Aus der Medizinischen Klinik, Innenstadt
der Ludwig-Maximillians-Universität München

Direktor: Prof. Dr. med. M. Reincke

Endocrine Hypertension, Adrenal Steroids and Development of

a Saliva Based Aldosterone Assay as a Potential Screening Method

Dissertation
Zum Erwerb des Doktorgrades der Humanbiologie
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

vorgelegt von
Jenny Manolopoulou
aus
Hannover
2008

Mit Genehmigung der Medizinischen Fakultät

der Universität München

Berichterstatter: Prof. Dr. med. Martin Reincke

2. Berichterstatter: Priv. Doz. Dr. Christian Rust

Mitberichterstatter: Prof. Dr. Ulrich Pohl

Priv. Doz. Dr. Michael Vogeser

Mitbetreuung durch den

promovierten Mitarbeiter: Dr. med. Martin Bidlingmaier

Dekan: Prof. Dr. med. Dr.h.c. M. Reiser

Tag der mündlichen Prüfung: 09.10.2008

OUTLINE of CONTENTS

Abbreviations

ACE	angiotensin-converting enzyme
ACh	acetylcholine
ACN	acetonitrile
ACR	aldosterone to cortisol ratio
ACTH	adrenocorticotropic hormone
AGT	Angiotensinogen
Ang I	Angiotensin I
Ang II	Angiotensin II
ANP	atrial natriuretic peptide
APA	aldosterone producing adenoma
ARR	aldosterone to renin ratio
AT$_1$	Angiotensin II type 1 receptor
AVP	arginine vasopressin
BAH	bilateral adrenal hyperplasia
BK	bradykinin
BMI	body mass index
$[Ca^{2+}]_i$	intracellular calcium
CBG	cortisol binding globulin
CV	coefficient of variation
DCM	dichloromethane
DIN	Deutches Institut für Normung (German Industry Norm)
ECF	extracellular fluid volume
ED$_{50}$	Estimated Dose 50
EDTA	ethylene diamine tetraacetic acid
EH	essential hypertensive
ENaC	epithelial sodium channel
HCO$_3^-$	bicarbonate
H$_2$CO$_3$	carbonic acid
HPLC	high performance liquid chromatography
11β-HSD2	11β-hydroxysteroid dehydrogenase type 2
IgG	Immunoglobulin G
IHA	idiopathic hyperaldosteronism
JG	juxtaglomerular
kDa	kilodalton

LOD	limit of detection
LRH	low renin hypertension
mM	millimolar
ml/min	milliliter per minute
MeOH	methanol
MR	Mineralocorticoid receptor
NA	noradrenaline
ng/ml	nanograms per milligram
nm	nanometers
17-OHP	17-hydroxyprogesterone
ODS	octadecylcylsilane
PA	Primary aldosteronism
PEG	polyethyleneglycol
pg/ml	picograms per milligram
PLA	plasma aldosterone
POMC	proopiomelanocortin
RP HPLC	reverse phase high performance liquid chromatography
RT	room temperature
Rt	retention time
RAAS	Renin Angiotensin Aldosterone System
RAS	Renin Angiotensin System
RIA	radioimmunoassay
SA	salivary aldosterone
SD	standard deviation
SEM	standard error of the mean
SF	salivary cortisol
StAR	steroidogenic acute regulatory protein
TRFIA	time-resolved fluorescence immunoassay
UV	ultraviolet
ZG	zona glomerulosa

1. Introduction

The mineralocorticoid aldosterone

Adrenal cortex and production, mechanism of action

The steroid hormone aldosterone, synthesized in the outermost zone, of the adrenal cortex, the zona glomerulosa, has an essential role in electrolyte and fluid balance. However, this hormone is also known to stimulate cellular hypertrophy, matrix formation, and cell death (White, 2003; Rocha et al., 2000). Aldosterone is the final endocrine signal in the renin angiotensin aldosterone system (RAAS) (Booth et al., 2002). As for all steroid hormones, the substrate for aldosterone synthesis is cholesterol. The early pathway of aldosterone biosynthesis involves the initial enzymatic step converting cholesterol to pregnenolone. The mineralocorticoid biosynthetic pathway itself begins at the level of progesterone which is 21 hydroxylated by CYP21A2 to 11-deoxycorticosterone (Figure 1). The next two steps involve CYP11B2 for the conversion of 11-deoxycorticosterone to corticosterone and finally the "late pathway" of aldosterone biosynthesis of corticosterone to aldosterone (Williams, 2005).

Aldosterone is designated a mineralocorticoid because its actions involve reabsorption of Na^+ and release of K^+ by epithelial cells of the kidney, particularly in the distal convoluted tubule and cortical collecting duct, as well as in the intestine, colon and salivary glands. (Weber, 2001). Aldosterone's effect on sodium transport occurs in three phases, starting with the first, latent phase, where there is no increase in the transepithelial transport and lasts for only a few minutes up to one hour. This non-genomic effect, occurs in, for example, smooth muscle. The second occurs between 1 and 6 hours, and there is a rapid 4 to 6-fold increase in the sodium current. In the final late phase, after 3 hours, there is an additional 2 to 4-fold increase in the sodium current, but this requires gene transcription and the formation of new sodium channel mRNA (Booth et al., 2002; LeMoëllic et al., 2004). The classical, and longer term epithelial effects of aldosterone

on the mineralocorticoid receptor (MR) to retain Na^+ and excrete K^+ are genomic, whilst its
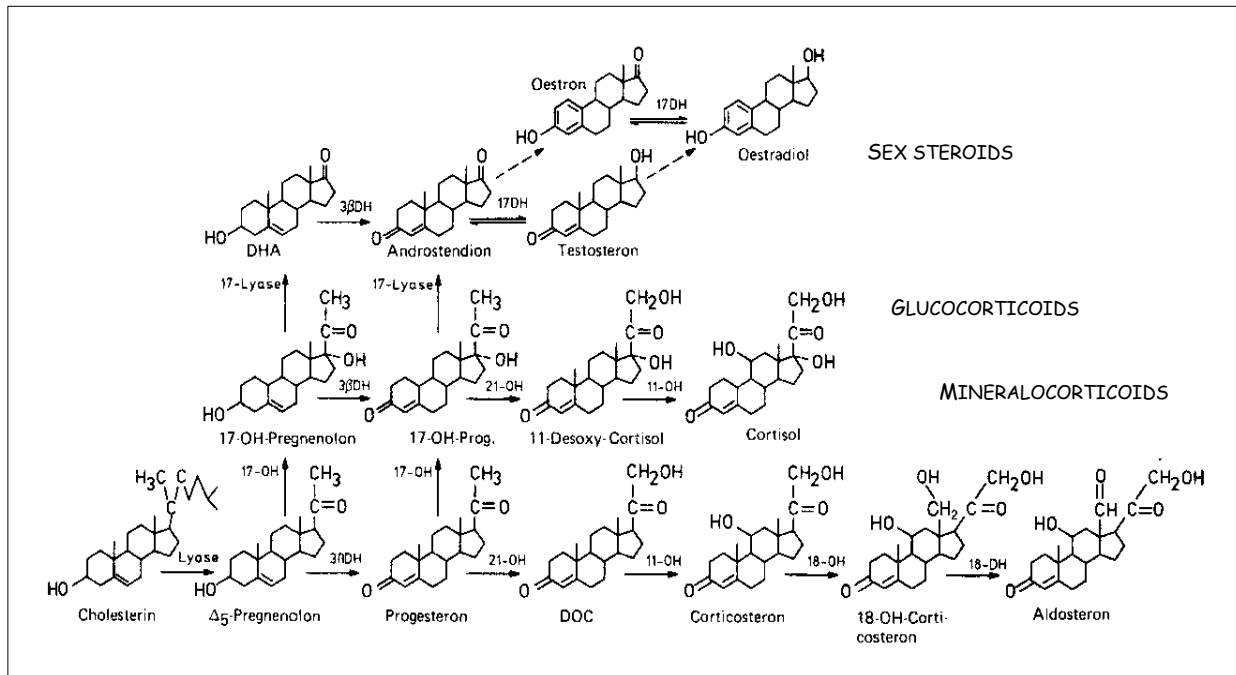
acute

Figure 1. Schematic diagram of steroid biosynthesis (reproduced from Dörner, 1999).

effects on the vasculature are clearly non-genomic. Some effects of the receptor activation, such as a central effect on blood pressure or cardiac fibrosis, have such complexity where the distinction between time courses, genomic or not, are not possible (Funder, 2006).

Rapid, non-genomic effects of aldosterone are believed to act on the Na^+/H^+ exchange via another (type 2) receptor and a 2nd messenger system. Several second messengers may be involved such as cAMP, inositol trisphosphate, and diacylglycerol. In human mononuclear leucocytes for example, aldosterone increases intracellular levels of Na^+, K^+, and Ca^{2+}, leading to volume increases in these and endothelial cells (Lösel et al., 2004). Changes in posture and blood pressure are rapid stimuli for aldosterone secretion which bring about direct vasoconstrictor effects. These non-epithelial sites of MR activation, such as vascular walls, are protected from activation by cortisol by expression of the enzyme 11-β-hydroxysteroid

dehydrogenase type 2 (11β-HSD2), which metabolizes glucocorticoids to products with reduced affinity for the MR, thus providing tissue specificity (Booth et al., 2002). This is important for aldosterone's acute, external rather than renal, blood pressure regulation effects. Evidence for this comes from the analysis of the effects of the selective MR antagonist, eplerenone, whereby there was no correlation found between its hypotensive effect of MR blockade and levels of plasma K^+ concentration, indicating its epithelial effects (Funder, 2006).

On a long-term basis, aldosterone acts by penetrating the cell membrane and binding to the specific type 1 cytoplasmic receptor. The hormone receptor complex moves to the cell nucleus, acts on nuclear DNA at the stage of transcription and initiates mRNA synthesis. Cytoplasmic proteins are then synthesized which may act by increasing luminal membrane permeability to sodium and potassium ions or by stimulating the Na^+/K^+-ATPase exchange pump in the serosal membrane. An active exchange of sodium ions in the tubular lumen for potassium or hydrogen ions in the peritubular fluid, is believed to occur at a ratio of two sodium ions for one potassium. Hydrogen ion excretion can also be influenced. In the excess presence of mineralocorticoids metabolic alkalosis may develop due to extreme loss of hydrogen ions as a consequence of increased re-absorption of sodium (Laycock & Wise, 1996). As a consequence of the exchange of K^+ or H^+ for Na^+, plasma sodium concentration rises, osmoreceptors in the anterior hypothalamus are stimulated, vasopressin is released from nerve terminals in the neurohypophysis, and water reabsorption from collecting ducts is stimulated. Water follows the movement of Na^+ via osmosis, so that chronic blood volume, and thus blood pressure, are established. When the plasma solute concentration is restored to normal and the extracellular fluid (ECF) volume has increased, then the osmotic stimulus for vasopressin ceases (Booth et al., 2002).

Even in the case of primary aldosteronism, ECF volume will not increase more than 15%, avoiding the occurrence of an oedema due to the existence of an 'escape mechanism' (Laycock

& Wise, 1996). In heart failure however, mineralocorticoid escape does not occur and even relatively low levels of aldosterone can cause sodium retention and volume expansion (Williams, 2005). The major physiological importance of the aldosterone system is to prevent loss of salt and water during periods of dietary sodium deprivation. (Weber, 2001), although just as important is aldosterone's role in maintaining potassium ion plasma concentration.

1.1.2. Control of release of aldosterone

Various factors control the release of aldosterone such as plasma sodium and potassium concentrations and aldosterone works constantly to keep these stable. A 10% decrease in sodium or a 10% increase in potassium stimulate the release of aldosterone by direct effects on the adrenal cortex. Potassium is a major physiological stimulus to aldosterone production and conversely aldosterone's ability to increase K^+ in the urine, feces, sweat and saliva secretion means it is integral to K^+ homeostasis. Aldosterone thereby serves to prevent hyperkaleamia during periods of high potassium intake. (Weber, 2001). Aldosterone excess, on the other hand, will lead to potassium loss and hypokalemia. Sodium adaptation is more complex, and it involves plasma volume changes as well as the Renin Angiotensin System (RAS), as discussed above. Although Angiotensin II (Ang II) is thought to be the main and final stimulus for the production of aldosterone in response to Na^+ deficiency, changes in the body's sodium content may also be affected by other mediators such as inhibitory stimuli from atrial natriuretic peptide (ANP) or a high extracellular sodium concentration (Müller 1995). Furthermore, the sensitivity of the zona glomerulosa (ZG) to different stimuli changes during different amounts of sodium in the body. The regulatory loops are equally as important in maintaining aldosterone secretion high when nutrition is sodium deficient and high in potassium or vice versa. Potassium and sodium concentrations and the RAS are all regulated by direct negative feedback loops in

contrast to other factors influencing aldosterone secretion such as ANP or serotonin which are not.
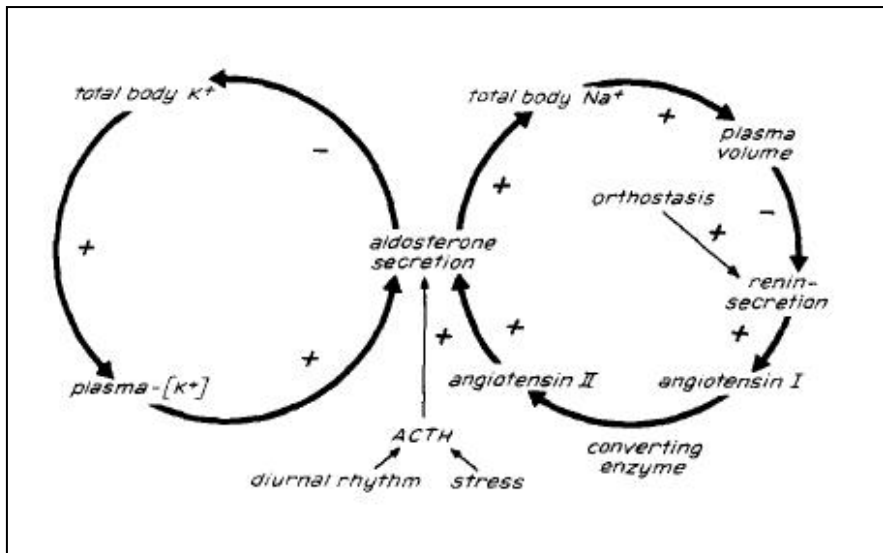
Figure 2. Regulation of aldosterone secretion in the mammalian organism (Müller, 1995).

Adrenocorticotropic hormone (ACTH) from the adenohypophysis, or anterior pituitary, is another such secretagogue that has both steroidogenic and tropic effects on the adrenal gland. The short-term action of ACTH involves the conversion of cholesterol to pregnenolone and therefore has a positive effect on aldosterone production as a short-term stimulator. This response is usually to stress, leading to production by zona glomerulosa cells in vitro as well as by the adrenal gland in vivo. The aldosterone stimulating effect of ACTH is thought to be transient. Prolonged or repeated administration of pharmacological doses in humans and animals has shown that plasma aldosterone returns to basal within a few days, in contrast to cortisol which remains elevated (Müller 1995, Gallo-Payet et al., 1996). On a long-term basis, ACTH has chronic effects that stimulate the biosynthesis of enzymes involved in steroidogenesis by increasing mRNA levels and by making more cells capable of conducting steroidogenesis (Vinson, 2003).

The rapid translocation of cholesterol to the inner membrane of the mitochondria is a rate-limiting step in the acute regulation of aldosterone production. ACTH works in conjunction with a factor called steroidogenic acute regulatory protein (StAR) to enhance this step. This is important since steroidogenic cells store minimal amounts of hormones, so that hormone levels are regulated primarily at the level of synthesis. The enzymes involved in the later steps of steroidogenesis are zone specific, so that the cells of the zona glomerulosa express the enzyme CYP11B2, producing aldosterone. The long-term regulation of aldosterone by K^+ or Ang II also involve stimulation of expression of this enzyme (Peters et al., 2006).

ANP is a hormone involved in sodium ion regulation which has an inhibitory effect on renin, and ultimately on aldosterone release, on catecholamines and on vasopressin. The main stimulus for its release being the stretching of myocytes, the site of its production, by increased atrial volume. ANP causes increased excretion of salt and water by the kidneys and a decrease in arterial blood pressure. Arginine vasopressin (AVP), a hormone originating from the posterior pituitary, has been thought to suppress renin secretion and thereby aldosterone secretion (Laycock & Wise, 1996). However there is increasing evidence that locally produced neurotransmitters and neuropeptides including AVP can participate in the regulation of steroidogenesis, and animal studies have shown that AVP produced within the adrenal gland can stimulate corticosteroid secretion via a paracrine mechanism. More recently, the presence of AVP secreting cells has been shown in the human adrenal gland and a direct stimulatory effect of AVP has been shown on the production of aldosterone in man. AVP-induced stimulation of human adrenocortical cells can be accounted for by activation of vascular V_{1a} receptors (Perraudin et al., 2006, Guillon et al., 1995). Catecholamines have been shown to stimulate mineralocorticoid production through activation of adrenergic β-receptors that are, like the ACTH receptor, positively coupled to adenylyl-cyclase (Perraudin et al., 2006).

1.1.3. Diurnal variation

The secretory pattern of aldosterone is influenced by ACTH and by PRA and there is evidence to show it has a circadian rhythm but with marked variations, making it difficult to define a normal range (James et al., 1976; Few et al., 1984; Few et al., 1986, Takeda et al., 1984). Peak secretion times for aldosterone and cortisol are significantly correlated according to some, with aldosterone secreted mainly at 2:15 and 6:30 am, under controlled conditions of sleep, posture and diet, preceding the secretion of cortisol by about an hour and following the secretions of melatonin and PRA. The study demonstrating these times by Hurwitz (Hurwitz et al., 2004) also confirmed the general peak times of aldosterone and cortisol previously shown by others and showed that prolonged bed rest does not change the intrinsic circadian rhythm or diurnal rhythms of the components of the RAAS, an observation which has also previously been reported for cortisol (Chavarri et al., 1977). Peak secretion times for aldosterone have been reported at around 10am by Takeda (Takeda et al., 1984) and between 8am and 11am by Few et al (Few et al., 1987). In a study by Katz (Katz et al., 1972) with frequent sampling it was observed that aldosterone, PRA and cortisol secretions are synchronous, they occur during late sleep and soon after rising and may depend on ACTH when the postural stimulus to renin is absent. A few years later the same authors reported that an external factor may control both ACTH and aldosterone, suggesting that renin is not the strongest determinant of aldosterone secretion (Katz et al., 1975).

Renin is inhibited when salt and water are taken in and activated when they are not. Therefore there can be periodicity in the activation of this system throughout the day depending on food intake, or over the course of many days, when periods of starvation are exchanged with the consumption of food and water. There is conflicting evidence on whether renin is driven by a sleep-wake cycle or occurs as an endogenous, circadian process independent of posture and diet (Hurwitz et al., 2004).

1.1.4. The classical and local tissue Renin-Angiotensin-Aldosterone System

The most important control mechanism for the release of aldosterone is the RAAS. Classically, the RAAS begins with the synthesis of renin, a glycoprotein enzyme of 44kDa, which is released by exocytosis from juxtaglomerular (JG) cells lining the afferent renal arterioles and nearby macula densa cells of the distal tubule (Carey & Siragy, 2003). Stimuli for the release of renin include decreased renal perfusion pressure, with the decrease in arterial blood pressure of the afferent arterioles being directly detected by JG cells or by adjacent renal vascular baroreceptors. Other stimuli such as haemorrhaging, salt and water loss, or abnormally long pooling of the blood in the legs when an upright posture is taken, in the case of postural hypotension, also stimulate release due to falls in blood pressure (Weber, 2001). Another suggested mechanism is via the direct effects of sympathetic stimulation to the kidneys. In this case JG cells may be innervated by adrenergic nerve fibers or by circulating catecholamines which may stimulate release by acting on α-receptors present on the cells (Laycock & Wise, 1996).

Following stimulation of the JG cells renin is released and it cleaves circulating Angiotensinogen (AGT) produced by the liver, forming the decapeptide precursor, Angiotensin I, which is biologically inert. AGT is also synthesized and released in other tissues including the heart, vasculature, kidneys and adipose tissue. Angiotensin-converting enzyme (ACE), bound to the plasma membrane of endothelial cells, cleaves 2 amino acids from Angiotensin I to form biologically active Angiotensin II (Ang II). Ang II is itself important in maintaining circulatory homeostasis. When it is present in large amounts it is a potent vasoconstrictor, constricting arterioles within the renal and systemic circulation. A further role of ACE is to metabolize bradykinin (BK), an active vasodilator and natriuretic substance, into the inactive metabolite BK(1-7). ACE therefore, not only increases the production of a vasoconstrictor, but at the same time also degrades a vasodilator (Carey & Siragy, 2003).

The role of Ang II in the RAAS is to stimulate the ZG to produce aldosterone thereby promoting the reabsorption of Na^+ in proximal segments of the nephron. Ang II is the principal stimulator of aldosterone production when intravascular volume is reduced (Weber, 2001). Blood pressure and Ang II are inversely related so that when blood pressure is decreased, Ang II production increases (Booth et al., 2002). Most of the cardiovascular, renal, and adrenal actions of Ang II are mediated by the type 1 receptor (AT_1) which is positively coupled to protein kinase C and negatively coupled to adenylyl cyclase. These receptors are responsible for mediating vascular smooth muscle contraction, aldosterone secretion, dipsogenic responses, renal sodium reabsorption, and tachycardic responses (Carey & Siragy, 2003). Aldosterone's response to Ang II depends on sodium balance. Aldosterone release in response to Ang II is increased within minutes from adrenal cells in vitro, and under sodium depletion the same amount of Ang II induces a greater increase in aldosterone production than under sodium load (Peters et al., 2007). Angiotensin and aldosterone are further involved in endocrine functions to do with circulatory homeostasis, namely the coagulation of blood, through increased production of plasminogen-activator inhibitor type1 and the activation of platelets, as well as the stimulation of thirst (Weber, 2001). Regarding thirst stimulation, a brain renin-angiotensin-system with all the relevant components has been identified (Parsons & Coffman, 2007). Other sites of action include the regulation of inflammatory processes, by stimulating cytokines, inflammatory-cell adhesion and activation of macrophages, and reparative processes by inducing growth of fibroblasts and synthesis of fibrillar collagens to aid the formation of scar tissue, after tissue injury (Weber, 2001).

In recent years studies have started to focus on the existence of the RAS locally within tissues and demonstrated its importance within the brain, heart, peripheral blood vessels, adrenal glands, and the kidney (Muller 1997; Engeli et al., 2000; Dostal, 2000; Takeda et al., 2004; Gomez-Sanchez et al., 2004; MacKenzie et al., 2000).

Local Renal Aldosterone system Investigators in one study demonstrated the local expression of aldosterone synthase, CYP11B2, gene as well as protein, and aldosterone production within the kidneys of normal rats (Xue & Siragy, 2005). The authors confirmed their hypothesis of the occurrence of these components of the RAAS by showing the presence of aldosterone in kidneys of adrenalectomised animals, and showed an increased renal aldosterone synthase expression after a low-sodium diet as well as after Ang II administration. They additionally confirmed that the AT_1 receptor regulates the production of this enzyme locally in the kidney by carrying out receptor blockade which reversed the Ang II up-regulation of the CYP11B2 enzyme. The study established that aldosterone production and its receptors exist in close proximity, pointing to a local renal autocrine or paracrine aldosterone system influencing renal function.

Local Adrenal RAS Adrenal renin and angiotensinogen mRNA and their formation within the adrenal gland ZG has proven the existence of a local adrenal RAS. Adrenal renin concentrations increase by sodium restriction and high-potassium diets, and nephrectomy increases adrenal renin via increased serum potassium. In zona glomerulosa cells where there has been inhibition of ACE or the AT_1 receptor, aldosterone stimulation by ACTH or potassium is also hindered (Gupta et al., 1995). However, it is not certain whether the adrenal RAS works as an autocrine/paracrine system to upregulate adrenal responses to Ang II originating from the systemic circulation, or if it has a role in pathophyiological disease states.

Cardiac RAS Evidence now exists that all the components of the RAS necessary for the production of Ang II exist, and that Ang II is in fact produced, within the heart. All of these components are distributed in myocardial fibroblasts and cardiomyocytes, the endothelium and vascular smooth muscle of coronary arteries and veins. Furthermore, renin, angiotensinogen, ACE and Ang II receptors are all present in the myocardium (Dostal & Baker, 1999). However, though myocardial concentrations of renin and angiotensinogen are at 1-4% of those in plasma, the levels of Ang I and II are over 100-fold those of plasma and the conversion is said to occur

via a heart chymase instead of ACE, which does not degrade BK, converting 90% of Angiotensin I (Ang I) to Ang II. Controversy still exists as to whether local Ang II production in the heart acts as a paracrine or autocrine regulator of cardiac function. Evidence for the possibility of the existence of a vascular RAS has shown that renin is probably not synthesized locally in blood vessel walls, because renin was not detectable by RT-PCR but rather that it is taken up by the endothelium. It is recognized, on the other hand, that Ang II is generated by endothelial and endocardial cells so that the endothelium mediates vascular Ang II formation by the cellular uptake of renin (Carey & Siragy 2003).

1.1.5. Aldosterone and endocrine hypertension

Aldosterone promotes hypertension, stroke, cardiac fibrosis, ventricular hypertrophy, and myocardial necrosis (Struthers & MacDonald, 2004), which is why understanding the regulation of its production is an essential goal in understanding the pathophysiology of cardiovascular diseases. Recent evidence has revived interest in aldosterone and its role in congestive heart failure (Adler & Williams, 2007). The importance of aldosterone in blood pressure homeostasis is particularly important since every known form of hereditary, or Mendelian, hypertension in humans results from anomalous aldosterone signaling or the increased activity of its final effectors, the luminal epithelial sodium (ENaC) and K^+ channel and the serosal Na^+/K^+-ATPase (Booth et al., 2002).

Hyperaldosteronism describes a state of sustained high secretion of aldosterone from one or both adrenal cortices and can be subdivided into two categories. In Primary aldosteronism (PA), the hyperfunction is due to autonomous adrenal secretion of excessive aldosterone, while in the secondary form the hypersecretion is due to increased levels of Ang II due to high plasma renin activity which may be occurring due to a variety of causes. The clinical picture is different in each case. In PA the clinical symptoms are those due to the pathophysiological actions of

aldosterone itself while in the secondary various causes of increased renin determine the clinical picture (Laycock & Wyse, 1996). Individuals with increased aldosterone are more prone to developing premature vascular diseases and therefore early diagnosis and treatment are essential. PA is the most common form of mineralocorticoid hypertension. It is increasingly common in 'essential' hypertension, present in about 10% of all hypertensives. Its diagnosis in hypertensive individuals is targeted by therapy such as by the removal of an adenoma or treatment with mineralocorticoid receptor antagonists (Schirpenbach & Reincke, 2007; Mulatero et al., 2005). Though hypokalemia was thought to be a mandatory finding in PA, studies later confirmed that most patients with PA are in fact normokalemic (Mattsson & Young, 2006; Schirpenbach & Reincke, 2006).

The two main causes of PA are due to aldosterone producing adenomas (APAs) in the classical Conn's syndrome, and bilateral adrenal hyperplasia (BAH), also known as bilateral idiopathic hyperaldosteronism (IHA) (Mattsson & Young, 2006). Other causes have also been identified, such as glucocorticoid remediable aldosteronism, under the control of ACTH, and adrenal carcinomas. APAs are unilateral tumours of the adrenal cortex which result in autonomous secretion of aldosterone, thought to be responsive to ACTH as opposed to Ang II although recently it has been suggested that up to 50% also respond to Ang II (Stowasser et al., 2001). In the case of BAH, both adrenals secrete high amounts of aldosterone in response to Ang II. Therefore the disorder may be considered an exaggeration of normality where excessive amounts of aldosterone are secreted in response to dominating levels of Ang II (Mulatero et al., 2005).

In PA, the mechanisms involved in the maintenance of autonomous aldosterone secretion in the absence of circulating Ang II remain to be elucidated. AVP may also play a role in the pathogenesis of aldosterone hypersecretion. The presence of AVP-secreting cells in APA tissue suggests that AVP released by intratumoral cells may exert an autocrine / paracrine stimulation

on the secretion of aldosterone. Indeed, data indicate that SR 49059, an AVP selective non-peptidic receptor antagonist, modifies tumor cell sensitivity to different stimuli including posture-sensitive hormones and ACTH (Perraudin et al., 2006).

A wide range of percentages has been reported for cases of PA among hypertensives from different centers, mostly due to the definition of the selection criteria for screening and confirmation. The most commonly used tests for screening the presence of PA is the upright serum aldosterone to upright plasma renin activity or plasma renin concentration ratio (ARR). According to a review by Montori and Young (Montori & Young, 2002) the ARR is the primary test of choice for predicting primary aldosteronism. This test is carried in the morning in order to avoid influences by diurnal rhythms of aldosterone and is dependable because it can diagnose aldosteronism even if aldosterone or renin are within normal values of the physiological range. Furthermore, this test can be carried out even when on anti-hypertensive medication. Due to the fact that low renin hypertension (LRH) can also produce an elevated ARR, there also needs to be a normal to high or pathologically high aldosterone value in order to confirm diagnosis of PA. Several factors are involved, however, in assigning an appropriate cut-off value for the ARR, such as the method used for the respective aldosterone and renin measurements. Giacchetti et al., for example, suggest a value of 40 giving best sensitivity and specificity, using the solid phase radioimmunoassay (RIA) kit (Biodata Diagnostics, Rome, Italy) for serum aldosterone and PRA was measured as the generation of Ang I *in vitro* also using a commercially available RIA kit (Radim, Rome, Italy). Furthermore, the role played by the nature of the cohort, in addition to false negatives due to ACE inhibitors, Angiotensin II and aldosterone receptor antagonists, and false positives due to β-blockers and excess dietary Na^+ intake all need to be taken into account (Schirpenbach & Reincke, 2006; Weinberger & Fineberg, 1993; Giacchetti et al., 2006).

Young in 2003 suggested that patients with hypertension and hypokalemia, and most with treatment-resistant hypertension should undergo screening for PA with the ARR and that a

high ratio is a positive screening test which warrants further confirmatory testing (Young, 2003). Dynamic tests typically used for confirmation of diagnosis of PA include the angiotensin-converting enzyme inhibitor captopril or the angiotensin receptor blocker losartan, in order to test the sensitivity of the adrenal gland to physiological and pharmacological stimuli. The high salt diet or saline infusion test, the deoxycorticosterone acetate suppression test, and the longer 4-day fludrocortisone suppression test are also used (Giacchetti et al., 2006; Valloton, 1996). The posture test, which has been widely used to discriminate between APA and BAH, has been found to be less reliable than previously thought since Ang II responsive adenomas have been found to respond to posture in a way that resembles BAH (Espiner et al., 2003).

In order to distinguish the subtype of PA tests such as the upright posture test, imaging of the adrenal glands, and adrenal venous sampling are used. In the upright posture test a serum aldosterone increase of 30% in upright posture, compared to supine, points to diagnosis of IHA while absence of a significant increase in response to posture points to the presence of APA (Giacchetti et al., 2006). Cortisol is also measured so that an increase after posture against the diurnal rhythm of cortisol renders the test invalid (Schirpenbach & Reincke, 2006). Adrenal vein sampling is used in the decision-making process leading to surgical intervention, but there are conflicting reports also arising from this method. Some studies exist showing its importance in identifying contralateral adrenal suppression to localize the abnormal gland while others report no discriminating ability (Doppman et al., 1992; Rossi et al., 2001; Espiner et al., 2003). Diagnosis of an adenoma can be made upon determination of aldosterone excess from one of the adrenal glands while there is suppression of the contralateral gland.

Patients with APAs have more severe hypertension, more frequently present hypokalemia, have higher plasma aldosterone (>25ng/dl), higher urinary aldosterone (>39µg/24hours), and in general are younger (<50 years) than those with idiopathic hyperaldosteronism (IHA) (Young, 2003). The study by Giacchetti (Giacchetti et al., 2006) was

in agreement with these findings, plasma K$^+$ levels were lower and urinary aldosterone higher in APA compared to IHA, while ARR's did not differ between the two subtypes. After a 4-hour saline infusion suppression test, serum aldosterone was higher in PA patients taking part in that study than in the essential hypertensives (EHs), but again there was no discrimination between APA and IHA. Accordingly, plasma K$^+$ was found lower in PA than in EHs, while urinary aldosterone and the ARR were higher. The authors further showed the importance of evaluating the serum aldosterone to cortisol ratio before and after a 4-hour saline infusion test to exclude the possibility that the aldosterone levels are being affected by ACTH stimulation due to circadian or stress-related factors. APA patients have a higher aldosterone to cortisol ratio at the end of the test due to the independently high production of aldosterone which remains unaffected by the stimuli coming from the RAS or by ACTH stimulation.

Saliva and monitoring of hormones

Saliva is a biological fluid containing many hormones, drugs and antibodies of interest, which is readily available and can be collected non-invasively. An extensive amount of research has been carried out showing the possibility of utilising saliva as a diagnostic tool. There are many distinct advantages of using saliva over serum as the diagnostic medium, mainly that whole saliva is easily available for non-invasive collection and analysis by individuals with limited training without the need for special equipment and, with certain devices, may be stored at ambient temperatures for extended periods. The nature of salivary collection means that multiple specimens can be collected from an individual, providing the further advantage of monitoring over longer periods of time, an important factor especially in the study of steroid hormones which have diurnal and monthly variations. Saliva may be used to monitor the presence of hormones, drugs, antibodies, micro organisms and ions. It has been analysed for use in monitoring of drug pharmacokinetic and metabolic studies, drug abuse testing, assessment in

endocrine studies such as testosterone in males (Tschop et al., 1998) and progesterone in females, and virus diagnosis of antibodies against for example measles, rubella, and mumps viruses (Hofman, 2001; Kaufman & Lamster, 2002).

Anatomy and physiology of the salivary gland

There are three pairs of salivary glands, the parotid, the submandibular and sublingual. Saliva secreted from the largest gland, the parotid, is serous or watery, whereas the submandibular produces a mixture of mucous and serous fluids and saliva from the smallest, the sublingual gland, is predominantly mucous due to the glycoprotein content. The salivary gland is composed of secretory end pieces, acini, and a branched ductal system (Figure 3). The secretory end pieces are supported and enveloped by a group of contractile myoepithelial cells which help to propel saliva into the ductal system (Edgar et al., 2004). A salivary gland itself can consist of a variation of end pieces comprised of mucous or serous cells and the lumen which is the start of the ductal system. There are three types of ducts through which the fluid passes, the intercalated, striated and excretory ducts (Höld et al., 1995).

