation of the HPA axis may underlie this relationship[491,492]. However, in most obese, insulin resistant subjects, circulating levels of cortisol are either normal or may even be slightly decreased[493]. Furthermore, there is growing evidence to suggest that metabolic clearance of cortisol may be enhanced in obese subjects as a consequence of an increase in 5α-reductase activity in hepatic and adipose tissue[395] which is accompanied by an increase in HPA drive in order to compensate for lower plasma cortisol concentrations[494,495]. These contradictory data have led to the proposal of a second hypothesis which suggests that relatively modest changes in the regulation of tissue sensitivity to glucocorticoid may result in increased levels of glucocorticoid hormone action which, in turn, promotes impaired insulin sensitivity, glucose intolerance, raised blood pressure and other features of the metabolic syndrome.

Levels of circulating glucocorticoid are determined principally by the rate of cortisol secretion, which is regulated by ACTH under the control of the HPA axis, and by its rate of metabolic clearance. In contrast, tissue sensitivity to glucocorticoid is determined not only by the levels of circulating cortisol to which the tissue is exposed but also by the abundance of GR and the availability of biologically active glucocorticoid in the intracellular compartment. Several studies have revealed compelling evidence to suggest that intracellular conversion of cortisone to cortisol by 11β-HSD1 is an important pre-receptor regulator of tissue sensitivity to glucocorticoid by modulating the availability of active ligand for binding with the GR[172].

Two isoforms of the GR have been described which comprise splice variants of the same gene[465]. Glucocorticoid receptor-alpha (GRα) is able to bind ligand whilst the truncated beta isoform (GRβ), which is unable to bind ligand, is thought to act as a dominant-negative inhibitor of glucocorticoid hormone action through heterodimerisation with GRα[465,496]. Moreover, the expression of GRα is regulated by its own ligand such that

cortisol induces down-regulation of GRα mRNA expression and stability and increases the post-translational turnover of GRα protein[496,497]. Thus, the tissue-specific expression and predominant 11-OR activity of 11β-HSD1 in glucocorticoid target tissues may be considered to play a key role in the regulation of tissue sensitivity to glucocorticoid through two interdependent mechanisms: the first is the direct regulation of intracellular levels of cortisol whilst the second is the corollary of increased intracellular levels of cortisol upon the regulation of GRα.

The significance of altered tissue sensitivity to glucocorticoid and the role of the GR and 11β-HSD1 11-OR activity in the aetiology of raised blood pressure, insulin resistance, hyperglycaemia and central obesity has been explored in a number of human studies[247,271,394]. Similarly, inhibition of 11β-HSD1 with pharmacological doses of carbenoxolone has been demonstrated to increase whole body insulin sensitivity in man[418] and to reduce fasting blood glucose levels in the rat[406]. Nevertheless, inhibition of renal 11β-HSD2 11-DH activity by carbenoxolone is also accompanied by raised blood pressure analogous to that seen in AME type II. Furthermore, studies using the 11β-HSD1 knockout mouse have revealed that these animals resist the hyperglycaemia associated with obesity and stress and do not exhibit glucocorticoid induction of hepatic gluconeogenesis even when circulating levels of glucocorticoid are elevated[172].

4.1.3 *Skeletal muscle is a key insulin and glucocorticoid target tissue*

Skeletal muscle is a key target tissue for insulin stimulated non-oxidative glucose utilisation represented by both increased glucose uptake and accelerated glycogen synthesis[446,463]. Moreover, as discussed earlier, skeletal muscle also represents the principal site of impaired insulin action in subjects presenting with the metabolic syndrome, obesity and non-insulin-dependent diabetes mellitus[462,463]. Importantly, whilst the molecular mechanisms underlying the processes of glucose utilisation in muscle have been extensively studied in both human and animal models the same is not true of the mechanisms underlying the regulation of glucocorticoid hormone action in this tissue.

Previous studies have demonstrated that the investigation of the molecular mechanisms underlying insulin responsiveness in skeletal muscle may be accomplished using human skeletal myoblasts in culture[451,463,498,499]. Moreover these studies have also revealed that muscle cells in culture retain the features of glucose intolerance and insulin resistance which characterise the subjects from which they originate and reflect the metabolic behaviour of intact skeletal muscle[463]. Therefore, these data suggest that cultured human skeletal myoblasts represent an important model with which to investigate the mechanisms underpinning the pathogenesis of glucocorticoid induced insulin resistance in the human population.

4.1.4 *Aims of this study*

The introduction to this chapter reviews the evidence in support of a relationship between increased glucocorticoid hormone action and insulin resistance and suggests that the regulation of tissue sensitivity to glucocorticoid is regulated by the availability of active glucocorticoid to the GR through intracellular conversion of cortisone to cortisol by 11β-HSD1. Chapter three of this thesis presents evidence which demonstrates glucocorticoid-induced up-regulation of 11β-HSD1 in human skeletal myoblasts which is directly mediated through the interaction of cortisol with the GR. Concomitant glucocorticoid-induced down regulation of GR expression may represent an important mechanism whereby tissue sensitivity to glucocorticoid is maintained in an environment in which cortisol is relatively more abundant. Moreover, dysregulation of this dynamic equilibrium may underpin many of the pathological features associated with altered tissue sensitivity to glucocorticoid. Thus, the aims of this study were to investigate the relationship between 11β-HSD1 and GR expression in human skeletal myoblasts from a cohort of male volunteers with contrasting levels of insulin resistance, blood pressure and adiposity.

4.2 Materials and Methods

4.2.1 *Study subjects*

Fourteen adult male volunteers aged 40 –60 years were recruited to the study. Recruitment, anthropometric measurement and estimation of glucose tolerance were performed by the Molecular And Metabolic Programming Unit, MRC, Southampton General Hospital. The subjects were segregated into lean-moderately overweight (n = 8) and obese subjects (n = 6) according to World Health Organisation (WHO) guidelines. Two of the subjects were classified as having type II diabetes but none of the subjects exhibited evidence of other disease nor were any receiving medical treatment. Ethical committee approval was granted by the Southampton and Southwest Hampshire National Health Service Trust and written informed consent was obtained from each subject. Glucose tolerance was estimated for each subject following a standard oral dose of 75g glucose. All of the non-diabetic subjects were found to have glucose tolerances within the normal range which was defined by a plasma glucose below 126mg/dl whilst fasting and a plasma glucose of less than 140mg/dl 2 hours post a standard 75g oral dose of glucose. Four weeks prior to each component of the study and hypoglycaemic or related agents were withdrawn. The subjects were admitted to the Clinical Research facility, Southampton General Hospital where they consumed a standard weight-maintenance diet which comprised calories as 55% carbohydrate, 30% fat and 15% protein for a period of 24 hours prior to commencement of the study. During this period the subjects completed a lifestyle and health questionnaire. Measurement of blood pressure, weight, height, waist and hip circumference, and skin fold thickness was performed by the same trained observer for each subject and body mass index (BMI), waist/hip ratio and body fat (%) were calculated.

4.2.2 *Hyperinsulinaemic-euglycaemic clamp*

Insulin resistance indices were measured using the hyperinsulinaemic-euglycaemic glucose clamp technique[500] by Dr. D E Flanagan at the Clinical Research facility, Southampton General Hospital. All subjects were fasted for 12 hours overnight prior to the procedure. Insulin and glucose infusions were administered into an anticubital vein. Blood sampling was performed from a dorsal vein on the opposite hand. This hand was

warmed to enable sampling of arterialised blood. After priming infusion of insulin, a continuous infusion of insulin was commenced at a fixed rate of 60mU/m^2/minute. The infusion was continued for a period of 2 hours. Plasma glucose was maintained at 5mmol/L by variable glucose infusion and the amount of infused glucose required to maintain euglycaemia was taken to represent the amount of glucose which had been metabolised (M). The mean plasma insulin concentration (I), measured by radioimmunoassay at the Endocrinology Unit Southampton General Hospital, was calculated. The M/I ratio (mg/m^2/minute x μU/ml) was used as the measure of tissue sensitivity to insulin[500].

4.2.3 *Immunohistochemical analysis of glucocorticoid receptor expression in human skeletal myoblasts*

Adherent myoblasts in 10cm^2 petri dishes which had been air dried and fixed with either 10% formol saline or 4% paraformaldehyde were immunohistochemically stained for the presence of human GR using a primary antisera for human GR at 1:100 – 1:1000 (affinity purified rabbit polyclonal antibody raised against a 16 amino acid peptide corresponding to the amino terminus of GR common to both the 95kDa GRα and GRβ isoforms obtained from Santa Cruz Biotechnology Inc., CA, USA) as previously described (3.2.10.6). Immunostaining was detected following brief incubation with diaminobenzadine and visualised under light microscopy.

4.2.4 *Glucocorticoid receptor and 11β-hydroxysteroid dehydrogenase mRNA and activity in human skeletal myoblasts from subjects with contrasting levels of insulin resistance and obesity*

Human myoblasts were obtained from fourteen male subjects recruited to the study as previously described (3.2.2). Monolayer cultures of myoblasts were grown to 90% subconfluence in 25cm^2 flasks and exposed to insulin (20-100mU/ml), cortisol (50-1000nmol/L) and cortisone (50-1000nmol/L) separately and in combination for 48-96 hours prior to assay of 11β-HSD1 activity as described in chapter three (3.2.4). Close agreement between changes in 11-OR activity, 11β-HSD1 immunoreactive protein and

mRNA expression observed in previous studies of the regulation of 11β-HSD1 (chapter 3) and the limitations of resource and time precluded an exhaustive investigation of the kinetic regulation of 11β-HSD1 in myoblasts from all fourteen subjects in this study. Therefore, experimental and statistical analysis of the differential effects of glucocorticoid and insulin on 11β-HSD1 11-OR activity in myoblasts from the subjects taking part in this study was made principally by examining changes in 11β-HSD1 mRNA expression using Northern blot analysis. Nevertheless, kinetic analysis of 11β-HSD1 11-OR activity was also performed using myoblasts from the most insulin sensitive (L/Is cells) and insulin resistant (Ob/Ir cells) of the subjects taking part in the study. Estimation of 11β-HSD1 11-OR activity, measurement of GR and 11β-HSD1 immunoreactive protein by Western blot analysis and measurement of GR and 11β-HSD1 mRNA by Northern blot analysis was performed as previously described (3.2.9 & 3.2.10). Analysis of the molecular determinants of glucocorticoid hormone action in skeletal myoblasts was performed by qualitative RT-PCR using primers specific for 11β-HSD1, 11β-HSD2, GRα, GRβ, mineralocorticoid receptor (MR) and Na/K-ATPase α1 subunit as previously described (3.2.8). Statistical comparison of 11β-HSD activity (expressed as the rate of conversion of cortisone to cortisol), 11β-HSD and GR mRNA was made using the unpaired 't' test. The significance of linear regression analysis was determined using Pearsons correlation. Where appropriate the data was normalised by logarithmic transformation.

