AMPEROMETRIC DETECTION OF ALDOSTERONE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH COPPER(II) BIS-PHENANTHROLINE

by

Rakesh Bose

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Signature: Rakesh Bose 8/21/95
Student Date

Approvals:

Leonard S. Kriegel 8/21/95
Thesis Advisor Date

James H. Nash 8/21/95
Thesis Co-Advisor Date

Michael A. Serra 8/21/95
Committee Member Date

John J. Kasinski 8/21/95
Dean of Graduate Studies Date

Abstract

A sensitive method for the determination of aldosterone in human urine has been developed. The method uses high performance liquid chromatography (HPLC) with amperometric detection for the estimation of urinary aldosterone.

The method utilizing electrochemical detection has been developed to overcome problems of low sensitivity and low selectivity that are often encountered in HPLC, particularly in the analysis of complex matrices such as biological fluids.

Essential steps to the method include acid hydrolysis of the aldosterone-18 conjugate in order to release the aldosterone in a 24 hr. urine sample, followed by solid-phase extraction with cyclohexyl and silica cartridges. This was followed by high performance liquid chromatography using a C_{18} silica based reversed phase column with detection of current in an electrochemical cell by the oxidation of the synthesized copper (II) bis-phenanthroline reagent. There was a linear correlation between the amount of aldosterone and the area under the aldosterone representative peak on the chromatogram. The method was able to detect aldosterone concentrations as low as 20 ng. This technique provided an accurate means of assaying urinary aldosterone.

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I would like to thank Nicole, for supporting me throughout my thesis. To my friends and graduate colleagues, I wish them God-speed.

Finally, I would like to thank God, for his guidance.

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LIST OF ABBREVIATIONS

ABBREVIATIONS	DEFINITION
HPLC	High-Performance Liquid Chromatography
hr	Hour
ng	Nanogram
DOC	11-deoxycorticosterone
OH	Hydroxy
ACTH	Adrenocorticotropic hormone
CBG	Corticosteroid-binding globulin
SHBG	Sex hormone-binding globulin
CMO	Corticosterone methyl oxidase
GC-MS	Gas Chromatography-Mass Spectrometry
DMB	1,2-diamino-4,5-methylenedioxybenzene
ABEI	Aminobutylethyl isoluminol
THALD	3α,5β-tetrahydroaldosterone
DPH	4,5-diaminopthalhydrazide
min	Minute
μg	Micogram
pg	Picogram
SP-RIA	Solid Phase Radioimmunoassay
UV	Ultra-Violet
CBP	Copper(II) Bis-Phenanthroline
CV	Cyclic voltammetry
SPE	Solid-Phase Extraction

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CHAPTER I

INTRODUCTION

A. Biochemistry of Aldosterone:

The human adrenal cortex secretes a variety of steroid hormones that are intimately concerned with a wide range of metabolic processes. The steroids that contain the cyclopentanoperhydrophenanthrene (fig.1) ring system constitute one of the more complex group of hormones (1). They include natural products such as sterols, bile acids, corticosteroids, cardiac glycosides, sapogenins and some alkaloids. Although these compounds are structurally similar, the biochemical effects produced by the various steroids differ significantly. Aldosterone, one of the major corticosteroids, plays a primary role in regulating electrolyte and fluid balance and blood pressure (2).

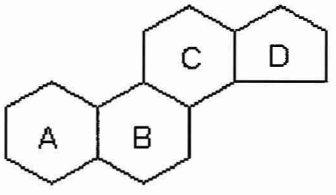
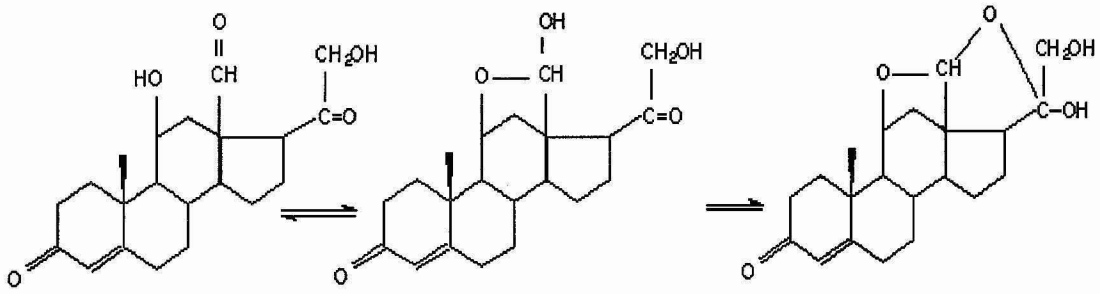


Figure 1: Cyclopentanoperhydrophenanthrene Ring (1)

In 1953, an Anglo-Swiss team, which included Simpson, Tait, Wettstein, Neher, Von Euw and Reichstein isolated the pure form of aldosterone. In 1954, the same team successfully established the chemical formula of the new compound (3). It was found that the new steroid, unlike other known corticosteroids, possessed an aldehyde group (-CHO) in position eighteen instead of the more typical $-CH_3$ group. Therefore the compound was named aldosterone. The tautomeric forms of aldosterone (Δ^4-Pregnen-18-al-11β,21,diol-3,20-dione-(11-18)- lactol) are shown in figure 2. Aldosterone in solution exists in an equilibrium between the aldehyde structure and the hemiacetal, with the hemiacetal form being favored.



ALDEHYDE HEMIACETAL HEMIKETAL

Figure 2: Tautomeric forms of Aldosterone (1)

B. Biosynthesis, Transport, Metabolism:

Aldosterone, the most potent mineralcorticoid, under physiological circumstances is synthesized in the adrenal zona glomerulosa of the adrenal cortex. Ayres et al in 1958 discovered that the isolated glomerular zone of human adrenal glands is able to produce aldosterone whereas the isolated fascicular and reticular zones are not (3). The synthesis of aldosterone requires 11 β-hydroxylation of 11-deoxycorticosterone (DOC) to form corticosterone, followed by the hydroxylation at position C18 to form 18-hydroxy-corticosterone, finally leading to oxidation at position C18. All three reactions in the zona glomerulosa are catalyzed by a single cytochrome P450 enzyme. The gene encoding this enzyme is termed CYP11B2 (4). Various phases are involved in aldosterone synthesis which take place in different structural elements of the adrenal cells such as the mitochondria, microsomal fraction and the cytoplasm (fig.3).

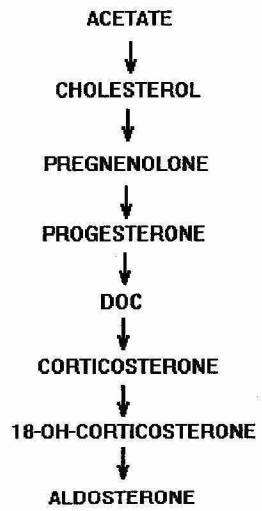


Figure 3: Intracellular Localization of Aldosterone Biosynthesis (3)
(DOC: Desoxycorticosterone)

With the use of various labeled precursors, the possible pathways of aldosterone synthesis have been confirmed (fig.4). Studies show that special 18-OH corticosteroids, such as 18-OH-progesterone, 18-OH-corticosterone and 18-OH-DOC, also play an intermediate role in the biosynthesis of aldosterone (3).

A recent study by Vecsei et al (5), postulates 21-deoxyaldosterone to be a precursor of aldosterone in an alternative biosynthesis pathway. Besides the main route of aldosterone biosynthesis via 11-deoxycorticosterone, corticosterone and 18-OH-corticosterone, data suggests an alternate biosynthetic pathway exists via 21-deoxycorticosterone among healthy and hypertensive patients. The data also shows similarities between the regulation of 21-deoxyaldosterone and the regulation of aldosterone.

In the zona fasciculata of human adrenal cortex, the steroid 11β-hydroxylase (P450C11) enzyme helps in the conversion of 11-deoxycortisol to cortisol. It is also responsible for the successive 11β and 18-hydroxylation and 18-oxidation of deoxycorticosterone, required for aldosterone synthesis in the zona glomerulosa. Two distinct 11β-hydroxylase genes in man, CYP11B1 and CYP11B2, have been predicted by Natarajan et al (6), to encode proteins with 93% amino acid identity. The same study shows that the CYP11B1 product could only 11β hydroxylate 11-deoxycortisol or deoxycorticosterone, whereas the CYP11B2 product could also 18-hydroxylate cortisol or corticosterone. It has been shown that CYP11B2 is also required for the final steps in

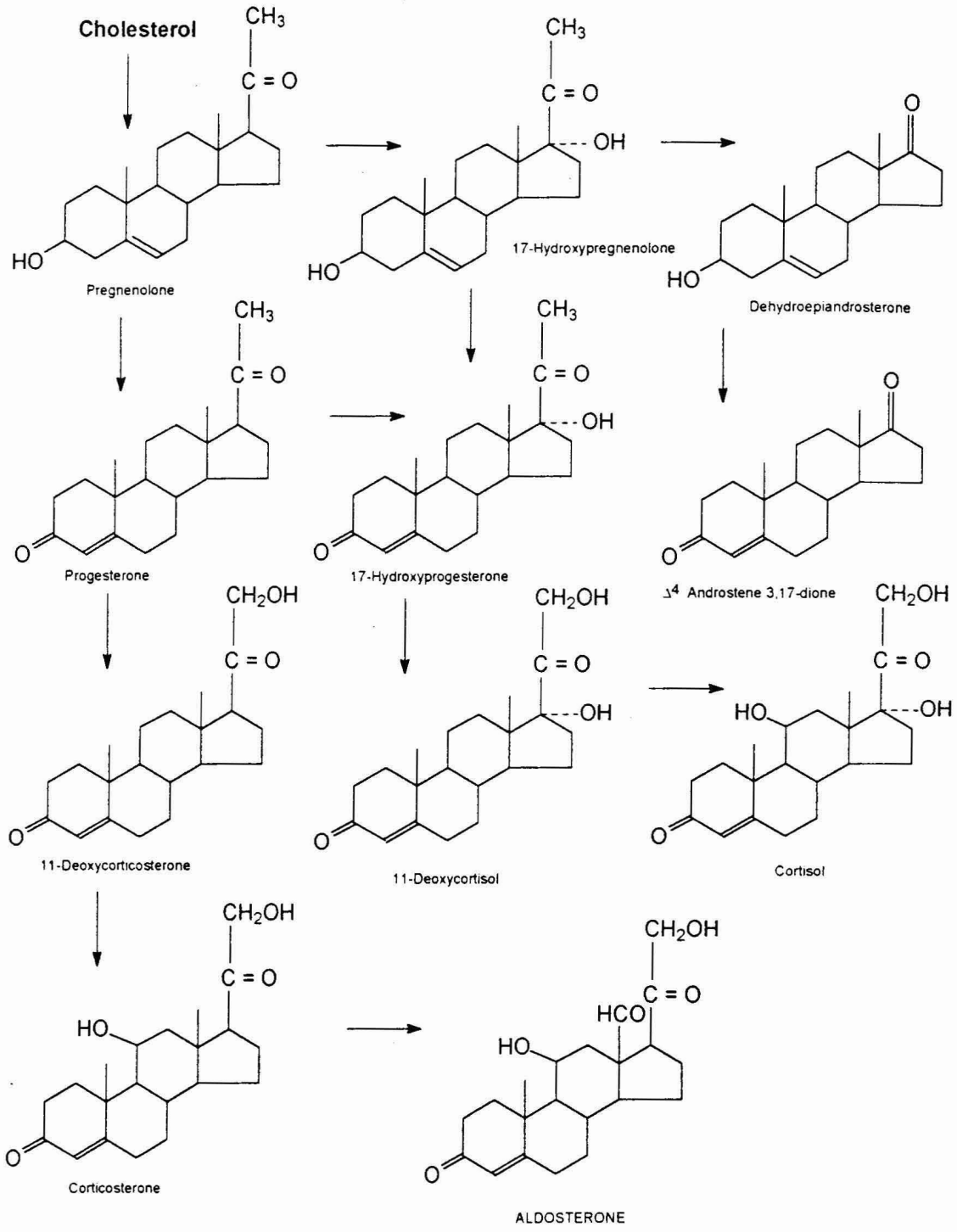


Figure 4: Pathways of Aldosterone Synthesis (17)

aldosterone synthesis.

In mammals, the final two steps in the biosynthesis of aldosterone play a major role in the complex physiological adaptation of aldosterone secretion to changes in sodium ion and potassium ion concentration. Muller (7) investigated the nature and the identity of the enzyme catalyzing these steps. In the human adrenal gland, two types of cytochrome P-450 (11β) enzymes are encoded by two different genes. In the adrenal cortex, the main enzyme has been found to catalyze only the conversion of deoxycorticosterone to corticosterone or 18-hydroxy-11-deoxycorticosterone whereas the second enzyme catalyzes the three steps involved in the conversion of deoxycorticosterone to aldosterone and occurs only in the zona glomerulosa (7).

Aldosterone is secreted in the zona glomerulosa of the outer layer of the adrenal gland known as the cortex. This layer contains an obligatory enzyme, 18-hydroxysteroid dehydrogenase, responsible for the formation of aldosterone. Adrenocorticotropic hormone (ACTH) stimulates steroidogenesis in the zona glomerulosa from cholesterol to progesterone and corticosterone. The renin angiotensin system then subsequently transforms corticosterone to aldosterone (8). Other factors such as adrenergic and dopaminergic systems, sodium and potassium ion concentration and ACTH levels modulate aldosterone synthesis either directly or indirectly by affecting the renin angiotensin system (fig.5).