Modified interstitial fluid originating from blood in the capillaries surrounding the acinar
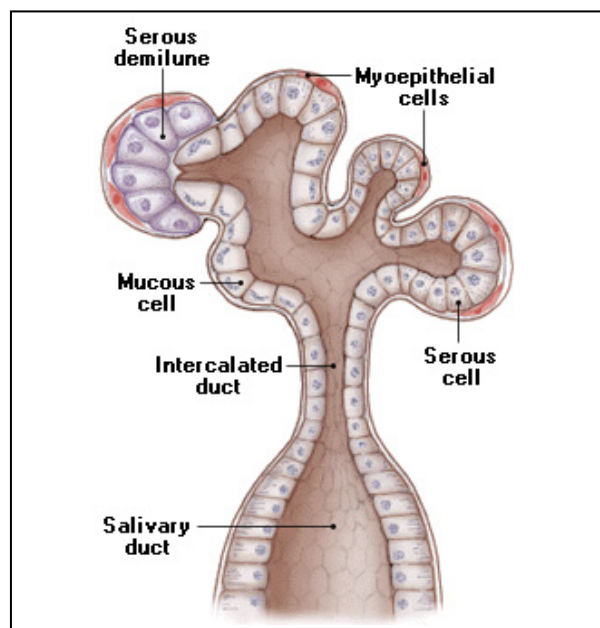
Figure 3. Anatomy of the salivary gland.

cells is secreted into the lumen and is then further modified within the ducts. Ion exchange takes place in the striated ducts changing the solution from an isotonic to a hypotonic one, and excretory ducts further modify the fluid before it enters the mouth. Before a substance can enter the salivary duct, however, it must pass the capillary wall, the basement membrane and the membrane of the glandular epithelial cells, the rate-limiting step occurring at the latter lipophilic layer of the epithelial cell membrane. For this reason, saliva is not simply an ultrafiltrate of plasma, but a complex fluid formed by passive diffusion, ultrafiltration through pores in the membrane, or by pinocytosis (Caddy, 1984). Furthermore, adjacent acinar cells within the end piece are coupled by gap junctions and these allow the transcellular exchange of ions and small molecules. Small molecules of a molecular weight up to 200Da can be transported via ultrafiltration (Madara, 1988).

1.2.2. Mechanisms of Innervation and Secretion

Salivary gland secretion is controlled mainly by parasympathetic nerve supply, originating from salivary nuclei at the juncture between the pons and the medulla, but also by the sympathetic nervous system, although to a lesser extent. Parasympathetic innervation is activated by taste and mechanical stimuli from the tongue and other areas of the mouth via afferent sensory nerve fibres, the facial (VII) and glossopharyngeal (IX), to the solitary nucleus. To innervate the glands themselves, efferent pathways arising from the facial nerve, and via the submandibular ganglion, release acetylcholine (ACh) at the acinar cells of the sublingual and submandibular gland. Similarly, efferent parasympathetic pathways of the glossopharyngeal nerve via the otic ganglion release ACh and innervate the parotid gland. Sympathetic stimulation affects more the salivary composition itself by increasing exocytosis from certain cells, which is also influenced by many androgen, oestrogen, glucocorticoid and other peptide

hormones. Sympathetic innervation regulates secretion of macromolecules via the release of the neurotransmitter noradrenalin (NA) (Turner & Sugiya, 2002).

1.2.3. Factors influencing composition and flow rate

Saliva leaving the end piece lumen is isotonic with plasma. As the fluid moves down the striated duct, sodium and chloride are reabsorbed, and potassium, bicarbonate and lithium are secreted into saliva. However, because the ductal membranes are relatively impermeable to water, the saliva becomes hypotonic as it moves down the duct (Ganong, 2003; Vining et al., 1983). The striated ducts transport electrolytes from the primary saliva by active transport so that when salivary flow rate is slow it is able to modify the composition of saliva greatly and in stimulated, high, flow rates the saliva resembles more the primary saliva coming from acinar cells (Edgar et al., 2004).

The contribution to whole saliva from each gland varies according to the level of stimulation. Un-stimulated saliva, in the absence of exogenous stimuli from taste or chewing, consists of 25% parotid, 60% from the submandibular, 8% from the sublingual, and 8% from the minor mucous glands and also contains gingival crevicular fluid, epithelial cells, bacteria, leukocytes, blood and viruses. In stimulated saliva the parotid contribution rises to 50%, the submandibular to 35%, and the sublingual and minor mucous contributions remain at 8% (Edgar et al., 2004).

Normal flow rates are between 0.3 and 0.4ml/min in un-stimulated and between 1.5 to 2.0ml/min in stimulated states, with a maximum flow rate of 7ml/min after masticatory or gustatory stimuli. A total of 0.6 to 1.5 litres of saliva are secreted per day. Because of the circadian rhythm of flow rate, measurements which are repeated should be taken at the same time of day. Flow rate is greatly influenced by the degree of hydration, so that when body water

content is reduced by 8% the salivary flow decreases to almost zero, and by body posture so that in a standing person flow rate is higher than when lying down or when seated (Ganong, 2003).

The pH of saliva depends on the bicarbonate concentration, and with increasing flow rates pH, bicarbonate (HCO_3.), chloride and sodium ions, and proteins increase (Dawes & Kubieniec, 2004). The ionic composition of saliva, when flow rate is constant, depends on duration of stimulation and circadian rhythms. Circadian changes in the mineralocorticoid aldosterone plasma concentration probably account for the circadian rhythms in salivary Na^+, K^+, and Cl^- ion concentrations. The acrophase in the secretory rhythm of these ions fits in with the morning to noon-time acrophase of plasma aldosterone, thought to be the steroid with the greatest influence on electrolyte composition. Furthermore, authors have shown that salt restriction as well as injection of aldosterone can cause reductions in salivary sodium concentrations (Dawes, 1972; Pawan, 1955).

Parameter	Mixed Saliva	Plasma
Volume	500-1500ml/day	4.3% body weight
Rate of Flow	0.6 (0.1-1.8) ml/min	
pH	6.7 (5.6-7.9)	7.4
% Water	98 (97-99.5)	91.5 (90-93)
Total protein (g/dl)	0.3 (0.15-0.64)	7.3 (6-8)
Albumin (g/dl)		4.5 (4-5)
Mucin (g/dl)	0.27 (0.08- 0.6)	
Amino acids (mg/dl)	0.1-40	0.98
Potassium (mmol/l)	8-40	3.5-5.5
Sodium (mmol/l)	5-100	135-155
Calcium (mmol/l)	1.5-2	4.5-5.2
Phosphate (mmol/l)	5.5-14	1.2-2.2
Chloride (mmol/l)	5-70	100-106
Cholesterol (mg/dl)	7.5 (3-15)	150-300

Table 1. Correlation of saliva to plasma (Ritschel and Thompson, 1983)

The concentration of protein in saliva is low, at about $1/13^{th}$ that of plasma, approximately 2mg/ml. However many of these proteins found in saliva have important antimicrobial, lubricative and digestive functions (Edgar et al., 2004). On the other hand, other proteins, such as steroid binding proteins, are unable to pass from blood to saliva and this has drawn attention to the measurement of steroid hormones in saliva by those interested in measuring the non-protein bound fraction of that found in plasma (Höld et al., 1995). Many proteins which are of clinical interest are present in saliva in trace amounts, such as choriogonadotropin, pituitary hormones, or albumin. However the plasma to saliva ratio is around 10000:1 and the origin is probably due to contamination from plasma or gingival fluid. Some steroids conjugated to proteins, such as DHEAS and conjugated estrogens, are lipid insoluble but small amounts do enter the saliva through the tight junctions making these concentrations flow rate dependent and so small that they are easily affected by plasma or gingival fluid contamination. These concentrations therefore provide no clinically useful indication of plasma concentrations. In contrast, the lipid-soluble, un-conjugated steroids, such as cortisol, testosterone, and progesterone, enter saliva by passing through the cell membranes, are independent of flow rate, and concentrations give a close approximation of the plasma concentrations, giving a clinically useful index of unbound plasma levels (Vining et al., 1983).

1.2.4. Diagnostic application of saliva in measurement of steroid levels

Endocrine function is frequently evaluated and analyzed by looking at hormone levels in saliva. The availability of this biological fluid and the non-invasive nature of its collection compared to blood sampling has made salivary steroid assays an appealing alternative. Most steroid hormones are lipid-soluble and cross acinar cell membranes entering the saliva via passive diffusion, unlike other polar, small molecules (molecular weight up to 100-200) which enter via ultrafiltration through the tight junctions between cells (Quissell, 1993). Hormones are

not believed to enter the saliva via active transport. When a constant correlation exists between salivary and serum hormone levels, neutral steroids which can diffuse easily into saliva represent the free, non-protein-bound serum hormone level. Conversely, protein hormones do not enter the saliva by passive diffusion due to their large size and would only be found in this fluid by contamination from serum due to oral wounds or from gingival crevicular fluid (Kaufman & Lamster, 2002).

Cortisol is a typical example of a steroid hormone commonly used for diagnosis and the identification of patients with Cushing's syndrome and Addison's disease (Hubl et al., 1984), monitoring the hormone response to physical exercise (Lac et al., 1997) and the effects of stress (Obminski et al., 1997). The levels of this hormone in saliva are highly correlated to plasma and not affected by changes in serum binding protein concentrations. Importantly, because cortisol is the most prominent stress hormone and repetitive salivary sampling was not shown to induce its release (Kirschbaum & Hellhammer, 1989), it is fair to assume that this sampling method does not affect the levels of other steroid hormones either. Cortisone is a hormone without diagnostically significant salivary levels which are actually higher than those found in serum, due to the enzyme 11β-HSD which is present in saliva and converts cortisol to cortisone (Vining & McGinley, 1986). Salivary testosterone and dehydroepiandrosterone have also been measured at 1.5-7.5% of serum concentrations (Gaskell et al., 1980). Similarly, estimation of salivary testosterone levels has been suggested for the assessment of testicular function (Walker et al., 1980), behavioural studies of aggression, depression, and anti-social behaviour (Granger et al., 1999), with a high correlation between salivary and serum-free testosterone concentrations ($r = 0.97$) reported using a direct radioimmunoassay technique (Vittek et al., 1985). A significant correlation ($r = 0.78$) has also been reported for levels of serum and saliva estradiol (Wang et al., 1986) as well as salivary progesterone ($r = 0.47–0.58$), which both appear to reflect serum levels also during the menstrual cycle. Use of salivary estriol levels was suggested for the assessment

of feto-placental function and progesterone for the prediction of ovulation and ovarian function (Lu et al., 1999). A non-isotopic immunoassay for the determination of salivary 17-hydroxyprogesterone (17-OHP) is a further example of the advantage provided by measuring this hormone in, for example, children where stress-related increases in the hormone can be avoided. Salivary 17-OHP levels are used to monitor metabolic control in children, adolescents and adults with congenital adrenal hyperplasia (Dressendorfer et al., 1998).

Concentrations of protein hormones such as gonadotropins, prolactin, and thyrotropin, on the other hand, cannot be measured by salivary analysis due to their large size not allowing them to cross by passive diffusion (Vining & McGinley, 1986).

Several studies have reported the correlation of salivary aldosterone, and its biological significance, compared to the levels found in plasma although a limited amount of work was done to show the clinical relevance. Unlike cortisol, there is little evidence for a specific protein to transport circulating aldosterone. An ultrafiltrate of plasma would contain up to 50% of circulating aldosterone, which, on an average sodium intake would secrete 0.1 to 0.7mmol per day. 75% of this aldosterone is inactivated by a single passage through the liver (Williams, 2005). In a study reporting an aldosterone-binding protein similar to cortisol binding globulin (CBG), increasing cortisol concentrations were shown to displace aldosterone (Davidson et al., 1962), and other studies such as the one by McVie (McVie et al., 1979) have also shown that under circumstances of increased or decreased cortisol levels with ACTH or dexamethasone administration, free salivary aldosterone concentrations will also increase due to the presumed decrease in availability of CBG. The aforementioned study speculates, however, that the hormone is not CBG as progesterone has a higher affinity for this protein than cortisol and also that the protein is not albumin, although progesterone can displace aldosterone from its binding sites (Davidson et al., 1962).

1.2.5. Evidence for aldosterone in saliva until now

Salivary hormones used to measure the non-protein bound, serum concentrations were first shown by Katz in the late 1960's. These authors showed that the sum of cortisol and cortisone in saliva would represent the free fraction of the plasma concentration and that this could also be possible in the case of salivary aldosterone. McVie et al in 1979 tried to establish that saliva is a biological fluid which can be used to evaluate the concentration of free aldosterone by showing a correlation to urinary aldosterone excretion, plasma aldosterone and plasma renin activity. They showed this under conditions of varying dietary sodium, during ACTH stimulation and under dexamethasone suppression using a radioactive immunoassay. Previous investigators showed that protein bound aldosterone is between 30 and 90% and that changes in aldosterone binding were dependent on availability of corticosteroid binding globulin (Davidson et al., 1962, Meyer et al., 1961). During dexamethasone administration, McVie et al found that the protein bound aldosterone increased, indicating increased available CBG. During ACTH stimulation, protein bound aldosterone decreased, with low CBG. This assay, though, which was of the first describing aldosterone in saliva, required large volumes (4ml) and involved both extraction and chromatographic purification of samples prior to use. Importantly, baseline values of aldosterone were not detectable by the assay.

Another assay which required solvent extraction was described by Hubl et al., in 1983. This group developed a solid phase enzyme immunoassay and looked at aldosterone levels before and after ACTH stimulation and in patients with Conn's syndrome and Addison's disease. They found an overall level of 0.2 to 8.2ng/dl in normal subjects which increased by 463% after ACTH. Although the assay was non-radioactive, required relatively low levels of sample (200μl) and there was a good correlation with previously reported plasma values at the time, there was no continuation using this assay in further studies.

The following year, another group of investigators developed another 'direct' radioimmunoassay, with use of extraction by dichloromethane (DCM) to investigate plasma aldosterone (Few et al., 1984). These investigators conducted many physiological tests showing daily fluctuations of aldosterone levels and concluded that the marked diurnal variation of the salivary aldosterone concentration made it difficult to define a normal range. However, in normal un-stimulated subjects they did not encounter anything above 150pmol/l (54pg/ml) and suggest that this be the upper limit of healthy subjects. When plasma levels fluctuated rapidly, such as in response to exercise, ACTH stimulation, standing, or morning levels, salivary levels retained a fairly constant relationship. Lack of follow-up also exists with regards to this assay, possibly due to the sample volume of at least 1ml which was required and is not always easy to obtain from all subjects.

A similar system was reported by another group shortly thereafter (Atherden et al., 1985), also using a RIA employing extraction of samples. This group also showed aldosterone levels rising in response to sodium restriction and lowest levels overall at 12pm. The abundance of saliva to plasma concentrations was at approximately 26% both on low and normal sodium diets. Average values an hour after waking were found at 108pmol/l. Salivary aldosterone reflected changes seen in plasma in response to time of day, sodium status, ACTH stimulation, and dexamethasone suppression. An important observation taken from this study was that an hour after waking, with the participants still in the supine position, salivary aldosterone levels remained at the same level as when first waking up. This fact is relevant to screening of subjects early in the morning where it has been suggested that levels may be unrepresentatively high, possibly due to reduced salivary flow rates, as was found in the case of testosterone, and can disprove this theory. Therefore the potential of out-patient screening of hypertensive patients, which is the major goal in establishing such assays, may be recognized.

The most recent study to use salivary aldosterone levels in a diagnostic context was conducted to assess adrenocortical reserve by the simultaneous measurement of salivary cortisol and aldosterone (Cardoso et al., 2002). This study re-confirmed that the salivary aldosterone response to ACTH stimulation mirrors that seen plasma. The investigators used a modification of a commercially available solid-phase RIA by increasing the sample volume to 400μl, twice that necessary for plasma assessment, however. In healthy subjects this study reports values of 100pmol/l after ACTH stimulation, corresponding to 36pg/ml, with basal values at an average of 35pmol/l. There is a clear discrepancy in these results compared to the findings of previous groups who found basal concentrations of salivary aldosterone in healthy humans at approximately three-fold higher concentrations. Many factors are involved and should be taken into account, especially when comparing commercially available to 'in-house' validated assays, which could explain the inconsistencies which are observed such as the simple difference in calibration of standard curves.

Immunoassay

1.3.1. Theory of competitive immunoassay design using time-resolved fluorescence

Immunoassays use reagents to generate a signal from a sample. In the immunometric design, an antibody is immobilized onto a plastic surface and captures the test analyte from the sample, and a different antibody which is labeled, for example with a radioactive isotope, specific for another part of the analyte molecule, is used as a basis for signal generation. The labeled component of the immunoassay is called the tracer. The analyte that the antibody is specific for is known as the antigen. The material that the antibody is irreversibly bound to is the solid phase (Wild, 2005).

For small molecules such as steroids though, a different immunoassay design is needed. Only one antibody is used and it is present in a limited quantity. The other key reagent, the

tracer, is the target analyte and is labeled with a suitable signal generation material. In the case of the in-house immunoassay being validated here, this is biotinylated aldosterone. The proportion of tracer that binds to the limited antibody sites is indirectly proportional to the concentration of analyte in the sample. This is known as a competitive immunoassay.

In competitive assays the labeled antigen binds to the antibody sites unoccupied by the sample antigen. The addition of unlabeled sample antigen in such a system causes a reduction in the number of unoccupied binding sites and therefore, a reduction in binding of labeled antigen, making the amount of sample analyte indirectly proportional to the signal obtained. Immunoassays do not give direct measurements. They estimate analyte concentration in unknown samples by comparison of signal strength from the labeled reagent to that of similarly treated standard samples. Calibration is the process used to assign values to unknown samples using a standard.

Samples of known antigen concentrations are therefore included as standards, or calibrators, and a calibration curve drawn of percentage of activity in the bound fraction compared to total activity, against the antigen concentration in the standards. The concentration of antigen in unknown samples may then be interpolated from the calibration curve (Figure 4). If the percentage bound is measured then the antigen concentration of an unknown solution can be estimated.

Signal generation system In the assay system presented here, the biotin-streptavidin-Europium^{3+} system was used in order to assess the amount of antigen that is bound. Biotin is a small, water-soluble vitamin (MW 244Da) with extremely high affinity for the egg-white protein avidin in free solution. A bacterial source of avidin, streptavidin (MW 60kDa) is generally used, though, which does not contain any carbohydrates and has a neutral isoelectric point. Those properties make it inert in assay systems so that there is lower non-specific binding and greater sensitivity. The strength and speed of binding between avidin and biotin is used to link

molecules together to create signal generation systems. Each molecule of streptavidin has four binding sites for biotin. Biotin acts as the label used in place of a radioisotope or enzyme, binding to streptavidin and consequently labeling the bound fraction with the signal source. At the end of the assay, a conjugate of streptavidin linked to a signal-generating substance, in this case Europium^{3+}-coupled streptavidin, is added. Streptavidin is incorporated into macromolecular   complexes containing many chelation sites for europium ions, producing a several thousand-fold amplification of each biotin label. The conjugate and biotin bind together, labeling the bound fraction with the signal source.

Detection system with time-resolved fluorescence Signal detection occurs via the principle of time-resolved fluorescence. Assay detection limits using conventional fluorescent tracers are limited by background interference, such as that due to the natural fluorescence from various compounds in biochemical samples such as blood serum. This interference can be avoided by using a fluorophore, like with lanthanide chelates, which has a comparatively long decay time and there is a time gap between excitation and measurement of the emitted light, ie. 10-1000μs vs 1-20ns, giving rise to time-resolved fluorescence (Soini and Kojola, 1985). In the case of the immunoassay presented here, streptavidin is labeled with the lanthanide Europium^{3+}. After the main assay incubation and separation, the europium ions are dissociated when an enhancement reagent is added and form a new, fluorescent complex with the ligand in the enhancement solution, a fluorinated β-diketone. In the DELFIA$^{®}$ (Wallac) system, a fluorometer supplies 1000 pulses of light per second each lasting less than 1μs and fluorescence is measured between 400 and 800 μs after each pulse (Wild, 2005).
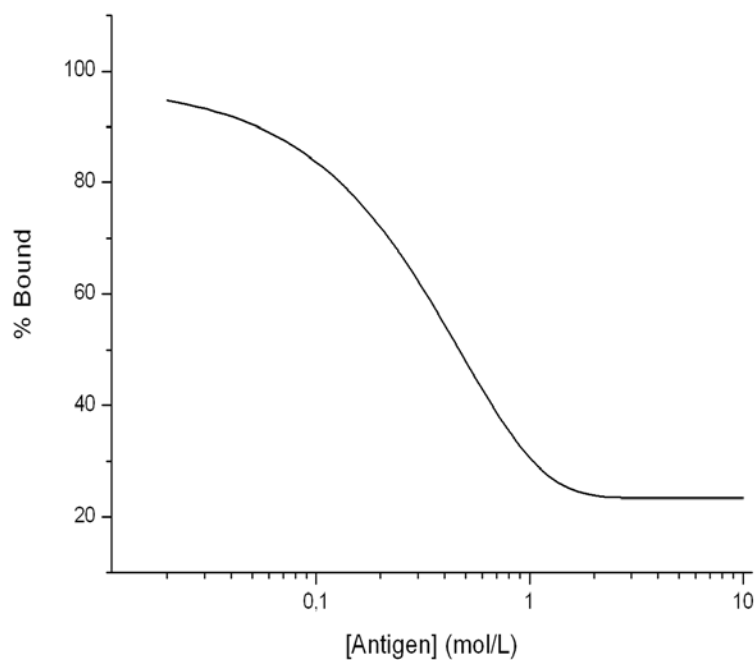
Figure 4. Competitive assay dose response curve.

1.3.2. Purification by reverse phase High Performance Liquid Chromatography (RP-HPLC)

Data published so far on the measurement of aldosterone in salivary samples has, for the most part, been achieved by using ^{3}H- or ^{125}I-labelled aldosterone derivatives. In order to avoid the undesirable health and environmental considerations, and to take advantage of the stability and specific activity of biotinylated tracers, the more innovative approach of biotinylating the steroid aldosterone, such as has previously been applied to cortisol, was chosen. However, the lack of commercially available biotinylated aldosterone required this conjugation step to be carried out in-house with a subsequent step to purify the final solution containing the conjugate tracer of excess educts still present which could potentially interfere with the specificity and lead to increased background noise. This step was carried out using chromatographic purification by the reverse-phase technique.

The definition of chromatography stated by Holman et al in 1993 is the separation of a mixture of compounds by continuous distribution between two phases moving with respect to one another. HPLC separates steroids by their partition coefficients between aqueous and oily phases, samples are injected onto a column and eluted by mixtures of organic solvents and water. The degree of hydrophilic and hydrophobic interactions within the molecule and hence its chemical structure, for example a ketone versus an alcohol, will effect the separation (Holman et al., 1993). The mobile phase used is liquid as opposed to gas, hence its name, and pressure drives this mobile phase through the fine particles of the stationary phase of the column. Reverse phase high performance liquid chromatography (RP HPLC) is also known as adsorption or partition chromatography (Tuomainen, 1997). It is used to define the state whereby the stationary phase in the column is less polar than the mobile phase. The stationary phase is the non-polar solid support material within the column. Silica particles are the most commonly used material for the stationary phase, onto which carbon chains (C_1-C_{18}) are chemically bonded (Holman et al., 1993). Octadecylcylsilane (ODS, C_{18}) has a long hydrophobic alkyl chain and is most appropriate, and therefore more frequently used, in capturing small molecules and peptides. In general, the fewer the carbon atoms on the alkyl chain the shorter the retention time on the column (Tuomainen, 1997).

The (silica) beads or particles making up the stationary phase are characterized by particle size and pore size, with particle sizes ranging between 3 and 50 microns. Smaller particles give higher separation efficiencies. Particle pore size is measured in angstroms (1 angstrom Å $= 10^{-10}$m). This stationary phase interacts with analytes based on their polarities so that they move down the column at a slower or faster rate along with the polar, aqueous, organic mobile phase. The mobile phase is an aqueous medium to which organic solvents, for example methanol or acetonitrile, added to lower its polarity, and other modifiers may be added (Kazakevich & McNair, 2002).

In RP HPLC retention is mostly influenced by polarity so that hydrophobic compounds are retained more than hydrophilic. Additional polar groups (-OH, -NH$_2$, -COOH) reduce retention. The relative polarity of certain compounds and side chains are given in Table 2. The time it takes for a compound to go from the injector through the column and into the detector is called the retention time (Rt). Column length, diameter, packing material all influence retention time but it is mostly influenced by the amount of interaction a compound has with the stationary phase. A compound which is not retarded at all by the stationary phase will move down the column at the speed of the mobile phase and produce what is referred to as the solvent front and represents the void of the column. Depending on the composition of the mobile phase, compounds interacting least with the stationary phase of the column will elute first and those interacting more will elute later (Holman et al., 1993).

Solvent Polarity Chart			
Relative Polarity	Compound Formula	Group	Representative Solvent Compounds
Nonpolar	R-H	Alkanes	petroleum, ethers, ligroin, hexanes
	Ar-H	Aromatics	toluene, benzene
	R-O-R	Ethers	diethyl ether
	R-X	Alkyl halides	tetrachloromethane, chloroform
	R-COOR	Esters	ethyl acetate
	R-CO-R	Aldehydes and ketones	acetone, methy ethyl ketone (MEK)
	R-NH$_2$	Amines	pyridine, triethylamine
	R-OH	Alcohols	methanol, ethanol, isopropanol, butanol
	R-COHN$_2$	Amides	Dimethylformamide
	R-COOH	Carboxylic acids	ethanoic acid
Polar	H-OH	Water	Water

Table 2. Solvent polarity chart reproduced from Chemical Technicians' Ready Reference Handbook Fourth Edition, Gershon Joseph Shugar, Jack T. Ballinger, 1996.

The mobile phase can be either of a uniform composition (isocratic separation) or applied through the column as a gradient. In the case of a gradient separation, increasing concentration of the organic modifier will bring about a reduction in the retention of non-polar compounds,

since the mobile phase competes more effectively with the hydrophobic surface of the stationary phase (Holman et al., 1993) and allows these compounds to elute through the column.

Detection of steroids after separation by HPLC in the fractions eluted and collected is possible through various techniques the most common being ultraviolet (UV) spectrophotometry which is accomplished due to specific UV absorbing chromophores within a steroid's structure. Functional groups responsible for absorbance, for example ketones, vary in wavelength as does maximal absorbance between steroids depending on their chemical structure. For instance, a conjugated ene-one in the A ring of corticosteroids absorbs at 240nm while benzene rings of oestradiols at 225 and 280nm. Solvents themselves are assigned cut-off values below which they absorb themselves, acetonitrile for example absorbs at 200nm, not interfering therefore when using an absorbance detector set to 245nm (Kazakevich & McNair, 2002).

1.4. Aims

It was the purpose of this study to develop and clinically validate a non-isotopic immunoassay for the determination of levels of the mineralocorticoid hormone aldosterone in saliva, which could potentially be applied as a screening method for the detection of PA involved in endocrine hypertension.

➢ Tracer production

The first goal of the study was to produce a non-isotopic aldosterone tracer according to previously established methods of biotinylating steroid hormones (Dressendorfer, 1992), purify it using a RP HPLC method, and validate its performance in the immunoassay to detect levels of aldosterone in saliva.

➢ Saliva assay

The assay design is based on the competition reaction principle, chosen due to the small molecular weight of the steroid hormone aldosterone, with biotin as the primary probe and

incorporating a commercially available streptavidin-Europium^{3+} chelate as the second step. The method works in conjunction with the DELFIA system for time-resolved fluorometric end point measurement (TR-FIA).

> Validation

A small sample volume of 100μl is sufficient for each duplicate measurement. Validation of the assay included: to ascertain whether an extraction step would be necessary, to optimize conditions of the assay including concentrations of tracer and capture antibodies utilized, and to optimize incubation times necessary for optimal binding.

> Clinical validation

The study aimed to validate, in a clinical setting, the salivary aldosterone assay by showing that increases seen in levels of plasma aldosterone in response to physiological stimuli, such as during a posture test or after stimulation of the HPA axis with ACTH, are also mirrored in saliva, making the assay a possible screening tool for discriminating between healthy and Primary aldosteronism(PA) patients.

> Aldosterone to Cortisol Ratio

Examination of the salivary aldosterone measurements in combination with salivary cortisol from the same sample, taking into account the circadian rhythm of cortisol and autonomous hyper-secretion of aldosterone in PA, gave rise to the possibility of using the ratio between the two hormones to improve this discrimination and suggest a cut-off which could be used for the differential diagnosis of the disease state.

> Application to rodent low sample serum/plasma volume

During the course of setting up the aldosterone-in saliva assay, the opportunity arose to obtain an extremely sensitive monoclonal antibody against aldosterone. This antibody was used to show that the sensitivity achievable with such a highly specific monoclonal antibody is adequate to measure aldosterone levels in small sample volumes taken from rodents. The assay was used to test animal model studies ongoing in our laboratory.

2. Materials and Methods

2.1. Experimental

2.1.1. Materials

2.1.1.1. Reagents

Acetic acid (glacial) 100% anhydrous (CH_3COOH), Merck 100063 (Darmstadt, Germany)

Albumin Bovine (BSA), Sigma A7906 (Taufkirchen, Germany)

Aldosterone ($C_{21}H_{28}O_5$), Fluka 05521 (Taufkirchen, Germany)

Aldosterone 3-CMO ($C_{23}H_{31}NO_7$), Steraloids Inc. Q2010-000 (Newport, USA)

Acetonitrile (CH_3CN), Chromasolv- Sigma 34851 (Taufkirchen, Germany)

Biotinamidocaproyl Hydrazide, Sigma B3770 (Taufkirchen, Germany)

Cyproterone Acetate ($C_{24}H_{29}ClO_4$), Sigma C3412 (Taufkirchen, Germany)

Dexamethasone ($C_{22}H_{29}FO_5$), Sigma D1756 (Taufkirchen, Germany)

N,N′-Dicyclohexylcarbodiimide (DCC), Fluka 36650 (Taufkirchen, Germany)

Dichloromethane (CH_2Cl_2), Merck 106044 (Darmstadt, Germany)

Diethylenetriaminepentaacetic acid (DTPA), Sigma D6518 (Taufkirchen, Germany)

N,N-Dimethylformamide (DMF), Sigma D8654 (Taufkirchen, Germany)

Dimethyl Sulfoxide (DMSO), Sigma D5879 (Taufkirchen, Germany)

Ethanol (C_2H_5OH), Merck 100983 (Darmstadt, Germany)

17α-Ethynylestradiol ($C_{20}H_{24}O_2$), Fluka 02463 (Taufkirchen, Germany)

Fludrocortisone acetate ($C_{23}H_{31}FO_6$), Sigma F6127 (Taufkirchen, Germany)

Fluorometholone ($C_{22}H_{29}FO_4$), Sigma F9381 (Taufkirchen, Germany)

γ-Globulin Bovine, Sigma G7516 (Taufkirchen, Germany)

1-Hexanesulfonic Acid Sodium, Sigma H9026 (Taufkirchen, Germany)

Hydrochloric acid (HCl), Merck 9057 (Darmstadt, Germany)

N-hydroxysuccinimide (NHS), Fluka 56480 (Taufkirchen, Germany)

Methanol (CH_3OH), Merck 106018 (Darmstadt, Germany)

Medroxyprogesterone 17-acetate ($C_{24}H_{34}O_4$), Sigma M1629 (Taufkirchen, Germany)

Norethisterone acetate ($C_{22}H_{28}O_3$), Aldrich R182265 (Taufkirchen, Germany)

d(-)-Norgestrel ($C_{21}H_{28}O_2$), Sigma N2260 (Taufkirchen, Germany)

Phosphate Buffered Saline tablets (PBS), Sigma P-4417 (Taufkirchen, Germany)

ortho-Phosphoric acid (H_3PO_4), Merck 100573 (Darmstadt, Germany)

Prednisolone ($C_{21}H_{28}O_5$), Sigma P6004 (Taufkirchen, Germany)

2-Propanol (C_3H_8O), Sigma-Chromasolv 34863 (Taufkirchen, Germany)

Polyethyleneglycol 10000 ($HO(C_2H_4O)nH$), Merck 817008 (Darmstadt, Germany)

Potassium carbonate (K_2CO_3), Merck 104928 (Darmstadt, Germany)

Potassium hydrogen phthalate ($C_8H_5KO_4$), Merck 104874 (Darmstadt, Germany)

Sodium azide (NaN_3), Merck 6688 (Darmstadt, Germany)

Sodium chloride (NaCl), Merck 6404 (Darmstadt, Germany)

di-Sodium hydrogen phosphate anhydrous (Na_2HPO_4), Merck 106586 (Darmstadt, Germany)

Sodium hydrogen carbonate ($NaHCO_3$), Merck 106329 (Darmstadt, Germany)

Sodium phosphate dibasic dihydrate (Na_2HPO_4 x $2H_2O$), Fluka 71638 (Taufkirchen, Germany)

Spironolactone ($C_{24}H_{32}O_4S$), Sigma S3378 (Taufkirchen, Germany)

Streptavidin-Europium, Perkin-Elmer Life Sciences (Turku, Finland)

2-Thenoyltrifluoroacetone ($C_8H_5F_3O_2S$), Sigma T27006 (Taufkirchen, Germany)

Tri-octylphosphinoxid ($C_{24}H_{51}OP$), Merck 8254 (Darmstadt, Germany)

Triton X-100, Sigma T8787 (Taufkirchen, Germany)

Trizma base (Tris-(hydroxymethyl)-aminomethane), Sigma T1503 (Taufkirchen, Germany)

Tween 20, Sigma P1379 (Taufkirchen, Germany)

Tween 40, Sigma P1504 (Taufkirchen, Germany)

Water for chromatography (H_2O), Merck 115333 (Darmstadt, Germany)

2.1.1.2. Antibodies

Anti-aldosterone polyclonal (rabbit) antibody, Acris Antibodies GmbH Catalog No. BP 233,
   (Hiddenhausen, Germany).

Anti-rabbit (goat) Immunoglobulins, 1H7 in-house O.D. 1.728 (1.23mg/ml)

Anti-mouse (rabbit) Immunoglobulins, DAKO Code No. Z 0109 (Hamburg, Germany)

Anti-aldosterone monoclonal mouse antibody, A2E11, IgG_1 kappa

   The cell culture supernatant was kindly provided by C.E. Gomez-Sanchez, Division of

   Endocrinology, G.V. Montgomery VA Medical Center and University of Mississippi

   Medical Center, Jackson, Mississippi, USA.)

2.1.1.3. Buffers

Primary antibody Coating Buffer

For 1 litre of buffer: 8.9g Sodium phosphate dibasic dihydrate (Na_2HPO_4 x $2H_2O$) was dissolved in 800ml of distilled water and the pH set to 7.4 using Phosphoric acid (H_3PO_4). The volume is then brought up to 1 liter with distilled water. The buffer was stored at room temperature for 1 month.

Secondary (Capture) antibody Coating Buffer (See Assay Buffer)

Assay Buffer for Immunoassay (LKC)

For 5 litres of buffer: 45.0g of sodium chloride (NaCl), 30.0g of Tris-(hydroxymethyl)-aminomethane, 2.5g of sodium azide (NaN_3), and 0.5g of Tween 40 were dissolved in 3litres of deionised water. Subsequently, the pH was adjusted to 7.75 using concentrated hydrochloric acid (HCl). 25.0g of Bovine Serum Albumin (BSA), 2.5g Bovine γ-Globulin, and 39.35mg Diethylenetriaminepentaacetic acid (DTPA) were then added and the volume was brought up to 5litres. The assay buffer was left overnight to fully dissolve and was then filtered first over a 8μ filter (Fa.Sartorius 11301-50-N) and subsequently over a 0.8μ filter (Fa.Sartorius 11304-50-N). The buffer was stored at 4°C for up to 3 months.