4.3 Results

4.3.1 *Physiological and anthropometric characteristics of the subjects*

A summary of the characteristics of the study group are illustrated in table 4.1 (page 206). When the subjects were stratified by body mass index according to World Health Organisation (WHO) criteria, seven of the subjects were categorised as being obese (BMI > 30Kg/m^2) and seven were categorised as being either lean or moderately overweight (BMI 25-29 Kg/m^2). These criteria were used to stratify the subjects throughout the remainder of this study.

4.3.2 *The molecular determinants of glucocorticoid hormone action in human skeletal myoblasts*

4.3.2.1 *Glucocorticoid receptor expression*

Analysis of gene expression by qualitative RT-PCR using primers specific for GRα, GRβ, mineralocorticoid receptor (MR) and Na/K-ATPase α1 subunit revealed expression of GRα and GRβ but not MR mRNA in skeletal muscle biopsies (figure 4.1a, page 207) whilst skeletal myoblasts cultured under glucocorticoid-free conditions revealed expression of GRα but not GRβ or MR mRNA. Northern blot analyses using a GRα specific cRNA probe (3.2.9.4) revealed the presence of a 7.0kb mRNA species in skeletal myoblasts (figure 4.1c). Western blot analyses revealed the presence of a 95kDa immunoreactive protein in these cells (figure 4.1c). Incubation of L/Is cells with increasing concentrations of cortisol (50-1000nM) revealed a marked dose dependent decrease in GRα mRNA and 95kDa immunoreactive protein (figure 4.1c/d/e). Down-regulation of GRα mRNA was abolished by co-incubation with a 10-fold molar excess of the GRα antagonits, RU38486 (figure 4.1b). Incubation of myoblasts with varying concentrations of insulin, IGF-1 and glucose had no effect on GRα mRNA expression. Immunohistochemical analysis revealed positive staining for GR in all cells which was distributed to both the nuclear and cytoplasmic compartments despite the absence of glucocorticoid in the growth medium (figure 4.2a, page 208). Western blot analysis of

nuclear and cytosolic protein fractions separated by differential centrifugation confirmed these observations (figure 4.2b).

Under basal, glucocorticoid-free conditions and following incubation with glucocorticoid there were marked between-subject differences in levels of GRα and 11β -HSD1 mRNA expression which were unaffected by cell passage number (passage 3-12) or cell density (up to 98% confluence). Variability attributable to sources of methodological error was minor compared with the marked differences evident between subjects, not only for GRα but also for 11β -HSD1 (table 4.2).

4.3.2.2 *11β-hydroxysteroid dehydrogenase activity and mRNA expression*

Molecular analysis of 11β-HSD1 activity, mRNA and 34kDa immunoreactive protein in skeletal myoblasts and their response to glucocorticoid and insulin are detailed in chapter 3 of this thesis (3.3.3, 3.3.4 & 3.3.5). Kinetic analysis of 11β-HSD1 11-OR activity from the most insulin sensitive (L/Is cells) of the subjects revealed apparent K_m and V_{max} for the conversion of cortisone to cortisol of 476nM and 94pmol/mg protein/hour and 437nM and 46pmol/mg protein/hour in the presence and absence of 20% FCS respectively while apparent K_m and V_{max} for the conversion of cortisone to cortisol in myoblasts from the most insulin resistant subject (Ob/Ir cells) was 419nM and 62pmol/mg protein/hour and 423nM and 32pmol/mg protein/hour.

Table 4.1 Summary of the physiological characteristics of 14 adult males aged between 40 and 69 years, recruited randomly as study subjects.

Characteristic	Arithmetic Mean (SD)	Range
Insulin Sensitivity (GDR)	5.7 ± 2.9	2.32 - 10.10
Body Mass Index (Kg/M2)	29.8 ± 4.3	25.2 - 39.3
Waist/Hip Ratio	0.967 ± 0.072	0.862 - 1.087
% Body Fat	26.3 ± 4.2	19.0 - 32.4
Systolic Blood Pressure (mm Hg)	147.6 ± 28.9	110 -206
Diastolic Blood Pressure (mm Hg)	86.9 ± 11.3	74 - 110
Age (Years)	58.6 ± 9.0	40 - 69

Insulin sensitivity was measured as glucose disposal rate (GDR mg/m^2/minute) by hyperinsulinaemic-euglycaemic clamp. All data were normally distributed and are represented by arithmetic mean ± standard deviation (SD).

Table 4.2 Methodological variability in GRα and 11β-HSD1 mRNA quantification

Methodological Variability	%CV	n
Intra-flask variation	<5	6
Temporal variation (9 subjects)	8.7 - 19.7	6
Intra-Northern blot variation	10.1 - 14.0	6
Inter-Northern blot variation	<13.6	4
Inter-passage variation (4 subjects)	12.7 - 22.5	5
Variation due to cell density (4 passages)	9.4 - 16.1	12

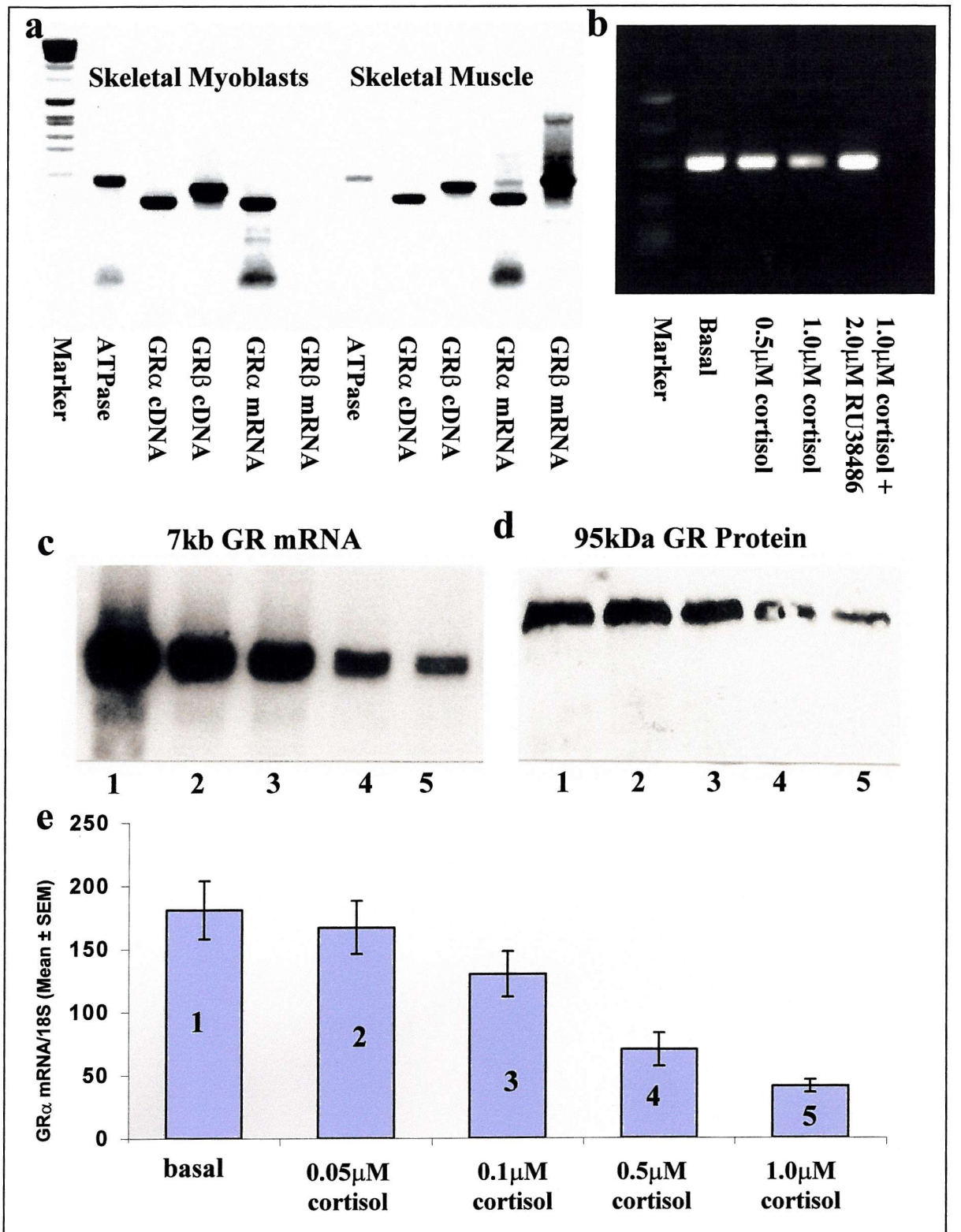


Figure 4.1 Characteristics of GR expression and regulation in human skeletal myoblasts:(a) RT-PCR analyses of Grα and GRβ mRNA expression in cultured skeletal myoblasts and representative skeletal muscle tissue (b) Semi-quantiative RT-PCR illustrating abolition of the dose dependent decrease in GRα expression by RU38486 (c) Northern blot analysis illustrating the decline in GRα mRNA expression and (d) Western blot analysis illustrating the decline in of 95kDa GR immunoreactive protein induced by physiological concentrations of cortisol

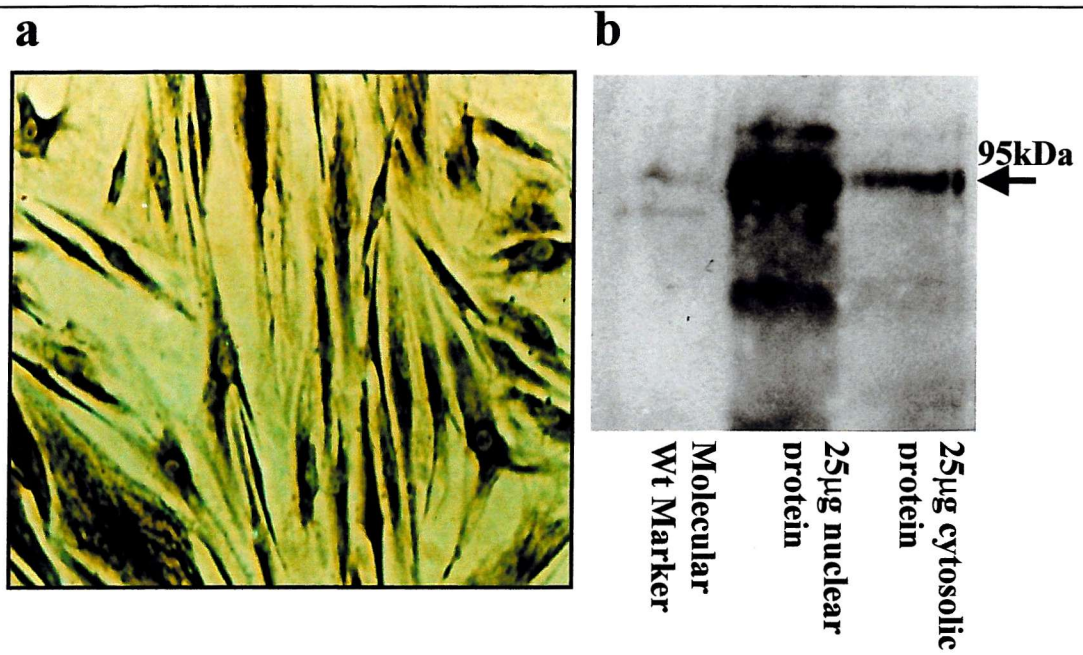


Figure 4.2 Glucocorticoid receptor expression in human skeletal myoblasts
(a) Nuclear and cytosolic positive immunohistochemical staining for human GR in human skeletal myoblasts (b) Western blot analysis of nuclear and cytosolic protein fractions following differential centrifugation revealed GR to be distributed to both the nuclear and cytoplasmic compartments.