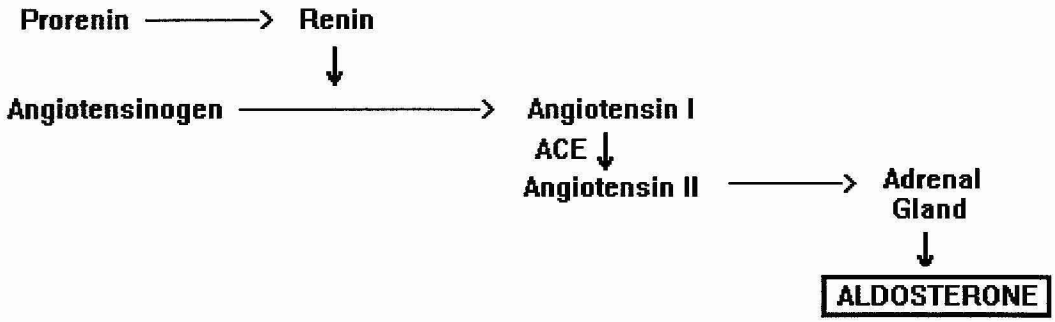


Figure 5: Renin-Angiotensin-Aldosterone System (2)
(ACE: Angiotensin-Converting Enzyme)

The control mechanism for the secretion of aldosterone involves the renin-angiotensin system. Renin, a proteolytic enzyme, is synthesized and stored in specialized cells called the juxtaglomerular cells, which are located along the terminal part of the afferent arterioles of the renal glomeruli (9). Stimulation of these cells releases renin into the blood, where it hydrolyzes its substrate, angiotensinogen, to produce a decapeptide known as angiotensin I. Angiotensin I is then rapidly converted to an octapeptide, angiotensin II. Angiotensin II is a potent vasoconstrictor and stimulates the cells of the zona glomerulosa to produce aldosterone by aiding in the conversion of cholesterol to pregnenolone. Studies performed by Aguilera (10) showed that the effect of angiotensin II is potentiated by serotonin or by increases in extra-cellular potassium ion concentration, while the effect is inhibited by dopamine, somatostatin, and atrial natriuretic peptide.

The chief effect of aldosterone is the promotion of sodium ion reabsorption by the kidney to maintain an appropriate $Na^+/K^+/H^+$ concentration balance (fig.6). This reabsorption process also affects water retention by the body. Aldosterone production is highly regulated by the renin-angiotensin system and to a much lesser extent by adrenocorticotropichormone (ACTH) concentrations. ACTH attains a more prominent role in regulating aldosterone secretion when the renin-angiotensin system is suppressed.

Aldosterone is secreted episodically, with the highest circulating levels at about the time of awakening and the lowest concentrations shortly after sleep onset. In healthy subjects, a low sodium diet, maintaing an upright posture and the use of diuretics have been found to increase plasma aldosterone levels, whereas a high sodium diet, angiotensin converting enzyme inhibitors and lying in the supine position decreases aldosterone secretion (11). Sodium restriction has been found to increase the number of adrenal glomerulosa angiotensin II receptors and the activity of enzymes of the early and late aldosterone biosynthetic pathway (10).

According to Muller et at (12), the secretion of aldosterone is directly influenced by various physiological agents. Their action includes acute stimulation or inhibition of early biosynthetic step, long term activation or suppression of late biosynthetic steps, and induction of growth or atrophy of zona glomerulosa cells. One of the agents, endothelin-1, has been found by Rossi et al (13), to stimulate aldosterone secretion by

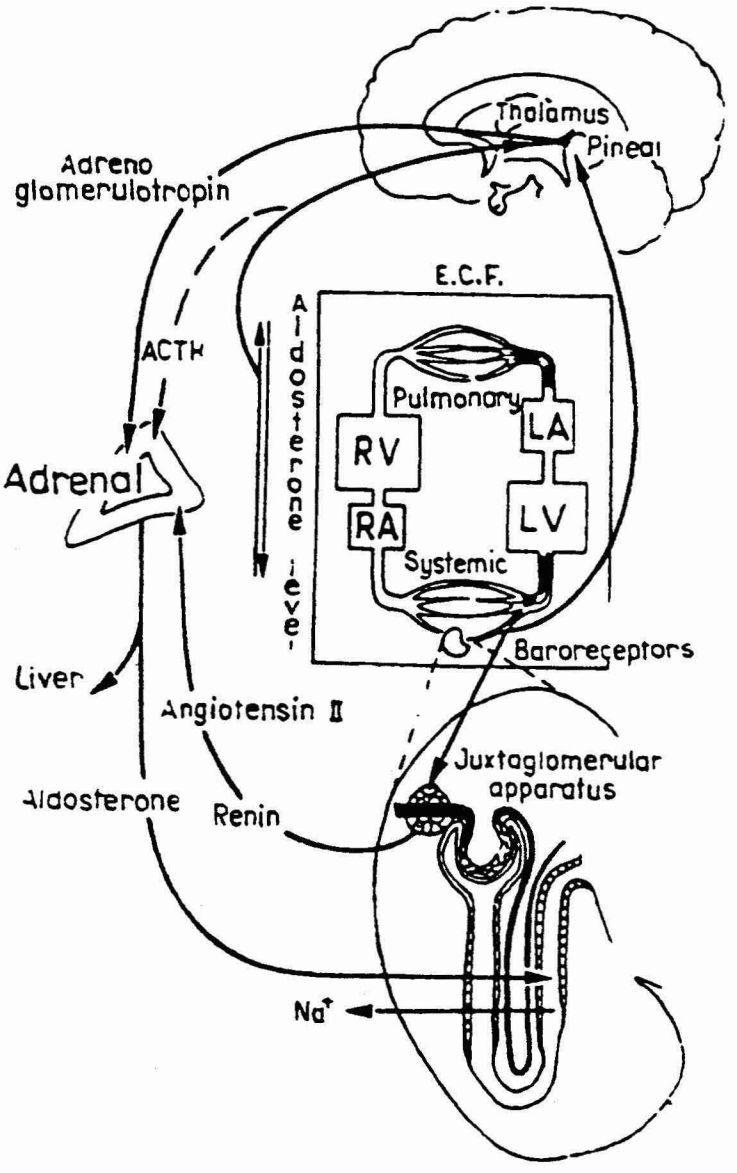


Figure 6: The mechanism controlling aldosterone secretion (3)

interacting with the specific receptors endothelin-A and endothelin-B at a greater level in aldosterone-producing adenomas than in the normal human adrenal cortex. Calcium ion has also been found by Ganguly et al (14), to mediate aldosterone secretion from the adrenal glomerulosa cells.

Steroid hormones circulate in the blood as free hormones or are bound to specific steroid binding proteins such as corticosteroid binding globulin (CBG), albumin, or sex hormone-binding globulin (SHBG) that are produced by the liver. Some steroid hormones are conjugated to glucuronide or sulfate. They are excreted via the kidneys or gastrointestinal tract, where they can be reabsorbed. Aldosterone has a higher affinity for CBG, although the latter is not a major carrier protein. Once it has been synthesized in the adrenal gland zona glomerulosa, aldosterone is released into the blood stream where it gains entry into the cytoplasm of responsive epithelial cells. Upon entry into the cell, aldosterone binds rapidly to cytoplasmic receptors and the receptor-aldosterone complex is then transferred to the nucleus of the cell. After it is bound to its nuclear receptor, DNA-dependent RNA synthesis is initiated, leading to the generation of several proteins (15). Aldosterone also binds to plasma protein molecules. According to Luetscher and Cheville (16), the aldosterone then enters the kidney where it can either return to the plasma, be excreted unchanged into the urine and form acid-labile conjugates or emerge as another unspecified metabolite. The liver is responsible for clearance of aldosterone from

the plasma and forms various conjugates and metabolites. Acid-labile conjugates are formed in the kidneys and in the liver. Studies performed by Peterson (3) confirmed that under normal circumstances the greatest amount of aldosterone is metabolized in the liver. Thus the majority of the degradation products of aldosterone, especially tetrahydro-aldosterone, are formed in the liver (fig.7). On the other hand, the kidneys are considered to take part in the formation of the 18-oxo-glucuronide of aldosterone. The presence of the metabolic products of aldosterone in blood plasma has been previously demonstrated by Pasqualini and Luetscher (18). About 50 to 55 percent of the administered aldosterone was found to be excreted as tetrahydroaldosterone glucuronide, acid-labile 18-oxo-glucuronide, and free aldosterone, with a large fraction being shared by other metabolites and derivatives of these metabolites (Kelly and Lieberman, fig.8)(18). A considerable number of the metabolites are the stereoisomers of tetrahydroaldosterone.

The tetrahydro derivatives of aldosterone are excreted in the urine as glucuronide esters. The acid-labile metabolites are made up of compounds conjugated at the eighteen position. Recent studies by Takeda et al (19), have shown the presence of other derivatives of aldosterone in the urine. One of those, 19-noraldosterone was shown to be synthesized and produced in the human adrenal gland, and it possesses a potent mineralcorticoid activity. 18,19-Dihydroxycorticosterone, a possible precursor of 19-noraldosterone, has also been identified in human urine (19). *In vitro* formation of

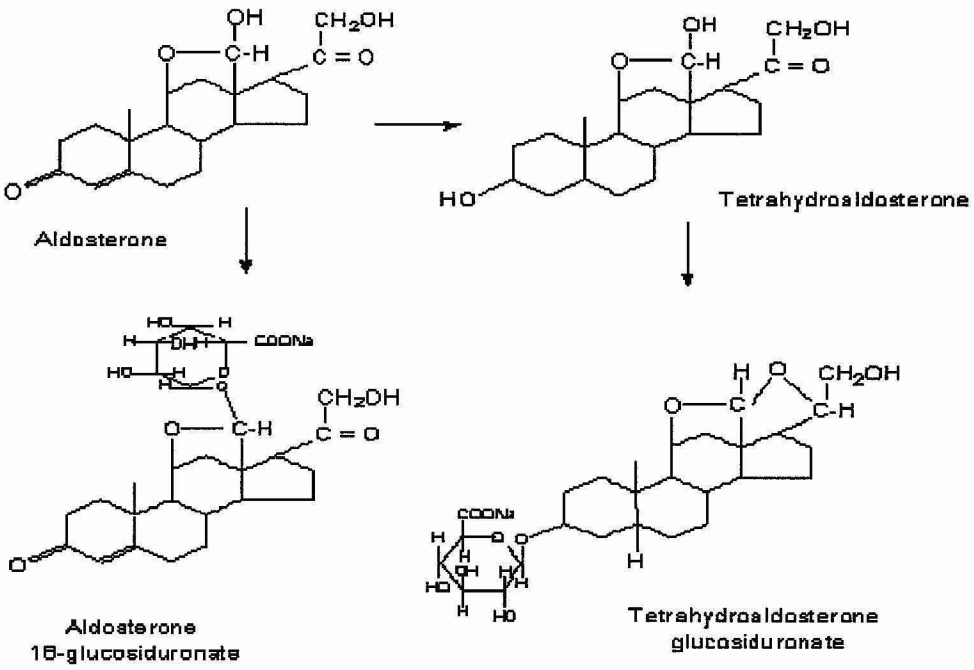


Figure 7: Metabolism of Aldosterone in the Liver (17)