Wash Buffer for Immunoassay

For 2 litres of wash buffer: 10 Phosphate Buffered Saline tablets were dissolved in deionised water along with 1ml of Tween 20. The buffer was stored at room temperature for up to one week.

Enhancement Solution

Stock solution A

5.55g 2-Thenoyltrifluoroacetone ($C_8H_5F_3O_2S$) and 966.5mg Tri-octylphosphinoxid ($C_{24}H_{51}OP$) were dissolved in 5ml of absolute ethanol. 50ml of Triton X-100 was added and brought up to

250ml with Aqua ad injectabilia water. This stock solution was stored in the dark at 4°C for up to 6 months.

Stock solution B

3.46g of Potassium hydrogen phthalate ($C_8H_5KO_4$) was dissolved in 15ml of Acetic acid (CH_3COOH) and taken up to 250ml with Aqua ad injectabilia water, pH set to 3.15. This stock solution was stored in the dark at 4°C for up to 3 months.

Working solution

2.5ml of Stock solution A and 25ml of Stock solution B were mixed and taken up to 250ml with Aqua ad injectabilia water. This solution was stored at room temperature for up to 3 months.

Artificial Saliva

Stock solution

For a 10x concentrate: 4.2g sodium hydrogen carbonate ($NaHCO_3$), 0.5g sodium chloride (NaCl), and 0.2g potassium carbonate (K_2CO_3) were added to 100ml distilled water. The buffer was stored at 4°C for up to 6 months.

Working solution

The stock solution was further diluted at 1:10 with distilled water. Stored at 4°C, always made fresh on the day of use.

Elution Buffers for Chromatography column

50mM Tris-HCl Buffer (Buffer A)

3.028g Tris-(hydroxymethyl)-aminomethane dissolved in 400ml distilled water. The pH was adjusted to 7.8 using Hydrochloric acid (HCl) and subsequently taken up to 500ml with distilled water.

86% Methanol/H_2O Buffer (Buffer B Knauer column)

430ml methanol (CH_3OH) were taken up to 500ml with chromatography gradient water.

86% Acetonitrile/ H_2O Buffer (Buffer B Phenomenex column)

430ml acetonitrile (CH_3CN) were taken up to 500ml with chromatography gradient water.

Dichloromethane Extraction Buffer

50mg Polyethyleneglycol 10000 ($HO(C_2H_4O)nH$) in 500ml Dichloromethane (CH_2Cl_2).

2.1.2. Equipment

Automatic microtiter plate multi washer, TECAN, SLT Labinstruments (Crailsheim, Germany)

Victor^3, Perkin Elmer (Wiesbaden, Germany)

Jouan GR 422 Centrifuge, Thermo Fischer Scientific, (Germany)

ÄKTA™ purifier UPC-900, Amersham Biosciences Europe GmbH, (Freiburg, Germany)

Synergi 4u Fusion-RP 80Å reverse phase chromatography column, Phenomenex Inc., (Aschaffenburg, Germany)

Vertex-Säule Eurospher 100 C18 5μm, Knauer Advanced Scientific Instruments, (Berlin, Germany)

DU$^®$-62 Spectrophotometer, Beckman Coulter GmbH, (Krefeld, Germany)

2.1.3. Miscellaneous materials

Maxisorp-microtiter plates (Nunc, Roskilde, Denmark, No. 442404)

pH meter (Wissenschaftlich-Technische Werkstätten, Weilheim, Germany)

2.2. Clinical Studies

2.2.1. Materials

NaCl isotonic solution 0.9%, Braun, (Melsungen, Germany)

Disinfectant spray, Cutasept® FBode Chemie (Hamburg, Germany)

250µg synthetic human ACTH 1-24 (Synacthen; Novartis)

2.2.2. Equipment

Salivette® Sarstedt, No. 51.1534 (Nümbrecht, Germany)

Venous access: VenflonPro 18 Gauge, Becton Dickinson, (Heidelberg, Germany)

Multidirectional Discofix® stopcocker for infusion therapy, Braun, (Melsungen, Germany)

Cannula Fixation Dressing, Curapor® i.V. Lohmann Rauscher, (Regensdorf, Germany)

BD Discardit™ II 2ml and 10ml Syringes, Becton Dickinson, (Heidelberg, Germany)

Elastic bandage, Nobafix®, (Wetter, Germany)

Multi-Adapter for S-Monovette, Sarstedt, (Nümbrecht, Germany)

Combi-Stopper, closing cone combi blue, Braun, (Melsungen, Germany)

I.V. administration set for gravity infusion Intrafix® Air, Braun, (Melsungen, Germany)

S-Monovette Blood Collection System, Sarstedt 2,6ml (Serum) and 7,5ml (Plasma EDTA)

      Sarstedt, (Nümbrecht, Germany)

Mandrin, Becton Dickinson, (Heidelberg, Germany)

RR-machines

Powder-free examination gloves, Sempercare® Semperit Technische Produkte

      Ges.m.b.H & Ko KG, (Vienna, Austria)

2.3. Methods - Experimental

2.3.1. Synthesis of aldosterone 3-CMO-biotin conjugate tracer

Commercially available derivatives of aldosterone and biotin were coupled as described previously in Dressendörfer et al., 1992, for the synthesis of the aldosterone3-CMO-biotin conjugate, over three consecutive days. On day 1, preparation of aldosterone 3-CMO active ester derivative was as follows: 5mg (11.5μmol) of aldosterone3-CMO (Steraloids Inc., Newport, USA) was dissolved in 100μl of dry, amine-free N,N-Dimethylformamide (DMF) (Sigma). 5.6mg (50μmol) N-hydroxysuccinimide (NHS) (Fluka) and 10.3mg (50μmol) N,N′-Dicyclohexylcarbodiimide (DCC), (Fluka) were dissolved in 40μl and 60μl of DMF respectively, and added to the aldosterone 3-CMO. The reaction mixture was stored at room temperature under light-exclusion for 24hours. On day 2, coupling of aldosterone-NHS ester to biotin was as follows: 9.3mg (25μmol) Biotinamidocaproyl Hydrazide, (Sigma) was dissolved in 100μl of Dimethyl Sulfoxide (DMSO), (Sigma). The completely dissolved biotin was then added to the initial reaction mixture and left at room temperature for another 24hours under light-exclusion. On day 3, the final volume (300μl) of the reaction mixture was further diluted at 1:10 with a 50mM Tris-HCl buffer (pH 7.8), to a final concentration of 1.6 mg/ml, aliquoted and stored at -20°C.

2.3.2. Reverse phase chromatographic purification of the aldosterone tracer

The aldosterone 3-CMO-biotin conjugate (1.6 mg/ml), was further diluted with 50mM Tris-HCl buffer to a concentration of 0.1 mg/ml and 120μl aliquots, corresponding to 0.012mg, were injected into the column. The aldosterone conjugate was applied to two reverse phase chromatography columns, the "Vertex-Säule Eurospher 100 C18 5μm" provided by Knauer Advanced Scientific Instruments and the "Synergi 4u Fusion-RP 80A, 250 x 4.6mm" from Phenomenex.

2.3.2.1. Vertex-Säule Eurospher 100 C18 5μm"- Knauer Advanced Scientific Instruments

The elution buffer used to run the column was a linear gradient of 0 - 100% Buffer B (86% Methanol/H_2O) starting with 100% Buffer A (50mM Tris-HCl pH 7.8). The elution was run at a flow rate of 0.7ml/min in 20 column volumes (Figure 5 and 6).

2.3.2.2. Synergi 4u Fusion-RP 80A, 250 x 4.6mm - Phenomenex Ltd.

Two different elution systems were used to purify the conjugate using the Phenomenex column. In the first instance, the elution buffer used to run the column was also a linear gradient as described above, of 0 - 100% Buffer B (86% Acetonitrile/H_2O) starting with 100% Buffer A (50mM Tris-HCl pH 7.8). The elution was run at a flow rate of 0.7ml/min with a gradient length of 20 column volumes (Figure 7). In the second instance, an isocratic gradient of 33% Tris-HCl/ 67% Acetonitrile-H_2O(86-14%) buffer was run, with a step to 100% Acetonitrile-H_2O(86-14%) buffer after 60 minutes, run at 0.7ml/min, also in 20 column volumes (Figure 8).

For both columns and in all runs, a volume of 100μl was injected into the column via the injection loop, corresponding to 0.01mg of the biotinylated aldosterone conjugate. The wavelength on the absorbance detector was set to 254nm and fractions of 500μl were collected. There was a set column pressure of 9.8 MPa. At the end of each run the columns were washed through with 5 column volumes of the final running elution, i.e. 86% Methanol/H_2O for the Knauer column and 86% Acetonitrile/H_2O for the Phenomenex column.

2.3.3. Evaluation of fractions

2.3.3.1. Aldosterone content with RIA MAIA Adaltis

Highest absorbance(mAU) peak fractions at 254nm were diluted in Assay Buffer at 1:1000, so that values would fall within the measurable range of the assay, and added as samples for analysis of aldosterone content using a commercially available radio-immunoassay (Aldosterone MAIA, Adaltis Italia S.p.A.), see Table 4. Peak fractions that corresponded to two fractions were pooled and measured together. A selection of baseline fractions were also added for measurement to ascertain that there was minimal or no aldosterone within these fractions.

2.3.3.2. Fractions as assay tracer

As a preliminary protocol the anti-aldosterone capture antibody was diluted at a 1:1000 and the incubation time was set to 1.5hours. In subsequent assays, the concentrations of capture antibody and incubation time were modified in order to optimize the assay procedure accordingly. The concentration range of the calibrators used in the assays to test activity of the tracer fractions, incubation times and concentration of antibodies was initially reported in the ng/ml range for reasons of simplicity and clarity in the graphic representation. Calculations and ED_{50} values are reported in pg/ml. Once the assay range was established, for example in Figures 13, 14, and 15, the x-axis of the standard curve is presented in the pg/ml range.

As an initial screening step, the fractions found to have highest aldosterone content were added, at different dilutions, and used as tracer in the assay in order to assess overall maximum counts, displacement capability, and to assess whether this correlated to concentration of aldosterone obtained from the radioimmunoassay. Fractions not showing peaks were also assessed to ensure that biotinylated aldosterone was present. Only a selection of the fractions tested are shown in

graphic representation in the 'Results' section, although several more were tested in order to ascertain the optimum tracer for use in the immunoassay.

Peak fractions 93, 95 and 97 from the linear gradient Knauer Run 1 were tested at dilutions of 1:10, 1:100, 1:1000, and 1:10000 as tracer in the assay (ex. Fraction 97, Figure 5). Peak fractions 92/93, 94/5, and 96/97 from linear gradient Knauer Run 2 were tested at dilutions of 1:100, 1:1000, 1:5000 fraction 94/95, and 1:10000 respectively (ex. Fraction 96/97, Figure 6). Fractions 68/69, 70 (Figure 7) and 64/65, 81/82 (not shown), Phenomenex Run 1 were tested at 1:1000. Peak fractions 67, 68 and 69, from Phenomenex Run 2, as well as pooled fractions 63/64, 65/66, 72 and 73 were tested likewise, as were fractions 25, 29, 30/31, and 34 and 50, pooled 51/52 and 55 from isocratic Phenomenex Run 3 were all used at 1:1000. Similarly, fractions 34, 35/36, 37, 38, 39, 40, 50/51, 90/91 from isocratic Phenomenex Run 4 were also run at a 1:1000 dilution to ascertain activity (ex. Fractions 39, 40, 50/51 and 90/91, Figure 8).

2.3.3.3. Direct comparison of activity at equal concentration

A previously established suitable tracer concentration for the cortisol in saliva assay described in Dressendoerfer et al 1992, was found to be 20pg/well. For this reason, after initial screening, the fractions were each diluted accordingly to correspond to a final concentration of 20pg/well in order to acquire a direct comparison of binding activity (Figure 9, Table 5). Furthermore, this step was carried out in order to find the fraction with a combination of highest aldosterone content and lowest amount of excess biotin, that is, the most purified fraction giving least amount of background producing non-specific binding.

2.3.3.4. Optimising concentration of chosen tracer fraction

For competitive assays the rate of change of signal upon addition of sample hormone or of labelled antigen tracer, is greatest when a small amount of antigen tracer is added as this limits the "signal-to-noise-ratio". For this reason it is optimum to have the lowest amount of tracer possible so that small changes in sample antigen can be detected, as the rate of change is more visible. Two tracer fractions from Knauer Run 1 and from isocratic Phenomenex 4, (Figure 10) were used at 2 and 20 pg/well with varying antibody concentrations and incubation time. Fraction 40 from Phenomenex run 4 was also tested at 2pg, 5pg and 20pg per well (Figure 11).

2.3.4. Conditions of the assay

2.3.4.1. Optimisation of incubation time between tracer and standards

Fraction 40 from the isocratic elution run on the Phenomenex column (2.19μg/ml) was used as tracer at a concentration of 2pg/well to look at the effect of an extended incubation time between the tracer and the standards. Assay times of 1.5 hours, overnight, and 48 hour incubations were compared, and total counts, total displacement and ED_{50}'s were assessed (Table 6). A combination of an overnight incubation with the Knauer fractions 97, and 96/97 and Phenomenex fractions 51/52 and 40 at 2 and 20pg/well, again with the capture antibody at 1:1000 (Figure 12) was applied to determine optimum concentration of the tracer at the longer established incubation time of an overnight duration.

2.3.4.2. Optimising concentration of polyclonal anti-aldosterone capture antibody

For similar reasons which concentration of tracer needs to be at lowest concentrations possible, the antibody concentration also needs to be as low as possible in order to minimise errors in estimating the unoccupied sites. The displacement capability of the tracer Phenomenex fraction 40 at 2pg/well and an overnight incubation, was evaluated at higher, 1:5000, and lower, 1:100,

titration of the polyclonal Acris capture antibody (Table 7). Total counts, total displacement and ED_{50}'s were assessed.

2.3.4.2. Optimising concentration of monoclonal anti-aldosterone capture antibody

The concentration of the monoclonal A2E11 capture antibody was optimised by coating at increasing dilutions from 1:2500 to 1:50000 and the total counts, displacement and ED_{50}'s were assessed (Figure 13, Table 8). The tracer Phenomenex fraction 40 was at 2pg/well with an overnight incubation time.

2.3.5. Working dilution of antibodies

Direct coating of the capture antibody to the solid phase, i.e. the microtitre plate well, can result in conformational changes that reduce its affinity for the analyte. To avoid this problem a 'primary' antibody is coated first, containing immunoglobulins against the corresponding species in which the antibody is raised. This antibody, which is specific for the Fc, constant, region of the capture antibody, allows for correct orientation, leaving the antigen-specific Fv region available for binding. This will then allow for highest binding capacity of the immobilized antibody and for good reproducibility of coating conditions.

2.3.5.1. Primary coating goat-anti-rabbit immunoglobulins

A goat anti-rabbit immunoglobulin (IgG) antibody, produced in-house, (1H7), was immobilised onto Maxisorp-microtiter plates (Nunc, Roskilde, Denmark) at a concentration of 300ng per 200µl well in 50mM sodium phosphate (Na_2HPO_4 $x2H_2O$) pH 7.4 coating buffer and left for a minimum of one overnight incubation.

2.3.5.2. Primary coating rabbit anti-mouse immunoglobulins

A rabbit anti-mouse IgG antibody (DAKO, Hamburg, Germany) was immobilised onto Maxisorp-microtiter plates (Nunc, Roskilde, Denmark) at a concentration of 300ng per 200µl well in 50mM sodium phosphate (Na_2HPO_4 x $2H_2O$) pH 7.4 coating buffer and left for a minimum of one overnight incubation.

2.3.5.3. Secondary coating - polyclonal rabbit anti-aldosterone (Acris Antibodies GmbH)

Goat-anti-rabbit IgG coated plates were washed 3 times and subsequently commercially available rabbit anti-aldosterone capture antibody (Acris, Hiddenhausen, Germany) was used at a 1:1000 dilution in Assay Buffer, i.e. 100ng/well, was pipetted into each well and left for at least one overnight incubation. Plates could be stored for up to one month without loss of activity.

2.3.5.4. Secondary coating - monoclonal mouse anti-aldosterone antibody (A2E11)

Rabbit anti-mouse IgG antibody coated plates were washed 3 times and subsequently mouse anti-aldosterone antibody (A2E11) was used at a 1:10000 dilution in Assay Buffer was pipetted into each well and left for at least one overnight incubation. Plates could be stored for up to two weeks without loss of activity.

2.3.6. Preparation of aldosterone calibrators

A stock solution of aldosterone (Fluka, Taufkirchen Germany) was reconstituted in pure ethanol to 10mg/ml and stored at -80°C. This was consequently serially diluted with artificial saliva, prepared according to the German Industry Norm (DIN 53 160) standards, to 10000pg/ml to give the highest calibrator and further to give 5000, 2000, 1000, 500, 200, 100, 50, 20, 10 and 5pg/ml. The calibrators were kept at -20°C for several months.

2.3.7. Final Assay Procedure

Microtitre plates were coated with a combination of either the goat anti-rabbit immunoglobulin (IgG) antibody produced in-house and subsequently the polyclonal rabbit anti-aldosterone capture antibody (Acris, Hiddenhausen, Germany), or the rabbit anti-mouse IgG antibody (DAKO, Hamburg, Germany) and subsequently the mouse anti-aldosterone monoclonal capture antibody (A2E11). Previous to the assay procedure, plates were washed three times in an automatic microtiter plate washer (TECAN, SLT; Crailsheim, Germany) using an in-house prepared 0.05% PBS/Tween-20 buffer and subsequently 50 µl of each sample were pipetted in duplicate, directly onto the plates along with aldosterone calibrators and controls. Subsequently, 100 µl of biotinylated aldosterone tracer were pipetted into each well, diluted in assay buffer at 5 pg/well, the plate was sealed with self-adherent foil to avoid evaporation and incubation proceeded for an hour at room temperature on a horizontal microtiter plate shaker (TECAN, SLT Labinstruments, Crailsheim, Germany) before being left overnight at 4°C.

The following day, the incubation was terminated by washing three times on the automatic microtiter plate washer. 200 µl per well of a Streptavidin coupled to Europium conjugate (PerkinElmer/Wallac, Turku, Finland) were then added and incubated for thirty minutes on the horizontal shaker at room temperature. After a 6-fold wash of the plate, an in-house „enhancement solution" was added and incubated on the shaker for fifteen minutes, used to transfer the Europium conjugate into a fluorescent complex. Amount of biotinylated aldosterone bound was then measured using a fluorometer (VICTOR[3], PerkinElmer).

2.3.8. Extraction of aldosterone from saliva and plasma/serum

For the extraction of aldosterone from saliva samples, 200µl of each saliva sample were vortexed at a low speed with 2ml of dichloromethane (DCM)/Polyethylene glycol (PEG) 10000 for 30 minutes. For the extraction of aldosterone from human plasma samples, 200µl of plasma were

vortexed with DCM as for saliva. For the extraction of aldosterone from rodent plasma or serum samples where a volume of 200µl was not available, 50µl were used instead.

When the two phases were separated, the organic phase (1.7ml) was removed, transferred to another tube and left to evaporate to dryness overnight. The following day, the sample was reconstituted with 10µl methanol and 160µl DIN artificial saliva. Duplicate samples of 50µl were then directly pipetted onto the microtiter plate. In the case of the smaller volume of serum or plasma samples used for rodent aldosterone level analysis, the concentration (pg/ml) given by the assay was multiplied 4-fold in order to compensate for the dilution factor after reconstituting the sample.

2.3.9. Saliva sampling

The sampling device used to collect saliva was the Sarstedt salivette, containing a 40 x 9mm cellulose tampon (see Appendix 7.3). The salivette is held at the rim of the suspended insert so that the stopper is removed. The swab is removed from the salivette and gently chewed for 2 minutes, or kept in the mouth until the patient or participant could no longer prevent himself/herself from swallowing the saliva produced. Participants were asked not chew the salivette for 30 minutes after eating or drinking, immediately after taking any kind of oral medication or tablets, immediately after smoking, or immediately after brushing teeth to avoid contamination from blood and to ensure there was no contamination of saliva with interfering substances. The saturated swab was then returned to the suspended insert and the salivette is closed firmly with the stopper. Time and date of collection, as well as name or code were labeled on each tube. Before analysis, the sample was thawed and centrifuged so the saliva was transferred to the outer tube, the inner tube and swab were discarded. Saliva was pipetted directly from the outer tube after the inner tube was discarded.

2.3.10. Assay Validation

2.3.10.1. Cross –reactivity

Assay specificity is the ability of an antibody to produce a measurable response only for the analyte of interest and cross-reactivity is a measurement of antibody response to substances other than the analyte. A pure sample of the cross-reacting substance is spiked into an analyte-free matrix to give a suitably wide range of concentrations. Cross-reactivity is defined at the point where the reduction in signal corresponds to 50% of the signal achieved in the absence of analyte (B/Bo of 50%, ie ED_{50}), as a percentage of the analyte concentration giving the same fall in signal (Wild, 2005).

% cross-reactivity = $\dfrac{\text{concentration of analyte giving 50\% B/Bo}}{\text{concentration of cross-reactant giving 50\% B/Bo}}$

Polyclonal Rabbit anti-aldosterone (Acris)

A series of both endogenously found and synthetic steroids were tested for potential cross-reactivity with the anti-aldosterone capture antibody. These steroids were added as samples in the assay in increasing concentrations and standard displacement curves were obtained. Comparison of the ED_{50} values allowed us to acquire the % cross-reactivity (Table 9).

A2E11 monoclonal anti-aldosterone antibody

The monoclonal antibody against aldosterone used in the immunoassay was prepared and cross-reactivities were tested as previously described in Gomez et al 1987. Briefly, three different tracers and separation procedures were used, namely, ammonium sulphate separation and charcoal adsorption with tritium label, and charcoal separation with [125I]iodohistamine-aldosterone tracer (Table 10).

2.3.10.2. Sensitivity – Lower limit of detection

Sensitivity can be defined as the concentration at which one can be certain that a sample is not zero. There are two ways sensitivity can be evaluated, analytical and functional. Analytical sensitivity is determined most commonly by repeatedly assaying the zero standard and defining the limit of sensitivity as being the concentration corresponding to two or three standard deviations above the mean. This equates to the probability that a single replicate of sample is not part of the distribution of the zero analyte values (Wild, 2005).

Polyclonal Rabbit (Acris) and A2E11 monoclonal anti-aldosterone

The analytical sensitivity or lower limit of detection (LOD) of the assay, was determined by assessing the zero standard in a typical assay a total of 30 times and the mean (± standard deviation; SD) value of the counts was calculated. A statistically significant difference from the zero standard was taken to be more than 2 times the standard deviation from this repeated mean. This corresponding aldosterone value was thereafter determined from its intercept with the displacement curve (polyclonal Acris antibody Figure 14, A2E11 monoclonal antibody Figure 15).

2.3.10.3. Linearity

Linearity is an additional check of the accuracy of the assay and is used to judge whether or not dilutions of a sample lie parallel to the calibration curve. Often called dilution experiments, this experiment answers the question "if a sample is diluted will it give the same result or not". Assessed by assaying samples diluted in an analyte-free matrix (Wild, 2005).

Polyclonal Rabbit anti-aldosterone (Acris)

Four samples of pooled saliva were spiked with pure aldosterone (ethanol stock of 10mg/ml), previously diluted in artificial saliva to a concentration of 1000pg/ml, and measured. The

'spiked' pools were then serially diluted again with artificial saliva and the values obtained upon measurement with the assay were compared to the expected calculated concentration (Table 11).

A2E11 monoclonal anti-aldosterone antibody

A plasma sample which was measured above the highest standard point of the linear range of the assay was diluted (1:2) in the matrix medium used to produce the calibrators and measured. This sample was then further serially diluted (1:4, 1:8, 1:16, 1:32) and the average recovery rate was determined (Table 12).

2.3.10.4. Recovery

Recovery is the test most commonly used to demonstrate the accuracy of an assay. Accurate quantities of analyte are added to samples and the incremental increase in concentration is determined. It assesses the calibration of the assay and the influence of differences between sample and calibrator matrices (Wild, 2005).

% recovery = $\dfrac{\text{measured increase in concentration}}{\text{predicted increase in concentration}}$ x 100%

Polyclonal Rabbit anti-aldosterone (Acris)

A saliva sample, which was measured for an initial aldosterone concentration, was serially diluted at 1:2 with each of the 9 standards and the mean % recovery was calculated (Table 13).

A2E11 monoclonal anti-aldosterone antibody

A plasma sample, measured for initial aldosterone concentration, was extracted and reconstituted a total of nine times and the sample was then pooled. Each of the standard points was then added at a 1:1 dilution to an aliquot of the sample. Average recovery for each sample was found at 102% (Table 14).

2.3.10.5. Precision (Reproducibility)

Precision describes the repeatability of an analytical technique and is an estimate of the error in the technique, expressed as the coefficient of variation (%CV) at a particular analyte level, and occurs due to combined effects of antibody characteristics, separation, detection, and manipulation errors. Within-run precision of a sample is determined when the same sample is run on several occasions within the same assay. Twenty replicates of sample are included spaced equidistantly throughout the assay. Between-run precision is an index of the ability of the assay to reproduce the same result on the same sample from run to run and from day to day. %CV was determined by the standard deviation of the mean / mean x100 (Wild, 2005).

Polyclonal Rabbit anti-aldosterone (Acris)

The intra-assay coefficients of variation were determined after a 10-fold measurement of pooled saliva samples of known aldosterone high, intermediate and low concentrations, also before and after extraction (Tables 15). The inter-assay coefficients of variation were calculated by a 20-fold measurement of pooled saliva samples of known aldosterone high, intermediate and low concentrations and were determined respectively (Table 16).

A2E11 monoclonal anti-aldosterone antibody

Intra-assay coefficients of variation were determined by 20-fold measurements of pooled plasma samples with high, intermediate and low aldosterone concentrations which were extracted, reconstituted, pooled again and then added as samples on the same plate (Table 17). Inter-assay coefficients of variation were determined by 20-fold measurements, on consecutive days, of plasma samples extracted and reconstituted on each occasion (Table 18). Inter-assay coefficients of variation were also determined for three pools of non-extracted saliva samples with low, intermediate and high aldosterone concentrations (Table 19).

2.3.11. Salivary aldosterone determination with and without salivette

In order to determine whether or not levels of aldosterone are affected in any way by using the salivette device, such as by the interference or cross-reaction of substances which may be present in the tampon insert, ten pairs of samples collected using the Sarstedt salivette and collected by spiting directly into the external tube of the salivette, were measured using the Acris assay. Samples were frozen at -20°C once and then thawed and centrifuged before pipetting onto the plate. Participants were asked to abide by sampling guidelines prior to collection. No stimulation of salivary flow rate was applied for collection without the salivette.

2.3.12. Pre-analytical storage stability of salivettes

As a test of storage stability, five healthy volunteers were asked to provide four consecutive saliva samples within a few minutes. One of the samples was immediately centrifuged, mixed thoroughly and subsequently aliquoted (200µl) and stored at 4°C as well as room temperature for up to 7 days (see Figure 16, 'standard'). After 1, 3, and 7 days at the same conditions the other samples were also frozen, thawed and then centrifuged before finally being analyzed for aldosterone content on the same day and in the same run.

2.3.13. Correlation of calibrators with commercially available RIA

A set of aldosterone calibrators prepared in-house (0 to 2000pg/ml) were added, without any prior treatment, as samples and measured with the DPC Biermann (Coat-A-Count) radioimmunoassay (Figure 17, Table 20). Similarly, a set of DPC calibrators taken directly from the kit were added as samples and measured in the in-house TRFIA using the Acris polyclonal antibody before and after extraction using dichloromethane/PEG (Figure 18, Table 21). These same calibrators were also measured using the A2E11 assay after extraction (Figure 19, Table 22).

2.3.14. Extraction of saliva and plasma – 'Acris assay'

2.3.14.1. Salivary aldosterone

A series of 188 saliva samples were measured using the Acris assay before and after extraction using dichloromethane/PEG 10000. Samples were thawed and extracted on the day previous to the assay and an extra aliquot was kept in an eppendorf cup for the following day for the 'no extraction sample' in order to avoid repeat freeze thawing of the original sample. Two examples of day profiles from healthy participants as well as a morning to mid-afternoon profile from a patient suspected of Primary aldosteronism (Figure 20a, b, c, d) are shown.

2.3.14.2. Plasma aldosterone

A series of plasma samples were tested for aldosterone content using the DPC RIA and correlated to the results obtained by the Acris assay. 200µl from each of the samples underwent extraction and reconstitution, with the standard protocol described above, for measurement in the Acris assay. Plasma samples collected from two healthy participants, one taking part in a posture test study which was carried out in the clinic, to show that steep increases may still be monitored after extraction, and another collecting a normal day profile, are shown in Figure 21, correlation between the two assays Figure 22. A set of samples was also further evaluated using a decreased volume of 100µl. Extraction and reconstitution were carried out according to the procedure above and aldosterone values acquired upon completion of the assay were multiplied by a factor of 2 to compensate for the dilution factor. These samples were measured alongside a standard 200µl extraction and compared to this and the DPC assessment (Figures 23a and b, 24a and b).

2.3.15. Extraction of plasma – 'A2E11 assay'

The A2E11 antibody was used to determine whether or not plasma aldosterone concentrations may be measured in smaller volumes than the 200μl and 100μl already established. Initially, a set of plasma samples selected from a healthy subject carrying out a day profile collection were assessed by the standard extraction method to determine if plasma aldosterone measurement is in fact possible using the A2E11 antibody. Following to this, a smaller volume of 50μl was used while all the following steps of the procedure remained unchanged, and a factor of 4 was added to the end results after measurement of the plate (Figure 25a, b, and c).

2.4. Methods - Clinical

Samples included for salivary and plasma aldosterone, as well as salivary cortisol measurements were obtained from PA patients admitted to the Ludwig Maximilians University Clinic, and from randomly chosen healthy volunteers. Salivary samples were also obtained from nine PA patients admitted to the Hospital Europeen Pompidou in Paris, France. Samples collected from patients and volunteers taking part in the ACTH stimulation test were obtained from an ongoing study concurrently taking place at the clinic. For all studies involving the collection of human samples, approval of the study protocol was obtained by the Medical Faculty of the University of Munich Ethical Committee (Project Nr. 069/04). Written informed consent was obtained from all patients and healthy volunteers participating in all studies and consent forms, questionnaires and description of the study were handed out to each individual beforehand (see Appendix 7.1 and 7.2). Where PA patients admitted to the LMU Clinic participated, confirmation of the disease was by failure to suppress aldosterone to less than 80pg/ml in the saline infusion test.

2.4.1. Clinical validation of salivary aldosterone

2.4.1.1 Acris assay clinical validation – Day profile study

It was the purpose of this study to obtain simultaneous saliva and plasma samples from healthy participants throughout the course of the day in order to show that the pattern according to diurnal rhythms which is seen in plasma aldosterone can also be monitored in saliva. Nine healthy participants (4 men and 5 women) were recruited from the staff of the clinic as well as from random volunteers (mean ± (SD), aged men: 35 ± 9.35, women: 24 ± 2.4, BMI men: 23.7 ± 2.0, women: 20.6 ±1.2, Table 23). These participants were on no medication apart from thyroxin or the contraceptive pill and did not report any endocrine disorders. At 08:00 an indwelling catheter was inserted into the antecubital vein and serum and saliva samples were obtained simultaneously every two hours thereafter up to and including 18:00. During this time individuals were allowed to go about normal daily activities. Figure 26 shows the mean (± SEM) results for salivary and plasma aldosterone.

2.4.1.2. Mean aldosterone levels in PA and healthy participants

Salivary sample collection for the accumulation of total salivary aldosterone comparisons between PA and healthy volunteers (Figure 27) as well as for separate day profiles from PA and healthy (Figures 28 and 29 respectively) was as described in the section above 'Salivary sampling'. For the determination of a total salivary aldosterone and plasma correlation (Figure 30), plasma and saliva samples from single as well as day profiling studies were used. For single time point samples and day profiles, plasma samples were collected as described above 'Day Profile Study' by an indwelling catheter inserted into the antecubital vein. Plasma samples were assayed for aldosterone content by the DPC Biermann RIA.

2.4.1.3. Posture test study

A cohort of 31 healthy, normotensive, volunteers with no medical history of renal failure or hypertension were recruited (15 male, 16 female, age ranged from 18 to 30 years, BMI ± (SD) 22.2 ± 1.8). Sixteen of the volunteers had a history of regular cigarette consumption. These participants smoked an average of 12.2 cigarettes per day (range, 4 to 30) and smoked for an average of 7 years (range, 1 to 15). The volunteers were on no medication excluding thyroxin or the contraceptive pill. Demographics for the participants are shown in Table 3.

	Male		Female	
	Smokers	Non-smokers	Smokers	Non-smokers
n	7	8	8	8
Age	25 ± 2.4	23.4 ± 3.2	21.6 ± 2.8	21.6 ± 3.1
BMI	24.1 ± 1.3	22.2 ± 1.4	21.8 ± 1.9	20.9 ± 1.3
OC	-	-	6/8	5/8
RR Syst BL	128.7 ± 10.6	123.3 ± 12.7	120.9 ± 7.8	115.4 ± 10.5
RR Syst Std	122.9 ± 12.5	130.0 ± 19.3	118.5 ± 7.8	110.5 ± 16.4
RR Diast BL	81.0 ± 8.5	69.3 ± 8.0	74.9 ± 7.5	64.0 ± 8.9
RR Diast Std	77.6 ± 11.2	80.9 ± 7.6	69.6 ± 6.7	67.4 ± 10.7

Table 3. Posture study demographics. Data are shown as mean ± SD
n, No. of participants, OC, Oral Contraceptive, Syst BL and Diast BL, systolic and diastolic blood pressures at baseline, Syst Std and Diast Std, systolic and diastolic bloop pressures after standing.

Study design and sample collection

The study was approved by this institution's local ethics committee. Subjects were asked to give written informed consent. The participants were asked to come in to the hospital in a fasting state at 08:00 and were given a standard breakfast provided by the clinic. An indwelling catheter was inserted into a forearm vein and subsequently participants were asked to assume the supine position before the first simultaneous plasma and saliva samples were taken at 10:00. This was designated as time point t0. Samples were collected every 15 minutes for one hour until 11:00

and then one hour later at 12:00. This was the last supine sampling time point, t120, after which the subjects rose to a standing position. Samples were then collected again at 15 minute intervals for the next hour, and subsequently at the next two hour time points, t240 and t300 respectively. During the last two hours of the study the participants were allowed to leave the hospital and return for subsequent collections. Blood pressure was measured manually from the other arm after the first supine hour as well during the first hour after standing.

Hormone assays

Blood samples collected were used for the measurement of plasma aldosterone (Coat-a-Count, Diagnostic Products Corporation Biermann GmbH, Bad Nauheim Germany), and salivary aldosterone was measured using the Acris assay. Salivary aldosterone responses before and after change in posture were compared to the plasma aldosterone at each time point (Figure 31) and overall data was analysed for differences found between males and females and differences related to smoking status (Figures 32 and 33 respectively).