4.3.3 *Glucocorticoid induced down-regulation of glucocorticoid receptor mRNA expression in human skeletal myoblasts is correlated with levels of insulin resistance and obesity*

Northern blot analyses revealed marked between-subject differences in GRα mRNA expression in myoblasts under basal, glucocorticoid-free conditions (figure 4.3a, page 210). Western blot analyses also revealed marked between-subject differences in 94kDa GR immunoreactive protein which was in close agreement with levels of GRα mRNA ($r = 0.92$, $P < 0.001$). A strong inverse association was observed between GRα mRNA expression in myoblasts *in vitro* and glucose disposal rate *in vivo* (figure 4.3b), while strong positive associations were observed between GRα mRNA expression in myoblasts and body mass index (figure 4.3c), percentage body fat (figure 4.3d) and systolic blood pressure (figure 4.3e).

Following pre-incubation of myoblasts with 200nM cortisol for 48hours the associations between GRα mRNA expression in myoblasts and *in vivo* glucose disposal rate (figure 4.4b, page 211), body mass index (figure 4.4c), percentage body fat (figure 4.3d) and systolic blood pressure (figure 4.4e) were similar to those observed under basal, glucocorticoid-free conditions. However, there was a strong positive association between the magnitude of the decline in GRα mRNA expression in myoblasts (expressed as a percentage decline) and *in vivo* glucose disposal rate ($r = 0.85$, $P<0.001$), and strong inverse associations between percent decline in GRα mRNA and body mass index ($r = 0.78$, $P<0.01$) and percentage body fat ($r = 0.63$, $P<0.01$).

When the subjects were stratified on the basis of BMI (4.3.1) into obese or leaner subjects, median levels of GRα mRNA expression were three-fold greater in myoblasts from obese subjects under basal, glucocorticoid-free conditions and five-fold greater following incubation with 200nM cortisol compared with myoblasts from leaner subjects (figure 4.5a/c, page 212). Furthermore, the decline in GRα mRNA expression effected by 200nM cortisol in myoblasts from obese subjects was three-fold smaller than that which was observed in myoblasts from leaner subjects (figure 4.5b/d) .

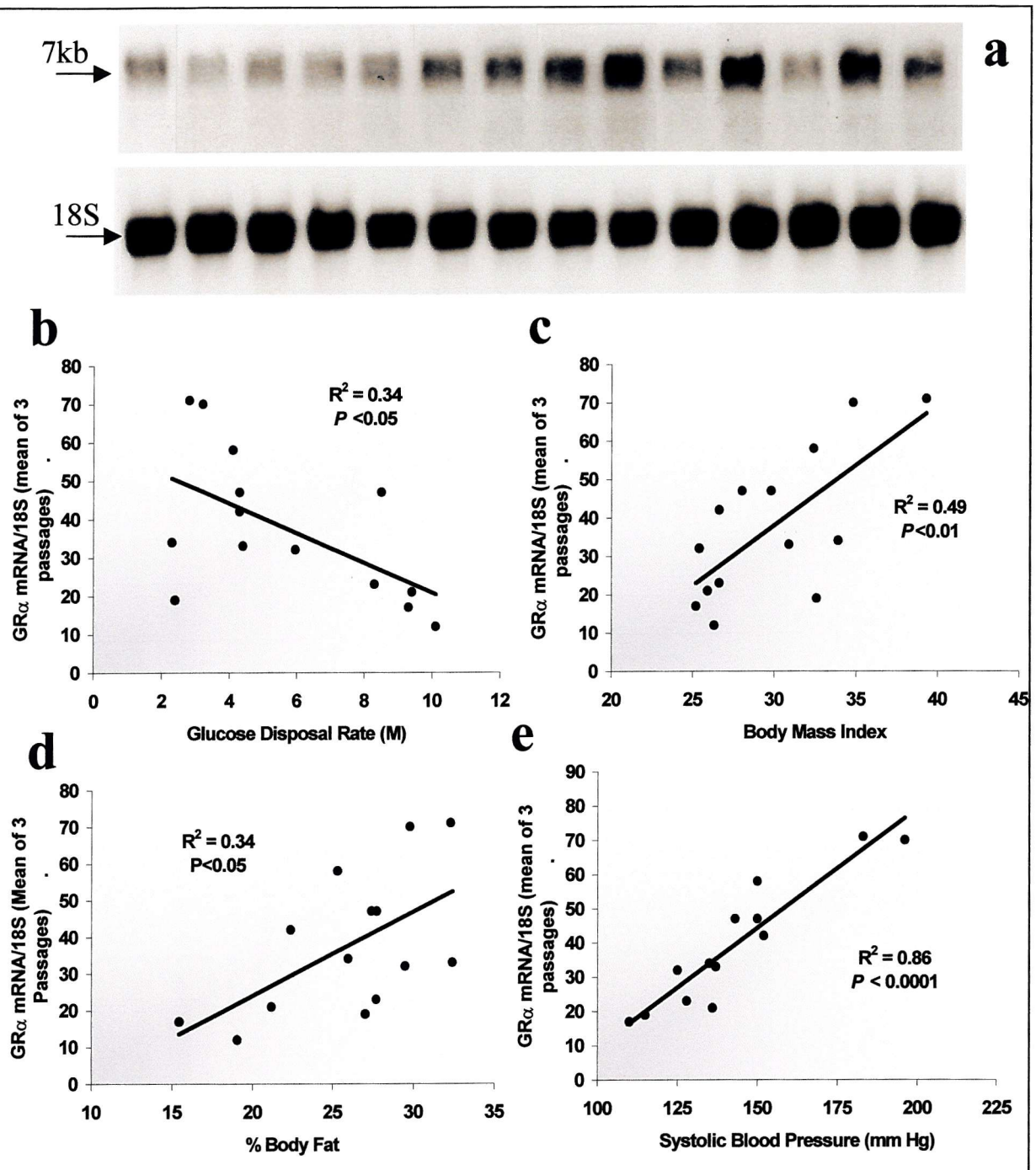


Figure 4.3 Associations between GRα mRNA expression in human skeletal myoblasts under basal, glucocorticoid free conditions and *in vivo* insulin sensitivity, adiposity and systolic blood pressure in lean, moderately overweight and obese men. (a) Representative Northern blot of GRα mRNA expression (lean-moderately overweight to the left, obese subjects to the right) (b-e) Regression analyses depicting associations between constitutive GRα mRNA expression and glucose disposal rate (b), BMI (c), % body fat (d) and systolic blood pressure (e)

Each data point represents GRα mRNA expression in RNA pooled from three flasks from each of three different passages. The systolic blood pressure for one of the leaner subjects was >3 SD above the arithmetic mean for the subject group and was excluded from the regression analysis relating to blood pressure

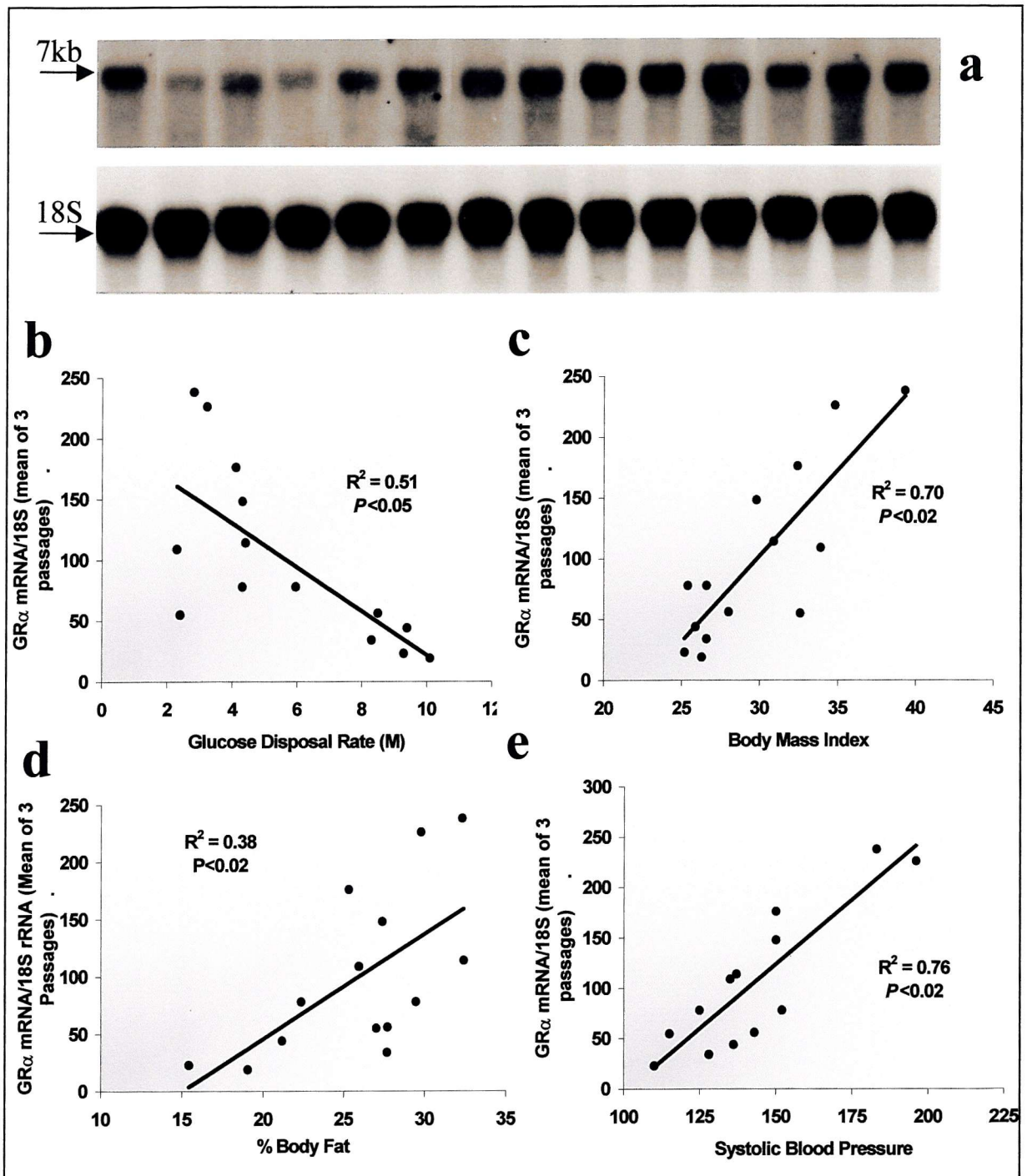


Figure 4.4 Associations between GRα mRNA expression in human skeletal myoblasts following incubation with 200nM cortisol and *in vivo* insulin sensitivity, adiposity and systolic blood pressure in lean, moderately overweight and obese men. (a) Representative Northern blot of GRα mRNA expression (lean-moderately overweight to the left, obese subjects to the right) (b-e) Regression analyses depicting associations between constitutive GRα mRNA expression and glucose disposal rate (b), BMI (c), % body fat (d) and systolic blood pressure (e)