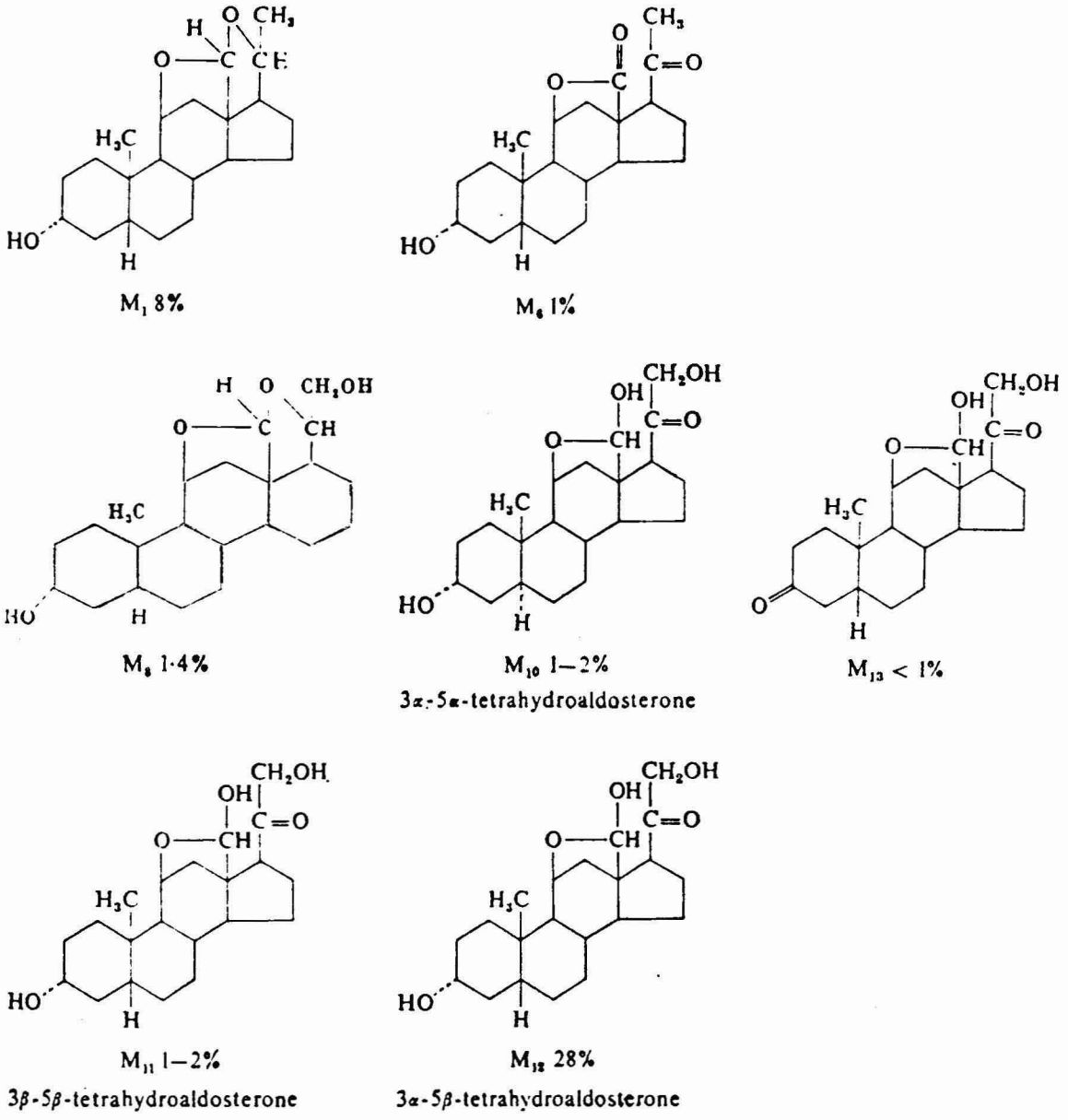


Figure 8: Metabolites of aldosterone in man (3)

18-deoxy-19-noraldosterone has been found by Takeda et al (20), to be a potent aldosterone antagonist and plays a key role in primary aldosteronism. Earlier identification by Zennaro et al (21), of 2-alpha-hydroxylated metabolites formed in liver led to the chemical synthesis of potent mineralcorticoids such as 2-alpha-hydroxy and 2-beta-hydroxy derivatives of aldosterone.

C. Biological Effects and Clinical Significance:

The biological effects of aldosterone depend not only on the activity of eventual counter-regulating mechanisms but also on the state of the cell receptor sites, their age, pathological alterations, etc. The principal effects are as follows:

1) Influence on electrolyte (and water) metabolism mediated by both renal and extrarenal target organs

2) Effect on circulatory system

a) effect on blood pressure (peripheral effect)

b) cardiac effect

3) Anti toxic effect

It is evident that aldosterone diminishes the excretion of sodium ions and increases the output of potassium ions by salivary, sweat, gastric and intestinal excretory glands. Aldosterone has also been shown to diminish magnesium ion output in urine among primary and secondary hyperaldosteronism patients (22). Elevation of the aldosterone

level in the blood plasma due to increased formation of aldosterone by the adrenal gives rise to conditions termed "primary" aldosteronism, whereas a rise in aldosterone levels, secondarily induced by pathogenic factors, are termed "secondary" aldosteronism. A "mixed" form of aldosteronism, or mineralocorticism, is characterized by an increase in the secretion of other corticosteroids with some mineralcorticoid effect along with the increase in aldosterone secretion. Decreased production of aldosterone leads hypo-aldosteronism. According to Rao et al (23), some of the prohormones, such as 18-hydroxycorticosterone, are elevated in aldosterone-producing adenoma, which along with idiopathic hyperplasia are considered as the two main causes of primary hyperaldosteronism.

Other conditions due to abnormal aldosterone activity include Conn's syndrome or primary aldosteronism, which is caused from increased formation of aldosterone and is characterized by hypertension and hypokalemia, Bartter's syndrome or secondary aldosteronism, characterized by primary hyperplasia of the juxtaglomerular apparatus leading to an increased production of renin and angiotensin, and Cushing's syndrome as a result of autonomous, excessive production of cortisol.

Several unusual conditions are characterized by chemical abnormalities that suggest aldosterone excess or deficiency; however, these deficiencies are not connected to the renin-angiotensin aldosterone system. Liddle's syndrome (pseudo-hyperaldosteronism)

has been found to resemble primary aldosteronism clinically, the aldosterone production level has been found to be low with the absence of hypertension. Similarly in Bartter's syndrome, excess prostaglandin secretion induces renal potassium ion wasting, but there is an increment in the aldosterone and renin levels.

The two inborn errors of terminal aldosterone biosynthesis that are characterized by over production of corticosterone and deficient synthesis of aldosterone are corticosterone methyl oxidase deficiency type I (CMO-I), characterized by decreased production of 18-hydroxycorticosterone and corticosterone methyl deficiency type II (CMO-II), characterized by over production of 18-hydroxycorticosterone and an elevated plasma ratio of 18-hydroxycorticosterone to aldosterone (24). CMO-II deficiency has been determined by Pascoe (25) to be an autosomal recessive disorder of aldosterone biosynthesis and has been linked genetically to the CYP11B1 and CYP11B2 genes that encode two cytochrome P450 isozymes, P450XIB1 and P450XIB2, respectively. It was Kooner et al (26), who found that the 18 hydroxycorticosterone/aldosterone ratio is highly correlated with that in plasma whereas it varies considerably in saliva.

CHAPTER II

HISTORICAL

The determination of aldosterone and its metabolites in urine and plasma provides an excellent index of aldosterone secretion and has been useful for clinical diagnosis and/or treatment. Recent studies in the measurement of aldosterone, directly or indirectly, in various clinical conditions have produced a variety of methods of assaying aldosterone. The measurement of aldosterone still presents a technical challenge due to the minute amount of the steroid present in body fluids.

Modern techniques, such as solid phase radioimmunoassay, chemiluminescence immunoassay, HPLC with chemiluminescence detection, and thin-layer chromatography with fluorimetry have been used. Estimations of aldosterone in blood and urine based on double-isotope derivative formation or GC-MS has also been feasible in specialized laboratories. Some of the latest techniques will be discussed.

Methods for the determination of aldosterone:

(1) HPLC with Fluorescence Detection

HPLC with fluorescence detection was used by Yamaguchi et al (27), to detect aldosterone along with eighteen other 21-hydroxycorticosteroids. The corticosteroids were oxidized by cupric acetate to form the glyoxal derivatives. They were then converted into fluorescent quinoxalines by reaction with 1,2-diamino-4,5-methylene-dioxybenzene (DMB). The quinoxalines were subsequently separated within 70 minutes on a reversed-phase column by a stepwise elution with mixtures of methanol, acetonitrile and 1.0 M ammonium acetate.

The purpose of the experiment was to establish a rapid and sensitive HPLC method for the determination of free and total 21-hydroxycorticosteroid levels in human urine. The study provided a method for the simultaneous determination of hydrocortisone, cortisone, tetrahydrocortisol, tetrahydrocortisone, along with aldosterone in human urine.

The mean retention time (n=10) for aldosterone was found to be 16.1 minutes (+/- 0.3) with a 95% recovery. The detection limit was 0.51 pmol/50 μL. The method was sensitive enough to measure other corticosteroids in 500 μL of normal urine.

(2) Chemiluminescence Immunoassay of Aldosterone in Serum

This method by Stabler et al (28), was developed with aminobutylethyl isoluminol (ABEI) as the label, for the direct measurement of aldosterone in serum. In this assay, the samples were incubated with antibody, aldosterone-carboxymethyl oxime (CMO)-ABEI, and paramagnetic particles coated with a second antibody. After magnetic separation and washing, the samples were incubated with 200 µl of NaOH at 60°C for 30 min. The chemiluminescence in the luminometer was produced by the serial injection of 150 µl each of microperoxidase and H_2O_2 solutions.

Concentrations of aldosterone up to 0.03 nmol/L could be assayed (n=20). The study was also in excellent agreement with RIA results from 0 to ~2 nmol/L. The label was stable with a shelf-life of at least two years. The method was simple and avoided the hazards and costs associated with isotopic waste.

(3) HPLC with Chemiluminescence Detection

HPLC with chemiluminescence detection has been used by Ishida et al (29), to measure 3α,5β-tetrahydroaldosterone (THALD), a major metabolite of aldosterone found in human urine. Though it was not sensitive enough to measure aldosterone precisely, it was able to provide an index of aldosterone secretion. Urinary THALD, released by enzyme hydrolysis, was isolated and concentrated using a Sephadex G-25M column and Bond-Elut C_1 cartridges. It was then oxidized by copper(II) acetate to form the

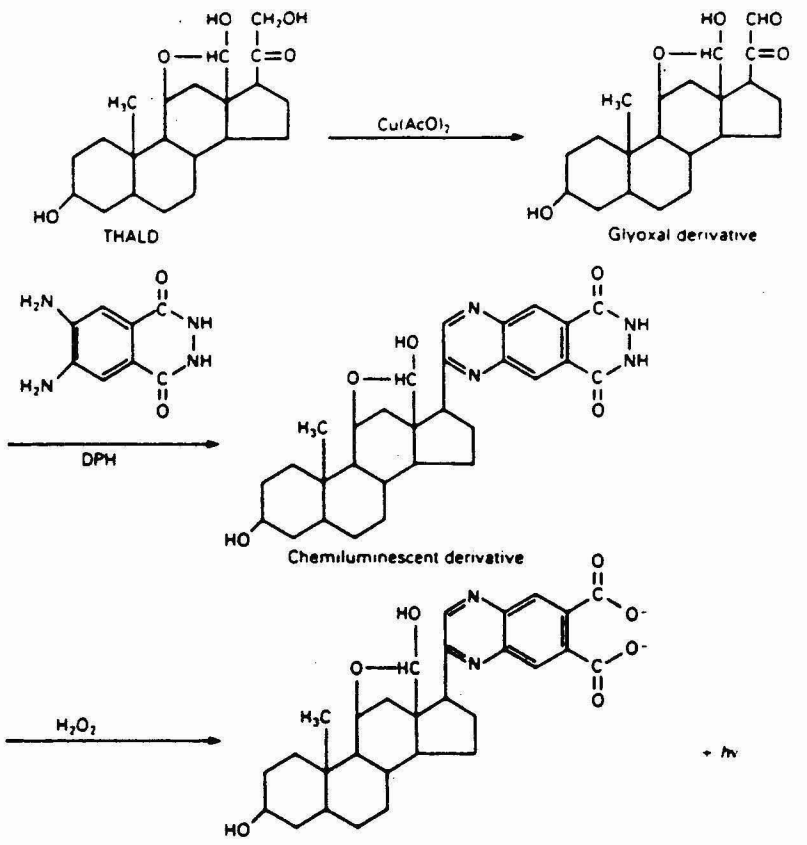


Figure 9: Derivatization and chemiluminescence reaction of THALD with DPH

corresponding glyoxal derivative (fig.9). The glyoxal derivative was then converted into the chemiluminescent quinoxaline by reaction with 4,5-diaminopthalhydrazide (DPH). It was then separated within 50 min on a reversed-phase column with isocratic elution followed by chemiluminescence detection.

The detection limit of THALD was found to be 0.6 pmol (220 pg) /mL. The mean THALD concentration (n=12) was found to be 36.71 μg/dL. The retention times for THALD and aldosterone were found to be 37.0 and 25.0 min, respectively. The authors of this study were optimistic of this method's application to the determination of aldosterone in plasma. They were also able to utilize this procedure for the determination of THALD and cortisol in human urine with fluorescence detection.

(4) Thin-layer Chromatography with Fluorimetry

Thin-layer chromatography with fluorimetry was developed by Mattingly et al (30), for the estimation of urinary aldosterone and to investigate the use of overnight samples for detecting hyperaldosteronism in 24 hr. urine specimens. The steps involved were hydrolysis of the 18-conjugate to release aldosterone and its oxidation with Benedict's solution, followed by thin-layer chromatography on silica gel and the development of fluorescence on the plate with sulfuric acid. The mean aldosterone excretion among 67 adults was 15.7 nmol/24 hours, whereas the the mean overnight excretion among 65 adults was 2.6 nmol/8 hours. This technique indicated that overnight

estimations seemed to be as effective as 24 hour assays for identifying patients with hyperaldosteronism.

(5) Solid Phase Radioimmunoassay (SP-RIA)

A solid phase radioimmunoassay was developed for the determination of conjugated aldosterone in urine samples by Torok (31). The urine was hydrolyzed with acid and then extracted with dichloromethane and the extract was analyzed by SP-RIA. The two different anti-aldosterone antisera used to coat the plates were S-3: against aldosterone-21-hemisuccinate-BSA and 58: aldosterone-3-(O-carboxymethyl)-oxime-BSA. The method was found to be time-saving, and did not require paper chromatography. It was an adequate method for many clinical purposes.