2.4.1.4. ACTH stimulation test

Saliva samples were collected along with plasma sampling carried out during an ACTH stimulation test, as part of an ongoing study taking place at the clinic, looking at the response of patients with suspected Conn's syndrome to various stimuli. Twenty participants, 12 Conn's syndrome patients (6 men and 6 women, aged 57 ± 11.5) and 8 healthy volunteers (2 men and 6 women, aged 26 ± 2.9) were studied for basal and post-ACTH aldosterone levels in plasma and saliva. Baseline salivary and plasma samples were collected an hour before participants were given 250µg synthetic human ACTH 1-24 (Synacthen; Novartis) as a baseline measurement and subsequently simultaneous saliva and plasma samples were taken at 15 minutes after injection and then every 15 minutes following that (Figure 34).

2.4.1.5. Aldosterone to Cortisol Ratio

Saliva samples were collected from a total of 68 subjects who participated in the study. 27 of whom were confirmed with PA in the form of aldosterone producing adenoma (APA) or bilateral adrenal hyperplasia (BAH), and 41 healthy volunteers (control). PA patients were aged 14 to 82 years, 16 were female and 21 male, and had a mean (± SEM) BMI of 25.4 ± 1.03. Control participants were aged between 19 and 65 years, 12 were female and 33 male, and they had a mean BMI of 23.4 ± 0.5. Concentrations of salivary aldosterone were analyzed by the Acris in-house TRFIA and salivary cortisol by a commercial luminescence competitive immunoassay (Cortisol Luminescence Immunoassay, IBL; Hamburg, Germany) and the ratio between the two hormones was thereby determined, aldosterone to cortisol ratio (ACR). Daytime saliva samples, taken between 7 and 12am, and evening samples between 6 and 10pm, were collected using Sarstedt salivettes. Furthermore, in a subgroup of 10 PA and 8 Control subjects an ACTH stimulation test was performed to investigate the influence of stimulation of the pituitary adrenal axis on the ACR (Figure 36). ACRs in saliva found in the morning were compared to those in the evening in order to establish the ratio which could be set as a cut-off and used to discriminate between PA patients and healthy subjects. Furthermore, comparison of the ACR determined using morning samples to that given in the evening was used to demonstrate the most appropriate time of day for carrying out sampling (Summary Table 24, Figures 35-37).

2.4.1.6. 'A2E11 assay' clinical validation in saliva

For the clinical validation of the A2E11 assay in saliva two sets of day profile samples collected from patients with PA at half-hour intervals were measured using the assay according to the standard procedure described above, 'Final Assay Procedure' without pre-treatment of the samples with DCM/PEG extraction. Samples were also evaluated using the Acris in-house assay

according to the same procedure and using the same set of calibrators so that a comparison could be made between aldosterone levels given by the two assays (Figures 38).

2.4.2. Clinical validation in rodent serum/plasma using the A2E11 assay

The studies presented here were conducted in accordance with institutional guidelines for the humane treatment of animals using mice from the indicated strains. All animals were housed in a room lighted 12hours per day at an ambient temperature. Animals were allowed 1week to recover after arrival and had free access to rodent diet and tap water ad libitum until the initiation of the experiment.

2.4.2.1. Basal aldosterone in male and female wild type mice

All mice serum samples used for the validation of the aldosterone assay in small volumes of rodent serum or plasma were obtained from the Institute of Molecular Animal Breeding and Biotechnology, LMU. Mice were kept under standard (specified pathogen-free, SPF) conditions and had free access to rodent diet (V1534; ssniff, Soest, Germany) and tap water *ad libitum*. Serum samples were stored at -20°C in eppendorf tubes until the time of assessment.

In order to determine mean, baseline serum aldosterone concentrations in male and female wild type mice, serum samples from 75 mice (C57BL/6 x NMRI) aged between 3 and 11 weeks, were assessed using the A2E11 assay according to the standard procedure incorporating a 50µl volume (as described in the section above "Extraction of plasma – A2E11 assay**"**), and results were separated according to gender differences (Figure 39). Aldosterone concentrations were subsequently analyzed according to mice of different ages (at 3, 5, 7, 9 and 11 weeks) in order to determine differences occurring during development (Figure 40, Table 25). Statistical significances between the different age groups of mean male and female mice values shown in Table 26.

2.4.2.2. Suppression and stimulation of the HPA axis in mice

Serum samples were collected from twenty-one mice which were tested for stimulation with ACTH, dexamethasone suppression and a control group for comparison to baseline values. Seven mice (aged 11 weeks) were injected intraperitoneally (i.p.) for 10 consecutive days with a daily dose of 250 μl ACTH solution (Synacthen ®, Novartis Germany, 0.25 mg/ml). A second group (n = 7) received daily i.p. injections of 200 μl Dexamethasone solution (Vetoquinol, Firma, Germany; 4 mg/ml). A control group (n = 7) received 250 μl vehicle only per day (0.9 % v/v NaCl; B. Braun, Melsungen, Germany) i.p. On day 10, 3 hours after the last injection, mice were sacrificed and blood samples were collected in the afternoon. As above, serum samples were stored at -20°C in eppendorf tubes and were assessed using the A2E11 assay according to the standard procedure using 50μl (Figure 41).

2.4.2.3. Effect of an increased potassium diet on the adrenal RAAS

Mice serum samples used for the assessment of aldosterone levels in mice given a high or low potassium containing diet were collected from the Institut für Molekulare Medizin und Zellforschung, Universität, Freiburg. Twenty-six, 12 week-old male wild type mice (CD1), were divided into 3 groups and were fed for 5 weeks with a low potassium chow diet and distilled water in the low potassium group(n = 9), regular chow and distilled water in the control group (n = 10), and high potassium chow (141mg/kg Na^+, 11381mg/kg K^+, 179 mg/kg Cl^-) and distilled water containing 2% v/v KCl in the high potassium group (n = 7). Mice were anaesthetized using isoflurane before decapitation and subsequently trunk blood was collected and serum samples were transferred in aliquots to 1.5ml Eppendorf tubes containing 10μl 0.5M EDTA. Blood was spun down at 5000rpm for 10minutes at 4°C, and the supernatant (serum) was transferred to new 1.5ml Eppendorfs. Samples were stored at -20°C until the time of assessment.

Samples were measured using the A2E11 assay with a 50μl sample (Figure 42a), as well as the DPC RIA (Figure 42b).

2.5. Statistical analysis

For reasons of clarity in figures and text means ± SEM or ± SD are provided, as indicated. No assumptions were made about the probability distribution of the data sets and therefore non-parametric methods were applied. Comparison of two paired groups was carried out using the Wilcoxon signed-rank test, independent samples and comparison of two unpaired groups was tested using the Mann-Whitney U test, comparison of three or more unmatched groups was done by Kruskal-Wallis, and Spearmann rank correlations were used to quantify the association between two variables. Only results with $P < 0.05$ were considered statistically significant.

3. Results

For the chromatographic separation of the biotinylated aldosterone 3-CMO conjugate (1.6 mg/ml) from excess biotin and other educts which could potentially still be present in the final reaction solution we utilized two Reverse Phase HPLC columns and applied two different gradient systems in order to optimize the separation.

3.1. Reverse phase chromatographic purification of the aldosterone 3-CMO-biotin conjugate

3.1.1. Vertex-Säule Eurospher - Knauer Advanced Scientific Instruments

Chromatogram Knauer column - Run 1 (Figure 5): Fraction peaks collected and tested were peaks 93, 95, and 97, the highest peak fractions occurring between 70 and 80 minutes. Knauer column - Run 2 (Figure 6): Fraction peaks collected and tested were peaks 92/93, 94/95, and 95/96, the highest peak fractions occurring between 75 and 85 minutes. Fractions corresponding
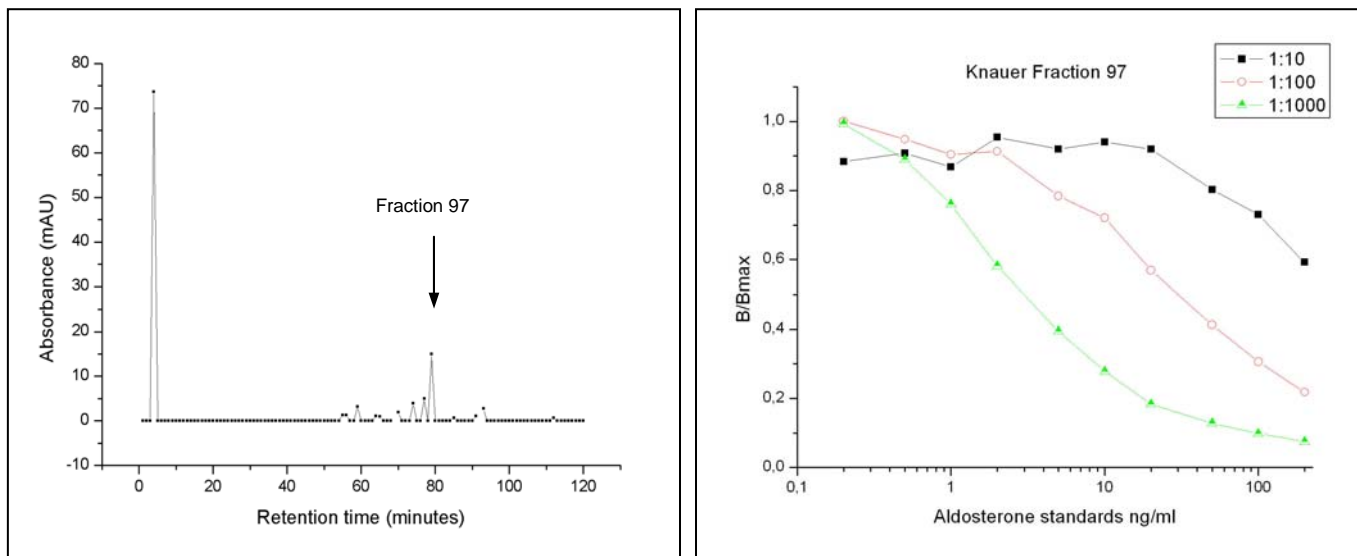
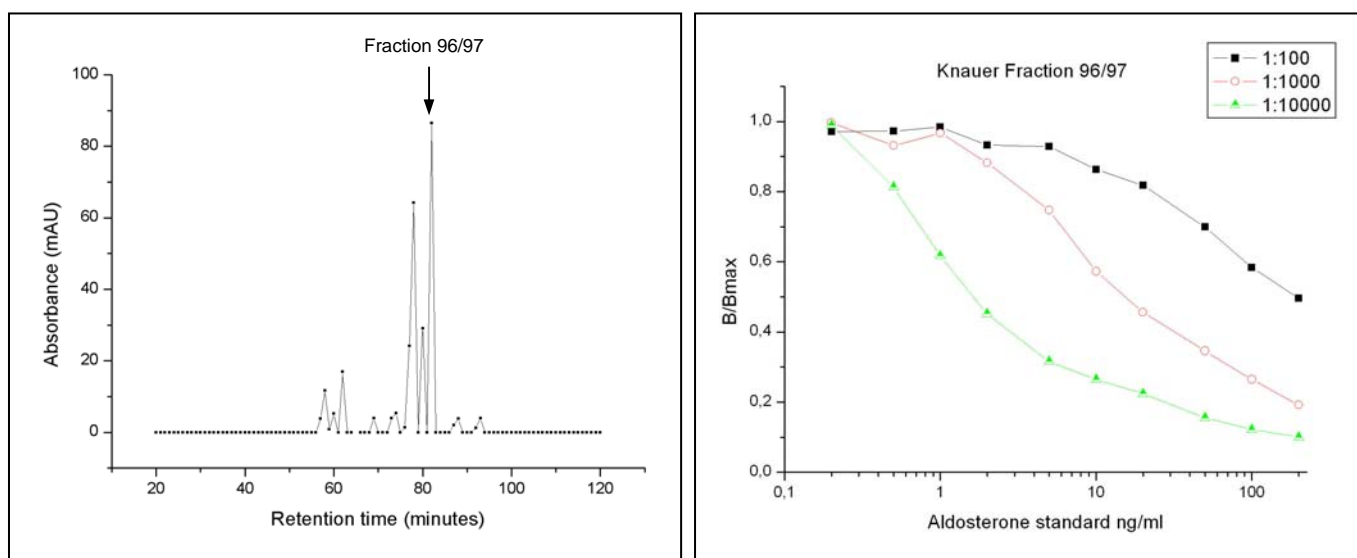**Figure 5.** Run 1 using the Knauer column with a linear gradient solvent change.

Figure 6. Run 2 using the Knauer column with a linear gradient solvent change.

to smaller peaks and to baseline fractions were also tested for both Knauer runs in order to ascertain if aldosterone is still present, or present in higher concentrations than that found in the highest peaks. Most other peaks observed in the chromatogram occurred previous to the three major ones seen at around 80 minutes. These smaller peaks contain more polar compounds that were eluted first from the column and are likely to correspond to other educts in the solution such as the excess biotin or the aldosterone ester created on day 1.

3.1.2. Synergi 4u Fusion-RP - Phenomenex

The two linear gradient runs using the Phenomenex column were carried out first (ex. Run 1, Figure 7). Both of these yielded two major peaks at around 50 and 35 minutes, peaks 68/69 and 70 in the first, and 67, 68, and 69 in the second. Two isocratic gradient runs were also carried out following to this (ex. Run 4, Figure 8). Major peaks were found at around 35 and 30 minutes respectively. For Run 3 these were peak fractions 47, 48/49, and 51/52, and for Run 4 fractions 35/36, 37, and 40. As with the Knauer column, most other peaks observed in the chromatogram occurred previous to these highest absorbance peaks and were assumed also to correspond to educts or possibly impurities present in the solution or the eluent buffers. Minor peaks and baseline fractions were again tested for aldosterone content.
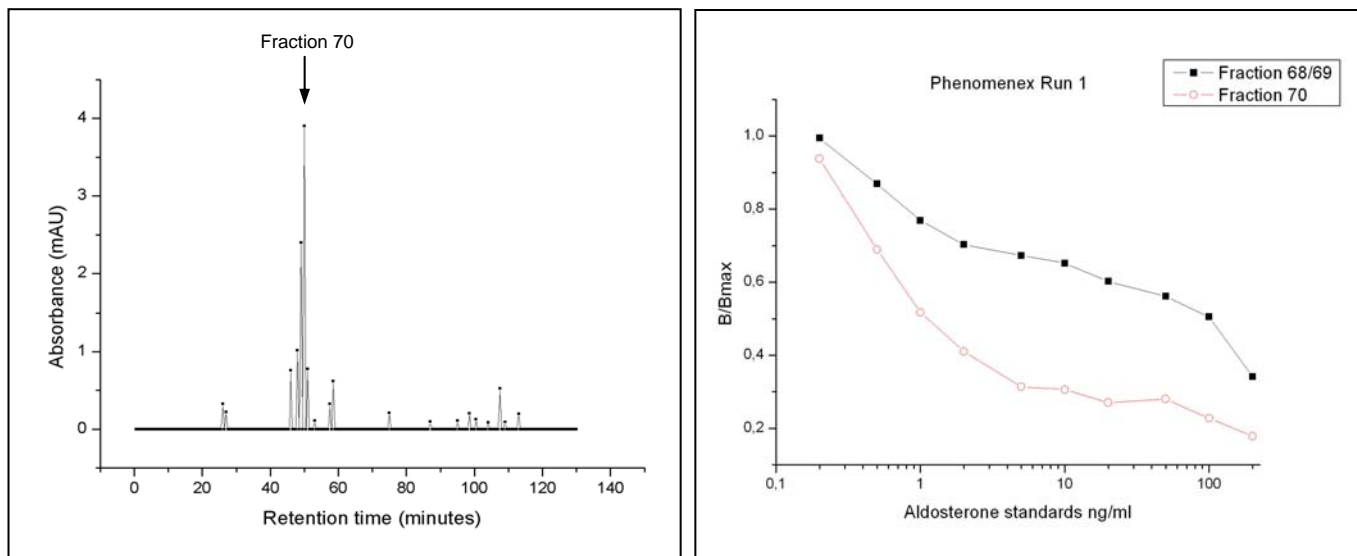
Figure 7. Run 1 using the Phenomenex column with a linear gradient solvent change. Fractions at 1:1000.

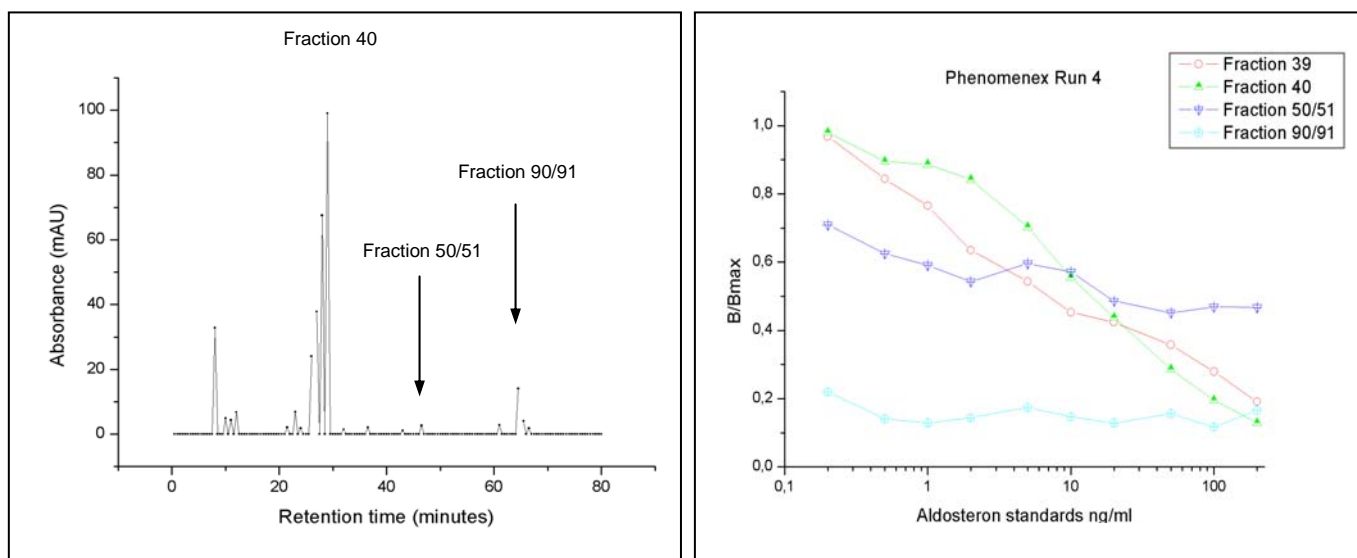**Figure 8.** Run 4 using the Phenomenex column with an isocratic gradient solvent change. Fractions at 1:1000.

3.2. Evaluation of Fractions

3.2.1. Aldosterone content with RIA MAIA Adaltis

The MAIA assay showed that peak fractions 97 and 94/95, 96/97 from Knauer Run 1 and 2 respectively, and peak fractions 68, 69 from Phenomenex Run 2, 48/49 and 51/52, Phenomenex

Run 3, and 40 from Phenomenex Run 4, had the highest aldosterone content (Table 4). After

dilution to obtain values within the working range of the assay, concentrations were extrapolated

to the 'true value' and found to range from 13 to 2190 ng/ml. Smaller peaks from all the runs

that were tested did not exceed 2000 pg/ml in each case, some baseline fractions contained

between 300 and 1200 pg/ml. Though some were tested for confirmation of a negative result,

most of these fractions were not tested further in assay runs due to the, at least thousand-fold,

difference in aldosterone content.

Summary of chromatography runs – fractions with highest Aldo content					
RP Column	Chromatography Gradient	Fraction	MAIA pg/ml	Max counts at 1:1000	pg/ well at 1:1000
Knauer	Linear; 0 - 100% B	Peak 93	12700	280325	1.3
Run 1	50mM Tris pH 7.8[a] - 86% MeOH[b]	Peak 95	154700	102074	15.5
		Peak 97	200000	82299	20.0
Knauer	Linear; 0 – 100% B	Peak 92/93	85400	296145	8.5
Run 2	50mM Tris pH 7.8 [a] - 86% MeOH[b]	Peak 94/95	325000	392651	32.5
		Peak 96/97	1253000	181214	125.3
Phenomenex	Linear; 0 – 100% B	Peak 68/69	36300	105901	3.6
Run 1	50mM Tris pH 7.8 [a] - 86% ACN[c]	Peak 70	15300	35786	1.5
Phenomenex	Linear; 0 – 100% B	Peak 67	145000	312060	14.5
Run 2	50mM Tris pH 7.8 [a] – 86% ACN[c]	Peak 68	1579000	368214	158.0
		Peak 69	1920000	314306	192.0
Phenomenex	Isocratic with step to 100% B	Peak 47	14710	253403	1.5
Run 3	33% (Tris pH 7.8 [a] - 86% ACN[c])	Peak 48/49	948000	363062	94.8
		Peak 51/52	1431000	115483	143.1
Phenomenex	Isocratic with step to 100% B	Peak 35/36	187000	296122	18.7
Run 4	33% (Tris pH 7.8 [a] - 86% ACN[c])	Peak 37	1255000	347389	125.5
		Peak 40	2190000	110442	219.0

Table 4. Aldosterone content and maximum counts of each fraction at a 1:1000 titration used as tracer in the immunoassay. [a] Buffer A for both linear and isocratic runs, 50mM Tris HCl at pH 7.8, [b] Buffer B for linear gradient runs, 86% Methanol/H_2O, [c] Buffer B for isocratic gradient runs 86% Acetonitrile/H_2O.

3.2.2. Fractions as assay tracer

To determine if the aldosterone present in the chosen fractions was coupled to biotin and functions as a tracer and to see if the displacement activity correlates with concentration of aldosterone given in the MAIA, fractions were added at several dilutions as tracer. Comparison of displacement curves obtained upon completion of the assay, showed that concentration of aldosterone present in the fraction did not always correlate to the maximum counts seen in the assay, possibly due to too much biotin still present in the fraction which produces extra background counts.

However, the displacement curves also showed that the aldosterone present in the highest peak fractions was in fact biotinylated and functions as tracer to displace aldosterone present in standards, with higher or lower sensitivity. Displacement was assessed by the B/Bmax relationship, corresponding to binding activity compared to total binding. Since a higher dilution than 1:1000, ie 1:10000, did not increase sensitivity of the tracer and decreased the total counts to lower than adequate this was not repeated for the Phenomenex run fractions. Knauer Run 1 fraction 97, Run 2 fraction 96/97, Phenomenex Run 1 fraction 70, Run 3 fraction 51/52, and Run 4 fraction 40 revealed the highest displacement down to 0.1 of maximum binding, Bmax.

Several smaller peaks and baseline fractions which were chosen randomly and added as tracer confirmed that they did not contain biotinylated aldosterone but a substance which was not in competition with the aldosterone in the standards and gave little or no displacement.

3.2.3. Direct comparison of activity at equal concentration

At 1:1000 the concentration of the tracer corresponded to different amounts for each fraction, some above and some below 100pg/well. For this reason it was necessary to have a direct comparison of all the fractions at the same concentration in order to find the fraction containing highest counts overall with the lowest background, non-specific binding. All fractions were therefore diluted to 20pg/well and total counts and maximum displacement were compared (Figure 9, Table 5).
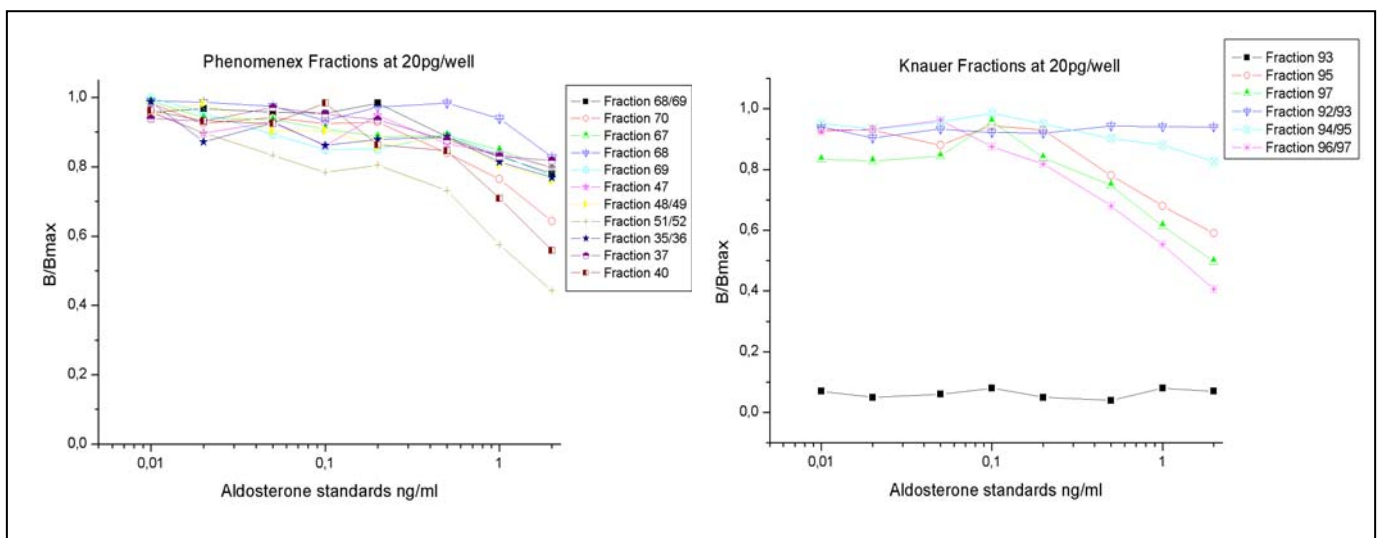

Figure 9. Direct comparison of all Knauer and Phenomenex fractions at 20pg/well.

Knauer fraction 97 and 96/97, from Runs 1 and 2, showed a high displacement but overall low counts at 20 pg/well. Other fractions, such as Knauer 94/95 and Phenomenex 48/49 for example, which had a high aldosterone content and high counts showed a poor displacement of only 12% and 20% respectively. Isocratic Phenomenex run fractions 51/52 and 40 had a high displacement of 54% and 46% which, though slightly lower than Knauer fraction 96/97, had total counts which were almost twice as high. Fractions 51/52 and 40 were more sensitive than other fractions, for example 68 and 69 from the linear gradient runs which displaced only 22% and 13% of the aldosterone present in the standards.

Displacement capability of tracer at equal concentrations				
RP Column	**Fraction**	**pg/well**	**Max counts**	**Maximum % displacement**
Knauer	Peak 93	20	197895	7
	Peak 95	20	105298	32
	Peak 97	20	61034	41
Knauer	Peak 92/93	20	188174	0
	Peak 94/95	20	214736	12
	Peak 96/97	20	57231	57
Phenomenex	Peak 68/69	20	143960	20
	Peak 70	20	72028	31
Phenomenex	Peak 67	20	224937	21
	Peak 68	20	185193	22
	Peak 69	20	118572	13
Phenomenex	Peak 47	20	291280	14
	Peak 48/49	20	228024	20
	Peak 51/52	20	90170	54
Phenomenex	Peak 35/36	20	259688	15
	Peak 37	20	225081	18
	Peak 40	20	111807	46

Table 5. Comparison of total counts and total displacement when fractions are added as tracer in the immunoassay at an equal concentration of 20pg/well.

3.2.4. Conditions of the assay

3.2.4.1. Optimisation of tracer concentration and incubation time between tracer and standards

A combination of extended incubation times with the tracer at 20 pg and a lower, 2pg, per well concentration was assessed using the fractions with highest sensitivity, Knauer fractions 97 and 96/97, and Phenomenex fractions 51/52 and 40 (Figures 10-12).
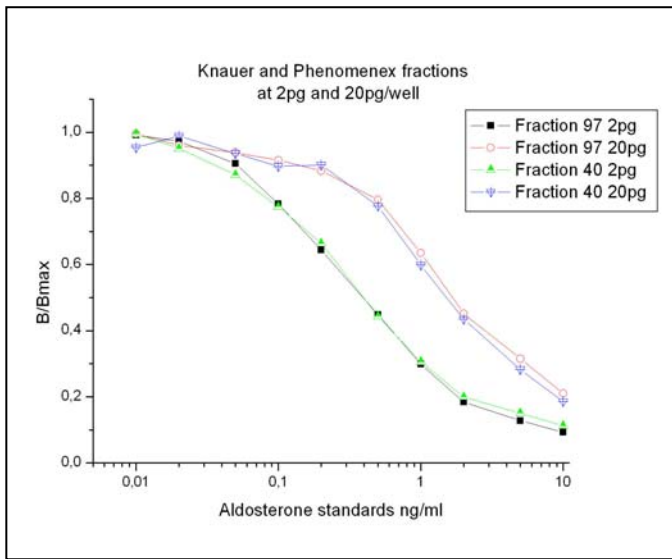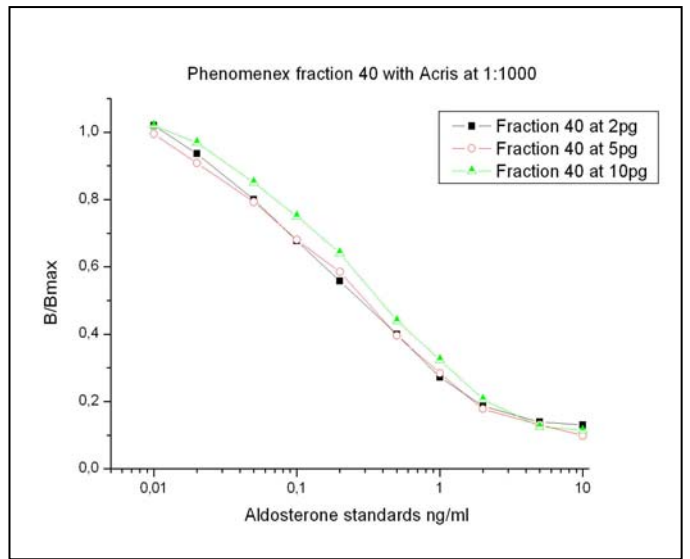
Figures 10. 48hour incubation of tracer fractions.

Figure 11. Overnight incubation and decreasing concentration of aldosterone tracer.
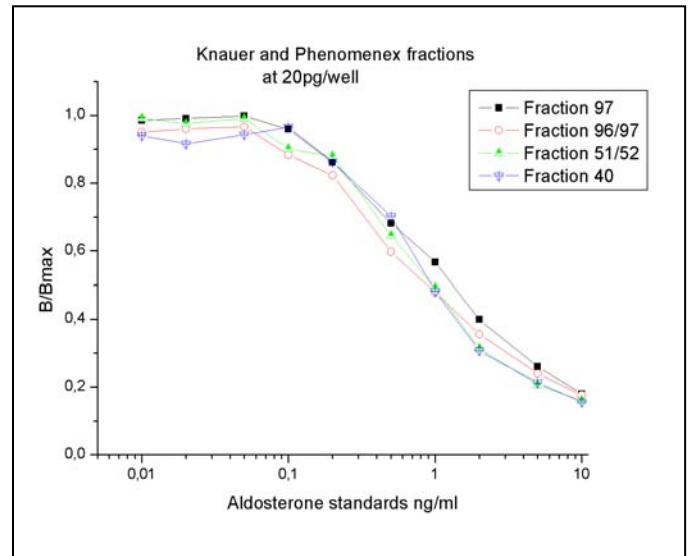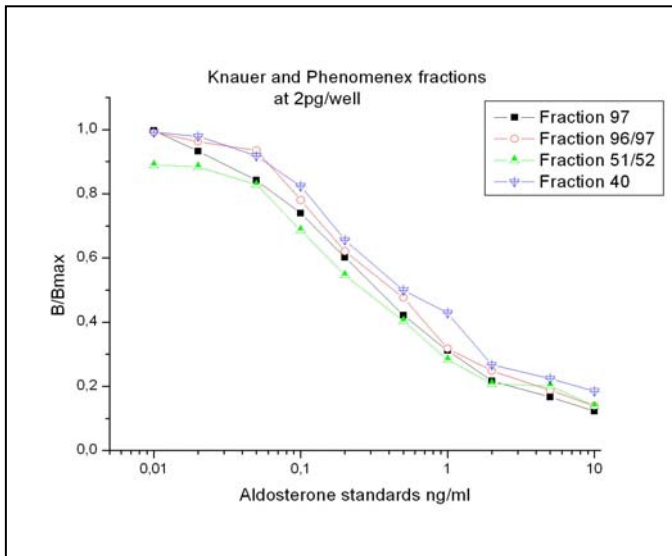

Figure 12. Overnight incubation of Knauer and Phenomenex fractions, Acris at 1:1000

Assay incubations times of 1.5 hours, overnight, and 48 hours were compared (Table 6). An overnight incubation compared to 1.5 hours significantly increased total counts by 20000. Although increasing the incubation time further, to 48 hours, did increase the displacement potency of the tracer by a small amount, this was not considered enough to justify the increased total assay time for practical reasons. From the same set of assays it became apparent that decreasing the concentration of the tracer from 20pg to 2pg/well increased the sensitivity of the tracer and increased total displacement from 67% to 75%, though there was an overall drop in counts of 22000, which could be compensated for by increasing the incubation time.

Effect of increasing incubation time			
	Max counts	ED_{50} (pg/ml)	Maximum % displacement
1.5 hours	14000	321	81.3
Overnight	37000	505	81.5
48 hours	38000	379	88.7

Table 6. Displacement capability of tracer (Phenomenex fraction 40) at 2pg/well and Acris at 1:1000, with increasing incubation times.

3.2.4.2. Optimising final concentration of polyclonal capture antibody and tracer.

Comparison of an increased concentration of the Acris antibody from 1:1000 to 1:100 showed that total counts did not increase overall, with the added disadvantage that the ED_{50} also increased from 200pg/ml to 470pg/ml. Total counts decreased drastically to 13000 upon further dilution of the capture antibody down to 1:5000, with a concurrent decrease in the total displacement. Data is summarised in Table 7.

Effect of varying concentration of capture Ab			
	Max counts	ED_{50} (pg/ml)	Maximum % displacement
1:100	21500	465	85.4
1:1000	22000	201	86.9
1:5000	13000	149	76.9

Table 7. Displacement capability of tracer (Phenomenex fraction 40) at 2pg/well and an overnight incubation, at a higher, 1:5000, and lower, 1:100, titration of the Acris capture antibody.

Following this set of experiments it was decided to utilize Phenomenex fraction 40, as opposed to fraction 51/52 or Knauer fraction 97, although it had equal displacement capability, because fraction 40 had a higher concentration of aldosterone and could be used at a lower dilution. Therefore, further dilution of the capture antibody was combined with increasing concentrations of tracer fraction 40 to 5 and 10 pg/well to see if this would increase its sensitivity. Increased counts of 43000 and 56000 were seen at 5 and 10 pg/well respectively. At 5 pg/well ED_{50}'s were almost identical at 255 and 273 pg/ml, whereas there was a significant increase when the tracer was used at 10 pg/well to 390. The fact that at 5 pg/well the tracer showed practically the same displacement capability as at 2 pg/well but had significantly higher counts, meant that it was chosen as the optimum tracer concentration (Figure 11).