Each data point represents GRα mRNA expression in RNA pooled from three flasks from each of three different passages. The systolic blood pressure for one of the leaner subjects was >3 SD above the arithmetic mean for the subject group and was excluded from the regression analysis relating to blood pressure

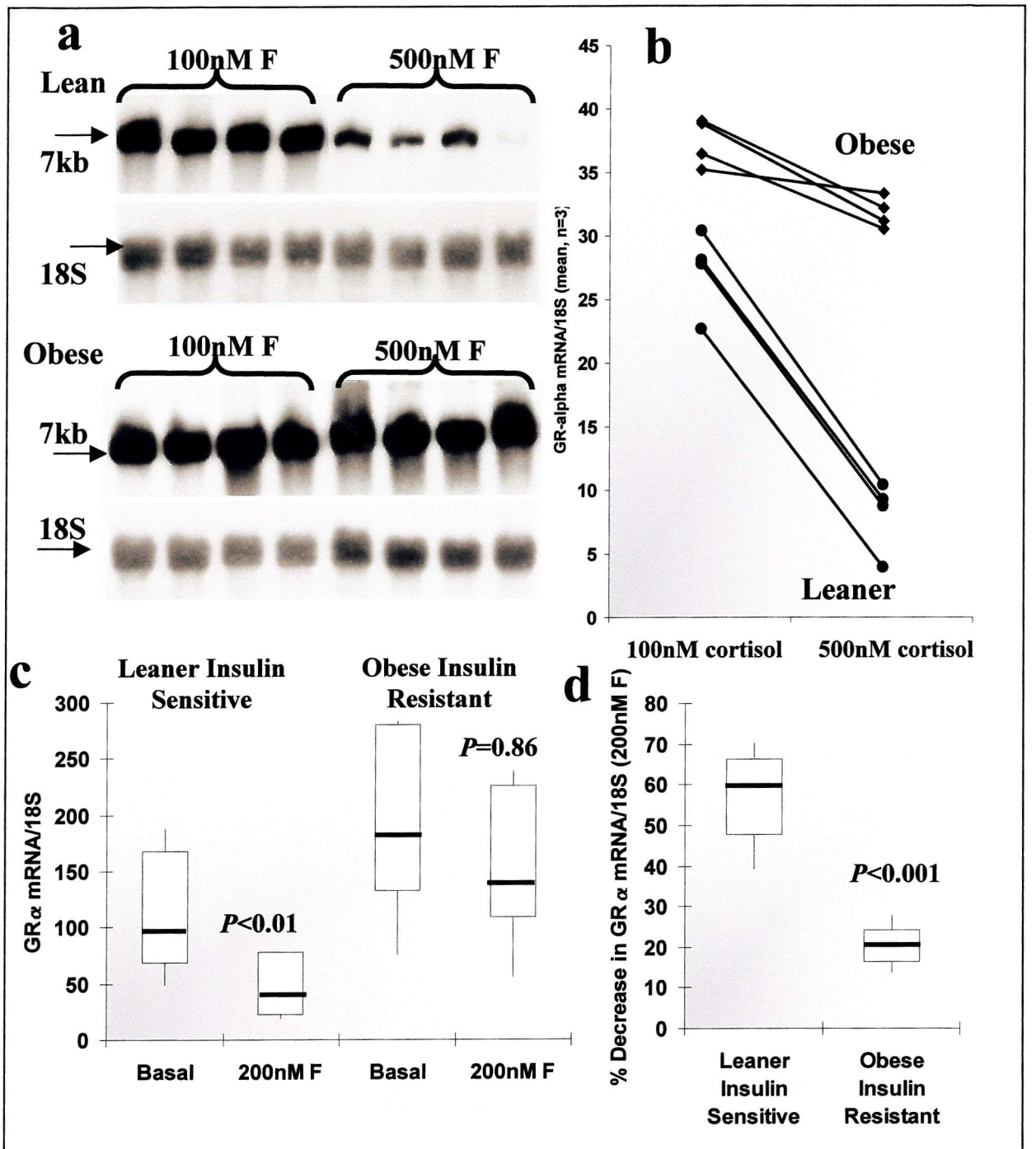


Figure 4.5 Sensitivity of GRα mRNA expression to down-regulation by cortisol in subjects stratified as obese (n=7) or lean-moderately overweight (n=7) according to WHO criteria

(a) Representative Northern blot of GRα mRNA expression from lean-moderately overweight & obese subjects following incubation with cortisol. Lanes 1-4 show GRα mRNA expression from the same subjects as lanes 5-8. (b) Comparative effects of a five-fold increase in cortisol on GRα mRNA expression in myoblasts from four of the most obese and leanest subjects (c/d) Box & whisker plots comparing GRα mRNA expression (c) and decline in GRα mRNA expression (d) in myoblasts under basal, glucocorticoid-free conditions vs 200nM cortisol in lean-moderately overweight and obese subjects.

Each data point represents GRα mRNA expression in RNA pooled from three flasks from each of three different passages.

4.3.4 *Glucocorticoid induction of 11β-hydroxysteroid dehydrogenase mRNA expression in human skeletal myoblasts is correlated with levels of insulin resistance and obesity*

Under basal, glucocorticoid-free conditions, analysis of 11β-HSD1 11-OR activity in intact myoblasts revealed marked inter-subject variability in 11β-HSD1 11-OR activity. Northern blot analysis revealed parallel marked inter-subject variability in 11β-HSD1 mRNA expression. However, under basal, glucocorticoid-free conditions no significant associations were observed between 11β-HSD1 mRNA expression in skeletal myoblasts and *in vivo* glucose disposal rate (M score) ($r = 0.24$; $P = 0.45$), BMI ($r = 0.07$; $P = 0.84$), systolic blood pressure ($r = 0.20$; $P = 0.83$) or percentage body fat ($r = 0.10$; $P = 0.98$).

In contrast with the observations made under basal, glucocorticoid free conditions, Northern blot analysis of 11β-HSD1 mRNA expression in myoblasts which had been pre-treated with 500nM cortisol for 48 hours (figure 4.6a, page 215) revealed a strong inverse correlation between 11β-HSD1 mRNA expression and *in vivo* glucose disposal rate ($r = 0.82$; $P < 0.0001$) (figure 4.6b), and strong positive associations between 11β-HSD1 mRNA expression and BMI ($r = 0.79$; $P < 0.005$) (figure 4.6c), percentage body fat ($r = 0.47$; $P < 0.05$) (figure 4.6d) and systolic blood pressure ($r = 0.52$; $P < 0.05$) (figure 4.6e). Moreover, Northern blot analysis also revealed a strong inverse correlation between the magnitude of the glucocorticoid-induced increase in 11β-HSD1 mRNA expression (expressed as a percentage increase) and *in vivo* glucose disposal rate ($r = 0.69$; $P < 0.01$) (figure 4.7a, page 216), and strong positive associations between 11β-HSD1 mRNA expression and BMI ($r = 0.72$; $P < 0.02$) (figure 4.7b), percentage body fat ($r = 0.59$; $P < 0.05$) (figure 3.7c) and systolic blood pressure ($r = 0.60$; $P < 0.05$) (figure 4.7d).

When the subjects were stratified on the basis of BMI (4.3.1) into obese or leaner subjects, median levels of 11β-HSD1 mRNA expression were two-fold greater in myoblasts from obese subjects following incubation with 200nM cortisol compared with myoblasts from leaner subjects (figure 4.8a, page 217). Furthermore, the decline in 11β-

HSD1 mRNA expression effected by 500nM cortisol in myoblasts from obese subjects was two-fold greater than that which was observed in myoblasts from leaner subjects (figure 4.8b).