(6) HPLC and UV Detection

Recently Chang et al (32), described a method for the simultaneous measurement of nine steroids, including aldosterone. The assay used isocratic HPLC with acetonitrile and water (40:60, v/v) along with UV absorbance detection at 210 nm and 240 nm to obtain good specificity. The absorbance of the estrogens compared at 210 nm was over 10-fold of that at 240 nm, whereas the rest of the steroids had higher absorbance at 240 nm than at 210 nm. The range of this assay was found to be linear extending from 10 to 80 ng for all the steroids including aldosterone tested at 210 nm and 240 nm.

CHAPTER III

STATEMENT OF PROBLEM

The determination of urinary aldosterone provides an excellent index of aldosterone secretion and can thus be useful in the diagnosis and/or treatment of the mentioned diseases (ref. chap.I). Various methods such as solid-phase radioimmunoassay (SP-RIA)(31), radioimmunoassay (RIA), gas chromatographic and mass spectometric (GC-MS), chemiluminescence(29), chemiluminescence immunoassay(28), thin-layer chromatography (30) and high performance liquid chromatography with fluorimetric detection (27) have been used for aldosterone determinations. Some of these methods are time consuming, require a radioactive compound, and are not sensitive enough for the low levels of aldosterone present in samples. They require large amounts of sample (10 mL of urine) along with expensive equipment and/or they require rather tedious purification techniques (29). These techniques often suffer drawbacks, such as insufficient sensitivity and selectivity, and the reagents used are either unstable or highly corrosive.

The purpose of this research was to produce an alternative detection method that would be more sensitive and highly selective. The detection of reducing sugars with an amperometric in HPLC was developed by Watanabe et al (33). The redox reaction of

copper(II) bis-phenanthroline was coupled with the reducing ability of the sugars in alkaline solution at high temperature on post-column reactor for the determination of glucose. The method was sensitive and selective due to an allowance of the applied potential to the working electrode to be as low as possible. Applications to urine and serum samples were also demonstrated. The same procedure has been employed to assay aldosterone in human urinary samples using high performance liquid chromatography, in which the reducing ability of aldosterone is coupled with the redox reaction of copper(II) bis-phenanthroline (CBP) as a mediator.

$$Cu(phen)_2^{+2} \xrightarrow[OH^-,\ heat,\ post\text{-}column]{reducing\ aldosterone} Cu(phen)_2^{+} \xrightarrow[oxidation]{electrochemical} Cu(phen)_2^{+2}$$

It is the CBP, reduced by aldosterone in alkaline solution at high temperature in a post-column reaction that is re-oxidized with the amperometric detector, that provides an accurate measurement of the aldosterone sample.

The method has been found to be highly selective and sensitive for the above assay. It is easy to perform and the reagent used was stable for a long period of time thus making it a less time consuming procedure.

CHAPTER IV

MATERIALS AND APPARATUS

Reagents and Solutions

All reagents and solvents were of analytical-reagent grade, or of the highest purity available. Double de-ionized ultra-filtered water was used for the preparation of all reagents and solvent systems. The chemicals and their manufacturers have been listed below.

Copper (II) sulfate, pentahydrate crystals (Mallinckrodt Inc., Paris, Kentucky), and 1,10 phenanthroline, monohydrate (GFS Chemicals, Columbus, Ohio, ACS reagent grade) was used without further purification. Sodium phosphate, dibasic, anhydrous quality was purchased from General Chemical Division, Morristown, New Jersey. Trimethylchlorosilane was bought from Sigma Chemical Company, St. Louis, Mo., whereas methylene chloride, dimethylsulfoxide, propylene glycol and triethylamine were all bought from J. T. Baker, Philipsburgh, NJ.

Methanol, acetonitrile, and acetone (Fisher Scientific, Fairlawn, NJ., HPLC grade), were used without further purification.

Concentrated hydrochloric acid (36.5-38.0%) was obtained from VWR Scientific, Norwood, Ohio. Sodium hydroxide was obtained from EM Science, Gibbstown, NJ.

For the solid-phase extraction, Varian Bond Elut cyclohexyl and silica cartridges were bought from Phenomenex Co., Torrance, CA.

Prepurified nitrogen and helium (Airco Inc., New York, NY.) were used to dry extracted urine specimens and for the HPLC mobile phase respectively.

Steroids

The steroids, d-aldosterone (anhydrous) and 3β,5β-tetrahydroaldosterone were both purchased at the purest quality available from Sigma Chemical Co., St. Louis, Mo. They were stored in a dessicator at 5°C.

Apparatus

High performance liquid chromatography was carried out with an LC/9533; Ternary Gradient Liquid Chromatograph purchased from IBM Instruments Inc. Danbury, CT. Chromatographic separations were performed using an Econosphere C-18, 5μ reversed phase column (250 mm, I.D. 4.6 mm) protected by a guard column purchased from Alltech Associates Inc.(Deerfield, IL), as were all connectors, mixing tee, teflon tubings, nuts, and ferrules. Helium was used to degas the mobile phase prior to its introduction in the system. The chromatograph was equipped with a Rheodyne sample injector valve (20 μL loop).

Model M-6000 Pump (Waters Associates, Inc. Milford, MA) was used to deliver the reagent.

For the electrochemical detection, BAS Model CV-27 (Bioanalytical System, Inc., Lafayette, IN) was used as the detector. A propylene glycol bath was set up using an Isotemp Constant Temperature Ciculator (Fisher Scientific, Fairlawn, NJ). The Back Pressure Regulator (Alltech, Deerfield, IL) was connected to the system to reduce the background noise. The chromatograms were obtained using a HP 3396 Series II Model integrator (Hewlett Packard, Wilmington, DE).

The UV measurements of the steroids were done on a Diode Array Spectrophotometer, Model 8452A, purchased from Hewlett Packard Co.(Wilmington, DE).

All the chemicals were weighed on a Mettler H20 balance (Mettler Instrument Corporation, Princeton, NJ). Hamilton Microliter Syringes were used to make all the injections. The experimental set up has been illustrated in figure 10.

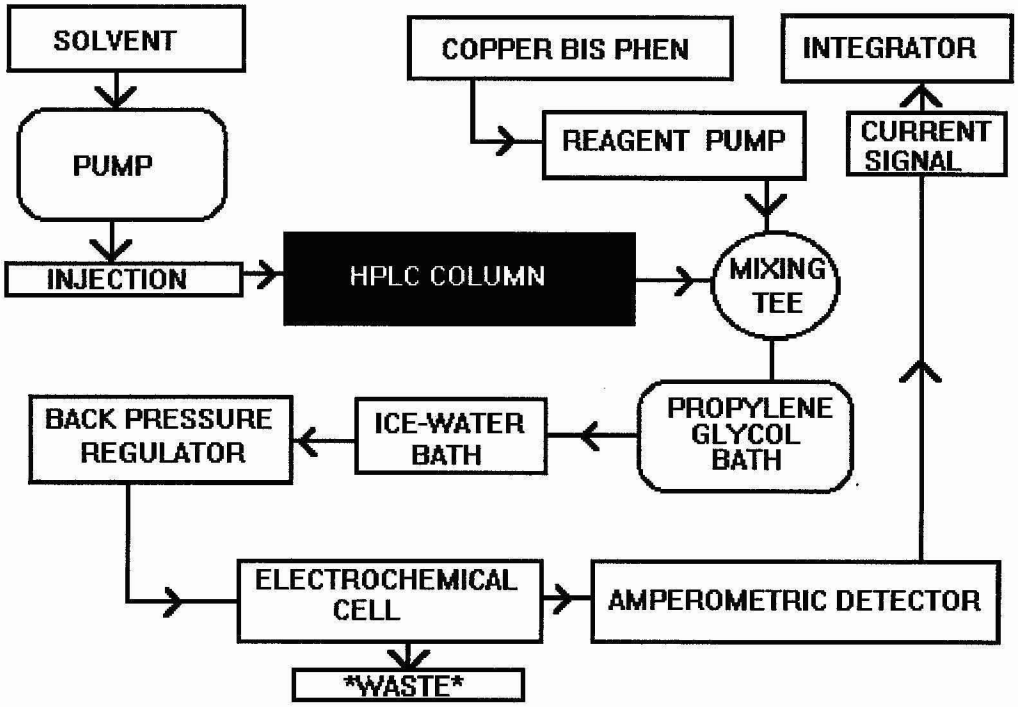


Figure 10: Experimental Set Up

CHAPTER V

EXPERIMENTAL

High Performance Liquid Chromatography

Chromatography is a separation technique where complex mixtures are separated into their individual components by differences in their distribution between two phases, a mobile phase and a stationary phase. There are two kinds of liquid chromatography, one being column chromatography and the other being thin layer chromatography. Column chromatography has been further subdivided into five major types. They all constitute high-performance liquid chromatography (HPLC) and include: liquid-solid chromatography (LSC), liquid-liquid chromatography (LLC), bonded phase chromatography (BPC), ion-exchange chromatography (IEC), and size exclusion chromatography (SEC).

In HPLC, a precision pump pushes the mobile phase at relatively high pressure through a stainless steel column that is packed tightly with small particles that act as the stationary phase. The mobile phase then passes through a detector where the sample concentration is measured. The detector generates a signal that is then fed to a recorder

producing a chromatogram with a series of peaks, each ideally representing a single component of the original mixture. The main characteristics of HPLC are its use of high pressure pumps, short narrow columns packed with small particles, and a detector that continously records some physical property of the eluent. Speed, high resolution, relatively low percentage errors ~1%, high sensitivity and automation are some of the advantages of HPLC.

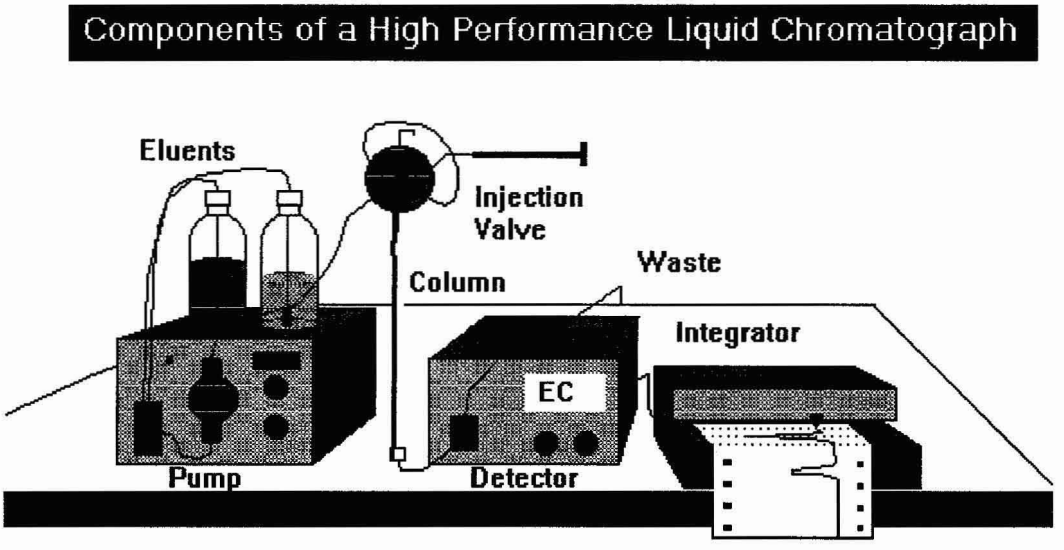


Figure 11: Components of HPLC

The basic components of an HPLC system are shown in fig. 11. Before the injection of the sample, the mobile phase is pumped from the reservoir, through the injector, the column, and the detector until a stable baseline has been obtained. The sample is then injected and with the help of the mobile phase, it is carried onto the column. Based upon partitioning between the mobile and stationary phases, the column retains

some components longer than others, and eventually each component, diluted with the solvent, will pass into the detector. Peaks are recorded on the chromatogram for each component that is separated in the column (34).

The solvent is the mobile phase and its purpose is to carry the sample through the column. Although the solvent must be able to dissolve the sample completely, it should be of highest purity. Other solvent factors include cost, viscosity, toxicity, boiling point and its miscibility with other solvents. Savant (Fullerton, CA) has developed a chart that correlates the miscible properties of different solvents among each other (fig.12). Other important components include pumps, injectors, columns, detectors, and recorders.

In this study, bonded phase chromatography has been employed to carry out the separation. The column containing the stationary phase, consists of a C-18 hydrocarbon covalently bound to the silica surface. This is a non-polar group and is termed as reversed phase. It is called so, because the stationary phase is non-polar and the mobile phase is polar. A detailed discussion can be found elsewhere (35).