3.2.4.3. Optimising final concentration of monoclonal capture antibody

The monoclonal anti-aldosterone, A2E11, antibody was tested at a series of dilutions in order to determine the optimum concentration producing highest counts and highest displacement capability of the tracer used in the final assay procedure (Figure 13a and b, Table 8). Maximum displacement was reached only after a 1:10000 dilution but then remained the same up to 1:30000. Further diluting the antibody up to 1:50000 decreased the ED_{50} although not significantly and furthermore, this occurred at the expense of the decreasing counts and a decreased total displacement.
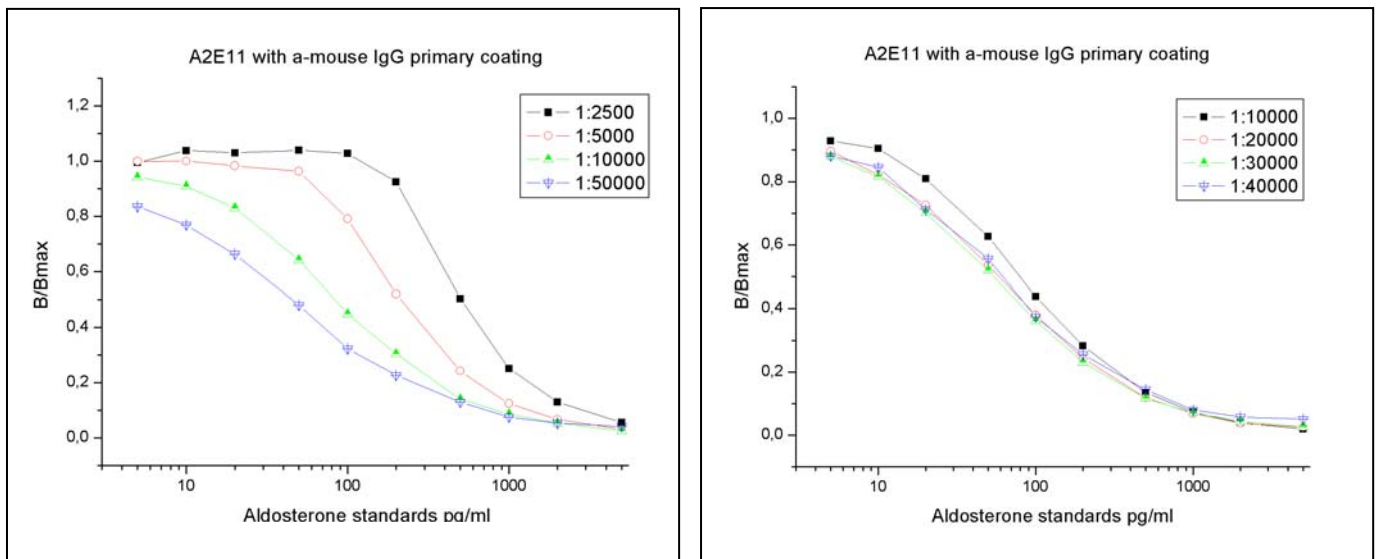
Figure 13a and b. Monoclonal antibody A2E11 at increased dilutions with Phenomenex fraction 40 at 5pg/well.

A2E11 with primary coating anti-mouse IgG			
Dilution factor	Max Counts	ED_{50} (pg/ml)	Maximum % displacement
1:2500	139593	494	94.4
1:5000	134772	212	96.8
1:10000	119566	88	97.4
1:20000	66119	58	97.4
1:30000	46646	55	97.4
1:40000	35266	61	94.9
1:50000	34386	46	95.8

Table 8. Optimisation of concentration of coating monoclonal capture antibody A2E11. Tracer used was Phenomenex fraction 40 at 2pg/well and an overnight incubation.

3.3. Assay validation

3.3.1. Cross-reactivities

3.3.1.1. Polyclonal rabbit anti-aldosterone (Acris)

Minimal cross-reactivity was found overall, also for substances present in high concentrations in the plasma such as cortisol (Table 9). The highest cross-reactivity, 0.01%, was found in the closest chemically related steroid, 18-OH Corticosterone. Cross-reactivity was also low for synthetic steroids, with the highest occurring in prednisolone, 0.009%. The aldosterone receptor antagonist spironolactone had a displacing potency of 0.002%.

Endogenous Steroids	% Cross-reactivity	Synthetic Steroids	% Cross-reactivity
Androstendione	0.003	Cyproterone Acetate	0.002
Corticosterone	0.006	Dexamethasone	0.001
18-OH-Corticosterone	0.01	Ethinyloestradiol	ND
Cortisol	0.004	Fludrocortisone Acetate	0.003
Deoxycorticosterone	0.003	Fluorometholone	ND
Deoxycortisol	0.001	Medroxyprogesterone	0.002
DHEA	ND[a]	Norethisterone Acetate	0.003
Estradiol	ND	Norgestrel	ND
Estrone	ND	Prednisolone	0.009
Pregnenolone	ND	Spironolactone	0.002
17-OH-Pregnenolone	ND		
Progesterone	0.005		
17-OH-Progesterone	0.001		
Testosterone	0.002		

ND[a] - not detectable

Table 9. Cross-reactivity – Acris antibody

3.3.1.2. Monoclonal anti-aldosterone antibody (A2E11)

The monoclonal antibody against aldosterone used in the immunoassay was prepared as previously described (Gomez-Sanchez et al., 1987). Cross-reactivities for cortisol and

corticosterone were found by those authors to be at <0.003% and were at <0.3% for other potentially cross-reacting steroids (Table 10). Highest % cross-reactivity was for the aldosterone metabolite tetrahydroaldosterone, found at 2.78%, 3.15%, and 5.0% with each of the RIA tracer separation methods but fortunately this is a metabolite which does not occur in saliva.

% Cross reactivity by tracer and separation method			
Endogenous Steroid	^{3}H AS[a]	^{3}H Ch[b]	^{125}I Ch[c]
3α,5β - THaldosterone	2.78	3.15	5.0
18-Oxocortisol	0.083	0.1	0.1
Cortisol	<0.0025	<0.003	<0.003
Corticosterone	<0.0025	<0.003	<0.003
DOC	<0.0025	<0.003	<0.003
Progesterone	<0.0025	<0.003	<0.003
18-OH- DOC	0.065	0.07	0.27
18-OH-Corticosterone	0.089	0.12	0.1
18-OH-Cortisol	<0.0025	<0.003	<0.003
Cortisone	<0.0025	<0.003	<0.003

Table 10. Cross-reactivity – A2E11 antibody (table reproduced from Gomez et al 1987)
[a]tritium label, ammonium sulphate separation; [b]tritium label, charcoal separation; [c]iodinated aldosterone-3-CMO-histamine, charcoal separation.

3.3.2. Sensitivity

3.3.2.1. Polyclonal rabbit anti-aldosterone (Acris*)*

The zero standard was measured in a typical assay a total of 30 times and the mean (± standard deviation; SD) value of the counts was calculated (39996 ± 1063). A statistically significant difference from the zero standard was taken to be more than 2 times the standard deviation from this repeated mean (ie. 39996 – 2128 = 37868 counts). This value was thereafter determined from its intercept with the displacement curve and found to correspond to 19pg/ml for a 50µl sample (Figure 14).

3.3.2.2. Monoclonal anti-aldosterone antibody (A2E11)

The zero standard was again measured in a typical assay a total of 20 times and the mean (±SD) value of the counts was calculated (50791 ± 3444). More than 2 times the standard deviation from this repeated mean (i.e. 43903 counts) was thereafter determined from its intercept with the displacement curve to correspond to 8 pg/ml (Figure 15).
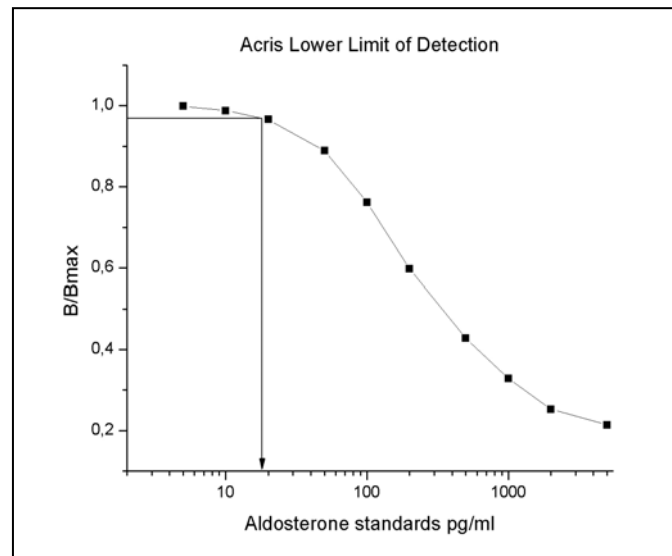

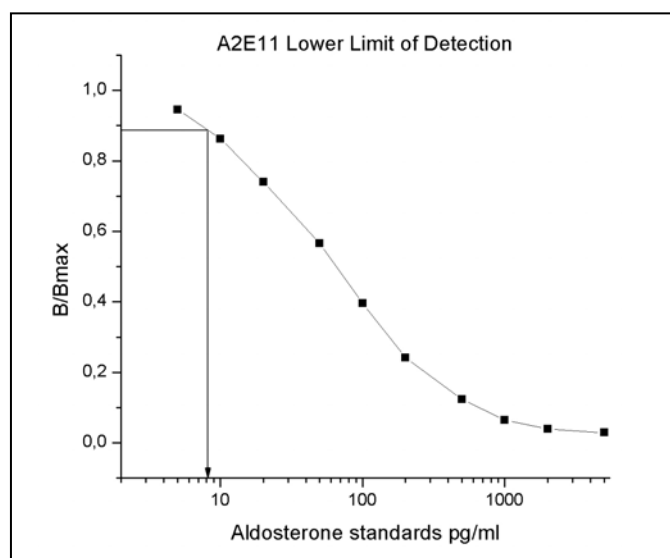**Figure 14.** Sensitivity – Limit of detection for the Acris antibody assay.

Figure 15. Sensitivity – Limit of Detection for the A2E11 antibody assay.

3.3.3. Linearity

3.3.3.1. Polyclonal rabbit anti-aldosterone (Acris*)*

Four samples of pooled saliva were spiked with pure aldosterone previously diluted in artificial saliva to a concentration of 1000pg/ml, and measured at 1355, 1815, 1628, and 1496pg/ml. After serial dilution of the 'spiked' pools with artificial saliva the values obtained upon measurement with the assay were compared to the expected calculated concentration (Table 11) and a mean recovery of 106.3% was found, showing that there is good linearity of diluted samples.

3.3.3.2. Monoclonal anti-aldosterone antibody (A2E11)

Measurement of a plasma sample after a 1:2 dilution in the matrix medium used to produce the calibrators yielded a concentration of 963 pg/ml. After further serial dilutions (1:4, 1:8, 1:16, 1:32) this sample had an average recovery rate of 105% (Table 12).

	Dilution factor	Measured value	Expected value	(%) Expected value
	original sample	>std	--	--
	1:2	963	--	100
Dilution in 0 std	1:4	509	481.5	106
	1:8	272	240.8	113
	1:16	125	120.4	104
	1:32	62	60.2	103
Avg % expected				**105**

Table 12. Linearity A2E11 assay - plasma sample serially diluted in the "0" standard after extraction.

	Dilution Factor	Measured value	Calculated value	% Expected value
Spiked Pool A	—	1355	—	100
	1:2	655	678	96.8
	1:4	332	339	98.1
	1:8	156	169	92.3
	1:16	94	85	111
	1:32	51	42	121.5
Mean %				**104**
Spiked Pool B	—	1815	—	100
	1:2	874	908	96.4
	1:4	521	454	114.9
	1:8	247	227	109
	1:16	120	113	106.3
	1:32	73	57	129.3
Mean %				**111**
Spiked Pool C	—	1628	—	100
	1:2	774	814	95.1
	1:4	362	407	88.8
	1:8	219	204	107.8
	1:16	112	102	109.8
	1:32	67	51	132
Mean %				**107**
Spiked Pool D	—	1496	—	100
	1:2	695	748	92.9
	1:4	368	374	98.4
	1:8	195	187	104.2
	1:16	104	94	110.9
	1:32	51	47	109.9
Mean %				**103**

Table 11. Linearity – Acris Antibody. Saliva sample serially diluted in the "0" standard after extraction.

3.3.4. Recovery

3.3.4.1. Polyclonal rabbit anti-aldosterone (Acris*)*

A saliva sample with an average concentration, after four consecutive measurements, of 443pg/ml, was diluted at 1:1, with each of the standards 0 to 2000pg/ml and the mean recovery was 100.9% (Table 13).

	Aldosterone added (pg/ml)	Measured value	Calculated value	% Expected value
Spiked Pool	_	444	_	100
	0	222	221.8	100.2
	10	237	226.8	104.6
	20	230	231.8	99.4
	50	277	246.8	112.4
	100	289	271.8	106.1
	200	317	321.8	98.4
	500	445	471.8	94.3
	1000	668	721.8	92.6
	2000	1219	1221.8	99.7
Avg % expected				**101**

Table 13. Recovery – Acris antibody. Each of the aldosterone standards were added to a saliva sample, which was previously spiked to 444 pg/ml, at a 1:1 ratio.

3.3.4.2. Monoclonal anti-aldosterone antibody (A2E11)

Two plasma samples, measured at 29 pg/ml and 20 pg/ml, were each extracted and reconstituted a total of nine times and each of the standard points was then added at a 1:2 dilution to the sample. Average recovery for each sample was found at 102% and 113% (Table 14).

1:1 Dilution with std (pg/ml)	Measured value	Expected value	% Expected value
Sample A	29	--	100
+ 5	20	17	117.6
+ 10	20	19.5	104.1
+ 20	27	24.5	108.6
+ 50	46	39.5	115.4
+ 100	65	64.5	100.3
+ 200	119	114.5	103.5
+ 500	239	264.5	90.4
+ 1000	476	614.5	77.5
+ 2000	1053	1014.5	103.8
Avg % expected			**102**

Table 14. Recovery – A2E11 antibody. Recovery of samples after extraction and spiked at a 1:1 dilution with each of the standard points.

3.3.5. Precision

3.3.5.1. Polyclonal rabbit anti-aldosterone (Acris*)*

The intra-assay coefficients of variation after a 10-fold determination for aldosterone concentrations of low sample pools 69 and 47, intermediate samples 124 and 94, and high samples 215 and 210pg/ml before and after extraction respectively, were 14.2 and 13.8, 10.1 and 9.7, and 7.2 and 6.0% respectively (Table 15). The inter-assay coefficients of variation were calculated by a 20-fold measurement of pooled saliva samples with aldosterone concentrations of 31, 33, 92, 94, 254 and 189 pg/ml and were determined at 35.1, 15.7, 16.3, 9.0, 14.2 and 8.7% respectively (Table 16).

	no extraction	after extraction		no extraction	after extraction		no extraction	after extraction
	204	205		125	93		82	56
	205	198		134	82		77	46
	200	213		134	85		69	51
	243	195		117	89		61	56
High Samples Pool 1	228	229	**Intrmd Samples Pool 1**	104	87	**Low Samples Pool 1**	78	40
	222	225		127	105		70	37
	228	208		108	100		56	48
	215	217		116	90		67	48
	210	218		145	97		54	50
	193	193		127	110		79	40
Mean	214.8	210.0		123.6	93.7		69.4	47.3
std dev	15.5	12.5		12.5	9.1		9.8	6.5
%CV	**7.2**	**6.0**		**10.1**	**9.7**		**14.2**	**13.8**

Table 15. Within-assay CV for Acris; validation before and after extraction of saliva samples with DCM/PEG (duplicate values).

	High Samples Pool 1	Intmd Samples Pool 1	Low Samples Pool 1
	253	80	29
	219	89	29
	284	97	43
	277	94	33
	247	104	31
	229	94	36
	266	105	36
	266	97	38
	228	82	31
	247	95	35
mean	251.4	93.5	32.5
std dev	21.9	8.4	5.1
%CV	**8.7**	**9.0**	**15.7**

Table 16. Between-assay CV for Acris antibody assay. Saliva samples without extraction (duplicate values).

3.3.5.2. Monoclonal anti-aldosterone antibody (A2E11)

Intra-assay coefficients of variation were determined by 20-fold measurements of pooled plasma samples with aldosterone concentrations of 18 pg/ml, 34 pg/ml, and 139 pg/ml which were extracted, reconstituted, pooled again and then added as samples on the same plate and found to be 7.3%, 6.3%, and 4.4%, respectively (Table 17). Inter-assay coefficients of variation were determined by 15-fold measurements, on consecutive days, of plasma samples of 14 pg/ml, 37 pg/ml, and 161 pg/ml and were found to be 15.2%, 15.1%, and 8.0 % respectively (Table 18). Non-extracted saliva samples which were measured at 9, 18, and 89 pg/ml had inter-assay coefficients of variation at 27.6, 14.4, and 11.4% respectively (Table 19).

	High Pool	Medium Pool	Low Pool
	144	34	19
	146	37	22
	138	34	19
	141	32	17
	145	33	18
	128	33	17
	134	35	16
	132	34	17
	134	33	19
	132	34	18
mean	138.8	33.5	18.1
std dev	6.1	2.1	1.3
%CV	**4.4**	**6.3**	**7.3**

Table 17. Intra-assay CV for A2E11 antibody on extracted plasma samples (duplicate values).

	High Pool	Medium Pool	Low Pool
	155	32	16
	168	28	15
	151	33	10
	141	37	11
	163	37	16
	170	38	14
	162	38	14
	170	43	18
	157	46	12
	152	45	14
	174	32	13
	145	43	14
	161	41	14
	152	35	12
	192	29	16
mean	160.8	37.1	13.9
std dev	12.9	5.6	2.1
%CV	**8.0**	**15.1**	**15.2**

Table 18. Inter-assay CV for A2E11 antibody on extracted plasma samples (singlicate values)

	High Samples Pool	Medium Samples Pool	Low Samples Pool
	84	16	9
	90	20	9
	91	17	9
	84	15	7
	72	17	11
	72	17	7
	104	20	12
	87	18	9
	100	15	8
	104	15	11
	83	23	9
	101	22	11
	88	18	7
	93	18	12
	84	21	9
mean	89.1	18.1	9.3
std dev	10.2	2.6	1.7
%CV	**11.4**	**14.4**	**18.4**

Table 19. Inter-assay CV for A2E11 antibody on non-extracted saliva samples (singlicate values).

3.4. Salivary aldosterone determination with and without the Sarstedt salivette

Samples of saliva which were collected without the use of the Sarstedt salivette had aldosterone levels which were highly correlated to those obtained by using the device (y = 1.0044x – 2.28; $R^2 = 0.9057$).

3.5. Pre-analytical storage stability of saliva samples

Aldosterone was stable if the saliva was kept in the original Sarstedt Salivette after both storage at room temperature as well as at 4°C for up to 7 days (Figure 16). In one of the saliva samples stored at ambient temperature within the salivette aldosterone showed a tendency towards increased values after 7 days, the study was not continued further after this point. 4°C would therefore be recommended when storing for longer periods. Saliva aliquoted and stored at 4°C in the eppendorf cups was also stable for up to one week. The salivary aldosterone levels were not stable however, when stored in eppendorfs for one week at room temperature. Increased values were seen as soon as after 3 days for two of the samples (subject 4 and 5) while for the remaining samples the increase was clear at 7days.
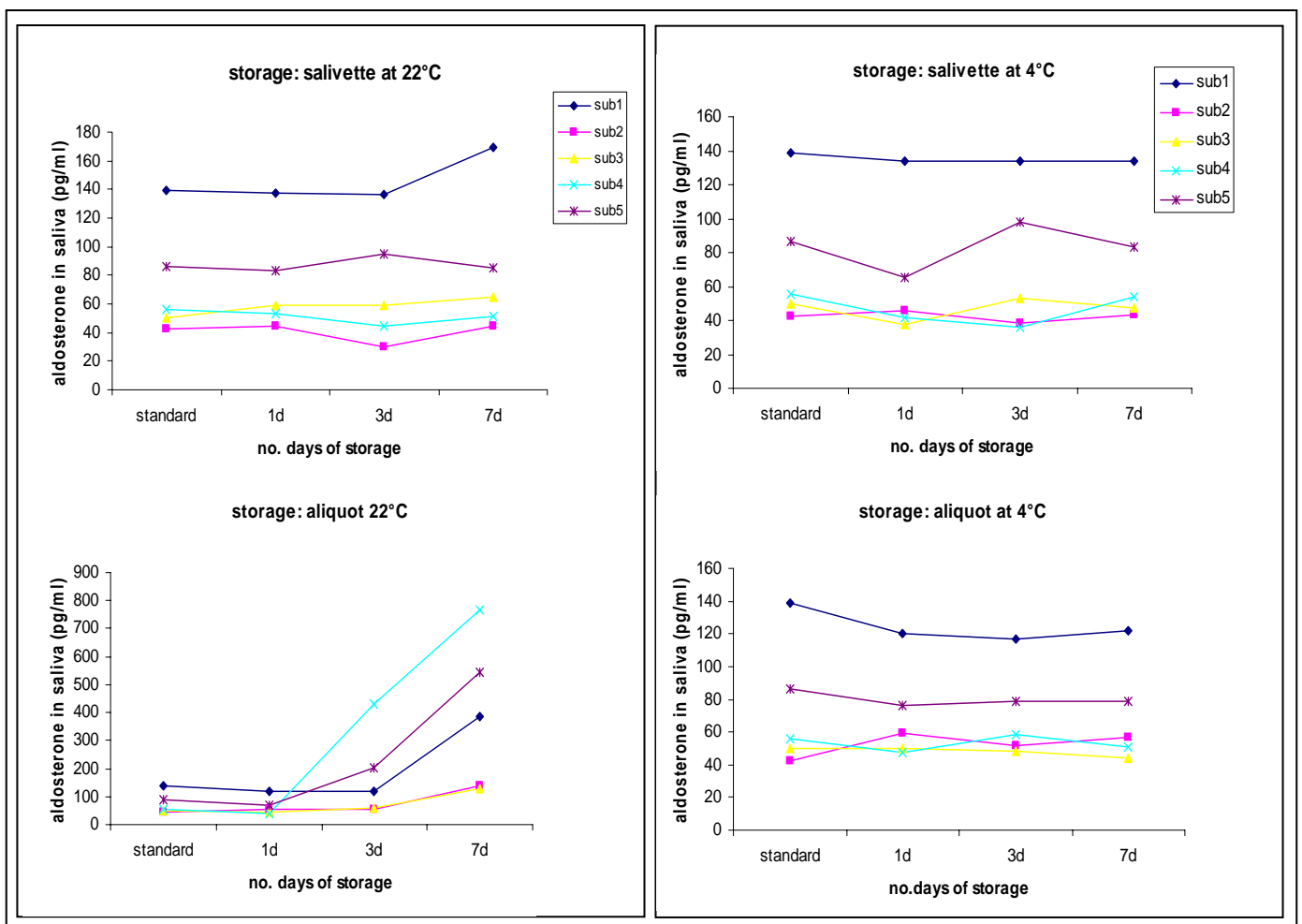
Figure 16. Stability of aldosterone in saliva, measured using the Acris assay, after storage in salivettes at room temperature and at 4°C, and after aliquoting and storage in eppendorf cups at room temperature and at 4°C.

3.6. Correlation of calibrators with commercially available RIA

In-house aldosterone calibrators (0 to 2000pg/ml) were added in two separate runs as samples and measured with the DPC Biermann (Coat-A-Count) radioimmunoassay and mean values were taken. Though the correlation between the two assays was very high, the in-house calibrators were measured by the DPC assay at approximately one third the value assigned in-house and therefore the first four calibrators (5 to 50 pg/ml) were not measurable in the linear range of the DPC assay ($y = 0.27x$, $R^2 = 0.98$; Figure 17, Table 20).
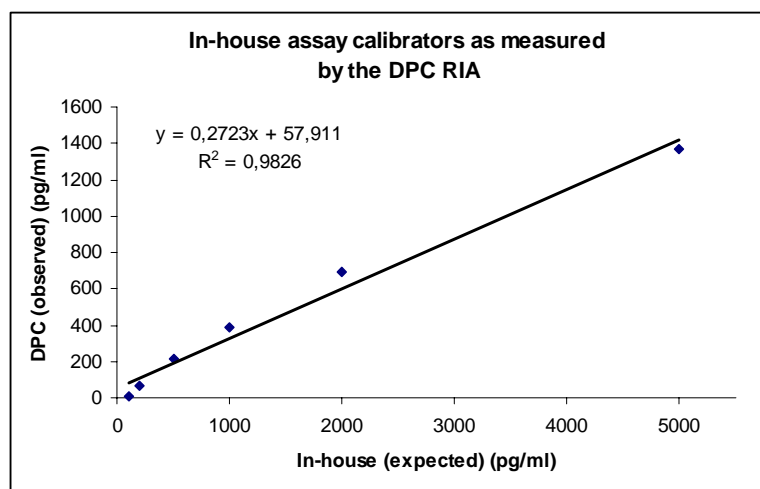
Figure 17. In-house aldosterone standards used for the Acris assay as observed by the DPC RIA kit when added as samples and measured in a typical assay.

In-House Std Curve	DPC measurement		
	Run 1	Run 2	mean
0	out	out	out
5	out	out	out
10	out	out	out
20	out	out	Out
50	out	out	Out
100	11.6	4.5	8.1
200	52.9	75.2	64.1
500	213.6	221.4	217.5
1000	388.6	384.4	386.5
2000	694.6	696.3	695.5

Table 20. DPC assessment of in-house Acris assay aldosterone calibrators.

Measurement of the DPC calibrators in the Acris assay corresponded to a factor of almost two times higher than the value given by the manufacturer ($y = 1.73x + 6.9$, $R^2 = 0.981$; Figure 18, Table 21), and after extraction of those calibrators using dichloromethane there was a decrease in the correlation to 0.71 ($y = 0.71x – 14.0$, $R^2 = 0.989$), where y = in-house assessment of the x DPC given concentration.
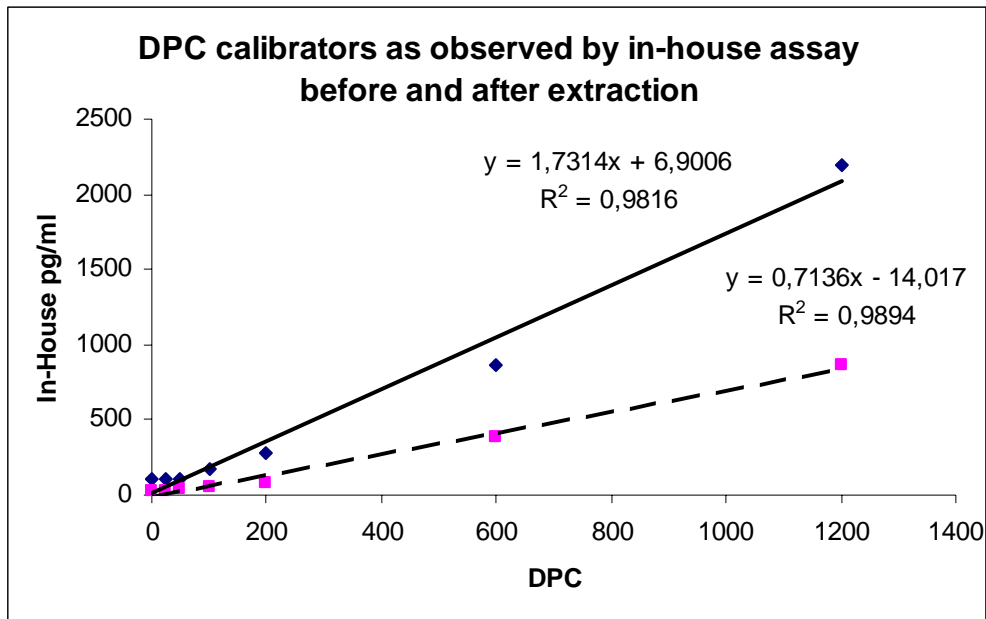
Figure 18. Correlation of DPC calibrators as measured by the in-house Acris assay before and after extraction (solid line=no extraction, dotted line=after extraction).

	DPC calibrators (pg/ml)	In-house assay (pg/ml)	In-house assay after extraction (pg/ml)
A	0	112	23
B	25	104	23
C	50	101	35
D	100	173	48
E	200	275	75
F	600	859	382
G	1200	2190	867

Table 21. DPC calibrators as measured by the in-house Acris assay before and after extraction.

Extraction and assessment of the calibrators using the A2E11 assay revealed a measurement by the in-house assay at around 60% that stated by DPC, with the relationship approaching 1:1 at higher concentrations due to the fact that concentrations lie within the linear portion of the curve in both assays (Figure 19, Table 22).

DPC calibrators as observed by in-house assay after extraction

$y = 0{,}9932x - 21{,}707$
$R^2 = 0{,}9814$

(In-House pg/ml vs DPC)
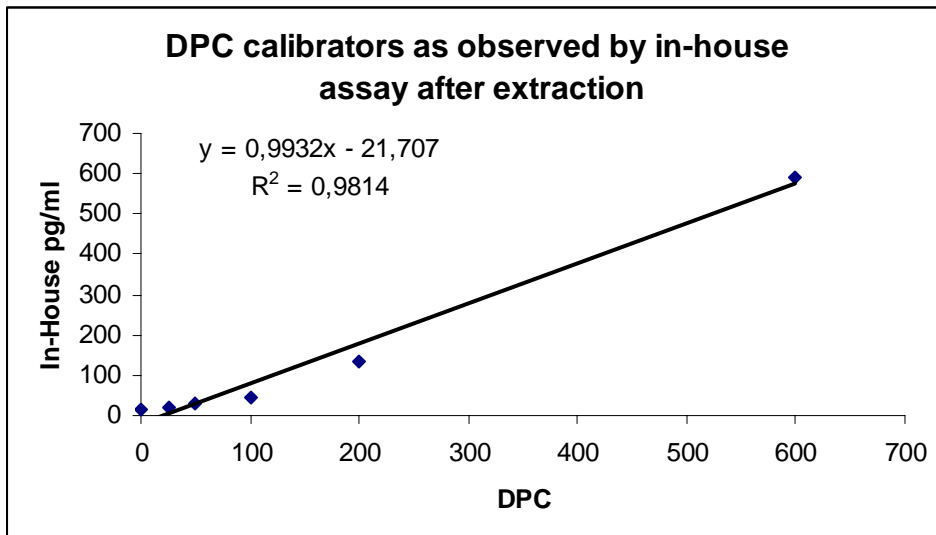
Figure 19. Correlation of DPC calibrators as measured by the in-house A2E11 monoclonal antibody assay after extraction (calibrator G not included as not in linear range of in-house assay, see Figure 24).

	DPC calibrators (pg/ml)	In-House assay after extraction (pg/ml)
A	0	14
B	25	21
C	50	31
D	100	44
E	200	135
F	600	593
G	1200	665

Table 22. DPC calibrators as measured by the in-house A2E11 assay after extraction.

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3.7. Extraction of saliva and plasma– Acris assay

3.7.1. Salivary aldosterone

After evaluation of 188 saliva samples before and after extraction, results following extraction were highly correlated at around 80% of aldosterone measured without pre-treatment (y = 0.7877x, R^2 = 0.75; Figure 20a, b, c, and d). Due to this high correlation and in order to minimize inter-assay CV's it was decided not to include the extraction procedure apart from situations where there were suspiciously high samples. Peaks at around 10am and 2pm in the PA patient are due to testing carried out as part of a study and corresponded to a physiological increase in the first case and an increase in response to ACTH stimulation in the second. There is an increase around mid-afternoon in both of the healthy participants which has also previously been reported by other authors and may be in response to normal daily rhythms. Levels do not differ overall between the healthy participants and the suspected aldosteronism patient in this case.