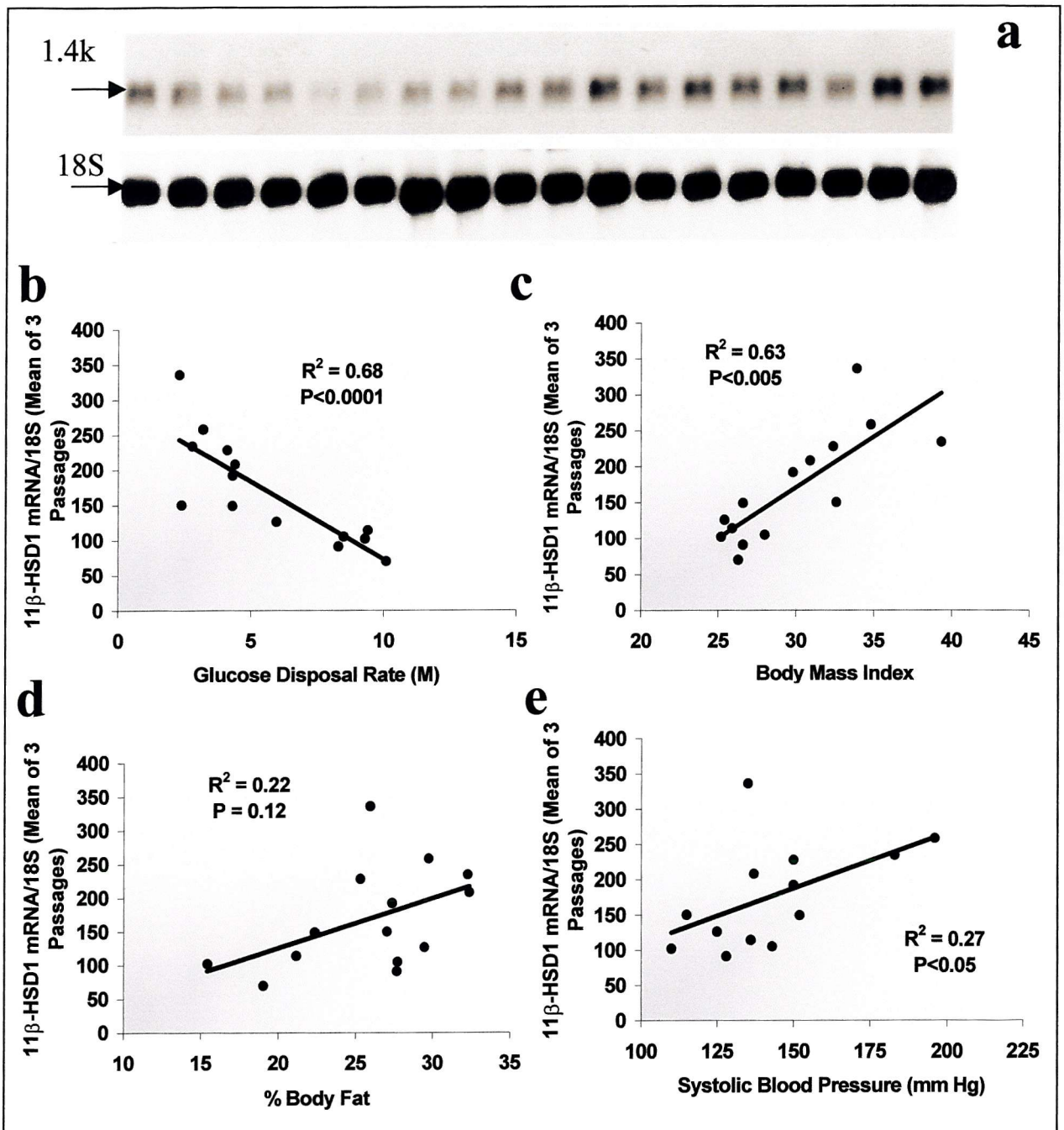


Figure 4.6 Associations between 11β-HSD1 mRNA expression in human skeletal myoblasts following incubation with 500nM cortisol and *in vivo* insulin sensitivity, adiposity and systolic blood pressure in lean, moderately overweight and obese men. (a) Representative Northern blot of 11β-HSD1 mRNA expression in myoblasts from 5 lean-moderately overweight subjects to the left, 4 obese subjects to the right (Each of the 2 lanes of RNA per subject reflect 11β-HSD1 expression in cells from 2 different passages pooled from 3 flasks) (b-e) Regression analyses depicting associations between 11β-HSD1 mRNA expression following incubation with 500nM cortisol and glucose disposal rate (b), BMI (c), % body fat (d) and systolic blood pressure (e)

Each data point represents GRα mRNA expression in RNA pooled from three flasks from each of three different passages. The systolic blood pressure for one of the leaner subjects was >3 SD above the arithmetic mean for the subject group and was excluded from the regression analysis relating to blood pressure

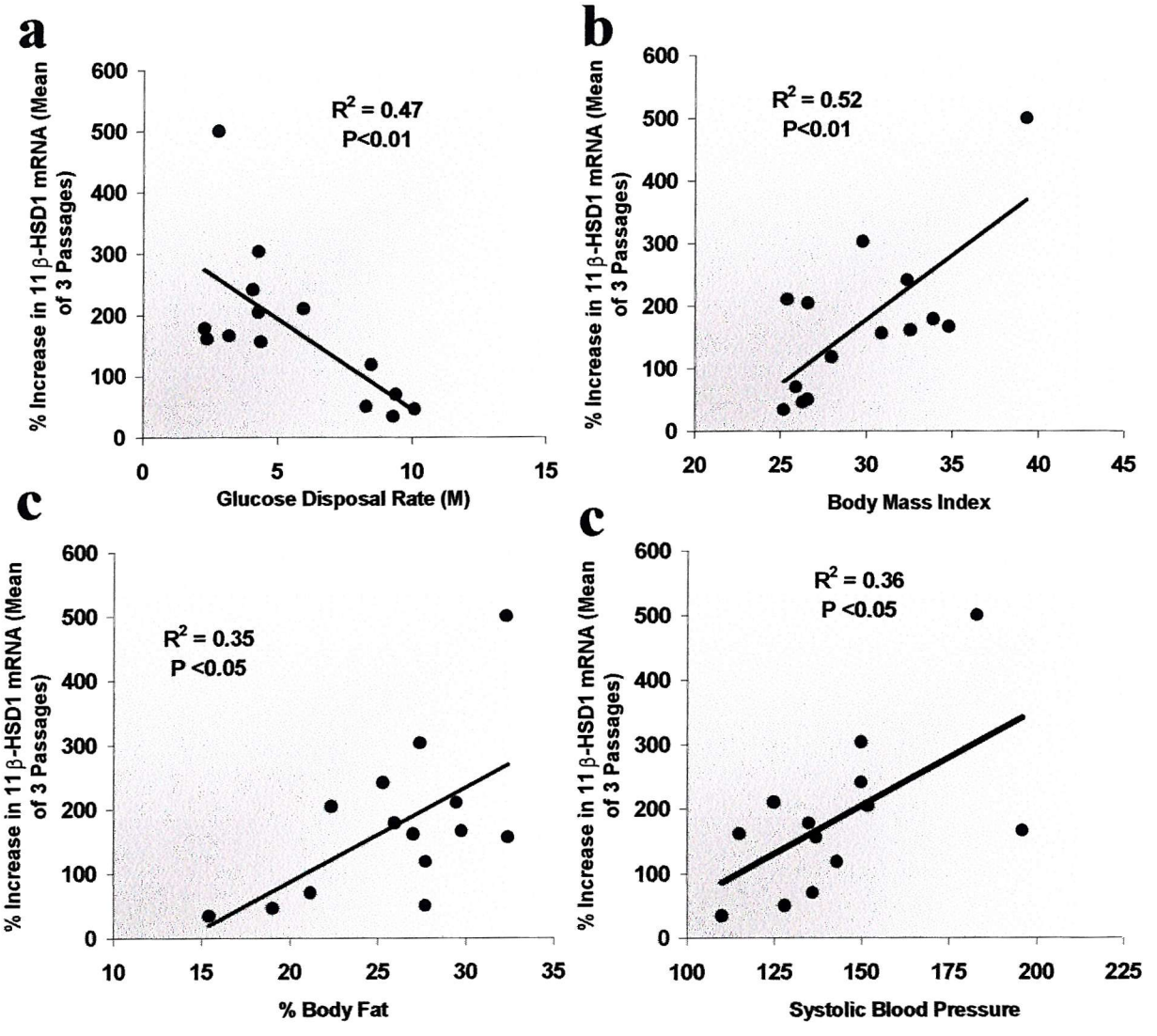


Figure 4.7 Associations between glucocorticoid-mediated induction of 11β-HSD1 mRNA expression in human skeletal myoblsts following incubation with 500nM cortisol and *in vivo* insulin sensitivity, adiposity and systolic blood pressure in lean, moderately overweight and obese men. (a-d) Regression analyses depicting associations between glucocorticoid-mediated induction of 11β-HSD1 mRNA expression following incubation with 500nM cortisol and glucose disposal rate (b), BMI (c), % body fat (d) and systolic blood pressure (e)

Each data point represents GRα mRNA expression in RNA pooled from three flasks from each of three different passages. The systolic blood pressure for one of the leaner subjects was >3 SD above the arithmetic mean for the subject group and was excluded from the regression analysis relating to blood pressure

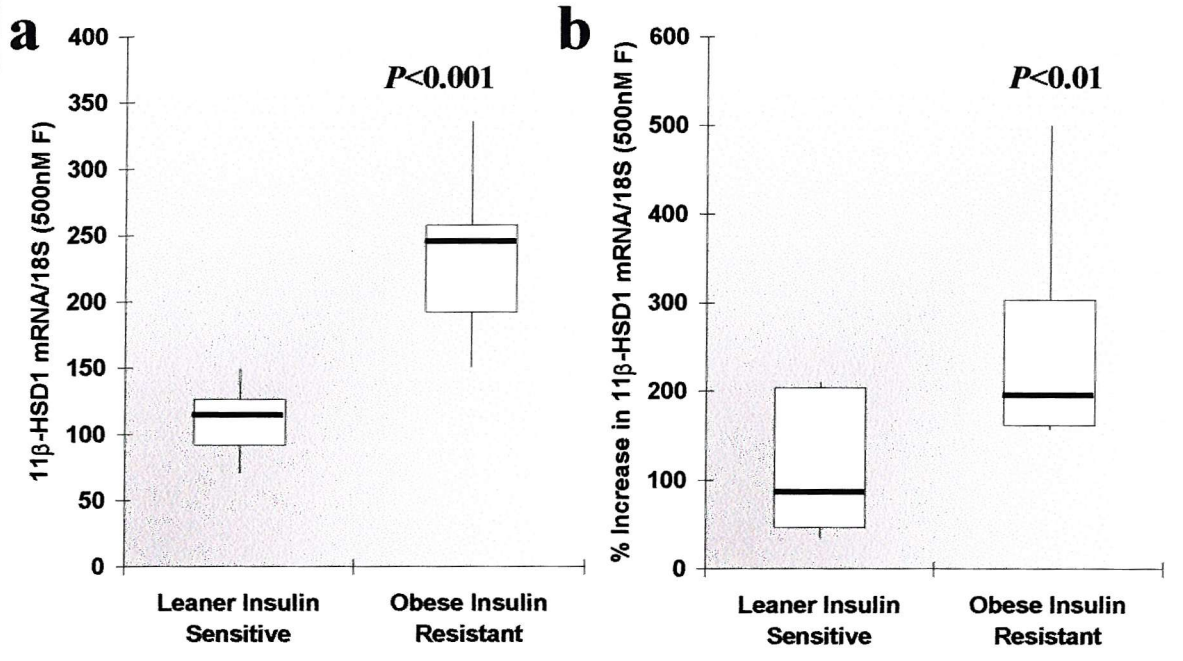


Figure 4.8 Box and whisker plot analysis comparing 11β-HSD1 mRNA expression in human skeletal myoblasts from lean-moderately overweight and obese subjects following incubation with 500nM cortisol for 48 hours (a) levels of 11β-HSD1 mRNA expression (b) the magnitude of the glucocorticoid-induced increase in 11β-HSD1 mRNA expression

4.4 Discussion

4.4.1 *The regulation of glucocorticoid hormone action in human skeletal myoblasts*