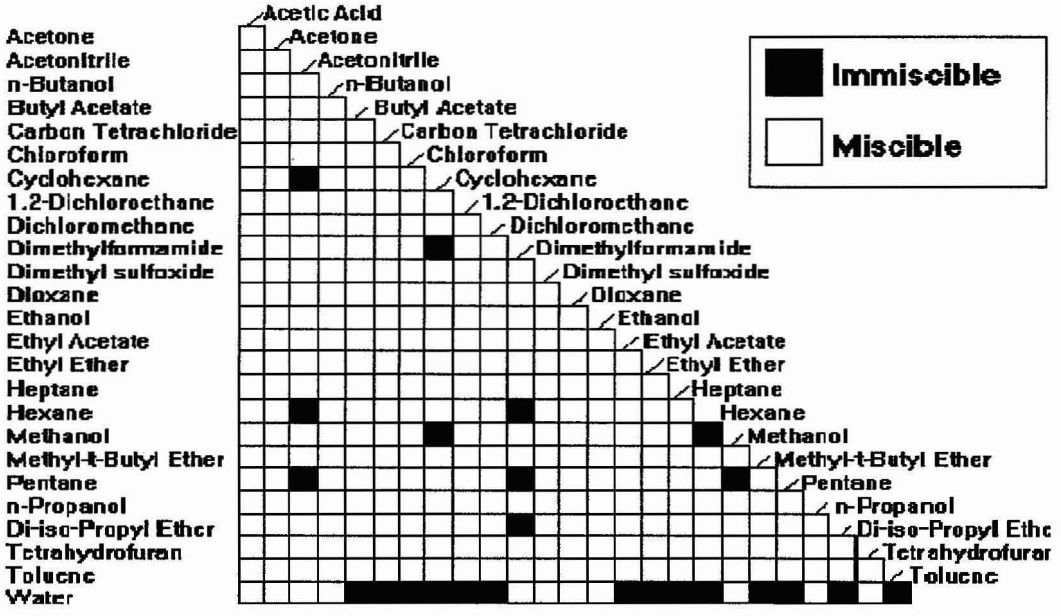


Figure 12: Solvent Miscibility Chart (Savant, Asymetrix Corp.)

Cyclic Voltammetry

The use of cyclic voltammetry (CV) has become increasingly popular in all fields of chemistry for studying redox states. By providing information regarding the stability of the product of the electrode reaction, it can be very useful in experiments involving liquid chromatography with electrochemical detection. CV has been effective in observing redox behavior over a wide potential range. Its ability to generate a new redox species during the first potential scan and then probe the species' fate on the second and subsequent scans, has proven advantageous for this system (36).

CV involves cycling the potential of an electrode immersed in a solution and then measuring the resulting current. The potential of this working electrode is controlled producing an excitation signal, relative to a reference electrode. The excitation signal causes the potential to first scan negatively starting from 0.00V and then reversing the scan causing a positive scan back to the original potential. Single or multiple scans can be performed using this technique. A cyclic voltammogram is obtained by measuring the current at the working electrode during the potential scan, and is considered as the response signal to the potential excitation signal. The voltammogram is a graphical illustration of current (vertical axis) versus potential (horizontal axis). As potential varies linearly with time, so does the current. The area under the resulting peak corresponds to the total charge transferred during the redox process. The important parameters of a

cyclic voltammogram includes the magnitudes of the anodic peak current (i_{pa}), the cathodic peak current (i_{pc}), the anodic peak potential (E_{pa}), and the cathodic peak potential (E_{pc}). the formal reduction potential ($E^{0'}$) for a reversible couple is found to lie between E_{pa} and E_{pc} and is formulated as:

$$E^{0'} = (E_{pa} + E_{pc}) / 2$$

and the number of electrons transferred in the electrode reaction (n) for a reversible couple can be calculated from the separation between the peak potentials from

$$\Delta E_p = E_{pa} - E_{pc} = 0.059 / n$$

CV requires a waveform generator for the production of the excitation signal, a potentiostat to apply the signal to an electrochemical cell, a current-to-voltage converter to measure the resulting current, and an x-y recorder/integrator to display the resulting voltammogram. A three-electrode configuration potentiostat has been employed in this study. The potentiostat applied the desired potential between a working electrode and a reference electrode. An auxiliary electrode was also present to provide the current required to sustain the electrolysis at the working electrode. This kind of a system prevented large currents from passing through the reference electrode, which could have changed its potential.

Solid Phase Extraction

Modern instrumental methods of analysis often require sample preparation for two main reasons,: cleanup and concentration. In liquid chromatography, the column life may be shortened due to the presence of impurities in the sample, thus making sample cleanup a mandatory procedure. Analyte concentration falls below the sensitivity range of the detector in many instances, and only with concentration can the analyte be brought into a practical range. Previous manual methods have proven to be inefficient, tedious, and costly.

Classical methods of clean-up include distillation, crystallization, precipitation, and centrifugation. Solid phase extraction (SPE) involves the use of small, disposable extraction columns, filled with one of a wide variety of sorbents. The columns are first conditioned with an appropriate solvent followed by conditioning with a typical sample matrix solvent, and then the sample is forced through the column by aspiration or pressure. The column is then washed to elute the impurities, leaving the analyte on the column. The purified analyte is finally eluted using a solvent strong enough to displace it from the adsorbent. Applications for the selection of various sorbents can be found or obtained from commercial companies or elsewhere (37).

When the impurities are more polar than the analyte, a reversed phase chromatographic sorbent may be most effective, where the sorbent is less polar than the

mobile phase or sample solution. The sample is dissolved in a polar medium and is passed through a nonpolar sorbent. The polar impurities remain strongly attached to the polar solvent system and thus pass through the column unretained, whereas the less polar materials are adsorbed by the nonpolar sorbent. The less polar analyte is then eluted by the addition of a nonpolar solvent in which the analyte is soluble, free from any polar imputrities.

On the other hand, when the analyte of interest is more polar than the impurities, normal phase conditions are utilized. Here the sample is dissolved in a nonpolar solvent, less polar than the adsorbent, silica. The solution then passes through the column where the polar analyte is adsorbed by the silica. The nonpolar impurities with greater affinity for the solvent passes through the column unretained. With the use of a more polar solvent, the polar analyte is then removed from the silica column.

SPE has been used in a variety of circumstances for faster and more efficient sample preparation. The technique has not only proven to be faster, but is much safer, more efficient, and economical than many other traditional techniques.

Experimental Protocol

1) Synthesis of Copper (II) bis-Phenanthroline (CBP):

CBP was synthesized according to the procedure by Hathaway et al (38). 0.02 mole of 1,10-phenanthroline was dissolved in 30 mL of methanol and then was added to a

warm aqueous solution of 0.01 mole of copper (II) sulfate pentahydrate salt in 30 mL of double deionized water. The entire mixture was refrigerated overnight in a cold room (5^0C) and the products crystallized. The crystals were washed with cold methanol and then dried in a vacuum dessicator. Recrystallization of the complex was carried out with double deionized water. It was redissolved in a minimum amount of double deionized water and filtered. The filtrate was reduced to a third of its volume by using a rotary vacuum evaporator (Rotavap ™) at 55^0C. The solution was stored in the cold room overnight. The crystals that formed were filtered and then placed in the dessicator under vacuum for overnight drying. The molecular weight of the CBP complex was calculated to be 591.95 a.m.u.

2) Reagent Solution Preparation:

The reagent solution was prepared by dissolving CBP crystals in a solution containing 0.05 M Na_2HPO_4 as the supporting electrolyte and the pH was adjusted to 11.2 by the addition of 2.0 M NaOH.

All steroid solutions were prepared by dissolving aldosterone and THALD in HPLC grade methanol. The stock solution of aldosterone (50 ppm) was prepared by dissolving 5.0 mg of pure aldosterone crystals in 100 mL of methanol. Subsequent standard solutions of aldosterone were prepared by diluting the above stock solution. The

THALD stock solution was prepared by dissolving 1.0 mg of pure THALD crystals in 20 mL of methanol.

3) Urine Sample Collection:

Urine specimen (24 hrs.) from a healthy volunteer was collected in a clean and dried brown glass bottle. 15 mL of 6.0 N HCl was used as the preservative and was added to the bottle before the collection. The total volume of 24 hr. collection was measured using a graduated cylinder and recorded.

A 100 mL portion of the urine was centrifuged for 10 min at 5000 x g. The upper layer (80 mL) was used for further extraction. To a 20 mL aliquot of the urine specimen, 40 µl of the aldosterone stock solution (50µg/mL) was added. The spiked aliquot along with three other unspiked aliquots (20 mL each) of the same urine specimen were taken and their pH values were adjusted to 1.0 using 6.0 N HCl. The samples were incubated at room temperature in the dark for 22 hrs. so that hydrolysis of the conjugated steroids could occur.

Before the hydrolyzed urine samples were adjusted to pH 6.0 with 15.0M NaOH, two 20 mL aliquots of the unspiked urine specimen were each spiked with 40 µl of the aldosterone stock solution (50µg/mL) . They were then loaded onto a 6 mL Cyclohexyl Bond Elut cartridge. The cartridge was washed with 10 mL of H_2O, followed by 5 mL of 25% CH_3COCH_3 and then with 5 mL of H_2O. It was then dried by vacuum aspiration for

5 minutes. The cartridge was then washed with 5 mL of hexane and eluted with two 3 mL aliquots of a mixture of CH_2Cl_2 / CH_3CN (90/10). The extract was dried under nitrogen (25°C).

The dried extract was dissolved in 6 mL CH_2Cl_2 and re-loaded onto a 6 mL Silica Bond Elut cartridge. It was sequentially washed with 2mL of CH_2Cl_2 and then 2 mL of CH_3CN. It was eluted with three, 2 mL aliquots of CH_3OH, and once again dried under nitrogen (25°C). It was finally re-dissolved in 200 µL of CH_3OH and the supernatant was injected into the chromatograph.

The reducing ability of aldosterone coupled with the redox reaction of CBP as a mediator in a post-column reactor was able to provide the following principle of detection.

4) Detection and Estimation:

Detection of the current was accomplished with a BAS-CV27 voltammograph, which was coupled to the Hewlett Packard integrator. The amount of aldosterone present in the sample was based on peak area. The net peak area due to aldosterone was plotted against the concentration of the standard stock solution.

CHAPTER VI

RESULTS AND DISCUSSIONS

Prior to HPLC use, a cyclic voltammogram of the CBP reagent was examined using a separate electrochemical cell different than that used for HPLC detection (fig.13). Glassy carbon was used as the working electrode, Ag/AgCl was used as the reference electrode and a platinum wire was used as an auxilliary electrode. The BAS-CV27 (Bioanalytical System, Inc., Lafayette, IN) voltammograph was used to measure the potential and was coupled to an x-y recorder. 10.0 mM CBP in 0.2 M Na_2HPO_4 at pH 11.2 with methanol/water (50/50) was used as the solvent at a scan rate of 50 mV/s. The oxidation potential of CBP vs Ag/AgCl was found to be 0.25V.

To the stock solution of aldosterone (100 mL), a few drops of 0.1% triethylamine was added to prevent any methyl ketal formation (39). A UV spectrum of the aldosterone stock solution was obtained to verify the purity of the steroid solution (fig.14). Two peaks were obtained (210 nm and 240 nm) according to the reference value (40).

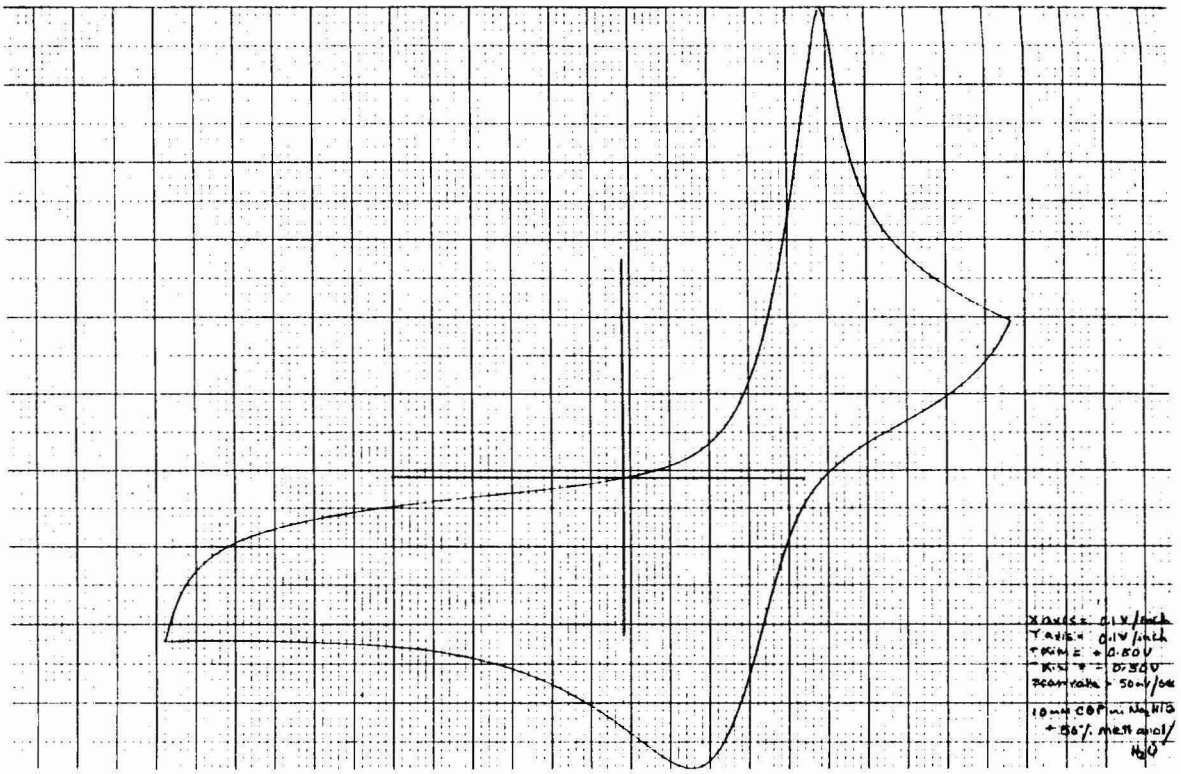


Figure 13: Cyclic Voltammogram of 10.0 mM CBP, 0.2 M Na_2HPO_4, in methanol/water (50/50), scan rate of 50 mV/s. Glassy Carbon Electrode(working electrode), Platinum wire(auxilliary electrode), and Ag/AgCl electrode(reference electrode).