3.7.2. Plasma aldosterone

Plasma samples tested for aldosterone content using the Acris assay with incorporation of the extraction method and comparison with values given by the DPC assay showed that there was an excellent correlation between the two assays (y = 1.007x + 16.236, R^2 = 0.85, n = 46, Figure 21 and 22). Using a reduced volume of 100μl, with assay values multiplied by a factor of 2, yielded aldosterone values that were at around 70% the value obtained by the in-house assay after extraction of 200μl and at approximately 80% those of the DPC RIA (Figure 23a and b). Though recovery was not complete the values still remained highly correlated (y = 0.7049x + 2.689 and y = 0,7942x -7.2087, R^2 = 0.91 and 0.88, respectively; Figure 24a and b).
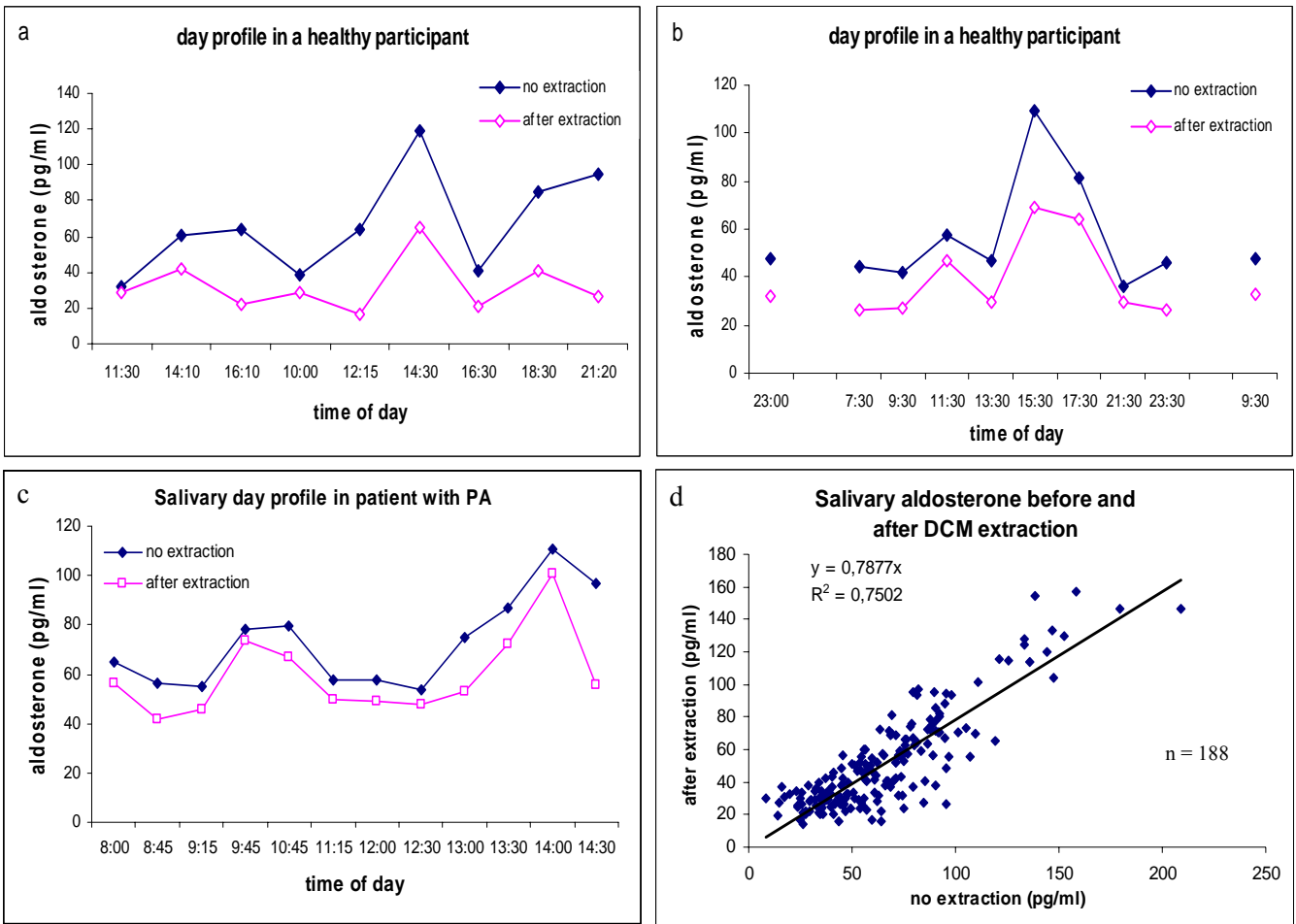
Figure 20. a, b and c. Daily saliva aldosterone profiles in two healthy participants and one suspected PA patient before and after extraction (200μl) as measured by the Acris in-house assay. **d.** Correlation before and after extraction of saliva samples as measured by the Acris assay.
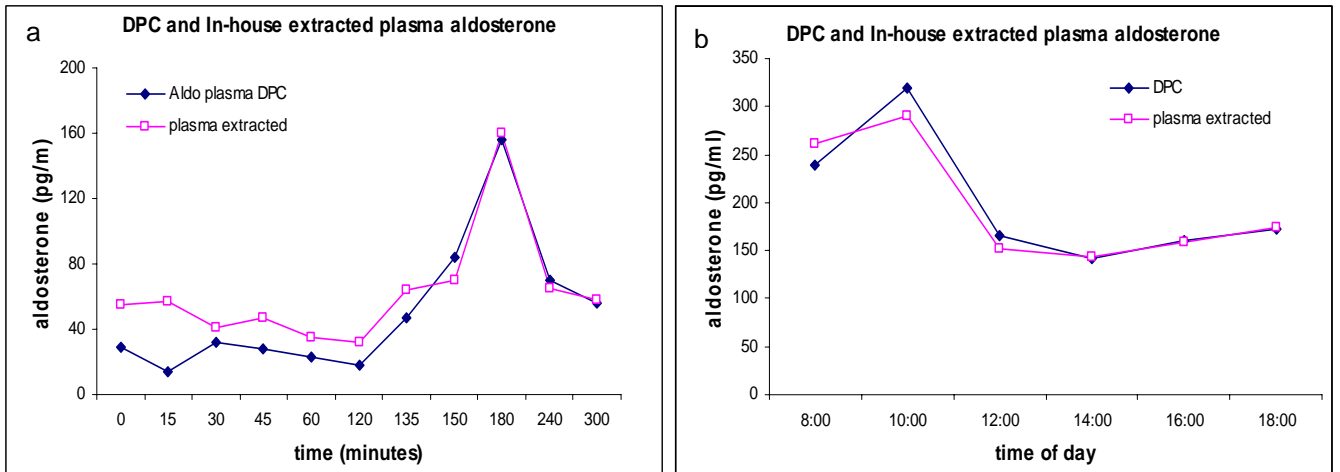
Figure 21. Daily plasma aldosterone profiles in healthy participants after extraction (200μl) as measured by Acris in-house assay compared to DPC assay.
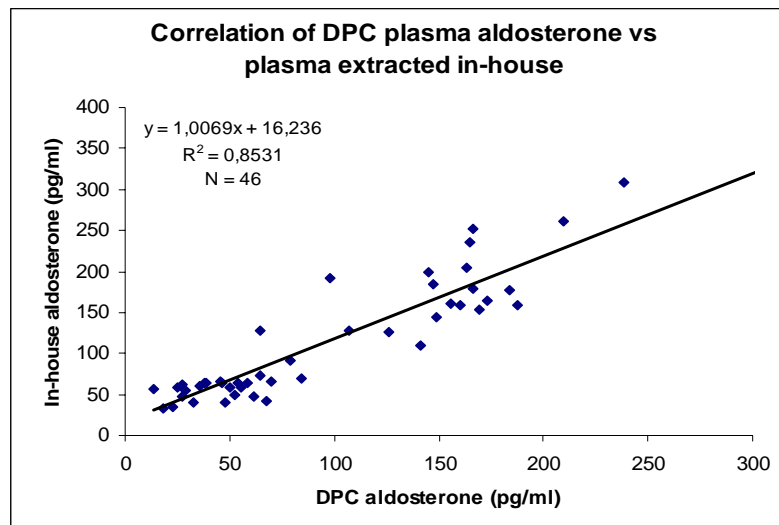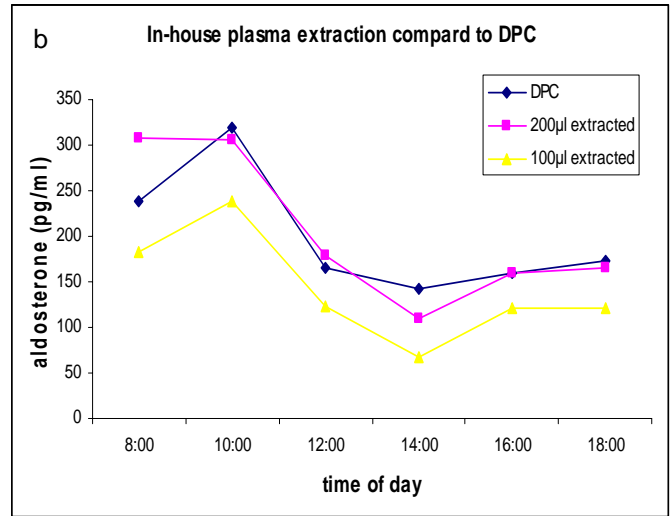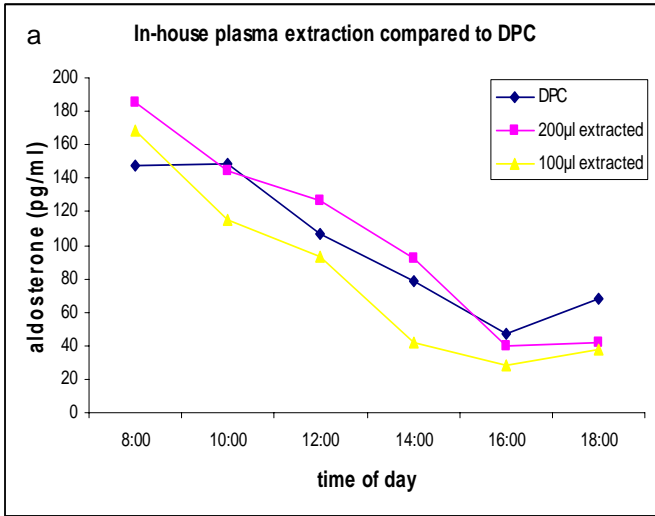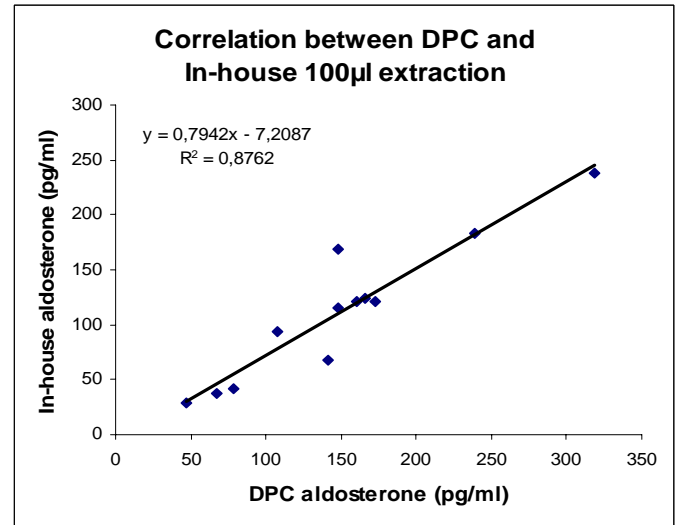

Figure 22. Correlation of plasma samples as measured by the Acris assay after extraction of 200μl of plasma and the DPC assay which incorporates no extraction step.
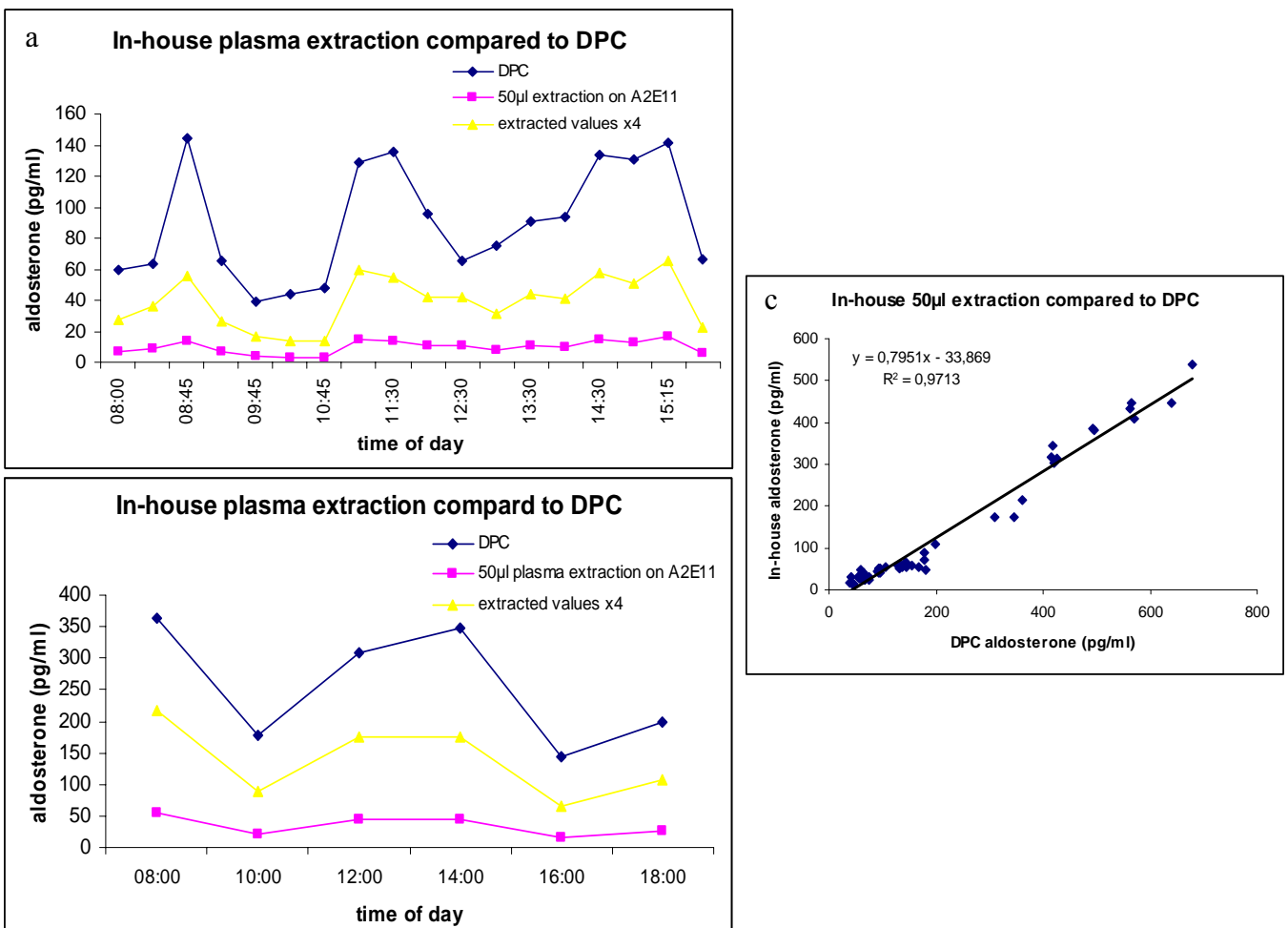
Figures 23a and b. Reduced volume plasma extraction for measurement in the Acris in-house assay compared to measurement by the DPC in two healthy participants.
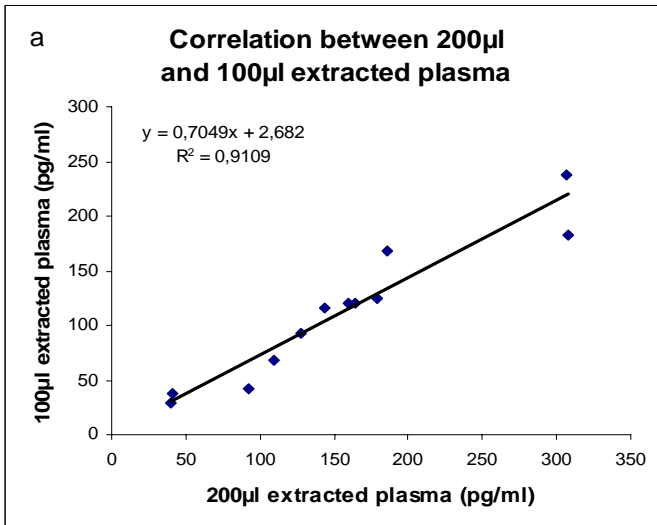
Figures 24a and b. a) Correlation between the standard volume used and the reduced 100μl and b) correlation for reduced volume plasma extraction and the measurement by the DPC in two healthy participants.

3.8. Extraction of plasma– A2E11 assay

Figures 25a, b, and c show the results of aldosterone measurements after plasma extraction using the A2E11 antibody assay using a smaller volume of 50μl. Using the standard volume it was ascertained that measurement of plasma aldosterone is possible, however at a lower level overall. When the smaller volume of plasma was used aldosterone values were still highly correlated to the DPC assay (R^2 = 0.97) and increases and decreases in response to various stimuli were clearly visible. Measurements were at a lower concentration of approximately 80% of those given by the DPC (y = 0.7951x – 33.9).

Figure 25 a, b, and c. Reduced volume plasma extraction (50μl) for measurement in the A2E11 in-house assay compared to measurement by the DPC in two healthy participants.

3.9. Salivary Aldosterone - Acris assay clinical validation

3.9.1. Day Profile Study results – saliva and plasma

Mean saliva values (±SEM) for 9 healthy participants (demographics Table 23) ranged from 91 ± 19pg/ml in the morning to 49 ± 5pg/ml at 6pm and plasma values from 157 ± 35pg/ml to 80 ± 22pg/ml (Figure 26), showing the general trend of increased levels in the morning that decrease during the course of the day, which has also previously been reported for healthy participants by several authors (Hurwitz et al., 2004; Few et al., 1987; Takeda et al., 1984; Chavarri et al., 1977; Katz et al., 1975). Though the salivary aldosterone decrease was not as pronounced as that seen in plasma, values were significantly lower at 6pm compared to the 8am values ($p < 0.05$, significance calculated by Wilcoxon paired t-test) as were plasma values at 6pm to 8am ($p < 0.01$).
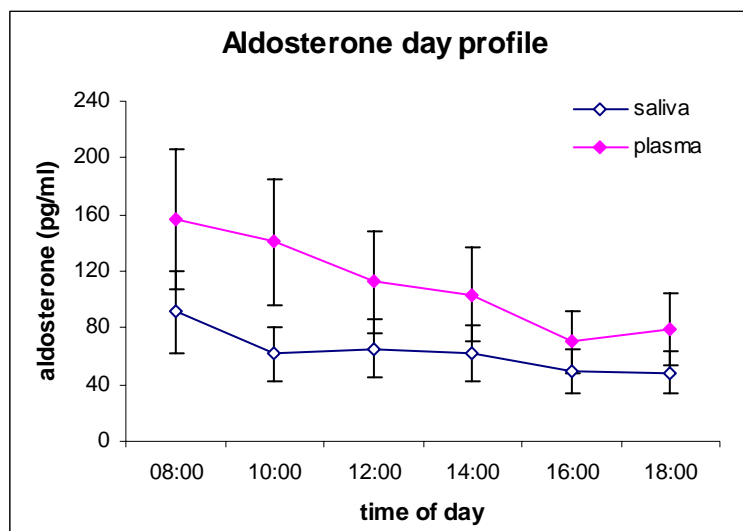

Figure 26. Mean (± SEM) saliva and plasma values for 9 healthy participants. Saliva values measured by Acris assay and plasma values measured by DPC RIA.

	Male	Female
n	4	5
Age (yrs)	35 ± 9.35	24 ± 2.35
BMI	23.7 ± 2.0	20.6 ± 1.2
OC	n/a	2/5

Table 23. Data are shown as mean ± SD. n, No. of participants; OC, Oral Contraceptive; All participants were non-smokers.

3.9.2. Mean levels in Primary aldosteronism and healthy participants

After measurement of 201 samples from confirmed PA patients, collected from patients admitted to the clinic, not taking any medication at the time of sampling, and 203 samples from healthy subjects and volunteers, baseline mean salivary aldosterone values, as measured by the Acris antibody assay, yielded concentrations of 120 ± 4 pg/ml and 59 ± 2 pg/ml respectively (p<0.0001, significance calculated by Mann-Whitney U test; Figure 27).
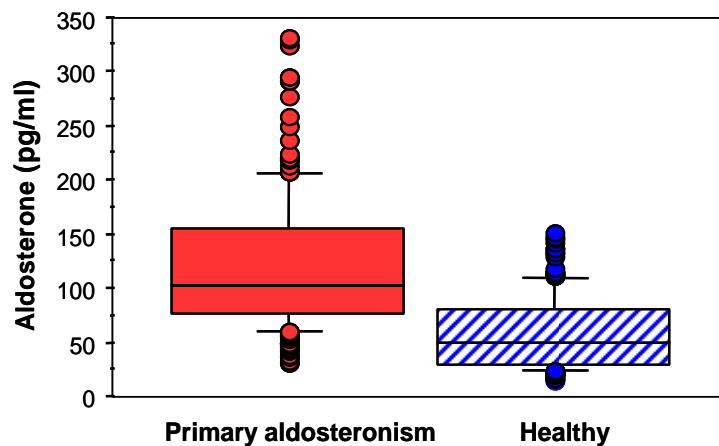
Figure 27. Salivary aldosterone levels in Primary aldosteronism and healthy participants. Mean value for PA = 120 pg/ml (n = 201) and for healthy participants =59 pg/ml (n = 203). Bars indicate 25th and 95th percentiles for each group with individual outliers given above and below; significance was calculated by Mann-Whitney U test, *p<0.0001.

Figure 28 shows mean salivary aldosterone values ($\pm$SEM) from nine PA patients collecting day profiles every four hours. Levels were sustained at a high concentration all day and did not vary between different time points (Kruskal-Wallis non-parametric test, p = 0.961). Similarly, salivary aldosterone samples collected from eight healthy participants between the hours of 8:45am and 15:45pm did not vary significantly though there seems to be an apparent increase between 10:45 and 11:00 and a tendency to decrease overall, although these were not found to be statistically significant (Figure 29, Kruskal-Wallis, p = 0.695).
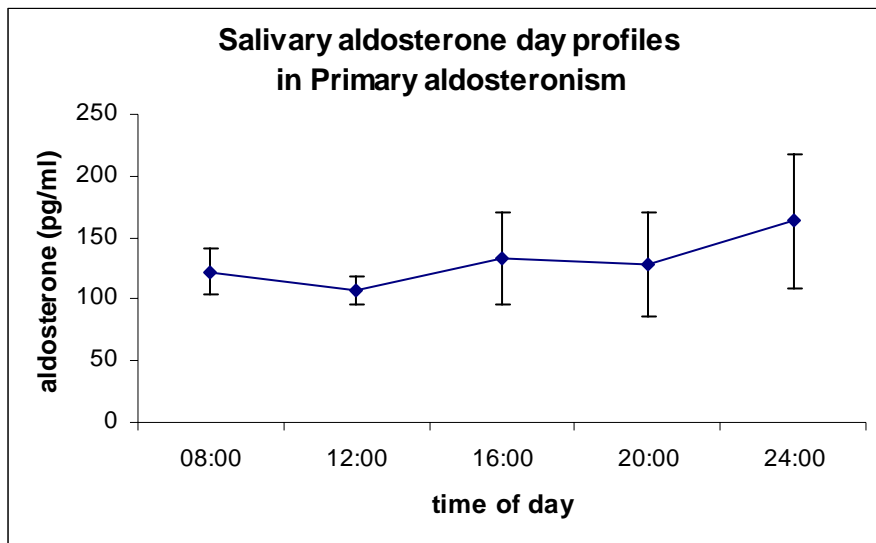
Figure 28. Daily profile of salivary aldosterone levels from eight patients with Primary aldosteronism, as measured by the Acris assay. Data is shown as mean (±SEM).
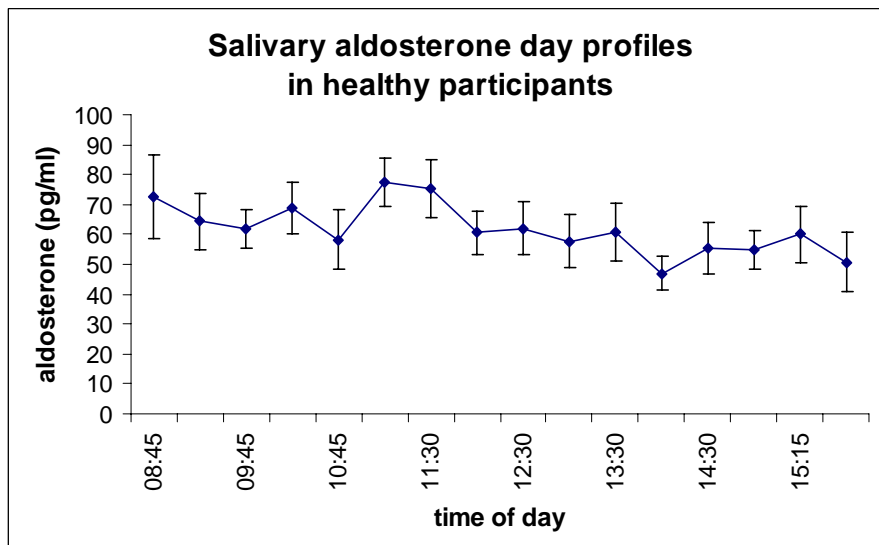

Figure 29. Daily profile of salivary aldosterone levels from eight healthy participants, as measured by the Acris assay. Data is shown as mean (±SEM).

3.10. Salivary aldosterone TRFIA measurements compared to plasma measured with a commercial RIA

After a measurement of a total of 912 paired saliva and plasma samples from PA patients and healthy control subjects, by the in-house TRFIA incorporating the Acris polyclonal antibody and the DPC RIA respectively, it was determined that aldosterone exists in saliva at around 28% that found in plasma, although there is considerable variability in this relationship, in accordance with previously reported correlations by other authors (McVie et al., 1979; Hubl et al., 1983; Few et al.,1984; Atherden et al.,1985) (Figure 30, $y = 0.275x + 54.6$, $R^2 = 0.5738$).
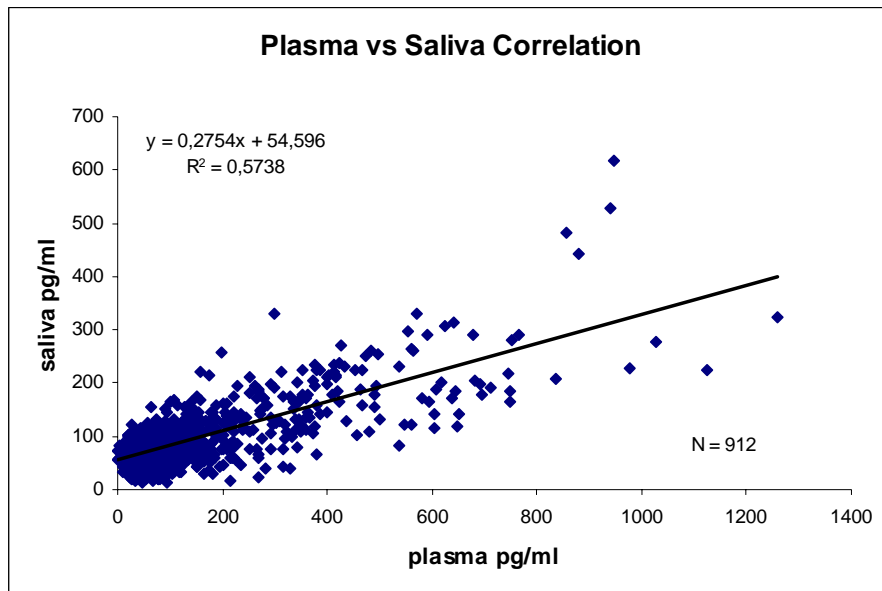

Figure 30. Correlation between the Acris aldosterone in saliva assay and the DPC aldosterone in plasma assay after measurement of 912 paired samples.

3.11. Posture test study

There was a significant increase in peak levels of salivary and plasma aldosterone after change in posture at 1hour following standing, when compared to mean baseline values at 2hours following a supine posture, ($p<0.0001$, Figure 31). Within the first hour after standing mean (±SEM) salivary aldosterone levels increased from 55 ± 4 pg/ml to 100 ± 8 pg/ml, while plasma levels also showed a marked increase from 33 ± 4 pg/ml to 145 ± 17 pg/ml. Salivary aldosterone

mirrored the increase seen in plasma with levels at approximately 26% of plasma (y = 0.259x + 52.38, where y= saliva and x= plasma).
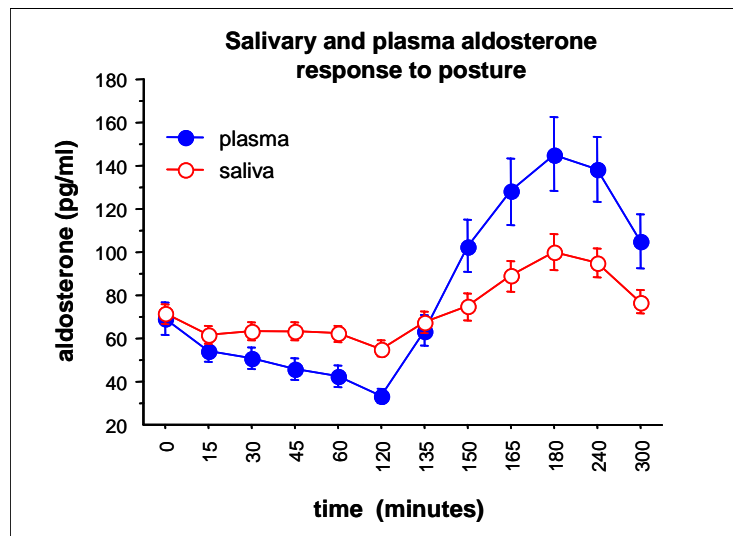

Figure 31. Mean (±SEM) salivary and plasma aldosterone responses to posture, as measured by the Acris in-house and DPC assays respectively, in 32 participants.
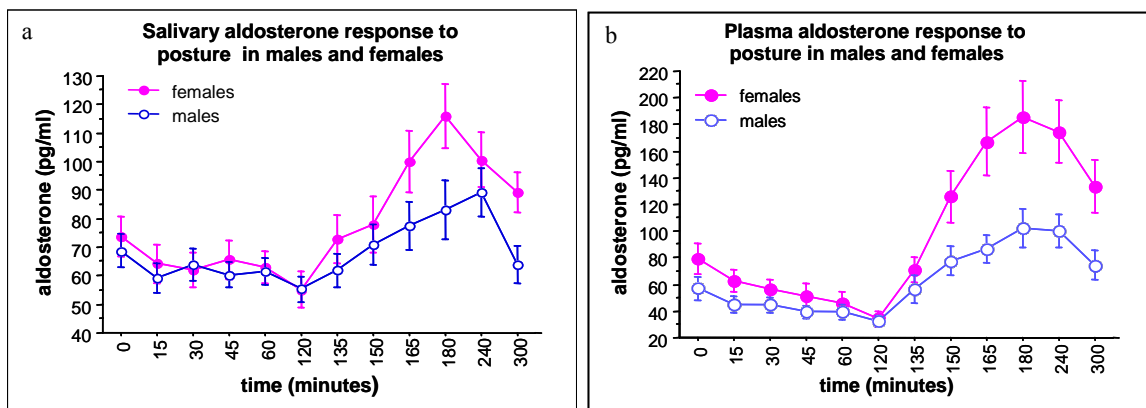

Figure 32a and b. Salivary and plasma aldosterone response to posture reached a higher value in females compared to males after change in posture.

Peak aldosterone after change in posture was clearly higher in females than in males, but independent of oral contraceptives (data not shown), in both salivary and plasma samples (Figure 32a and b). Univariate analysis showed there was an overall main effect of sex in plasma aldosterone, p= 0.028, females 99 ± 11 pg/ml compared to males at 63 ± 11 pg/ml (Fig 48b). In salivary aldosterone univariate analysis showed there was no main effect of sex (p= 0.230),

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although there was a time by sex interaction, p= 0.032, due to the increased values following the posture change in the females.

No difference in plasma aldosterone levels of smokers was found compared to the non-smokers (p= 0.669, univariate analysis). In salivary aldosterone univariate analysis showed there was a significant main effect of smoking (p=0.004) whereby smokers had a mean overall of 86 ± 6 pg/ml compared to a mean of 62 ± 5 pg/ml in the non-smokers (Figure 33b and a respectively).
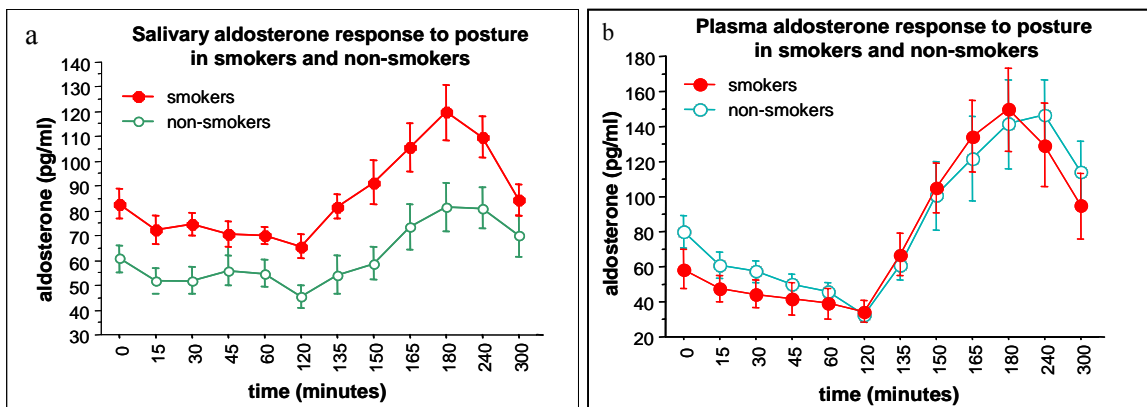

Figure 33a and b. Salivary and plasma aldosterone response to posture was higher overall in smokers compared to non-smokers at all time points. Similar results were not seen in plasma aldosterone between smokers and non-smokers.

3.12. ACTH stimulation test

In healthy participants mean baseline salivary aldosterone (SA) concentrations were 67 ± 7 pg/ml. After ACTH stimulation, mean SA were 128 ± 19 pg/ml, 121 ± 10 pg/ml, and 113 ± 14 pg/ml at 75, 105, and 135 minutes, respectively (Figure 34a). SA was significantly higher than basal at 75 and 105 (p<0.005), and 135 (p<0.02) minutes after ACTH stimulus. Most participants reached maximal aldosterone concentrations at 75 minutes after stimulation. Healthy participants showed a mean, max response increase of 61 ± 12 pg/ml at 75 minutes. Simultaneous plasma samples were taken and basal plasma aldosterone (PLA) concentrations determined using the DPC RIA were at 52 ± 10 pg/ml. After ACTH stimulation, mean PLA were 167 ± 24 pg/ml, 148 ± 23 pg/ml, and 136 ± 19 pg/ml at 75, 105, and 135 minutes,

respectively. As in the saliva samples, peak response was at 75minutes and declined thereafter and was statistically significant at each time point (p< 0.005, p< 0.01, and p<0.005 respectively). Statistical significances were calculated by Mann-Whitney un-paired t-test.
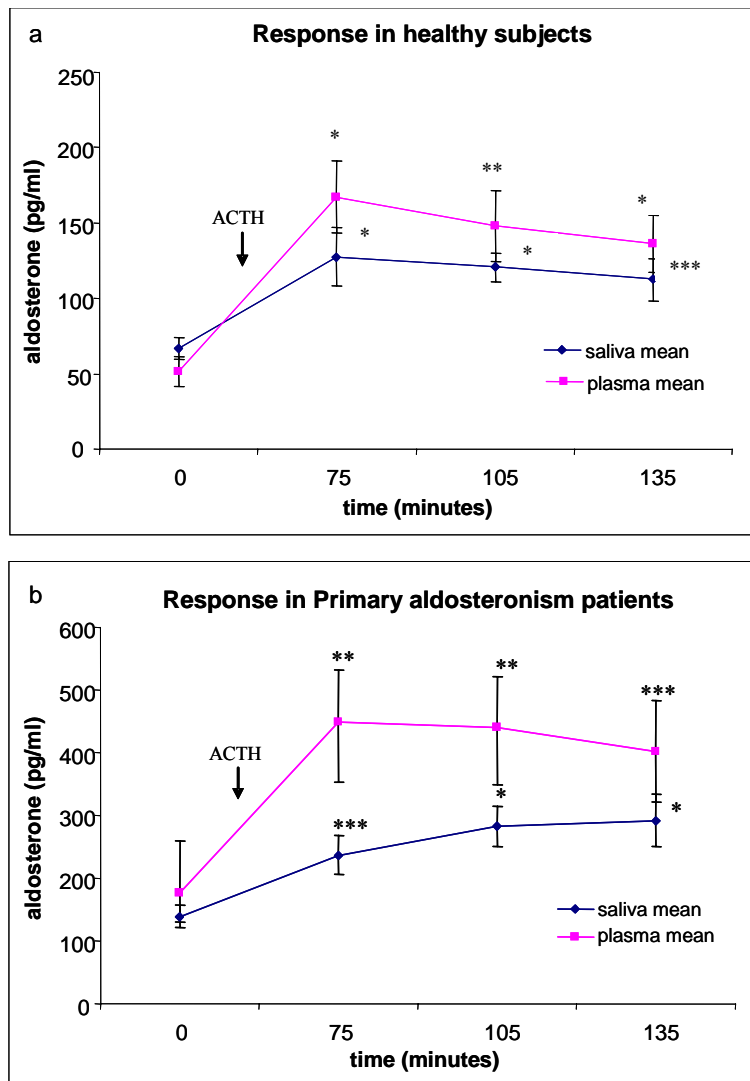

Figure 34a and b. Mean (± SEM) saliva and plasma aldosterone concentrations for normal healthy volunteers (upper panel) and suspected Conn's patients (lower panel) after stimulation with ACTH (250μg; Synacthen Novartis). * p<0.02, ** p<0.005 significant increase compared to baseline.

In PA participants mean baseline SA concentrations were 139 ± 18 pg/ml. After ACTH stimulation, mean SA increased to 237 ± 30 pg/ml, 282 ± 32 pg/ml, and 292 ± 41 pg/ml at 75, 105, and 135 minutes (Figure 50b). SA was significantly higher than basal at 75 (p<0.02), and at 105 and 135 (p<0.005) minutes after ACTH stimulus. Most PA participants reached maximal

SA concentrations at 105 and 135 minutes after stimulation, although the means of the two groups were not significantly different. PA participants showed a mean, max response increase of 143 ± 14 pg/ml at 105 minutes. Simultaneous plasma samples had basal PLA concentrations at 177 ± 47 pg/ml. After ACTH stimulation, mean PLA were 449 ± 97 pg/ml, 440 ± 91 pg/ml, and 402 ± 81 pg/ml at 75, 105, and 135 minutes (Figure 50b). Peak response was at 75minutes and declined thereafter and was statistically significant at each time point ($p < 0.01$, $p < 0.01$, and $p < 0.02$, respectively). All statistical significances between groups were calculated by Mann-Whitney unpaired t-test.