Previous investigations have clearly demonstrated that the regulation of glucocorticoid hormone action and of tissue sensitivity to glucocorticoid is achieved, at least in part, by the sensitive down-regulation of GR expression in response to exposure to glucocorticoid and that this is regulated at the level of mRNA transcription and stability and to a lesser extent as a consequence of increased protein turnover[496,497]. Furthermore, there is a growing body of evidence to suggest that isoforms of 11β-HSD may also play a role in the regulation of glucocorticoid hormone action[172,176,393] either by increasing metabolic clearance of cortisol through the action of renal 11β-HSD2 or by increasing the intracellular concentration of active glucocorticoid as a consequence of the conversion of cortisone to cortisol by 11β-HSD1. Moreover, evidence from studies of the regulation of 11β-HSD1 in skeletal myoblasts presented in chapter three of this thesis suggests that 11β-HSD1 in these cells is also subject to regulation by glucocorticoids such that exposure to glucocorticoids at concentrations equivalent with circulating levels of cortisol results in an increase in 11β-HSD1 mRNA and protein expression and 11β-HSD1 11-OR activity. Importantly, these investigations also suggest that the glucocorticoid-dependent up-regulation of 11β-HSD1 in skeletal myoblasts is mediated through the GR since these effects are abolished when these cells are incubated with glucocorticoid and the GR antagonist RU38486 in combination. Thus the regulation of intracellular glucocorticoid hormone action in skeletal myoblasts may be considered to be the product of two GR-mediated mechanisms characterised by: i) the sensitive glucocorticoid dependent down-regulation of GR expression which is accompanied by: ii) the sensitive glucocorticoid dependent GR-mediated up-regulation of 11β-HSD1 expression. Indeed, it is likely that these two mechanisms are mutually inter-dependent since failure of the former mechanism (glucocorticoid dependent auto-down-regulation of GR) might be predicted to result in a greater increase in glucocorticoid-dependent, GR-mediated 11β-HSD1 expression than would occur otherwise. Importantly, this might also be predicted to result in higher levels of intracellular cortisol and a persistent

amplification of glucocorticoid hormone action. These events, by analogy with the insulin resistance which accompanies Cushing's syndrome, might also be predicted to result in tissue specific glucocorticoid-mediated insulin resistance and may represent a mechanism underlying the insulin resistance of the metabolic syndrome

4.4.2 *The regulation of glucocorticoid hormone action in human skeletal myoblasts: associations with features of the metabolic syndrome*

4.4.2.1 *The regulation of glucocorticoid receptor expression*

Previous investigations of insulin signalling in skeletal muscle have confirmed the hypothesis that skeletal myoblasts in culture retain the features of insulin sensitivity which characterise the subjects from whom the cells originate[463]. Nevertheless, in the study reported in this thesis, experiments using skeletal myoblasts were restricted to the early passage numbers (3-7) in order to abrogate the possibility of metabolic changes away from their original phenotype. Whilst GR expression and binding and 11β-HSD expression have previously been investigated in human adipose tissue and cultured human adipose stromal cells[247,442] this study is the first to describe the regulation of glucocorticoid receptor and 11β-HSD1 expression in cultured human cells from skeletal muscle.

In common with previous reports[496,497] this study revealed a dose dependent down regulation of GR mRNA expression which was closely paralleled by changes in GR protein expression and which is consistent with the pre-translational regulation of GR expression in human skeletal myoblasts. Moreover, this effect was abolished by the GR antagonist RU38486 which confirms suggestions that regulation of GR expression by glucocorticoid is mediated through the GR itself.

This study revealed a marked between-subject variability in constitutive GR expression which was independent of passage number, cell density or periods of cryostorage and which was positively correlated with the degree of insulin resistance, adiposity and blood pressure exhibited by the subjects from whom the cells originated. Furthermore, the

differences in glucocorticoid receptor expression in skeletal myoblasts from different subjects observed under basal glucocorticoid-free conditions were maintained when these cells were exposed to glucocorticoids at concentrations which mimic those of the normal diurnal rhythm *in vivo*. However, while auto-down regulation of GR was evident in myoblasts from all of the subjects, when the subjects were stratified on the basis of BMI according to WHO criteria, the magnitude of the glucocorticoid-induced decline in GR mRNA expression in skeletal myoblasts from the obese subjects compared with that observed in cells from the leaner subjects was approximately three-fold smaller. Indeed, under conditions of glucocorticoid exposure the associations between GR expression in skeletal myoblasts and BMI, glucose intolerance, adiposity and blood pressure in the subjects from whom the cells were derived were stronger than under basal glucocorticoid-free conditions. These observations imply that not only are constitutive levels of GR expression higher in skeletal myoblasts from the more obese, insulin resistant subjects but that glucocorticoid induced auto-down-regulation of GR is also attenuated in these cells compared with skeletal myoblasts from their leaner, more insulin sensitive counterparts.

The strong associations between the metabolic syndrome *in vivo* and GR expression *in vitro* observed in this study, under both basal glucocorticoid-free conditions and following exposure to glucocorticoid are unlikely to be an artefact of tissue culture. Indeed, it is more likely that these data represent an under-estimate of the importance of GR auto-regulation by glucocorticoid in the pathogenesis of obesity and the metabolic syndrome. Thus, not only are constitutive levels of GR expression, and hence glucocorticoid sensitivity, higher in skeletal myoblasts from obese subjects with key features of the metabolic syndrome but, importantly, by extension of the observations made *in vitro* it is likely that in these subjects skeletal muscle GR expression *in vivo* is also higher. Furthermore, glucocorticoid-dependent auto-down regulation of GR is diminished in myoblasts from subjects with features of the metabolic syndrome and this might be predicted to maintain higher levels of glucocorticoid sensitivity in skeletal muscle throughout the diurnal variation in levels of circulating cortisol.

4.4.2.2 *The regulation of 11β-hydroxysteroid dehydrogenase*

Recent studies suggest that, in addition to the GR, 11β-HSD1 mediated regulation of intracellular conversion of inactive cortisone to active cortisol and hence glucocorticoid availability to the GR may also play a key role in the aetiology of insulin resistance[174,176], central obesity[247] and hypertension[271]. Indeed, mice in which the gene for 11β-HSD1 has been disrupted show attenuated expression of glucocorticoid responsive gluconeogenesis despite increased circulating levels of active glucocorticoid[172]. This suggests that 11β-HSD1 11-OR activity may serve to maintain intracellular glucocorticoid concentrations at a much higher level than those detected in the circulation and, as a consequence, increase intracellular levels of glucocorticoid hormone action beyond that which would be predicted from circulating levels of glucocorticoid. Moreover, further support for a role of 11β-HSD1 11-OR activity in the metabolic syndrome comes from human studies in which inhibition of hepatic 11β-HSD1 11-OR activity as a consequence of the oral ingestion of carbenoxolone markedly improves hepatic insulin sensitivity[174].

The study reported in this thesis has revealed abundant levels of 11β-HSD1 mRNA expression in cultured human skeletal myoblasts with rates of 11β-HSD1 11-OR activity equivalent to those reported in other human tissues[57,169,249]. However, in contrast with GR expression in human skeletal myobalsts , constitutive expression of 11β-HSD1 under basal, glucocorticoid free conditions was not associated with levels of insulin resistance, adiposity or other features of the metabolic syndrome examined in the subjects from whom the cells were derived.

Previous *in vitro* studies have suggested that 11β-HSD1 11-OR activity may be increased by glucocorticoid in cultured human adipose stromal cells[249] and hepatocytes[169]. Furthermore, data from the present study (discussed in chapter 3 of this thesis) indicates that 11β-HSD1 mRNA, immunoreactive protein and 11-OR activity in human skeletal myoblasts is sensitively up-regulated by physiological concentrations of cortisol in a dose dependent manner. Furthermore, abolition of the glucocorticoid dependent

induction of 11β-HSD1 mRNA, immunoreactive protein and 11-OR activity by the GR antagonist RU38486 confirms that this effect is mediated exclusively by the GR. As a consequence, it is likely that the magnitude of the glucocorticoid-mediated induction of 11β-HSD1 expression in skeletal myoblasts would be tempered by glucocorticoid-dependent down regulation of GR. Moreover, since constitutive levels of GR expression in skeletal myoblasts is positively correlated with features of the metabolic syndrome and the magnitude of the glucocorticoid-dependent down regulation of GR expression in these cells is negatively correlated with insulin resistance, adiposity and other features of the metabolic syndrome it is likely that the potency with which cortisol up-regulates the expression of 11β-HSD1 would be greater in myoblasts from the more obese, insulin resistant subjects.

Indeed, in this study, incubation of myoblasts with physiological concentrations of cortisol resulted in an increase in 11β-HSD1 mRNA expression and 11-OR activity the magnitude of which was strongly correlated with levels of insulin resistance, adiposity and blood pressure such that glucocorticoid-induced up-regulation of 11β-HSD1 was markedly greater in myoblasts from the more obese, insulin resistant subjects compared with cells from leaner subjects. Thus skeletal myoblasts from the more obese, insulin resistant subjects may be considered to exhibit a relative insensitivity to glucocorticoid induced down-regulation of GR accompanied by a highly sensitive glucocorticoid induced up-regulation of 11β-HSD1 expression. This effect might be predicted to maintain higher intracellular levels of active glucocorticoid in myoblasts from the more obese, insulin resistant subjects compared with their lean counterparts and represents a potentially powerful mechanism by which glucocorticoid hormone action may be amplified several fold within the cell.

Importantly, these data also suggest that key factors in the regulation of glucocorticoid hormone action are represented principally by the GR and secondly by the availability of 11β-HSD1 substrate, namely cortisone. Previous studies have demonstrated that that circulating levels of cortisone *in vivo* are derived principally from conversion of cortisol to cortisone by 11β-HSD2 11-DH activity in the kidney[17], distal colon[57] and adrenal

cortex[210,243]. However, metabolic clearance of cortisone as a consequence of its conversion to cortisol by 11β-HSD1 or hepatic hydroxylation to tetrahydrocortisone, predicts that circulating levels of cortisone, and indeed cortisol, are likely to constitute a relatively poor reflection of their intracellular concentration. Indeed, in most obese, insulin resistant subjects, circulating levels of cortisol and renal 11β-HSD2 activity are frequently normal[440,493].

In contrast, the data presented in the study reported in this thesis suggest that constitutive GR expression and GR responsiveness to glucocorticoid-dependent auto-down regulation are the principal determinants of the level of 11β-HSD1 expression and that the position of equilibrium between GR and 11β-HSD1 expression is the principal determinant of glucocorticoid hormone action in the skeletal muscle cell (figure 4.9, page 224). This hypothesis would predict that relatively modest dysregulation of GR or 11β-HSD1 expression may result in a disproportionately greater increase in levels of glucocorticoid hormone action in skeletal muscle cells and represents a plausible mechanism underlying the key role of glucocorticoid hormone action in the pathogenesis of features of the metabolic syndrome. Moreover, if this was to occur in other cell types, such as visceral adipocytes in which GR and 11β-HSD1 are also highly expressed[249] this may also promote the 'Cushing's like' obesity that is frequently associated with the metabolic syndrome[489].