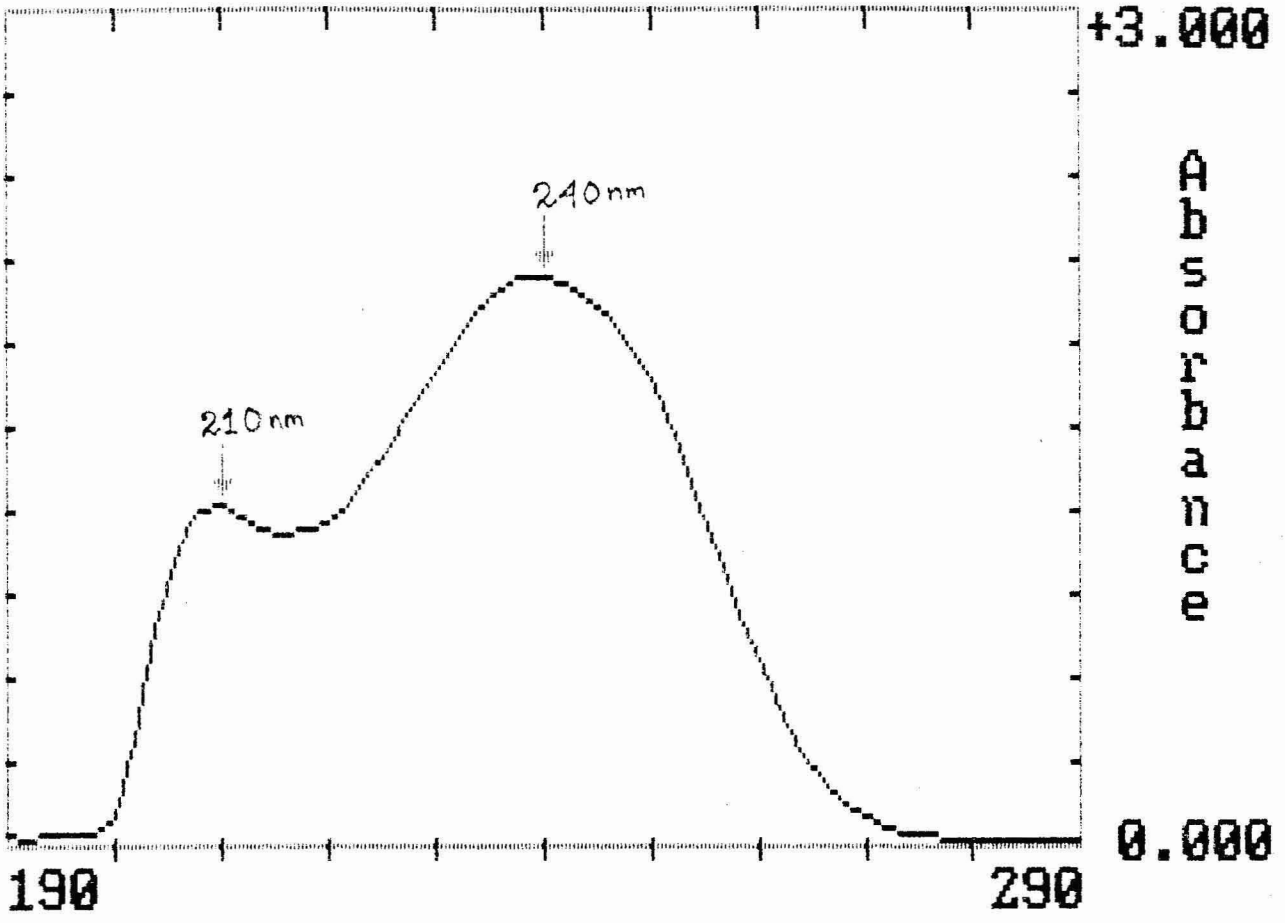


Figure 14: UV spectra of Aldosterone Stock Solution (50 ppm) in pure methanol.

The following HPLC configuration was obtained from previous studies (39,41) and was set up accordingly:

Sample: Aldosterone stock solution (50μg/mL).

Injection Loop: 20 μl.

Column: Econosphere C-18 preceded by a guard column.

Mobile Phase: Methanol/Water (55/45).

Temperature of Propylene Glycol Bath: 95°C.

Eluent Pump Flowrate: 1.0 mL/min.

Integrator Chart Speed: 1.0 cm/min.

Various studies were performed to find out the optimal parameters for the standard plot of aldosterone. They were as follows:

1) The dependence of peak response on applied potential;

2) The effect of Na_2HPO_4 concentration on peak response;

3) pH dependence of peak response;

4) The dependence of peak response on reagent flowrate;

5) The dependence of peak response on coil volume; and

6) The dependence of peak response on CBP concentration

Applied Potential

A hydrodynamic voltammogram was performed to study the optimum potential for the CBP reagent. The applied voltage vs. signal to noise ratio was plotted. Figure 15 shows the correlation between the applied potential to the working electrode and the detector response. The signal to noise ratio was maximum at an applied potential of 0.22V. Above 0.22V the background current increased with the potential. There was no signal obtained prior to 0.15V. Thus 0.22V was decided to be the optimum voltage to carry out the rest of the experiments.

Concentration of Na_2HPO_4

The concentration of the supporting electrolyte, Na_2HPO_4, affected the response considerably under a constant pH value of 11.2. The Na_2HPO_4 content in the reagent gave the maximum response at 0.05 M. Any concentration relatively higher or lower than that decreased the signal/noise ratio (fig.16).

pH Dependence

The reduction rate of CBP by sugars has been found to be highly dependent on pH and temperature (33). With the temperature of the post-column reactor being kept constant at 95^{0}C in a propylene glycol bath, the optimum pH occured at 11.2. The study was performed at four different pH values which included 10.3, 11.04, 11.2 and 12.0. Figure 17 shows the pH dependence on peak response.

HYDRODYNAMIC VOLTAMMOGRAM

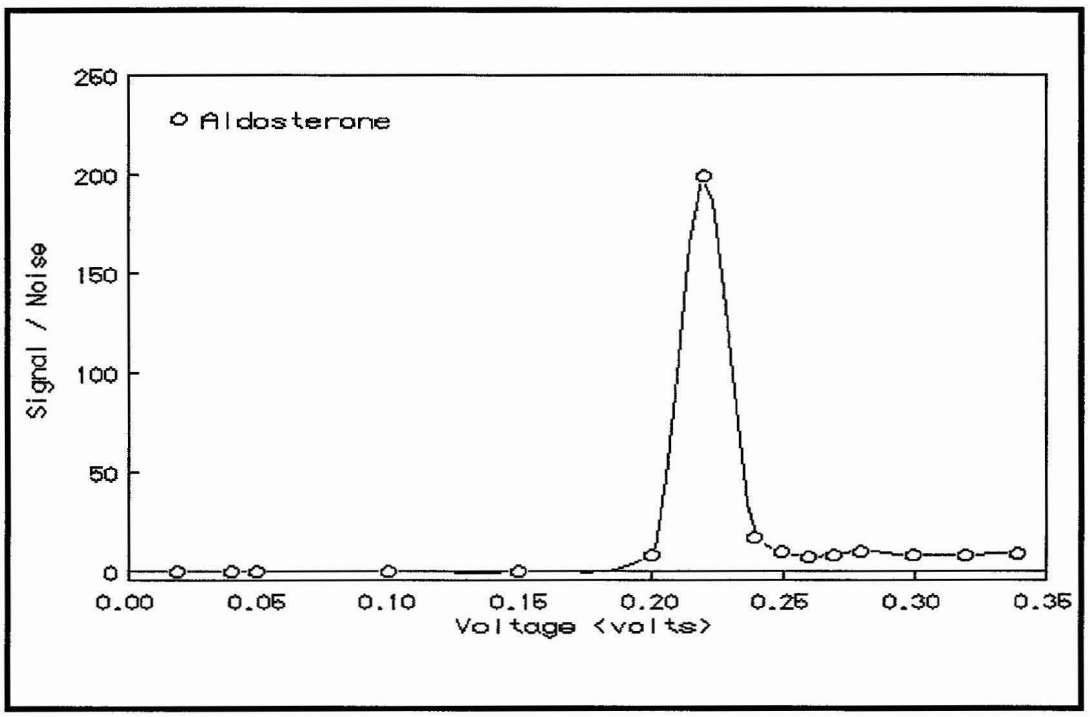


Figure 15: The dependence of peak response on applied potential: reagent; 3 mM CBP, 0.05 M Na_2HPO_4, pH 11.2; coil volume, 0.68 mL; reagent flowrate, 1.0 mL/min; sample, 10 ppm aldosterone.

BUFFER CONCENTRATION

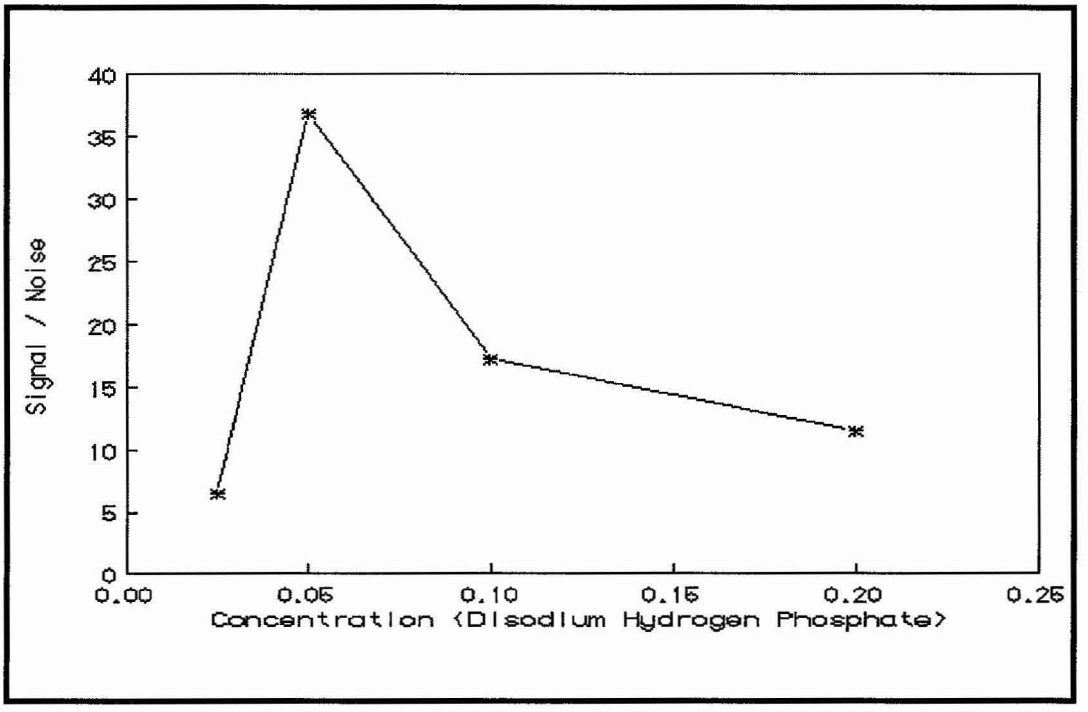


Figure 16: The effect of Na_2HPO_4 concentration on peak response;
3 mM CBP; applied potential, 0.22 volts;
pH 11.2; coil volume, 0.68 mL; reagent flowrate, 1.0 mL/min;
sample, 10 ppm aldosterone.

pH DEPENDENCE

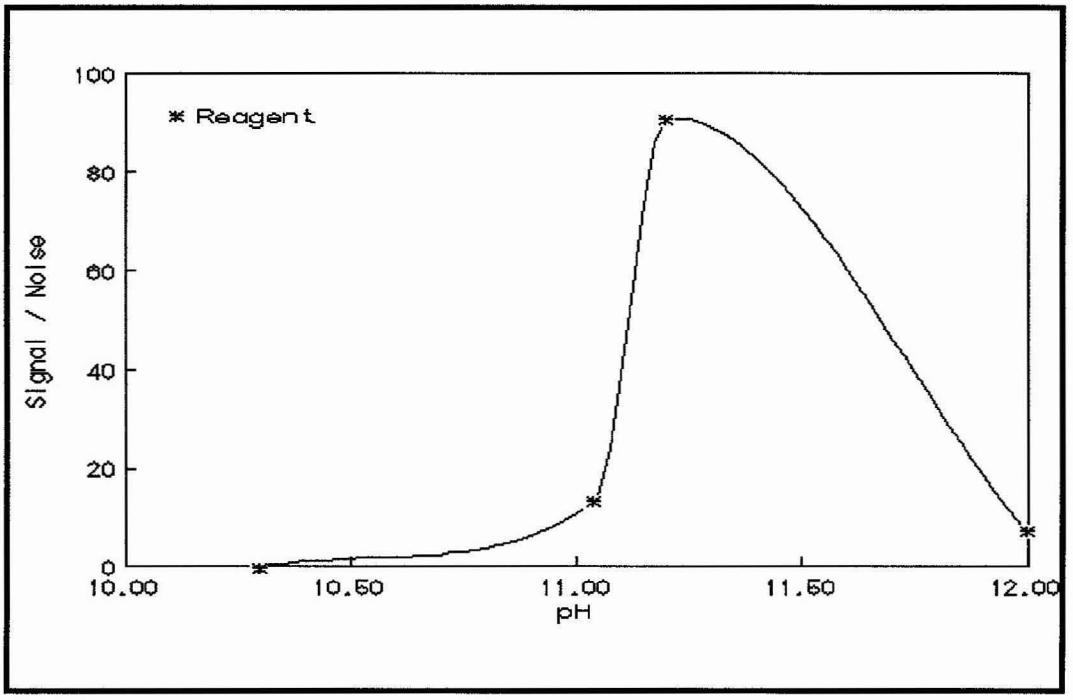


Figure 17: pH dependence of peak response; applied potential, 0.22V; reagent; 3 mM CBP, 0.05 M Na_2HPO_4; coil volume, 0.68 mL; reagent flowrate, 1.0 mL/min; sample, 10 ppm aldosterone.