3.13. Salivary Aldosterone to Cortisol Ratio

Our data in 27 PA subjects and 41 controls showed that the overall aldosterone to cortisol ratio (ACR $\pm$ SEM) was 0.180 ± 0.039 in PA compared to 0.033 ± 0.003 in controls ($p < 0.0001$, Figure 35a). The ACR is higher in the evening compared to daytime due to lower variation in aldosterone values together with decreasing cortisol. 7–12 am values in the PA patients yielded a ratio of 0.074 ± 0.01 compared to 0.318 ± 0.081 between 6–10pm. In the controls this difference is less pronounced, 0.021 ± 0.004 compared to 0.047 ± 0.004 in the evening (Figure 35b).
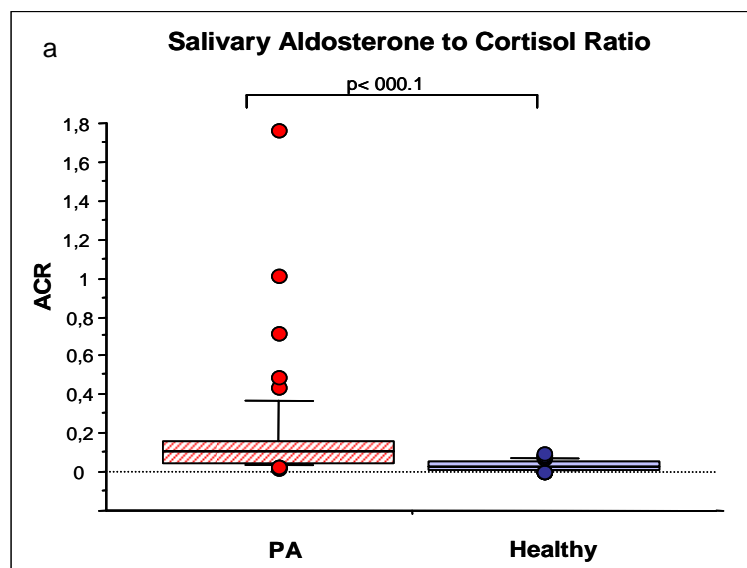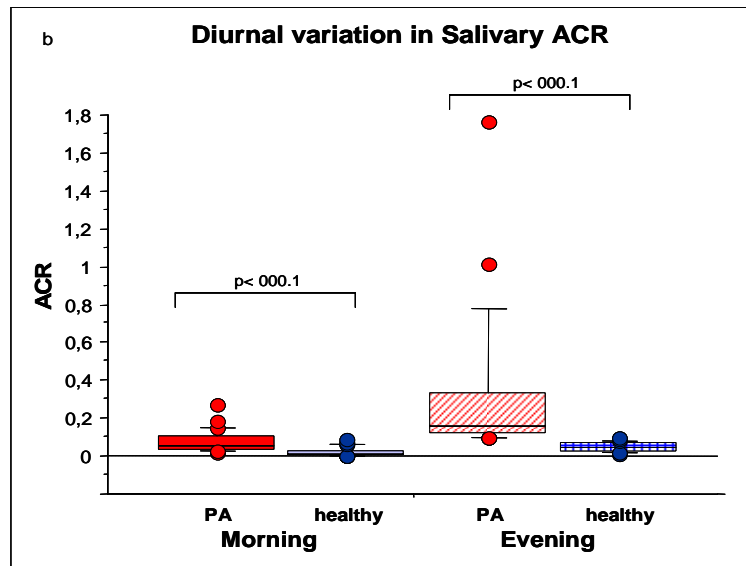
Figure 35a and b. a) Salivary aldosterone to salivary cortisol ratio (ACR) in Primary aldosteronism (PA; n = 53) and healthy control subjects (n = 60) b) ACR according to the diurnal variation seen with morning and evening sampling times.

	Primary Aldosteronism		**Healthy subjects**	
	morning	evening	morning	evening
ACR	0.019 to 0.268 (avg 0.074 ± 0.010)	0.093 to 1.762 (avg 0.318 ± 0.081)	0.004 to 0.086 (avg 0.021 ± 0.004)	0.013 to 0.098 (avg 0.047 ± 0.004)
SA (pg/ml)	35 to 368 (avg 96 ± 13)	22 to 527 (avg 130 ± 28)	20 to 109 (avg 59 ± 4)	19 to 79 (avg 42 ± 3)
SF (pg/ml)	324 to 4308 (avg 1618 ± 170)	155 to 942 (avg 426 ± 44)	843 to 14600 (avg 5079 ± 622)	300 to 3200 (avg 1174 ± 154)
N	30	23	33	27

Table 24. Summary of results from salivary ACR, salivary aldosterone (SA) and salivary cortisol (SF) in Primary aldosteronism and healthy subjects.

The overlap in salivary ACR seen between PA and healthy subjects in the morning is abolished in the evening. Levels are almost completely separated with only 1 out of 23 samples from PA below the upper limit of the control subjects (0.098). Therefore, at physiological conditions, a ratio higher than 0.1 at late night is suggestive of Primary aldosteronism.   Post-ACTH stimulation test the ACR decreased in the 10 PA to 0.015 ± 0.003 and to 0.014 ± 0.005 in the controls, compared to 0.104 ± 0.023 and 0.051 ± 0.009 at baseline respectively, due to the

approximately 10-fold increase in the levels of SC compared to the less pronounced increase in
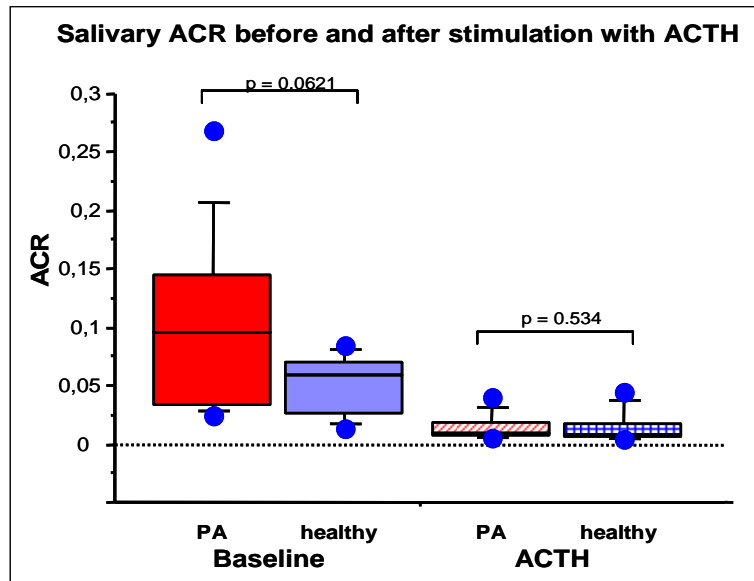
SA (Figure 36).

Figure 36. Salivary ACR in PA and healthy subjects after stimulation of the pituitary adrenal axis with ACTH is clearly diminished making it impossible to distinguish between the two groups.

Un-stimulated baseline SA was at an average of 111 ± 14 pg/ml in PA patients and 52 ± 3 pg/ml

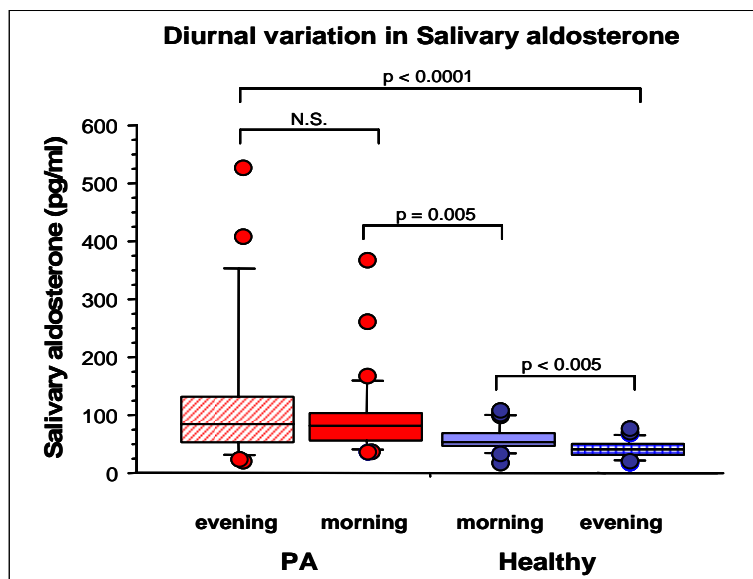in controls (Figure 37). The overlap in salivary aldosterone between PA and controls remains

Figure 37. Salivary aldosterone concentrations in Primary aldosteronism (PA) patients and healthy subjects. Morning and evening do not change significantly from morning to evening in the aldosteronism patients compared to controls where there is significant decrease ($p<0.005$).

even when evening samples are taken, although levels are overall significantly higher in the PA patients ($p < 0.0001$). SA and salivary cortisol (SF) (data not shown) levels both decreased throughout the day in the control subjects in accordance with expected circadian rhythms. Though there was a drop in SF in PA subjects, the decrease in SA was not observed, as would be expected with an autonomous hypersecretion of aldosterone.

3.14. A2E11 assay salivary aldosterone clinical validation

There was a highly positive correlation between the salivary aldosterone measurements given by the Acris assay to those given by the A2E11 assay ($R^2 = 0.94$), after measurement of 28 salivary samples, described by the equation $y = 2.7x + 25.7$ whereby aldosterone measured using the Acris assay gave values that were approximately three times those measured by the A2E11 (with a positive bias of 25.7, Figure 38).

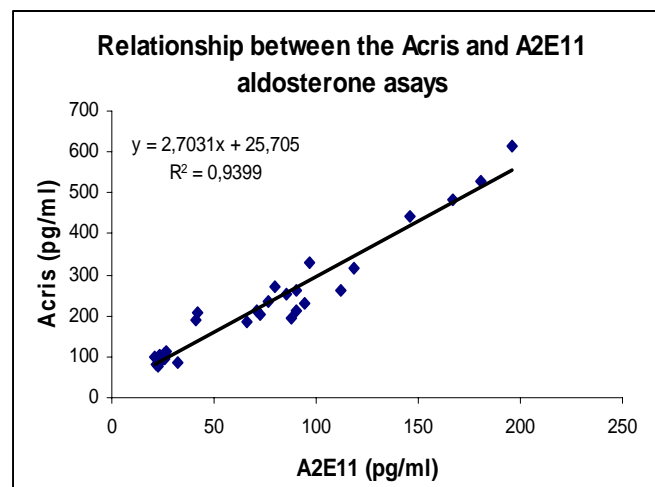**Figure 38.** Correlation between measurement of aldosterone by the two in-house saliva assays ($n = 27$).

3.15. A2E11 assay – validation in rodent serum/plasma

3.15.1. Basal aldosterone in male and female wild type mice

Values from all wild-type animals used in the study were examined in order to determine gender differences which may be present in mice. There was no overall significant difference (p =

0.396) found between female (n = 35) and male (n = 40) mice, whose serum values had mean (±

SEM) levels of 163 ± 34 pg/ml and 181 ± 27 pg/ml, respectively (Figure 39).
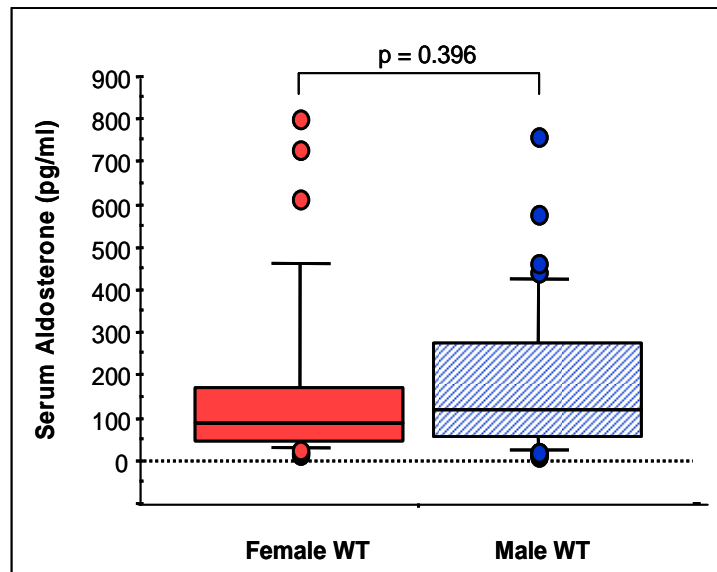

Figure 39. Mean serum aldosterone levels in female and male WT mice (n = 35 and n = 40 respectively). Bars indicate 25th and 95th percentiles for each group and significance was calculated by unpaired t-test.

In addition, there was no sex difference in any particular age group (p>0.05 at 3, 5, 7, 9 and 11

weeks, Table 26). Since no gender-dependent differences in serum aldosterone levels were

observed throughout the studied points of time, aldosterone values for female and male mice

were averaged at the different points of time (week 3: n = 8, week 5: n = 8, week 7: n = 27, week

9: n = 12 and week 11: n = 20; Figure 40, Table 25) and found to have mean (± SEM)

aldosterone concentrations of 52 ± 9 pg/ml, 57 ± 11 pg/ml, 272 ± 45 pg/ml, 107 ± 33 pg/ml, and

174 ± 34 pg/ml respectively. The Kruskal-Wallis non-parametric test displayed an overall

significance between the groups (χ^2 = 18,98; df = 4; p = 0.001). No significant difference in

aldosterone levels was found between weeks 3 and 5 (p = 0.753). At an age of 7 weeks

aldosterone rose by 377% compared to the 5-week old mice (p = 0.003). Although there was a

drop at week 9, serum aldosterone concentrations remained high overall and were not

significantly different between 9 and 11 weeks (p = 0.115). At week 11 the aldosterone levels

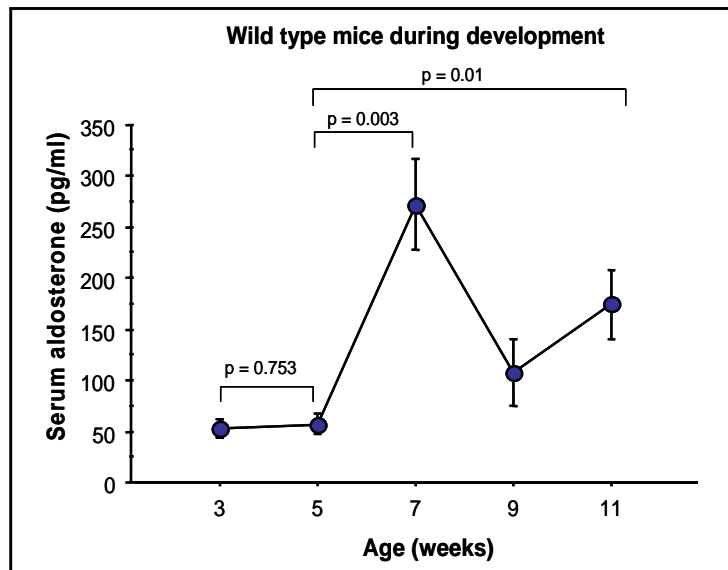were still significantly higher than at both weeks 3 and 5 (p = 0.007 and p = 0.011).

Figure 40. Mean (±SEM) serum aldosterone levels in male and female WT mice at indicated weeks of development. 3 weeks (n = 8), 5 weeks (n = 8), 7 weeks (n = 27), 9 weeks (n = 12) and 11 weeks (n = 20). Significances were determined by Kruskal-Wallis and unpaired t-test.

Age (weeks)	Female		Male		
	n	**aldosterone (pg/ml)**	**n**	**aldosterone (pg/ml)**	**p**
3	4	54 ± 9	4	51 ± 17	0.773
5	4	73 ± 16	4	42 ± 10	0.149
7	12	277 ± 77	15	268 ± 54	0.807
9	6	61 ± 15	6	154 ± 62	0.174
11	9	170 ± 62	11	178 ± 37	0.569

Table 25. Serum aldosterone concentrations in female and male mice at increasing weeks of development. There was no significant difference between the male and female mice throughout the 11 weeks.

weeks	p
3 - 5	0.753 n.s.
5 - 7	<0.005
5 - 9	0.354 n.s.
7 - 9	<0.05
7 - 11	0.220 n.s
9 - 11	0.115 n.s
5 - 11	<0.05

Table 26. Statistical significance between the different age groups of mean male and female mice values.

3.15.2. Suppression and stimulation in wild type mice

Kruskal-Wallis non-parametric test showed an overall significance between the three groups of control, dexamethasone suppressed, and ACTH stimulated mice (χ^2 = 15.866; df = 2; p < 0.001; Figure 41).   Animals treated with dexamethasone had significantly lower concentrations of aldosterone (35 ± 3 pg/ml) than the control mice (114 ± 33 pg/ml; p = 0.021). 6 out of the 7 mice treated with dexamethasone suppression had values below the LOD (8 pg/ml).   For the calculation of the mean, all these values were assigned a concentration of 8 pg/ml. Mice stimulated with ACTH showed significantly elevated values compared to the control group (603 ± 119 pg/ml; p = 0.003).
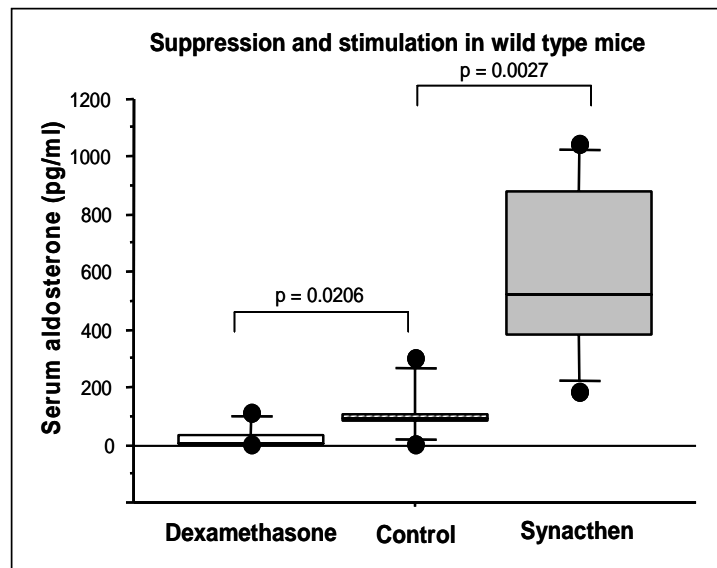

Figure 41. Mean (±SEM) serum aldosterone levels in grouped male and female WT mice treated with 10 days dexamethasone, 10 days NaCl (control group), and 10 days Synacthen ACTH. Significance compared to baseline control group determined by unpaired t-test.

3.15.3. Effect of an increased potassium diet – stimulation/suppression of adrenal RAAS

Serum samples measured from mice sustained on a high K^+ diet had mean aldosterone values of 1369 ± 703 pg/ml, the control group had a mean of 172 ± 36 pg/ml, and the low K^+ diet animals 287 ± 60 pg/ml, as measured by the in-house TR-FIA. The aldosterone values from the high K^+

group were significantly higher than those of the control group (p= 0.0025) while the control and low K$^+$ groups did not differ (p= 0.142, Figure 42). Similarly, the same samples from the same groups of mice when measured with the commercial DPC RIA kit gave values of 1744 ± 697 pg/ml, 294 ± 65 pg/ml, and 407 ± 76 pg/ml in each respective group. Again, the high K$^+$ fed animals were significantly higher compared to the controls (p= 0.0009) and the low K$^+$ were not statistically significantly higher (p= 0.369).
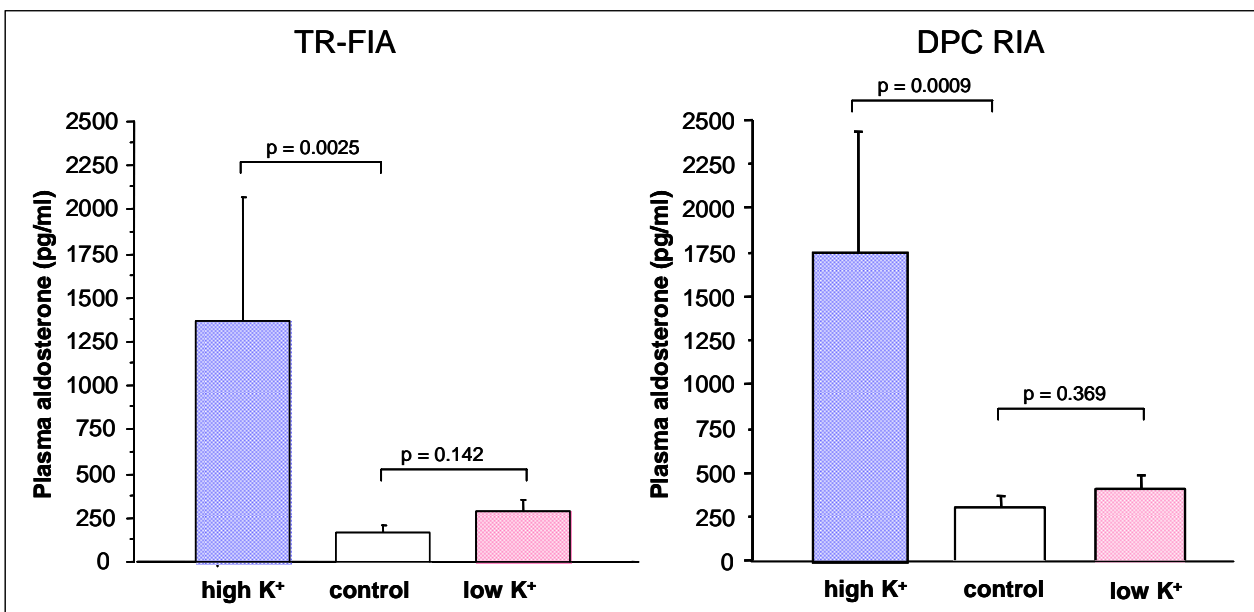

Figure 42. Aldosterone values in plasma of mice maintained on high and low potassium diets obtained using a) the A2E11 monoclonal antibody assay and b) the DPC RIA.

4. Discussion

4.1. Aldosterone in hypertension, Primary aldosteronism

The Renin Angiotensin Aldosterone System (RAAS) is the main effector mechanism in the equilibrium between vascular tone and intravascular filling pressure. It is activated if sodium intake is low and/or if intra-arterial volume and filling pressure fall. The RAAS maintains arterial pressure by increased angiotensin-induced constriction of vascular smooth muscles and increased angiotensin-induced stimulation of aldosterone thereby leading to sodium retention (Gavras & Brunner, 2007) and increased extracellular fluid volume. Cardiac hypertrophy, regulated by ventricular pressure and volume loading, and perivascular fibrosis have been positively correlated with the effector hormones of the RAAS (Takeda et al., 2000). Increased circulating levels of the mineralocorticoid aldosterone, the final hormone in the RAAS cascade, and its presence along with other components of the RAAS within cardiac tissues, are implicated in leading to tissue remodelling, heart failure and hypertension. An extensive amount of evidence links increased levels of aldosterone, even within physiological ranges, to the pathogenesis of hypertension in humans (Vasan et al., 2004). Evidence coming from recent large scale studies such as RALES and EPHESUS renewed interest in the role of mineralocorticoids in cardiovascular disease and in the risk of developing high blood pressure and showed the benefits of aldosterone blockade with mineralocorticoid receptor antagonists spironolactone and eplerenone, respectively, in patients with chronic heart failure (Pitt, 2004). Primary aldosteronism, where excess levels of aldosterone exist, most commonly due to an adrenal adenoma or to bilateral adrenal hyperplasia, is the most common form of essential hypertension and is now known to occur in approximately 10-15% of suspected patients (Stowassser et al., 2003; Young, 2003; Giacchetti et al., 2006).

This increase in recent years in the diagnosis of PA, justifies the development of widely applicable and easy to perform screening methods combining high levels of sensitivity for detecting increased levels of aldosterone in a wider range of suspected subjects who could then benefit from anti-mineralocorticoid treatment. The use of saliva as a diagnostic specimen has been used since the 1980s for the detection of steroid hormones such as cortisol, testosterone, estradiol and progesterone. However, measurement of salivary hormones can only be clinically relevant if they truly reflect the serum or plasma hormone levels or if a constant relationship exists between the concentrations in the two parameters. For neutral steroids which simply diffuse into saliva through epithelial cells, salivary hormones represent the free, non-protein bound serum hormone levels (Vining et al., 1983), which may be clinically relevant since it is this fraction which is biologically active. Salivary monitoring of hormones has many advantages compared to restraints imposed by serum or plasma analysis and the demanding 24hour urinary collection. It allows multiple sample collection in short intervals due to its non-invasive nature and sampling without supervision throughout the day removing the need to visit a specialized centre. In 1982 investigators showed that salivary cortisol levels correlate extremely well with free serum cortisol and since then many assays, mostly radioactive to begin with, have appeared for other steroid hormones (Kaufman & Lamster, 2002).

4.2. Aldosterone in saliva – application of the assay

The first report of a salivary aldosterone assay comes from the late 1970s but even preceding this authors had described the protein-free as opposed to protein-bound levels of aldosterone in plasma (McVie et al., 1979). Free levels existing at around 30-40% of bound and at a ratio of 0:2-0:3 (Few et al., 1986). Although some studies exist on a putative specific binding protein of aldosterone in plasma (Davidson et al., 1962), there has been no specific protein named until now. The same ratio of salivary to plasma aldosterone does not always exist

between all individuals and one reason for this may be that aldosterone concentrations are relatively low overall and therefore more likely to be affected by changes in binding proteins, although aldosterone is known to be weakly bound to proteins and exists at reasonably high free concentrations in serum and is therefore relatively unaffected (Few et al., 1986).

The low concentrations of aldosterone, compared to other hormones such as cortisol which is present at around 1000 times the concentration, has meant that previous assays have required large volumes of up to 2ml for each sample (Few et al., 1987). Alternatively, in one reported case the authors adjusted a commercially available assay designed for plasma level assessment, by increasing the volume twofold to 400μl (Cardoso et al., 2002). The assay described and validated here requires a small sample volume of 100μl for each duplicate measurement. Furthermore, it was established that results for salivary aldosterone correlate extremely well before and after extraction with DCM, and as this step is also often responsible for increasing inter-assay variability of results, it was decided that this step should be omitted. In cases where samples showed particularly high aldosterone values this step was applied to ensure that values are not increased due to interference from other cross-reacting substances.

Many of the previously established assays required not only extraction with DCM but also chromatographic purification (Hubl et al.,1983; McVie et al., 1979), and incorporated radioactively labelled tracers in the detection system (Few et al., 1987; Atherden et al., 1985). We have developed a non-isotopic immunoassay, avoiding the frequent production and between-assay variability of radioactive tracers, as well as the environmental, health, and practical aspects involved. The tracer used in our assay was designed according to a previously validated method for biotinylating steroids, providing practically unlimited stability and a "specific activity" which is not affected by storage time (Dressendorfer et al., 1992).

A further advantage offered by the non-radioactive, non-enzyme linked, biotinylated aldosterone tracer is that the biotin conjugate produced is of a relatively comparative size,

726.5Da, to the analyte and chances of binding to the anti-aldosterone antibody coated to the surface of the microtitre plate well are similar between the analyte and the tracer.

4.3. Establishment of the assay

The chromatographic technique used to purify the biotinylated aldosterone conjugate was reverse phase high performance liquid chromatography, chosen due to the non-polar nature of the adsorbant material of the column, octadecyl (C_{18}) ligand-bonded silica, ideal for binding small neutral molecules such as steroids. In this type of set-up the eluent's polarity plays an important role in the retention time and separation of the compound injected. For this reason two different types of elution profile were assessed, one in gradient, where the polarity of the solvent is steadily decreased, as well as an isocratic gradient pumped through the column equally during the whole analysis. The gradient approach, with increasing concentrations of methanol and acetonitrile respectively, was applied to both of the two different columns purchased from companies 'Knauer Advanced Scientific Instruments' and 'Phenomenex®', while the isocratic gradient only was applied using the Phenomenex column. Chromatography runs using the Knauer column yielded major peaks at approximately 80 minutes, with several smaller peaks occurring at around 60 minutes, presumably representing the elution of more polar educts still present in the sample, such as that due to the molar excess of biotin which was added to ensure complete biotinylation of the aldosterone-3CMO molecule. The fractions collected from this column yielded a tracer molecule with high specificity but concentrations were lower than those obtained by the Phenomenex column purification therefore further analysis was not carried out with this column. Use of an isocratic gradient as opposed to a linear gradient, with the Phenomenex column, yielded better separation of fractions containing higher concentrations of aldosterone with lower non-specific binding due to a lower amount of biotin still present in the solution, as was seen by fraction 51/52 of Phenomenex Run 3 and fraction 40 of Phenomenex

Run 4 which combined highest yield of concentrations, 1431ng/ml and 2190ng/ml respectively, and gave the highest displacement at 20pg/well of 54 and 46%.

During the course of the studies to validate the aldosterone assay for measurement in saliva, the opportunity arose to obtain a highly sensitive and specific monoclonal antibody against aldosterone from the University of Mississippi Medical Center (Jackson, Mississippi, USA.). Therefore, whilst continuing the ongoing studies with human saliva samples using the polyclonal antibody, development of an assay incorporating the monoclonal was likewise carried out. The superior sensitivity of the monoclonal antibody was taken advantage of in designing the assay method in order to measure aldosterone in small volumes of serum or plasma taken from small rodents. The polyclonal, commercially available anti-aldosterone antibody (Acris Antibodies GmbH, Hiddenhausen, Germany) was used to establish optimum conditions, such as concentration and incubation time necessary for optimum binding, for running the assay to measure aldosterone concentrations in saliva samples. Decreasing the concentration of the tracer approximately ten-fold as well decreasing the concentration of the antibody gave a significant increase in the sensitivity of the assay, sufficient to bring the measurable range of the assay down to 19pg/ml for each 50µl sample (well within the range of normal values and allowing distinction between healthy participants and those with PA). In combination with the monoclonal antibody the same tracer fraction from the Phenomenex run which was chosen for all consequent assay runs, gave a lower detection limit of 8pg/ml, yielding an even more sensitive assay. The tracer has been used and stored in our laboratory for 24 months without loss of activity.

The differences seen between the two assays using the two antibodies are to be expected when comparing the binding characteristics of a polyclonal compared to a monoclonal antibody. Discrepancies arise due to the methods involved in production of the antibodies. Whereas a polyclonal antibody involves the immune response of an individual immunized animal and will contain a heterogeneous mixture of antibody species of varying specificity, recognizing more

than one epitope, a monoclonal is derived from a specific clone which is isolated and expanded to produce antibodies which are homogenous and uniform in nature, with unique characteristics, epitope specific and with a fixed affinity.

The specificity of the monoclonal antibody had previously been certified when its production was published in 1987 by Gomez and co-workers (Gomez-Sanchez et al., 1987). Although the polyclonal antibody was checked for cross-reactivity with certain endogenously found steroids by the manufacturer, it was further verified in our laboratory and found to cross-react below 0.01% for endogenous and synthetic steroids. Cortisol, for example, which cross-reacts by only 0.004% with this antibody would require a concentration in the high microgram range in order to see an altered value in the aldosterone reading, unlikely since in the blood it rises to 0.25μg/ml even at peak levels. A concentration of a similar magnitude would be needed to interfere substantially with assay values in the case of progesterone which cross-reacted at 0.005% and is present at 26ng/ml at its highest luteal phase levels (Laycock & Wise, 1996).

Both the monoclonal and polyclonal assay were fully validated according to linearity, recovery, and reproducibility requirements. Measurements of human saliva samples were further validated after an extraction step using the solvent dichloromethane to remove any potentially interfering substances. The accuracy of the assay was confirmed by the excellent agreement of results obtained by the direct assay for saliva with those determined after extraction, and by plasma sample results obtained after extraction step which were compared to a commercial radioimmunoassay (DPC, Biermann) for measurement of plasma aldosterone levels.

4.4. Clinical validation

4.4.1. Salivary aldosterone monitors changes in plasma

Values for salivary aldosterone correlated well to those measured in concurrently obtained plasma samples as assesses by the DPC RIA. Assessment of the DPC calibrators using

the polyclonal antibody assay demonstrated that there are certain non-specific matrix effects occurring due to components present in the calibrators and interfering with the capture antibody. These interfering substances are not extracted into the DCM phase with values more closely correlated after extraction. With the monoclonal antibody assay there was a linear relationship with the calibrators after extraction. The naturally occurring low levels of aldosterone and the difference in calibrators of the two assays means that there is some overlap seen in the plasma and saliva values of the healthy participants at the lower range. This would also explain the differences in basal values reported by ourselves and some other investigators who report baseline values of 34.5pmol/L (12pg/ml) in healthy subjects (Cardoso et al., 2002).

Previous authors have suggested that frequency of sampling should be at least once an hour in order to detect short-term fluctuations in hormone levels which in aldosterone occur hourly, five samples per day giving a reasonable estimate of mean diurnal concentrations (Few et al., 1986). Though we did not collect samples as frequently as every hour we did observe from individual cases of sampling every two hours that salivary aldosterone indeed varies throughout the day and is often not found at its highest levels in the morning, as aldosterone is easily influenced by many external factors, such as diet and stress, and the regulation of its secretion is complex. Mean salivary aldosterone levels from 9 participants did show highest levels at 8am in the morning of 91pg/ml (255pmol/L) which decreased to significantly lower concentrations by 6pm, 49pg/ml (137pmol/L), and followed the pattern measured in concurrently collected plasma samples. This data is in agreement with previous observations on the circadian rhythm of aldosterone and the assumption that salivary aldosterone concentrations are a useful indicator of circulating aldosterone levels.

More frequent sampling from healthy volunteers which was carried out between the hours of 8:45am and 15:45pm did not show any diurnal rhythm. This may have been because 4pm is not late enough to detect the daily diurnal decrease in concentrations, or alternatively, that

the more constrained nature of the study, where participants remained in the clinic throughout the frequent sampling period in order to collect simultaneous plasma samples, kept levels of aldosterone elevated due to stress or other unknown factors.

In PA patients who collected samples from 8am until midnight there was no observed diurnal rhythm. Paradoxically, levels seemed to increase in the evening and deviated more from the mean though this was not statistically significant. Data on the circadian rhythm of aldosterone, or lack thereof, in PA patients of any of the subgroups is scarce. Several studies exist, though with conflicting results, describing the influence of pathological changes in the RAAS on diurnal rhythms of blood pressure and whether those correlate with levels of aldosterone. Evidence from one study suggests that while patients with APA have a preserved circadian blood pressure, as opposed to those with IHA, aldosterone levels do not correlate with the blood pressure changes (Zacharieva et al., 2006). The APA, though largely autonomous in its aldosterone secretion, is still influenced by ACTH and subsequently its diurnal rhythm and its secretion is synchronous with cortisol fluctuations regardless of changes in posture (Kem et al., 1973). Mean values from the patients with a diagnosis of PA due to an APA who were included in the studies shown here however, did not show a diurnal rhythm in aldosterone concentrations although there was a clear response to the ACTH injection.