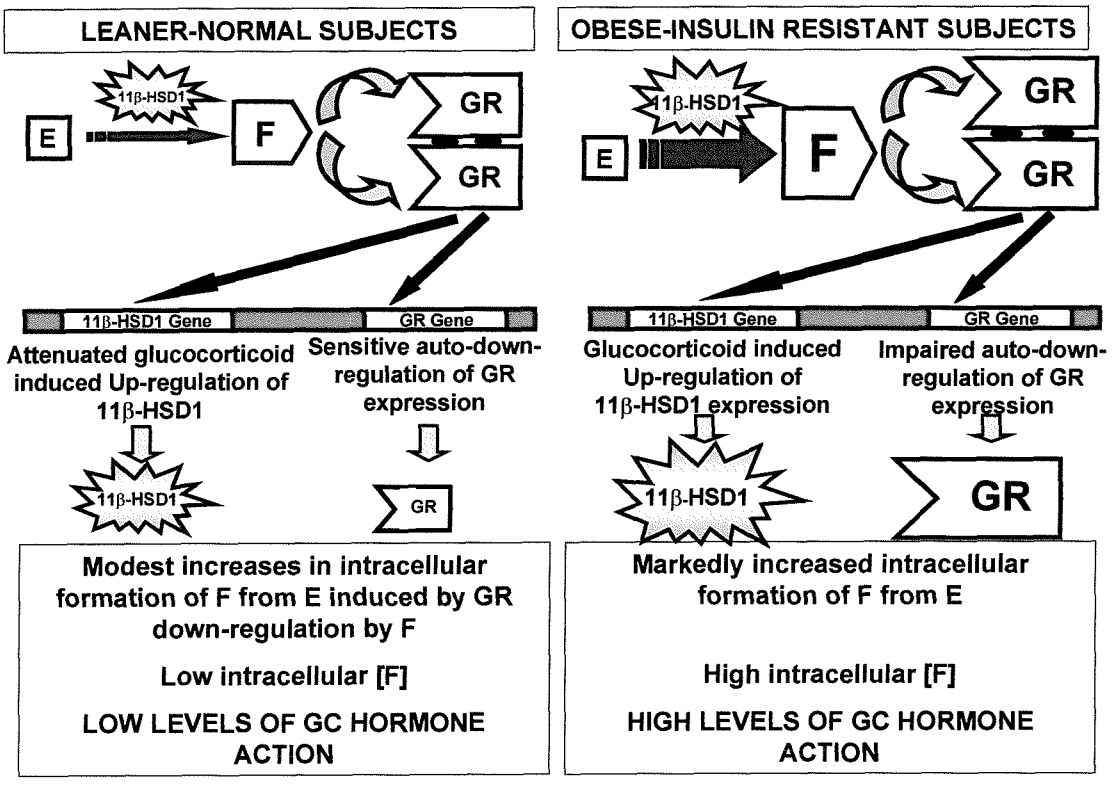


Figure 4.9 Schematic representation of the proposed glucocorticoid-dependent establishment of positions of equilibrium between 11β-hydroxysteroid dehydrogenase (11β-HSD1) mediated intracellular synthesis of cortisol (F) from cortisone (E) and expression of the glucocorticoid receptor (GR) in lean, moderately overweight subjects and obese-insulin resistant subjects

4.5 Suggestions for further research

The close associations between 11β-HSD1 and GR expression and key features of the metabolic syndrome, described in chapter 4 of this thesis, serve to highlight the significance of the dysregulation of isoforms of 11β-HSD in the pathogenesis of human disease. Importantly, these studies, in the light of evidence presented in chapters 3 and 4 of this thesis for the hormonal regulation of 11β-HSD1 and auto-down regulation of GR by glucocorticoid in skeletal myoblasts, suggest that dysregulation of 11β-HSD1 in skeletal muscle may play a key role in the aetiology of glucocorticoid dependent insulin resistance as a consequence of its central role in the regulation of tissue sensitivity to glucocorticoid. However, the observation that constitutive GR expression in skeletal myoblasts is closely associated with the degree of insulin resistance, adiposity and blood pressure in the subjects from who the cells were derived whilst constitutive 11β-HSD1 expression is not, would suggest that the primary abnormality, at least in skeletal myoblasts, is a failure of the mechanism of glucocorticoid-mediated GR auto-down-regulation. The mechanism underlying this phenomenon is unclear although genetic polymorphisms of GR have been reported which also have associations with features of the metabolic syndrome[501]. Nevertheless, more recent studies suggest that the BCl I restriction fragment length polymorphism for GR is not associated with blood pressure[502] and that GR phenotype is generally uninformative[503].

In the study reported in chapter 4 of this thesis, qualitative RT-PCR analysis of GR isoforms in skeletal muscle tissue and skeletal myoblasts revealed expression of the non-ligand binding isoform of GR, GRβ, in skeletal muscle tissue but not in skeletal myoblasts. However, preliminary investigations have revealed that GRβ may be expressed in these cells following incubation with glucocorticoid. Importantly Northern blot analyses have revealed that the magnitude of the glucocorticoid-mediated induction of GRβ mRNA expression appears to be inversely associated with the degree of insulin resistance, adiposity and blood pressure in the subjects from whom the cells were derived[504]. Since GRβ is generally considered to act as a dominant negative inhibitor of glucocorticoid hormone action through dimerisation with the ligand binding GRα

isoform[496] it may be predicted that failure of the mechanism of glucocorticoid-mediated GRβ up-regulation in insulin resistant cells may also contribute to increased levels of glucocorticoid hormone action in these cells. However, these data are only representative of gene expression at the level of mRNA. Whether this is translated to functional protein and to what extent this may affect glucocorticoid hormone action in these cells will be the subject of ongoing investigations using Western blot analysis utilising antisera specific to the GRβ isoform and by ligand binding studies.

Studies contemporary with the investigations reported in this thesis[248,249] suggest that central adiposity may be a reflection of increased levels of glucocorticoid action in omental adipose tissue. Moreover, in common with the regulation of 11β-HSD1 in human skeletal myoblasts by glucocorticoid reported in the chapter 3 of this thesis, 11β-HSD1 expression in omental adipose stromal cells is induced by glucocorticoid. However, in contrast with 11β-HSD1 in skeletal myoblasts, 11β-HSD1 in adipose stromal cells is unaffected by insulin when these cells are exposed to insulin in the absence of glucocorticoid[248]. Thus, since both skeletal muscle and adipose tissue represent insulin sensitive tissues, it is proposed that a comparison of the regulation of glucocorticoid hormone action in these two tissues may reveal the mechanisms underlying the heterogeneity of insulin resistance which is seen not only between subjects but also between tissues within an individual. Moreover, these studies could be expanded to include tissues which are not generally considered to be insulin target tissues, such as skin fibroblasts. This would enable a direct comparison of the hormonal and molecular regulation of glucocorticoid hormone action in insulin insensitive and insulin sensitive tissues.

Previous studies have revealed that 11β-HSD1 11-OR activity may up-regulated by IL-1β and TNF-α[444]. Moreover, circulating levels of TNF-α and IL-1β are frequently elevated in obese subjects and in subjects with non-insulin dependent diabetes mellitus. Importantly, investigations of the effects of IL-1β and TNF-α on 11β-HSD1 11-OR activity in human skeletal myoblasts reported in chapter 3 of this thesis, suggest that these pro-inflammatory cytokines potently up-regulate 11β-HSD1 expression in these

cells. Moreover, the close association between adipose tissue and skeletal muscle *in vivo*, together with the knowledge that these two tissues are capable of the synthesis and secretion of both IL-1β and TNF-α[468,469] suggests a mechanism of paracrine regulation of 11β-HSD1 in these tissues which may also contribute to the pathogenesis of insulin resistance.

The observation that the regulation of 11β-HSD1 in skeletal myoblasts by insulin may be modified by serum factors (discussed in chapter 3 of this thesis) presents the intriguing possibility that serum from insulin resistant and insulin sensitive subjects may also have differential effects upon the regulation of 11β-HSD1 in these cells. The investigation of these hypotheses would require an extension of the study described in chapter 4 of this thesis whereby primary cell cultures of both skeletal muscle and adipose tissue could be established from a cohort of subjects across a broad spectrum of phenotypes, to include both obese and lean insulin resistant subjects and subjects who are lean and insulin sensitive as controls. These investigations would include studies of the endocrine and paracrine interaction of these two cell types grown in isolation and in combination, and using conditioned growth medium across a spectrum of cells from phenotypically distinct subjects.

The regulation of human 11β-HSD2 by pro-inflammatory cytokines has not been previously reported. The observation that relatively high concentrations of IL-1β and TNF-α decrease 11β-HSD2 activity in JEG-3 cells suggests the possibility that human renal 11β-HSD2 may also be regulated in this manner. In the absence of an appropriate *in vitro* model of human distal tubular cells it would be interesting to investigate the role of IL-1β and TNF-α in the regulation of renal 11β-HSD2 in man *in vivo*. Chronic physiological stress is commonly accompanied by high circulating levels of pro-inflammatory cytokines[464]. Moreover, prolonged excessive exercise has been shown to result in a deterioration in immune function and an increase in HPA responsiveness[464]. An investigation of the rate of cortisol clearance in these subjects may identify changes in the activity of isoforms of 11β-HSD. Whilst urinary free cortisol:cortisone ratios may

reveal changes in renal 11β-HSD2 activity in these subjects it is likely that the increase in cortisol half life which accompanies an increase in hepatic 11β-HSD1 activity would confound the results of these analyses. It is likely therefore, that an accurate estimation of cortisol half life in these subjects using 11α-[^{3}H]-cortisol together with urinary free cortisol:cortisone estimation would be required in order to evaluate renal 11β-HSD2 activity in these subjects.

Finally, the significance of the investigations reported in this thesis and the results of further investigations into the role of dysregulation of isoforms of 11β-HSD in the pathogenesis of human disease suggest that there exists the potential for the pharmacological regulation of isoforms of 11β-HSD. The treatment of insulin resistance and other features of the metabolic syndrome by inhibition of 11β-HSD1 11-OR activity using carbenoxolone is unlikely to be acceptable, principally because of the profound side effects induced by the concurrent inhibition of renal 11β-HSD2 activity. These observations, therefore, highlight the requirement for pharmacological agents with the capacity for the specific inhibition of 11β-HSD1 11-OR activity whilst having no effect upon 11β-HSD2 11-DH activity. Importantly, the evaluation of these putative inhibitors of 11β-HSD1 would require an appropriate model of human 11β-HSD1 11-OR activity which maintain, *in vitro*, the phenotypic and metabolic features which characterise them *in vivo*. The human skeletal myoblasts, described in chapters 3 and 4 of this thesis, are an ideal model of 11β-HSD1 activity and are likely to represent a powerful tool for the rapid evaluation of these pharmacological agents.