CBP Reagent Flow Rate

The flow rate of the mobile phase, methanol:water (55:45, v/v), was kept constant at 1.0 mL/min as suggested by previous studies (39,41). Figure 18 shows the effect of the CBP reagent flow rate on peak response. From the various flow rates that were studied for the reagent, 1.0 mL/min was found to be the most suitable and was kept constant for the rest of the experiment. A slight decrease in the peak response was found at flow rates lower than 1.0 mL/min.

Volume (mL) of the Post Column Reactor Coil

The completion of the reaction depended upon the mixing coil volume and was found to be proportional to the total volume of the coil (fig.19). The greater the volume of the coil (mL), the higher the signal/noise ratio that was obtained indicating increased completion of the reaction. Four Teflon® coils of various lengths were used. They were all weighed with and without deionized water. The volume of the coils were calculated by taking the difference of those weights. The total volume of the four Teflon® coils was calculated to be 2.45 mL and they were placed in an Isotemp Constant Temperature Circulator, containing propylene glycol to prevent solvent loss by evaporation. The bath was maintained at 95^{0}C to obtain the maximum response.

CBP REAGENT FLOW RATE

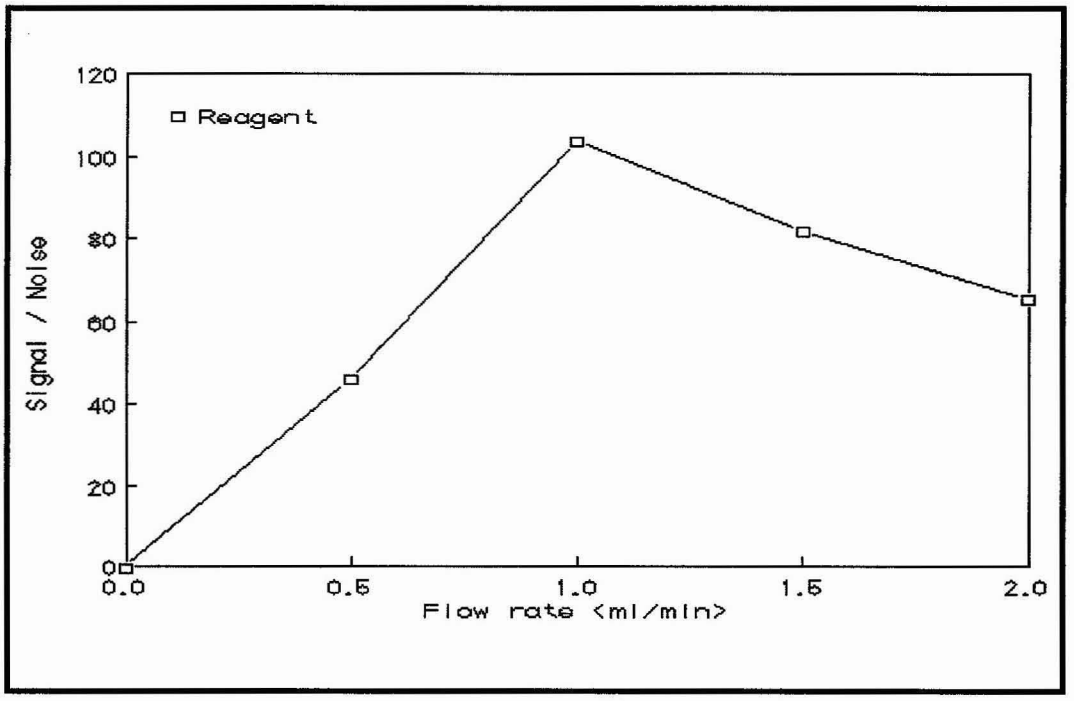


Figure 18: Effect of flow rate on peak response; applied potential, 0.22V; reagent; 3 mM CBP, 0.05 M Na_2HPO_4; pH 11.2; coil volume, 0.68 mL; sample, 10 ppm aldosterone.

COIL VOLUME (mL)

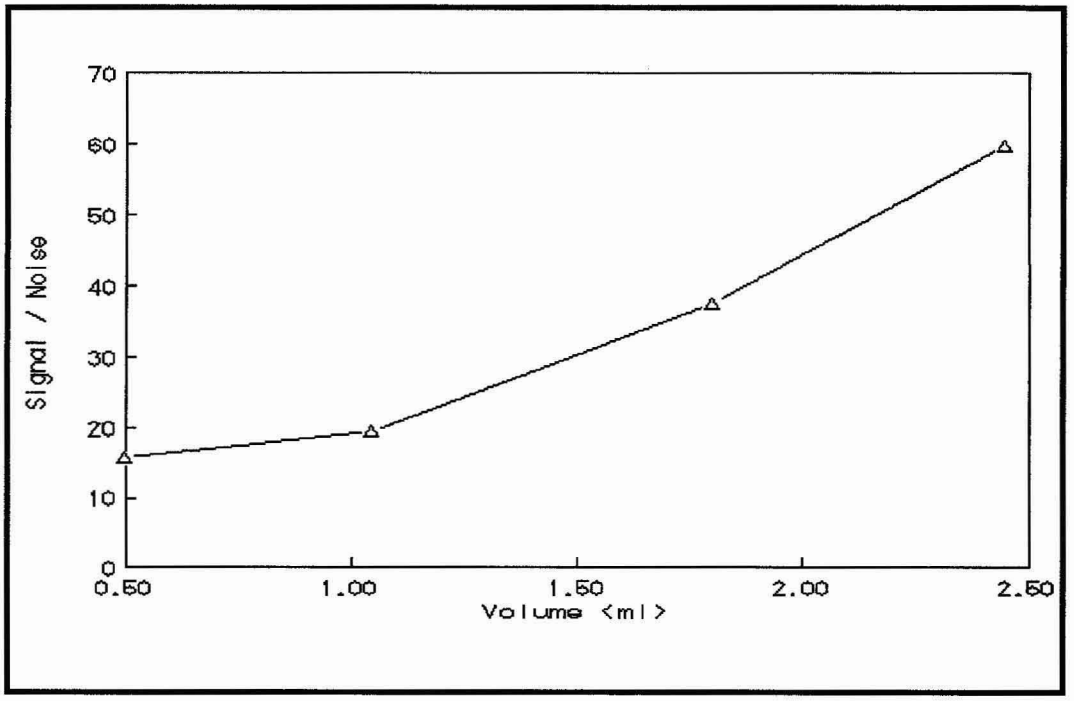


Figure 19: Effect of coil volume (mL); applied potential, 0.22V; reagent; 3 mM CBP, 0.05 M Na_2HPO_4; pH 11.2; reagent flowrate, 1.0 mL/min; sample, 10 ppm aldosterone.

CPB Concentration

The CBP reagent was light blue in color. It was degassed to remove dissolved oxygen by bubbling with helium. The signal/noise ratio was found to increase with the CBP concentration in the reagent (fig.20). The higher the CBP content, the greater was the peak response. For the standard plot of aldosterone, 3.0 mM of CBP was used, as less background current was obtained for that concentration.

After the studies were completed, the voltammogram was performed again to find a change in the applied potential, if any. The selected potential of 0.22V was still valid. With all the optimal parameters remaining constant, an aliquot (20 μL) of the stock solution of aldosterone was injected onto the HPLC. The retention time (t_R) of aldosterone was 9.4 minutes (+/- 0.1, n=18).

Once the desired retention time was reproducible, a standard plot for the aldosterone was constructed. The peak area detected by the integrator was used for the standard plot. The amounts used were 20, 40, 80, 120, 200 and 400 ng. All the runs for the standards were done in triplicates and the average peak area was plotted against the concentration. Table I shows the average peak area for each amount. The standard curve was found to be linear up to 400 ng (fig.21). Figures 22-24 represents chromatograms of standard aldosterone solutions of concentrations 20, 200, and 400 ng.

CBP CONCENTRATION

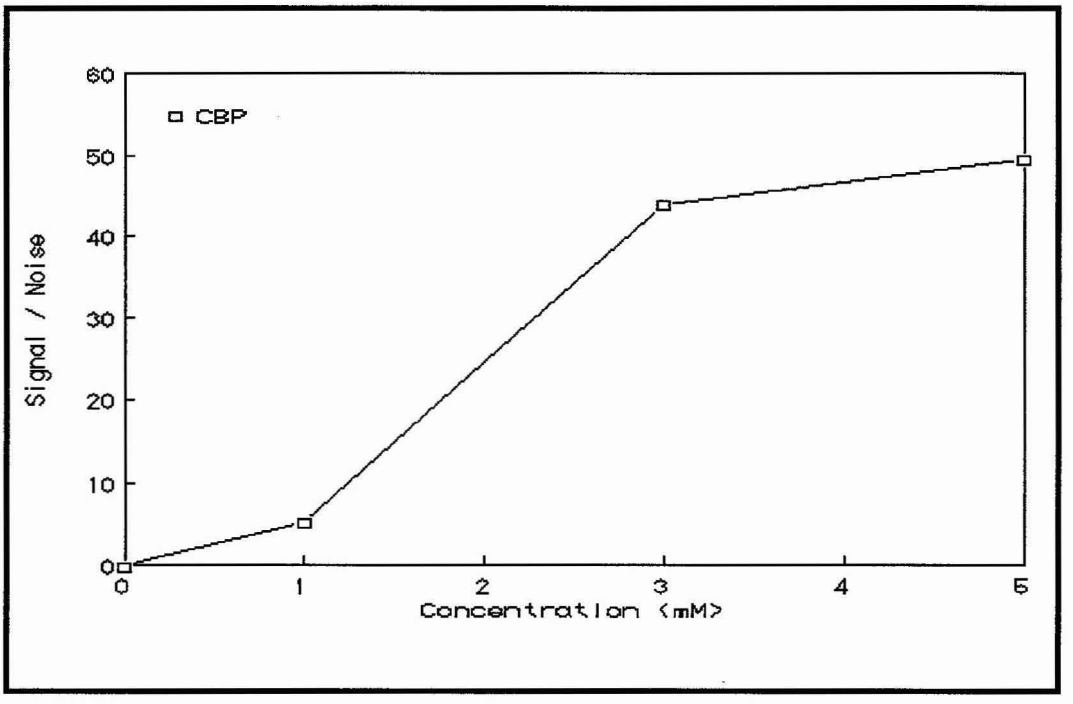


Figure 20: Effect of CBP concentration on peak response;
applied potential, 0.22V; reagent; 0.05 M Na_2HPO_4; pH 11.2;
coil volume, 2.45 mL; reagent flowrate, 1.0 mL/min;
sample, 10 ppm aldosterone.

Table I

Peak Areas For Aldosterone Standards

Aldosterone Amount (ng)	**Peak Area** (arbitrary unit)
20	2665814.3
40	4596497.8
80	7692310.3
120	9876023.3
200	16870944.0
400	29433808.0

STANDARD PLOT OF ALDOSTERONE

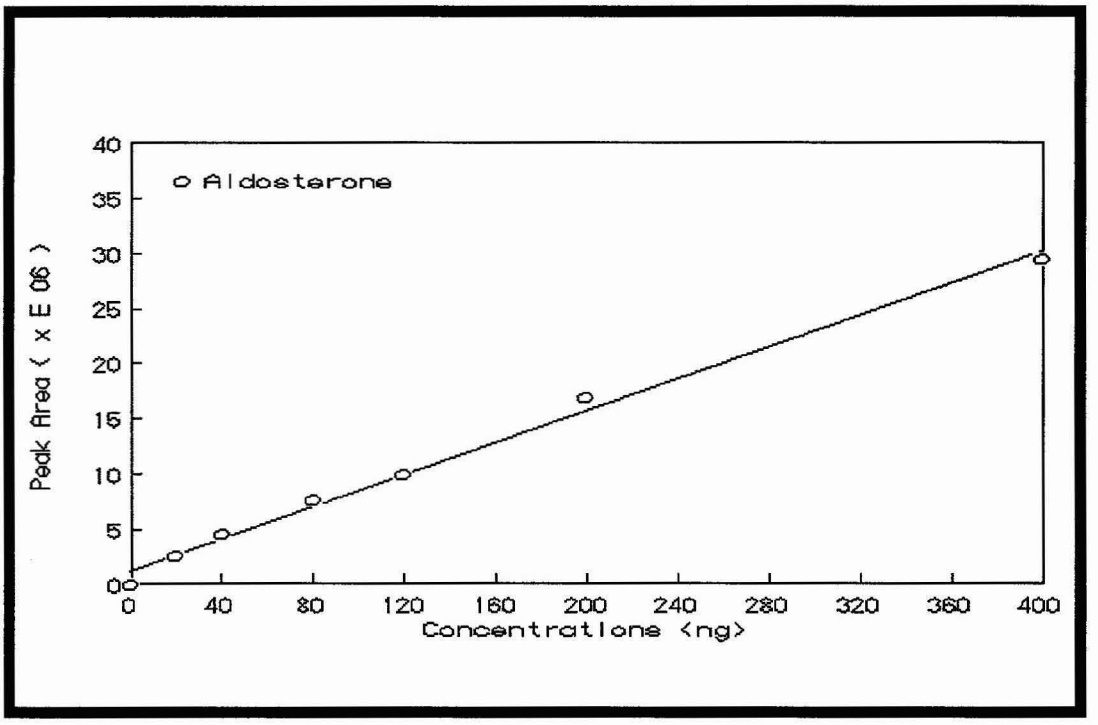


Figure 21: Aldosterone Standard Curve

applied potential, 0.22V; mobile phase, methanol:water (55:45);

reagent; 3 mM CBP, 0.05 M Na_2HPO_4; pH, 11.2;

coil volume, 2.45 mL; reagent flowrate, 1.0 mL/min.