4.4.2. Posture test findings and future application

The studies demonstrated overall that it was possible to apply the assay for measurement of aldosterone in saliva in order to monitor plasma aldosterone levels. The results correlated positively to plasma at 28%, in accordance with previous literature, mirroring its fluctuations under physiological conditions. An ideal setting to monitor a more drastic increase in aldosterone, typically used in clinical settings to study the responsiveness of the adrenal gland and distinguish the subtype of primary aldosteronism, is the posture test. The posture study was

used to demonstrate further that saliva as a medium can monitor aldosterone concentration reliably and that patients suspected of primary aldosteronism could potentially carry out this type of testing unsupervised in an out patient basis by collecting salivary samples instead and thereby avoiding the necessary admission to the clinic.

The study provided further potentially useful information in that females who participated in the study had a significantly higher aldosterone response to change in posture than males, in both plasma and saliva, and that the males reached mean peak aldosterone values an hour later than the females. The higher increase in the females could not be explained however by increased stimulation of the RAAS in these participants because this difference was not also observed in the renin results (data not shown). A clear sex difference that has been reported in both rats and humans is that there is a greater sensitivity of the adrenals to ACTH in females than in males, and this was also supported by our data where males had higher levels of ACTH following posture than females and may explain the higher response in aldosterone levels in the females (ACTH data not shown). Though this sex difference in response to posture has previously been reported in plasma values (Vernikos et al., 1993) it had not been shown before in studies involving salivary sample collection and serves as a further confirmation that gender differences should be taken into account when carrying out posture testing.

Another important finding was that the smokers taking part had higher salivary aldosterone throughout the duration of the study, as well as a higher increase compared to basal levels just before the change in posture. This was not seen, however, in the plasma values. It has also been well documented that chronic exposure to cigarette smoking brings about alterations in the responsiveness of the HPA axis, mainly due to the effects of nicotine, and additionally that smokers have higher free cortisol levels than non-smokers (Kirschbaum & Hellhammer, 2006). In our study we did not examine salivary cortisol concentrations but this would enhance the observations found in salivary aldosterone. The smokers also had higher

increases in ACTH following posture which were highly correlated to the increases in aldosterone (data not shown) and this is in agreement with the findings of previous workers such as Baer et al in 1985. Other studies report enhanced activation of the RAS in smokers (Laustiola et al., 1988) but this was not replicated in our study where smokers did not have higher renin levels than non-smokers. (A short study which was conducted in our laboratory to see if smoking affects salivary aldosterone levels due to changes in flow rate showed that this was not the case and that this is rather a long term effect.) We conclude that it is most likely that the increased ACTH, due to increased HPA axis activation and levels of cortisol, brings about higher levels of free aldosterone due to the decreased binding availability of aldosterone to CBG. Similar circumstances have previously been shown with aldosterone levels, after increased cortisol concentrations due to stimulation with ACTH (McVie et al., 1979).

4.4.3. ACTH stimulation test findings and future application

Not many studies exist describing the normal salivary responses of aldosterone to ACTH stimulation. The study by Cardoso et al in 2002 showed mean peak values of 193pmol/l (67pg/ml) at 60 minutes with a dose of 250μg synthetic β^{1-24} ACTH, whereas Few et al in 1984 showed a peak of approximately 50pmol/l (18pg/ml) but with a lower dose of only 50μg ACTH. In our study the peak values after ACTH stimulation in healthy subjects were at a mean of 128 ±19pg/ml (358pmol/l) at 75 minutes with 250μg synthetic β^{1-24} ACTH. Peak responses were also mirrored in the paired plasma samples which were collected concurrently. As far as we are aware there is no published data for salivary aldosterone levels in PA patient following stimulation with ACTH. Salivary aldosterone levels continued to increase for the 30 minutes following the peak seen in plasma levels in the PA patients, which could be explained by the overall higher levels of aldosterone giving rise to higher free concentrations due to decreased possibility of attachment to binding proteins. This information may be clinically useful to

delineate the total time necessary for collection of samples after the stimulation test, and potentially allowing for a diagnosis of adrenal function through the assessment of the salivary, free, active fraction of plasma aldosterone. The study reinforced the findings of previous investigations showing that in control subjects maximum responses will be found at 100 minutes following administration of corticotropin hormone (Cardoso et al., 2002).

4.4.4. Aldosterone to cortisol ratio – confirmation for diagnosis of PA

There is no reported set ratio between aldosterone and cortisol in saliva for healthy or aldosteronism individuals. Studies have shown that salivary glucocorticoids are positively correlated with salivary aldosterone levels (Kooner et al., 1991) and that the two hormones are more closely correlated during later periods in the day when fluctuations in aldosterone are smaller (Few et al., 1987). The preliminary data we have produced from twenty-seven PA patients and forty-one healthy controls suggest that using our assay combination a value of 0.1 for this ratio at basal physiological conditions in the evening may be used as a cut off for diagnosis of excess aldosterone production, as in the case of primary aldosteronism. During stressful situations such as was demonstrated by the extreme example of ACTH stimulation, however, the ratios decline in both groups, mainly due to the dramatic increase in cortisol which is less pronounced in aldosterone and renders the ratio inadequate. For this reason, combined with the normal diurnal decrease in cortisol in the evening, sampling for determination of the ratio to differentiate between aldosteronism and healthy individuals is proposed to take place in the evening between 6 and 10pm. The feasibility of using this ratio as a tool for the diagnosis of suspected Conn's syndrome is enhanced by a recent study where authors applied the aldosterone to cortisol ratio in serum for the distinction between aldosterone-producing adenoma and idiopathic hypertensive patients (Giacchetti et al., 2006).

4.4.5. Monitoring fluctuations of aldosterone in rodent plasma – potential for use in an experimental setting

In the second part of this thesis, three animal models were used to validate the aldosterone assay using the monoclonal antibody by using small volumes of serum or plasma to establish the measurement of aldosterone at physiological conditions, at various stages of pre- and post- pubescent development in mice, and after pharmacological and dietary manipulation of the HPA axis and the adrenal RAAS, respectively.

Values for serum aldosterone in mice are reported which are well within the measurable range of this assay. After assessment of samples taken from seventy-five C57BL/6 x NMRI mice we found no gender-dependent difference. This observation included male and female mice aged between 3 and 11 weeks after birth. To our knowledge so far, there has been no in-depth study describing gender-related levels of aldosterone in these or any other strain of mice. Though extragenital differences in mouse organs such as kidney, liver, and skeletal muscle are known to exist and differences in mouse adrenal weights have been reported, the exact nature of the differences within the adrenal gland have not as yet been established (Bastida et al., 2007). For example in one recent study (Sausbier et al., 2005) no differences were found in aldosterone levels between male and female wild type mice (SV129/C57BL6) and mice with mutations in voltage- and Ca^{2+}-dependent potassium channels. In another study looking at polyamines and adrenal sex dimorphisms however, female mice were found to have significantly higher basal aldosterone levels than male mice of the same (CD1) strain (Bastida et al., 2007).

Other authors have previously reported findings (Zhang et al., 2003; Jensen et al., 2004), that aldosterone does not change significantly in rodents during postnatal development, however these experiments were carried out in rats. In the present investigation we observed a highly significant increase in the levels of aldosterone between five and seven weeks of age and, though these levels decreased at weeks 9 and 11, they remained at a significantly higher level when

compared to 3- and 5-week-old mice. No difference in plasma aldosterone concentrations between neonate and adult 3-month-old mice (C57BL/6 strain) were reported by Cao et al (Cao et al., 2006). As mice older than 11-weeks were not included in the study, it is possible that the aldosterone levels decline thereafter. In another study showing aldosterone levels in 1 week- and 1 month-old wild-type and proopiomelanocortin (POMC) null animals, the authors reported aldosterone levels which were approximately twice as high in the wild types at 1 month compared to 1 week (793 ± 103 vs 357 ± 145 pg/ml) and significantly decreased at one month (203 ± 39 pg/ml) in the POMC null mice, while no difference occurred between wild-type and mutant mice at week 1 (89 ± 5 pg/ml) (Karpac et al., 2005). However, the different strain, assay, and blood sampling techniques used hamper a comparison between those studies and our own.

In order to show that the assay can be used to reflect changes in adrenocortical function after pharmacological or diet induced interventions in mice, aldosterone levels were investigated after suppression or stimulation of the HPA axis with dexamethasone and ACTH and after stimulation of the adrenal RAS by varied potassium diets. Aldosterone responses to ACTH stimulation compared to basal values have not been extensively documented so far in wild type mice. One study describes the response to ACTH treatment in wild type mice and mice lacking POMC, the precursor for ACTH, whereby the size of the adrenal gland as well as missing zonal structure, including the zona glomerulosa, returns after the 10 day treatment, with corticosterone levels rising in POMC null mice (to equal sham treated wild types) whilst aldosterone levels remain un-stimulated (Coll et al., 2004). However aldosterone data is not presented in this article describing the wild type values for response to ACTH. In the 11 week old mice which were included in the study assessing the response of the HPA axis, concentrations following stimulation with ACTH were, as expected, significantly higher compared to controls while those treated with dexamethasone suppression had concentrations significantly lower than the control group. Though ACTH was administered over ten days in these animals, the data most probably

demonstrate the effect of the last dose of ACTH on levels of aldosterone. This is most likely the case since studies have shown that ACTH has only short-lived effects on aldosterone, with a half-life of only 8 minutes, and the response to stimulation with ACTH decreases during chronic treatment unless it is given in a pulsatile fashion (Seely et al., 1989).

Elevated extracellular potassium concentrations directly stimulate aldosterone production by acting on the steroidogenic pathways occurring in the cells of the zona glomerulosa. This happens in a rapid effect within minutes at the level of cholesterol conversion to pregnenolone, but also on a longer-term basis of hours to days, by increased activity of aldosterone synthase at the level of corticosterone conversion to aldosterone (Muller, 1995; Williams, 2005). In our assessment of serum samples from mice administered a high K^+ diet for 5 weeks aldosterone levels were greatly increased as expected. The extent of the increase was confirmed as results were in extremely close correlation with a commercial assay requiring a much larger sample volume. Concentrations were not suppressed, however, on a low K^+ diet to below the control group values, and this was also observed using both assays. The findings of aldosterone levels in wild type mice on a normal potassium diet are in agreement with the findings of previous authors (172 ± 36 pg/ml vs 201.8 ± 26.2 pg/ml) (Arrighi et al., 2001), however the concentrations after a high K^+ diet are significantly lower but this may be expected as the mice were only given high K^+ chow without concurrently increased amounts of K^+ in the drinking water and the diet was only given for 2 weeks as opposed to 5 weeks as was the case in our study.

5. Summary

Recent evidence has shown the increased incidence of PA in approximately 15% of the hypertensive population, making a non-invasive and simple screening method for the measurement of aldosterone levels necessary. The use of saliva for determination of steroid hormones is now widely used and accepted and salivary aldosterone concentrations have previously been reported at around 30% of those seen in plasma. Furthermore, there is a current lack of longitudinal and systematic studies addressing the involvement of aldosterone in the regulation of the RAAS in rodents due to sample volume restrictions and the lack of sensitivity to detect the very low aldosterone concentrations in commercially available assays.

We developed a non-isotopic, competitive immunoassay for the determination of aldosterone levels in saliva, as well as in human and mouse plasma samples. The assay employs an aldosterone-biotin conjugate as the tracer and end-point determination through time-resolved fluorescence (TR-FIA) with Streptavidin-Europium as the detectable label. No pretreatment or purification of saliva is necessary while a simple extraction step is incorporated for the assessment of plasma levels. A polyclonal antibody was used for the development of the saliva assay giving a lower limit of detection of 19 pg/ml for each 50µl sample. Similarly, a highly specific monoclonal antibody against aldosterone, exhibiting a more sensitive linear working range starting from 8 pg/ml is used to detect aldosterone in 50µl of plasma. The monoclonal antibody could potentially also be used for the determination of salivary aldosterone levels, however this was not sufficiently evaluated in the studies shown here and further investigation of the exact assay conditions is needed.

Inter- and intra-assay coefficients of variation, mean recoveries, accuracy and linearity were validated for both assays and the assay results correlated significantly with a commercially available radioimmunoassay for plasma in both settings.

Overall, salivary aldosterone was found to correspond to approximately 28% of the concentrations seen in plasma and reflected the changes seen with posture and ACTH stimulation accurately. The assay presents the additional possibility of using salivary aldosterone levels, in combination with salivary cortisol, as a diagnostic tool in a clinical setting to screen suspected cases of PA and exclude healthy subjects. The salivary aldosterone to cortisol ratio remains elevated in PA persons due to autonomous hypersecretion of aldosterone throughout the day, alongside decreasing levels of cortisol, and can be clearly distinguished from healthy persons above a cut-off level of 0.1. Furthermore, as aldosterone concentrations are acutely affected by ACTH it was determined that sampling for this test should be carried out in the evening to avoid stress factors as well as diurnal fluctuations.

In addition, as basal aldosterone values and those after suppression and stimulation under different conditions were found within the linear range of the assay, it is proposed that the assay could be especially useful to monitor adrenocortical function in pharmacological and dietary intervention studies in rodent models where repeated sampling and volumes collected are limited and measurement of multiple blood parameters is desirable.

Zusammenfassung

Neue Untersuchungen zeigten, dass bei hypertensiven Patienten die Inzidenz des primären Hyperaldosteronismus höher als lange angenommen ist und bis zu 15% beträgt. Deshalb ist eine nichtinvasive und einfach durchzuführende Untersuchungsmethode zur Messung der Aldosteronkonzentration erstrebenswert. Die Verwendung von Speichel für die Bestimmung vieler Steroidhormone ist mittlerweile verbreitet und akzeptiert. Aldosteron zirkuliert in sehr niedrigen Konzentrationen im Plasma, und die Konzentration von Aldosteron im Speichel beträgt nach einzelnen Berichten ungefähr 30% der Plasmakonzentration. Daher ist die Sensitivität verfügbarer Aldosteronassays zur Messung im Speichel nicht ausreichend. Ein ähnliches Problem ergibt sich bei Studien, die den Einfluss von Aldosteron auf die Regulation des RAAS in Nagern untersuchen, da hier das Probenvolumen limitiert ist und kommerziell erhältliche Assays nicht sensitiv genug sind, um hier die sehr niedrigen Aldosteronkonzentrationen zu messen.

Daher wurde im Rahmen dieser Arbeit zunächst ein hochsensitiver, nicht-isotopischer kompetitiver Immunoassay zur Bestimmung der Speichelaldosteronkonzentration entwickelt. Der Assay verwendet ein polyklonales Antiserum als Fangantikörper und ein Aldosteron-Biotin Konjugat als Tracer, die Endpunktbestimmung erfolgt über Streptavidin-Europium durch zeitaufgelöste Fluoreszenz (TR-FIA). Für diesen Assay ist bei Speichelproben keine Vorbehandlung oder Reinigung notwendig, bei einem Probenvolumen von 50 µl wird ein unteres Detektionslimit von 19 pg/ml erzielt. Eine Abwandlung dieses Assays unter Verwendung eines spezifischen monoklonalen Antikörpers gegen Aldosteron erlaubt nach einer einfachen Extraktion mit Dichlormethan auch die Aldosteronbestimmung in kleinsten Volumina von humanem und murinem Plasma. Hierbei wird bei 50 µl Probenvolumen eine Sensitivität von 8 pg/ml erreicht. Ob dieser monoklonale Antikörper auch für den Speichelassay genutzt werden

könnte, wurde im Rahmen der hier gezeigten Untersuchungen noch nicht im Detail evaluiert. Die technische Validierung beider Assays wurde hinsichtlich Präzision, Reproduzierbarkeit, Wiederfindung, Linearität und Kreuzreaktivitäten durchgeführt. Die Meßergebnisse beider Assays korrelierten signifikant mit denen eines kommerziell erhältlichen Plasma-Radioimmunoassays, der allerdings ein vielfach höheres Probenvolumen benötigt.

Die klinische Validierung des Speichelassays bestätigte, daß die Speichelaldosteronkonzentration ca. 28% der Plasmakonzentration beträgt. Effekte durch Positionsveränderungen und ACTH-Stimulation, die sich im Plasmaaldosteron zeigen, werden akkurat auch in der Speichelaldosteronkonzentration widergespiegelt. Zudem wurde die Möglichkeit evaluiert, durch parallele Bestimmung von Aldosteron und Cortisol im Speichel und die Berechnung des Aldosteron/Cortisol-Quotienten Patienten mit primärem hyperaldsoteronismus von gesunden Subjekten abzugrenzen. Der Aldosteron/Cortisol-Quotient ist bei Patienten insbesondere spät nachts erhöht, da die autonome Hypersekretion von Aldosteron anhält, während die Cortisol-Werte absinken. Bei einem cut-off-Wert für den Quotienten von 0,1 können Patienten eindeutig von Gesunden unterschieden werden. Da die Aldosteron-Konzentration auch durch ACTH erheblich beeinflußt wird, wurde die abendliche Probennahme als bester Zeitpunkt ermittelt, da hier in aller Regel Streß und andere hypophysär vermittelte Einflußfaktoren am geringsten sind.

Der Assay zur Messung von Aldosteron aus kleinsten Probenvolumina wurde im Nagermodell validiert, wobei Werte nach Suppression der Nebennieren mit Dexamethason wie nach Stimulation mit ACTH und Kochsalzrestriktion innerhalb des linearen Messbereichs lagen. Deshalb könnte der Assay gerade im Nagermodel besonders nützlich sein, um die adrenocortikale Funktion in pharmakologischen und diätetischen Interventionsstudien zu

untersuchen. Angesichts der limitierten Probenvolumina gerade bei wiederholten Blutabnahmen sowie dem Bedarf, eine Vielzahl verschiedener Blutparametern zu bestimmen, sind bislang verfügbare Aldosteronassays hier wenig geeignet.

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7. Appendix

7.1. Consent Form for Posture Study Test

KLINIKUM DER UNIVERSITÄT MÜNCHEN

CAMPUS INNENSTADT
MEDIZINISCHE KLINIK
Lehrstuhl Endokrinologie / Diabetologie
Direktor: Prof. Dr. Martin Reincke

Klinikum der Universität München · Campus Innenstadt
Medizinische Klinik · Ziemssenstraße 1 · 80336 München

Endokrinologische Forschungslabors
Leiter: Dr. med. Martin Bidlingmaier

Tel. +49 (0)89 5160 – 2277
Fax +49 (0)89 5160 – 4457
E-Mail: martin.bidlingmaier@med.uni-muenchen.de

Internet:
http://mkl.medinn.med.uni-muenchen.de

Postanschrift:
Ziemssenstraße 1
80336 München

CONN-SYNDROM:
DIAGNOSTIK, BIOCHEMIE, MOLEKULARBIOLOGIE

Probandeninformation und Probandeneinwilligungserklärung
Version 16.11.2007

Liebe Probandin, lieber Proband,

Sie wurden von uns gefragt, ob Sie bereit sind als Probandin / Proband an einer wissenschaftlichen Studie teilzunehmen. Mit diesen Zeilen möchten wir Ihnen wichtige Informationen über diese Studie zukommen lassen.
Bitte lesen Sie die folgenden Informationen sorgfältig durch. Bitte wenden Sie sich bei Unklarheiten oder zusätzlichen Fragen an die / den für die Studie zuständige(n) Ärztin / Arzt.

Allgemeine Information

Durch diese Studie soll die Verlässlichkeit einer neuen Methode zur Messung des Hormons Aldosteron in Speichelproben untersucht werden. Das Hormon Aldosteron wird bei jedem Menschen in der Nebennierenrinde produziert und erfüllt wichtige Funktionen im Körper, kann jedoch bei übermäßiger Produktion auch nachteilige Wirkungen hervorrufen. Es spielt insbesondere eine große Rolle bei einer bestimmten Form des Bluthochdrucks, dem sogenannten Conn-Syndrom. Dabei wird durch einen Überschuss an Aldosteron der Elektrolytstoffwechsel gestört und so der Bluthochdruck bewirkt. Eine Erkennung dieser Ursache des Hochdrucks ist sehr wichtig, da man dann eine ursächliche Therapie der Erkrankung und somit oft eine Heilung erreichen kann. Bislang ist die Untersuchung der betroffenen Patienten jedoch sehr zeitaufwändig und mit vielen Blutentnahmen verbunden.

Wir hoffen, durch die Messung des Hormons in den viel einfacher, nämlich von den Patienten selbst auch ohne einen Arztes zu sammelnden Speichelproben, die Diagnostik dieser Erkrankung schneller und einfacher zu machen.

Als Probandin / Proband dürfen nur gesunde Personen teilnehmen. Sie sollten keine Medikamente regelmäßig einnehmen. Ausnahmen sind orale Kontrazeptiva (Pille), leichte

Das Klinikum der Universität München ist eine Anstalt des öffentlichen Rechts.
öffentl. Verkehr: U1, U2, U3, U6, U7, U8, Straßenbahnlinien 17, 18, 27
Stadtbuslinie 152 bis Haltestelle Sendlinger Tor

Behandlungseinrichtung
für Typ 1 und Typ 2 Diabetiker

UNION EUROPÉENNE DES MEDECINS SPECIALISTES
EUROPEAN UNION OF MEDICAL SPECIALISTS

Schmerzmittel (z.B. Aspirin) und Schilddrüsenhormone oder Jod. Sie sollen solche Medikamente lediglich dem Sie in der Studie betreuenden Arzt nennen.

Diese Studie wurde von einer unabhängigen Ethikkommission begutachtet und zustimmend bewertet.
Ihre Ärztin / Ihr Arzt wird Sie in einem ausführlichen Gespräch über Wesen, Bedeutung und Tragweite der Studie aufklären und Sie über den zu erwartenden Nutzen und über die möglichen Risiken der Studie informieren.
Diese Studie wird ausschließlich aus Mitteln des Forschungshaushalts der Universität und aus Mitteln der Deutschen Forschungsgemeinschaft finanziert.

Zielsetzung der Studie

In dieser Studie soll untersucht werden, wie stark die Konzentration von Aldosteron in den Speichelproben zu verschiedenen Zeitpunkten und unter verschiedenen Sammelbedingungen schwankt.

(a)   Wie stark schwanken die Aldosteronkonzentrationen in einem Individuum über einen Tag?
(b)   Wie stark sind die Konzentrationsschwankungen zwischen Individuen in einer großen Gruppe gesunder Personen, sowie zwischen Patienten mit Bluthochdruck unterschiedlicher Ursache?

Die Ergebnisse dieser Studie sollen es uns ermöglichen, Empfehlungen für die Untersuchung von Patienten, die an entsprechenden Erkrankungen leiden, zu erarbeiten. Zudem erfahren wir mehr über die Verlässlichkeit bzw. die Möglichkeit oder Unmöglichkeit einer Aldosteronmessung im Speichel.

Ablauf der Studie / Untersuchungen

Zunächst wird sich ein Arzt / eine Ärztin mit Ihnen über diese Information und die Studie insgesamt unterhalten. Es ist sehr wichtig, dass Sie schriftlich Ihr Einverständnis zur freiwilligen Teilnahme an dieser Studie erteilen, bevor die betreffenden Untersuchungen an Ihnen vorgenommen werden oder im Labor Speichel- oder Blutproben für Analysen verwendet werden.
Bevor Blutentnahmen stattfinden, wird sich ein Arzt / eine Ärztin ausführlich mit Ihnen über Ihren Gesundheitszustand bzw. über Ihre Krankheitsgeschichte unterhalten.

In allen Teilen der Studie werden die Speichelproben mit einem speziell konstruierten Sammelröhrchen, der sogenannten „Salivette®" gesammelt. Dabei handelt es sich um Plastikröhrchen, in denen sich ein kleiner Schwamm befindet. Diesen Schwamm sollen Sie zur Sammlung einer Speichelprobe ca. 3 – 5 min. kauen und dann – ohne ihn mit den Fingern zu berühren – wieder in das Plastikgefäß zurückspucken.

(a) Sammlung von Einzelproben:
Zur Untersuchung, der Hormonschwankungen über den Tag bitten wir Sie, alle zwei Stunden eine Salivette zu kauen und zu uns zurückzubringen. Die Speichelsammlung findet nur an einem Tag statt. Die Salivetten zum Sammeln des Speichels sind bereits beschriftet.

(b) Blutabnahme:
Nach dem Legen einer Verweilkanüle wird Ihnen nach Hinsetzen/-legen in der ersten Stunde alle 15 Minuten eine Blutprobe entnommen. Sie müssen danach noch eine weitere

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Stunde liegen bleiben. Nach Aufstehen wird Ihnen erneut in der ersten Stunde alle 15 Minuten eine Blutprobe entnommen. Nun müssen Sie noch 2 weitere Stunden stehen bleiben und umhergehen. Nach der ersten und nach der 3. Stunde wird Ihnen ebenfalls ein Röhrchen abgenommen. In der Zwischenzeit dürfen Sie die Klinik auch verlassen – wichtig ist nur, dass Sie umhergehen bzw. stehen und nicht sitzen oder liegen. Insgesamt werden Ihnen maximal 50 ml Blut entnommen.

Zusätzlich möchten wir Sie um Ihr Einverständnis bitten, eventuell verbleibende Reste der Blutproben aufbewahren und für zukünftige Messungen verwenden zu dürfen. Dies ist im Labor bei Einführung neuer Methoden oft wichtig, um Vergleichswerte zu bekommen. Selbstverständlich werden auch diese Reste der Blutproben nur den unten unter „Vertraulichkeit der Daten" genannten Richtlinien verwendet.

Fragen zum Gesundheitszustand
Der betreuende Arzt wird Sie bei jedem Besuch über Ihren Gesundheitszustand befragen. Zusätzlich bitten wir Sie einen Fragebogen bzgl. Ihres Gesundheitsstatus auszufüllen.

Risiken

Risiken der Sammlung von Speichelproben
Die Sammlung von Speichelproben stellt keinerlei Gefahr dar. Die Salivetten werden seit vielen Jahren hierzu verwendet, nachteilige Auswirkungen sind nicht bekannt.

Risiken der Blutabnahme
Die Blutentnahme von 50 ml stellt kein gesundheitliches Risiko dar. Blutspender des Roten Kreuzes beinhalten Abnahmen von bis zu 500 ml.
Zunächst besteht bei diesem Test wie bei jeder Blutentnahme das Risiko, dass beim Legen der Verweilkanüle an der Injektionsstelle vorübergehende Blutungen und Schmerzen, ein blauer Fleck sowie selten (meist durch das verwendete Pflaster bedingte) lokale allergische Reaktionen bzw. Entzündungen auftreten.

Vorzeitiger Abbruch der Studie
Wie bei jeder klinischen Studie ist es möglich, dass diese Studie frühzeitig abgebrochen wird. Sollte dies der Fall sein, ist Ihre Zustimmung hierzu nicht erforderlich. Zudem können Sie von einer weiteren Studienteilnahme ohne Ihre Zustimmung ausgeschlossen werden, z.B. wenn es Ihnen nicht möglich ist, die vorgesehenen Untersuchungstermine wahrzunehmen oder die Studienanweisungen zu befolgen. Sie können auch aus der Studie ausgeschlossen werden, wenn bei Ihnen nachträglich Ausschlusskriterien auftreten, wie z.B. eine Erkrankung, die eine weitere Studienteilnahme als zu riskant oder sinnlos erscheinen lässt.

Freiwilligkeit der Teilnahme
Ihre Teilnahme an dieser klinischen Studie ist freiwillig. Sie können Ihre Teilnahme jederzeit ohne Angabe von Gründen und ohne nachteilige Folgen beenden. Sie haben jederzeit das Recht, alle wichtigen Informationen über die Studie einzuholen.

Vertraulichkeit der Daten
Die personenbezogenen Daten und Informationen aus dieser Studie werden vertraulich behandelt. Die beteiligten Ärzte unterliegen selbstverständlich der ärztlichen Schweigepflicht.
Die im Rahmen der Studie erhobenen Daten werden in anonymisierter Form unter Einhaltung des Datenschutzes wissenschaftlich ausgewertet. Personenbezogene Daten

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werden nicht an Dritte weitergegeben. Nur vollständig anonymisiert werden die Daten für wissenschaftliche Veröffentlichung verwendet.

Stand dieser Information
Diese Information berücksichtigt alle Umstände und Daten, die im Februar 2004 über die im Rahmen der Studie verwendeten Analyse- und Diagnoseverfahren bekannt waren. Sollten sich wesentliche Änderungen ergeben, wird Sie Ihre Ärztin / Ihr Arzt umgehend darüber informieren.

Für weitere Fragen zu der beschriebenen Studie steht Ihnen Ihre Ärztin / Ihr Arzt,

Dr. med. Martin Bidlingmaier

Tel.: 089/5160 2277

zur Verfügung.

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CONN-SYNDROM:
DIAGNOSTIK, BIOCHEMIE, MOLEKULARBIOLOGIE

Einwilligungserklärung
Version 16.11.2007

Ich wurde von meiner Ärztin / meinem Arzt über Wesen, Bedeutung und Tragweite der Studie aufgeklärt. Ich habe alle Punkte der Probandeninformation gelesen und verstanden. Alle mich interessierenden Fragen hat meine Ärztin / mein Arzt in für mich verständlicher Weise beantwortet. Über den Studienablauf, die Risiken, meine Rechte und Pflichten bin ich informiert worden. Ein Exemplar der Probandeninformation und Einwilligungserklärung habe ich erhalten.

Ich erkläre mein Einverständnis zur Teilnahme an folgenden Teilen der Studie:

- ☐ Orthostasetest
- ☐ Sammlung von Tagesprofilen
- ☐ Kochsalzbelastungstest

Meine Teilnahme ist freiwillig und kann jederzeit von mir ohne Angaben von Gründen widerrufen werden, ohne daß mir daraus Nachteile entstehen. Über die Modalitäten der Honorierung wurde ich informiert.

Ich bin mit der im Rahmen dieser klinischen Prüfung erfolgenden Aufzeichnung von Krankheitsdaten (Daten zu meiner Erkrankung und deren Verlauf) einverstanden. Ich bin mit der elektronischen Datenverarbeitung und wissenschaftlichen Auswertung der Krankheitsdaten in anonymisierter Form einverstanden.

....
(Name, Vorname)   (Geburtsdatum)

....
(Ort, Datum)   (Unterschrift Patientin / Patient)

ERKLÄRUNG DES ARZTES
Die Probandin / der Proband wurde von mir über Wesen, Bedeutung und Tragweite der Studie aufgeklärt. Ein Exemplar der Probandeninformation und der Einverständniserklärung wurde der Probandin / dem Probanden ausgehändigt.

....
(Ort, Datum) (Stempel und Unterschrift der Ärztin / des Arztes)

Dr. med. Martin Bidlingmaier, Tel.: 089/5160 2277, Fax: 089/5160 4457
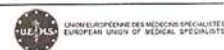
7.2. Questionnaire for Posture Study Test

J.A.W.S.

PROBANDENNUMMER: _____

Alter	
Geschlecht	

Körpergröße		cm
Gewicht		kg

Bekannte Krankheiten (allgem.)	
Regelmäßig einzunehmende Medikamente	
Blutarmut (Anämie)	
Kreislaufprobleme (Hypo-, Hypertonie)	
Erkrankungen des Stoffwechsels	
Erkrankungen des Herz-Kreislaufsystems	
Erkrankungen der Nieren und ableitenden Organe	
Erkrankungen des Bewegungsapparates	
Erkrankungen des Verdauungstraktes	
Neurologische Erkrankungen / Erkrankungen der Augen	
Frauen: Zyklustag / Hormonbehandlungen / Pille	
Nikotin / seit wann / Zigaretten pro Tag	
aktuell unter Diät (ggf. Diätform)	
Sport / - Art / h pro Woche	

Blutdruck	zw. 10 u. 11	
	12:15	

7.3. Instructions for use of Salivette®

Anleitung zur Speichelgewinnung mit der Salivette

Die genauen Sammelzeitpunkte bitte mit Ihrem Arzt absprechen.

Bei der Speichelgewinnung bitte beachten:
- Mindestens 30 min keine Nahrungsaufnahme, nichts Trinken (insbesondere nicht Kaffee, Kakao, Rotwein etc.)
- Kein Kaugummi kauen, nicht rauchen
- Vor dem Zähneputzen sammeln - keinesfalls direkt danach (Blutkontamination vermeiden!)
- Keine steroidhaltigen Tabletten / keine steroidhaltigen Cremes im Gesicht!

Wenn doch etwas getrunken oder Kaugummi gekaut wurde, ca 10min warten – bitte den Mund NICHT mit Wasser ausspülen (Verdünnung der Probe!).

Technik der Speichelsammlung:
- Verschlußstopfen entfernen - aber aufbewahren! Es ist günstig, das Einhängegefäß (siehe Abbildung) durch Druck am oberen Ende der Salivette im Zentrifugengefäß festzuhalten!
- Watterolle **ohne Hautkontakt** direkt aus dem Einhängegefäß in den Mund gleiten lassen.
- Ca 2-3min kauen, bis die Watterolle mit Speichel voll gesaugt ist (man sollte das Gefühl haben, dass die Watterolle wirklich voll ist und sich viel Speichel im Mund befindet).
- Watterolle direkt aus dem Mund ohne Hautkontakt wieder in das Einhängegefäß zurückspucken.
- Probenröhrchen mit dem Stopfen wieder gut und fest verschließen.
- Das Röhrchen unbedingt beschriften mit: **Name bzw. Probandennummer, Uhrzeit und Datum der Speichelprobe** (entweder mit Aufkleber oder wasserfestem Stift)

DIE KOMPLETTE SALIVETTE BESTEHT AUS STOPFEN, WATTEROLLE, EINHÄNGEGEFÄß UND ZENTRIFUNGENGEFÄß. BITTE UNBEDINGT AUCH SO KOMPLETT WIEDER ABGEBEN.

Zentrifugengefäß   Einhängegefäß   Watterolle   Stopfen

Acknowledgements

Curriculum Vitae

Name	Jenny Manolopoulou
Date of Birth	6th February, 1979
Place of Birth	Hannover, Germany
Address	Adelheidstr.34a, 80796, München
Marital status	single

Education

1984 - 1985	Makri Private Elementary School Athens, Greece
1985 - 1987	Greek Community School of Melbourne, Australia
1987 - 1988	Brighton Community School of Melbourne, Australia
1988 - 1990	Campion School of Athens, Greece
1990 - 1991	Glencoe Middle School Chicago, USA
1991 - 1994	Seoul Foreign School, S.Korea
1994 - 1996	International Baccalaureate American Community School of Athens
1996 - 1999	BSc (Hons) Medical Biochemistry University of Leicester
1999 - 2000	MSc Advanced Neuro- and Molecular Pharmacology University of Bristol

Work Experience

2000 - 2001	Research Scientist - Hellenic Pasteur Institute, Athens, Greece
2001 - 2002	Histologist/Immunohistochemist - GlaxoSmithKline, Essex, UK
2002 - 2003	Temporary Scientist - Merck Sharp and Dohme, Essex, UK
2003 - 2003	Biomedical Scientist - Pfizer Inc., Kent, UK
2004 - 2004	Research Scientist - "Olympic Games Athens 2004", Athens, Greece
2005 – 2007	(Stipendium) Ludwig Maximilians University Munich