Appendices

Appendix 1. Evaluation of the efficiency of derivatization of corticosteroids with CMMC

Quenching (q) was calculated using the expression: $q=(nH^a - n)/nH^b$ where:

(i) nH^b was given as the counts per minute (cpm) for the [^{3}H]-standard, after correction for tritium decay

(ii) n was given as the cpm obtained for the HPLC fraction collected 30 minutes after injection.

nH^a was given as the cpm obtained for the HPLC fraction collected 30 minutes after injection plus the [^{3}H]-standard

Conversion of cpm to decays per minute (dpm) was calculated using the expression: $dpm=cpm/q$.

Derivatization efficiency (E) was calculated using the expression: $E=n^a/n^b$ where:

(i) n^a was given as dpm obtained from the HPLC fraction collected 30 minutes after injection.

(ii) n^b was given as the total dpm added to the fraction.

Of the 200µl of prepared material, only 150µl was derivatized. 50µl of the derivatized product was analysed by HPLC and collected by fraction collector. The fraction was dried under a stream of dry nitrogen and reconstituted in 110µl acetonitrile/(50mM KH_2PO_4,10mM acetic acid) (40 –70% v/v). 50µl of the reconstituted fraction was counted by liquid scintiligraphy. Thus, the recovered fraction represented 5/11 of the total fraction derivatized and this factor was represented in all subsequent calculations.

Appendix 2. Analytical recovery of corticosteroids from plasma and urine by HPLC with solid phase extraction and fluorescence derivatization with CMMC.

	%Recovery (Urine)				% Recovery (Plasma)			
Steroid	**100nmol/L**	**SD**	**250nmol/L**	**SD**	**100nmol/L**	**SD**	**250nmol/L**	**SD**
6β-hydroxycortisol	not detected	*	not detected	*	not detected	*	83.9	16.3
20α-dihydrocortisol	not detected	*	73.4	23.3	73.4	21.8	70.4	14.9
20α-dihydrocortisone	not detected	*	95.6	26.8	83.5	10.7	88.6	5.0
20β-dihydrocortisol	86.6	18.9	95.4	14.1	86.8	9.4	89.1	8.7
20β-dihydrocortisone	80.2	21.6	85.6	9.7	94.1	6.3	79.6	6.1
α-cortol	106.4	10.7	101.5	8.9	97.1	11.3	84.6	8.4
β-cortol	Chromatographic co-elution							
α-cortolone	Chromatographic co-elution							
β-cortolone	76.8	18.3	86.9	5.9	98.4	3.5	86.4	6.2
cortisol	95.6	6.3	97.1	1.9	102.8	2.4	94.6	3.1
Cortisone	98.6	9.8	94.5	6.2	94.5	9.5	98.8	6.5
allo-tetrahydrocortisol	73.4	11.5	79.6	2.4	74.6	5.1	82.9	3.8
Tetrahydrocortisol	98.1	12.4	91.2	6.5	77	10.3	81.2	6.7
Tetrahydrocortisone	75.6	8.8	78.6	5.6	86.2	12.8	82.9	7.7
5α-dihydrocortisol	103.5	14.2	93.5	3.7	99.7	13.2	94.6	8.3
5β-dihydrocortisol	88.8	7.5	92.7	6	79.4	11.3	83.2	5.9
Corticosterone	99.7	4.2	94.3	5.4	86	14.7	86.1	4.8
5α-dihydrocortisone	86.4	9.8	102.4	5.1	95.4	10.3	79.4	5.7
5β-dihydrocortisone	98.2	10.1	83.1	6.7	81	5.7	77.3	6.3
11-deoxycortisol	89.4	6.1	97.1	2.4	86.7	13.1	97	4.1
11-deoxycorticosterone	103.1	7.6	95.6	3.7	96.4	6.2	101.4	3.2
17α-hydroxypregnenolone	96.4	6.8	102.8	5.8	89.5	7.4	94.2	3.7
Dehydroepiandrosterone	96.5	9.4	93.5	5.4	110.5	8.1	98.2	5.6
Pregnenolone	95.3	7.1	92.1	6.2	101.6	3.4	94.3	9.5

The data represents mean recovery of steroid (100 and 250nmol/L) added to three pooled plasma and urine specimens. 4ml urine and 1ml plasma were extracted and derivatized with 100µl CMMC(25mM), 50µl DMAP(50mM) and 50µl EDC(25mM) incubated at 70°C for 120 minutes.

Appendix 3. Analytical imprecision for the analysis of corticosteroids in plasma and urine by HPLC with solid phase extraction and fluorescence derivatization with CMMC.

Steroid	Urine		Plasma	
	Within Batch (CV%)	Between Batch (CV%)	Within Batch (CV%)	Between Batch (CV%)
6α-hydroxycortisol	not detected	not detected	34.6	42.1
20α-dihydrocortisol	not detected	not detected	41.3	43.2
20α-dihydrocortisone	not detected	not detected	28.1	32.3
20β-dihydrocortisol	16.3	21.8	12.7	16.7
20β-dihydrocortisone	21.4	22.3	10.4	12.9
α-cortol	10.1	8.6	8.6	12.1
β-cortol	Chromatographic co-elution			
α-cortolone	Chromatographic co-elution			
β-cortolone	6.8	8.4	9.7	14.3
cortisol	5.8	6.3	7.4	5.1
cortisone	9.7	9.9	10.8	12.1
allo-tetrahydrocortisol	16.4	21.6	11.9	13.2
tetrahydrocortisol	8.7	12.3	7.4	9.5
tetrahydrocortisone	10.5	11.6	13.8	12.9
5α-dihydrocortisol	16.3	13.2	11.4	15.8
5β-dihydrocortisol	12.9	14.7	10.1	12.8
Corticosterone	6.4	7.9	5.8	11.1
5α-dihydrocortisone	15.4	21.7	16.2	17.1
5β-dihydrocortisone	17.1	23.4	21.7	18.6
11-deoxycortisol	8.5	7.3	4.8	8.5
11-deoxycorticosterone	6.3	8.4	9.5	7.3
17α-hydroxypregnenolone	7.3	9.8	12.1	11.2
dehydroepiandrosterone	5.1	10.4	6.7	5.9
pregnenolone	6.3	5.9	7.4	8.2

The data represents mean recovery of steroid (100 nmol/L) added to pooled plasma and urine specimens. 4ml urine and 1ml plasma were extracted and derivatized with 100µl CMMC(25mM), 50µl DMAP(50mM) and 50µl EDC(25mM) incubated at 70°C for 120 minutes. Chromatography was performed using a Waters Nova-Pac C_{18} 60Å 4□m 300x3.9mm I.D. analytical HPLC column (Millipore, UK)

Appendix 4. Analytical recovery of corticosteroids from plasma and urine by HPLC with solid phase extraction and UV absorbance detection

Steroid	% Recovery (Urine)				% Recovery (Plasma)				% Recovery (DMEM)			
	100nmol/L	SD	250nmol/L	SD	100nmol/L	SD	250nmol/L	SD	100nmol/L	SD	250nmol/L	SD
6β-hydroxycortisol	98.8	6.3	94.3	6.2	100.1	2.7	99.4	3.4	98.7	1.4	97.6	2.7
20α-dihydrocortisol	92.1	8.4	93.5	3.6	100.4	3.1	96.5	5.8	99.2	1.6	98.7	1.9
20β-dihydrocortisol	94.2	6.4	91.0	5.8	97.5	8.9	94.0	5.4	101.4	0.8	91.4	3.4
20α-dihydrocortisone	91.5	7.7	91.9	8.1	95.1	10.7	92.4	2.6	96.8	2.4	98.4	4.9
20β-dihydrocortisone	90.2	9.5	93.7	9.1	96.1	7.4	90.9	8.4	96.4	6.7	95.6	4.5
cortisol	98.2	5.4	99.1	9.4	94.3	7.2	95.1	2.4	95.8	2.4	94.2	1.4
cortisone	94.1	6.8	96.4	7.0	99.2	1.9	96.0	4.3	99.7	3.7	102.4	1.7
corticosterone	91.7	9.5	93.4	8.2	96.5	8.8	94.1	7.2	95.7	4.1	97.4	3.2
11-deoxycortisol	96.0	6.3	94.2	4.8	99.2	9.1	98.4	1.9	97.6	5.7	98.7	1.8
11-deoxycorticosterone	94.2	10.3	91.8	3.8	94.0	6.8	91.5	2.3	102.4	4.1	95.7	2.1
17α-hydroxyprogesterone	102.4	11.5	99.4	9.6	105.7	3.8	108.6	3.4	98.5	3.2	97.6	0.8
Androstenedione	98.5	9.7	103.4	5.0	97.4	10.7	101.3	9.4	99.4	7.2	91.7	4.9
progesterone	99.2	2.6	98.9	6.2	95.2	5.0	93.1	1.1	103.4	6.5	95.4	2.4

The data represents mean recovery of steroid (100 and 250nmol/L) added to three pooled plasma, urine and tissue culture (DMEM containing 10% FCS) specimens. 4ml urine, DMEM and 1ml plasma were extracted. Chromatography was performed using a Waters Nova-Pac C_{18} 60Å 4μm 300x3.9mm I.D. analytical HPLC column (Millipore, UK)

Appendix 5. Analytical imprecision for the analysis of corticosteroids in plasma and urine by HPLC with solid phase extraction and UV absorbance detection

Steroid	Urine (n=10)		Plasma (n=10)		DMEM (n=10)	
	Within-Batch (CV%)	Between-Batch (CV%)	Within-Batch (CV%)	Between-Batch (CV%)	Within-Batch (CV%)	Between-Batch (CV%)
6β-hydroxycortisol	4.4	8.5	4.8	7.9	6.4	5.2
20α-dihydrocortisol	9.5	13.8	7.6	12.7	5.9	9.8
20β-dihydrocortisol	10.4	12.4	8.8	9.6	8.1	7.6
20α-dihydrocortisone	7.8	14.4	5.5	11.3	6.5	11.4
20β-dihydrocortisone	10.1	15.8	8.3	8.7	7.3	10.5
cortisol	6.8	9.5	5.7	7.1	6.2	8.2
cortisone	6.2	8.4	6.7	8.0	5.1	6.7
corticosterone	6.4	7.1	7.2	9.1	4.1	12.4
11-deoxycortisol	5.8	10.1	4.3	8.4	6.3	6.4
11-deoxycorticosterone	9.5	12.7	3.4	5.8	7.4	5.9
17α-hydroxyprogesterone	12.4	14.6	6.8	10.0	5.1	10.4
androstenedione	9.5	13.7	8.2	9.4	4.0	7.6
progesterone	4.8	9.2	7.0	7.1	6.4	5.2

The data represents mean recovery of steroid (100 nmol/L) added to three pooled plasma, urine and tissue culture (DMEM containing 10% FCS) specimens. 4ml urine, DMEM and 1ml plasma were extracted. Chromatography was performed using a Waters Nova-Pac C_{18} 60Å 4μm 300x3.9mm I.D. analytical HPLC column (Millipore, UK)

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