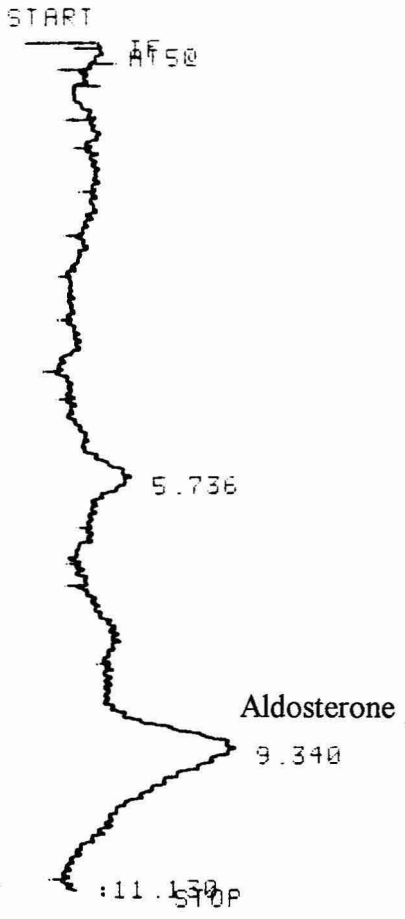


Figure 22: Chromatogram of Standard Aldosterone (20 ng)

applied potential, 0.22V;

reagent, 3 mM CBP, 0.05 M Na_2HPO_4; pH 11.2;

coil volume, 2.45 mL; reagent flowrate, 1.0 mL/min.

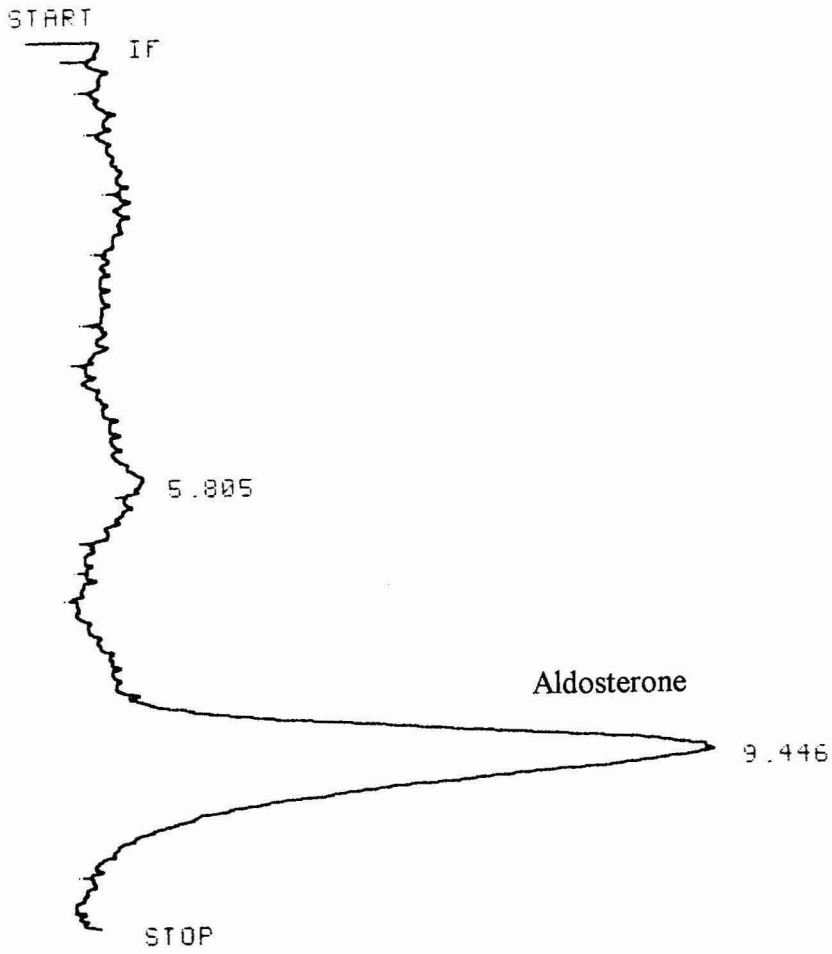


Figure 23: Chromatogram of Standard Aldosterone (200 ng)
applied potential, 0.22V;
reagent, 3 mM CBP, 0.05 M Na_2HPO_4; pH 11.2;
coil volume, 2.45 mL; reagent flowrate, 1.0 mL/min.

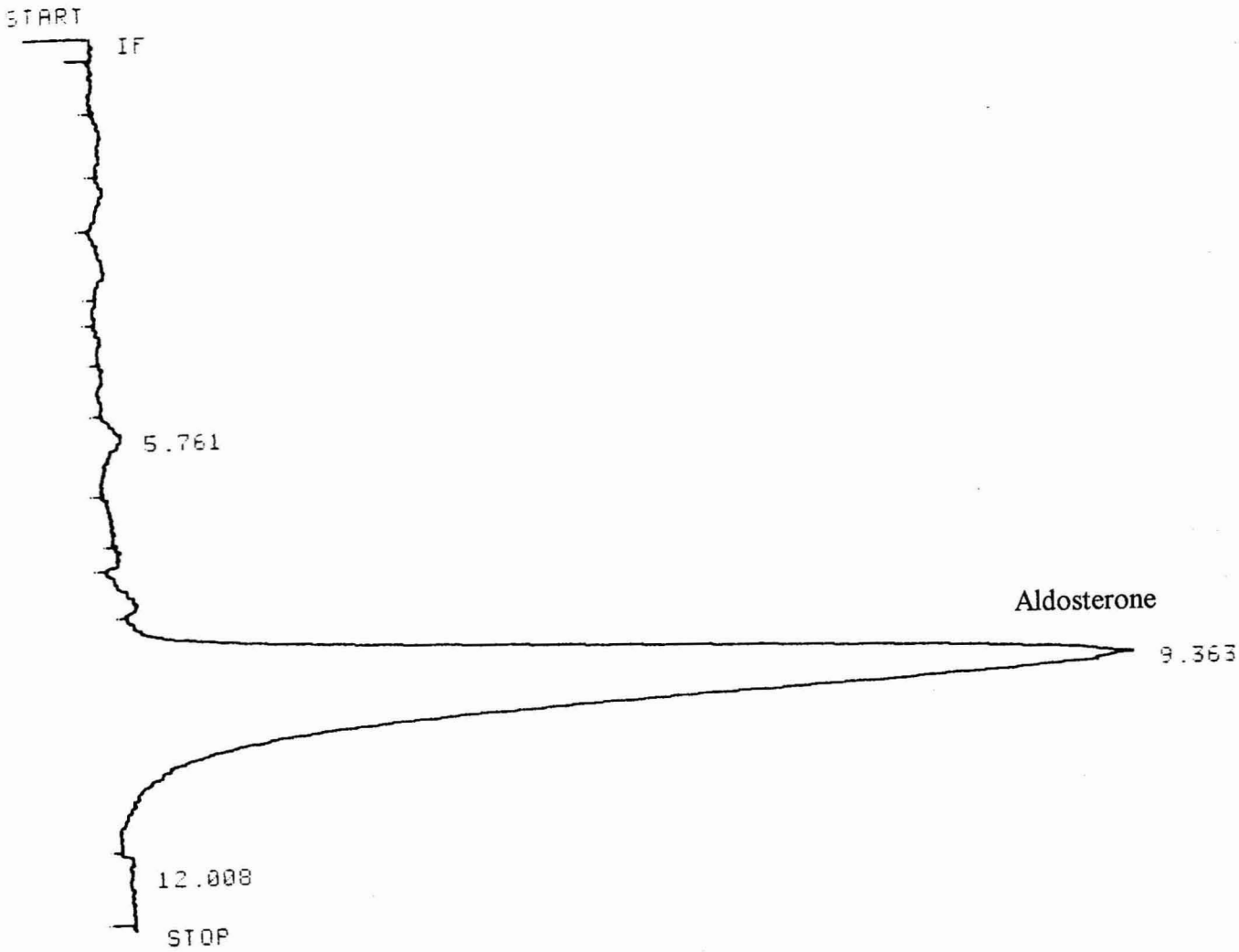


Figure 24: Chromatogram of Standard Aldosterone (400 ng)
applied potential, 0.22V;
reagent, 3 mM CBP, 0.05 M Na_2HPO_4; pH 11.2;
coil volume, 2.45 mL; reagent flowrate, 1.0 mL/min.

A 1:1 mixture of pure aldosterone (2 ppm) and THALD (2 ppm) was injected directly onto the column (fig.25). The retention time of THALD was around 10.6 min. Hence this metabolite would not interfere in the determination of aldosterone.

Urine Specimens and Chromatograms

A 24 hr. urine sample collected from a healthy volunteer, a 24 year old male, was prepared according to the method described in Chapter V. The total volume of the urine was measured to be 1260 mL. Four equal aliquots of the same urine specimen were obtained accordingly. One of the aliquots was spiked with 40 μl of the 50 ppm aldosterone stock solution before acid hydrolysis, the other two were spiked after the acid hydrolysis. The fourth aliquot was left unspiked. Solid-phase extraction procedures were performed on each of the aliquots and the eluates were reconstituted in methanol and injected onto the HPLC. Before every injection of the urine extracts, a chromatogram of a pure standard solution of aldosterone (200 ng) was recorded.

Figures 26 to 29 represents the chromatograms of all the four different urine specimens. Although the retention times for aldosterone were obtained around 9.4 minutes, no significant peaks were produced on the urine chromatograms. For the first chromatogram, where the sample was just run through the cyclohexyl cartridge, two prominent peaks were obtained at retention times 5.8 and 7.3 minutes (fig.26). For the unspiked urine specimen, the peaks were obtained at 1.3 and 5.8 minutes (fig.27). The

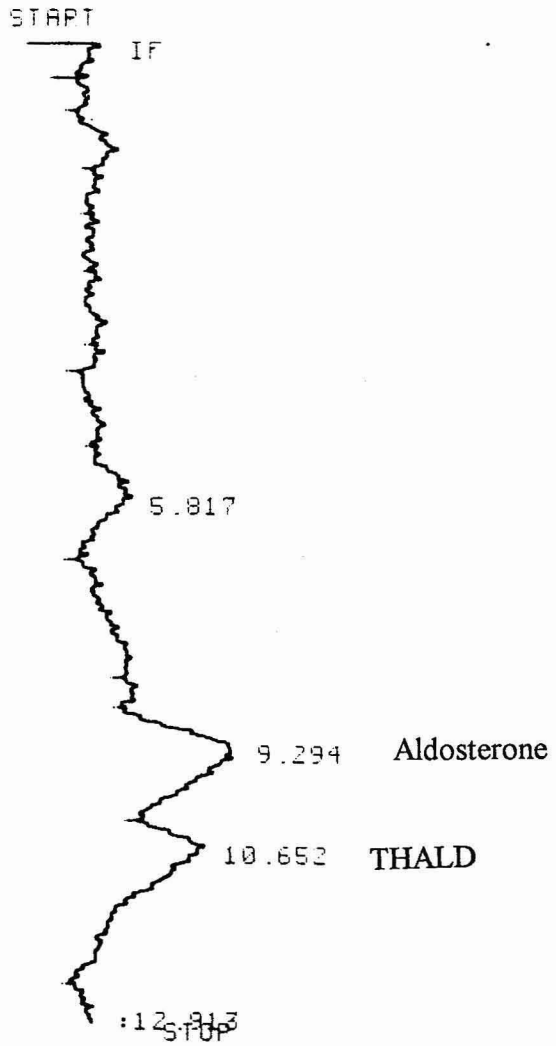


Figure 25: Chromatogram of a 1:1 mixture of Standard Aldosterone (2 ppm) and THALD (2 ppm);
applied potential, 0.22V;
reagent, 3 mM CBP, 0.05 M Na_2HPO_4; pH 11.2;
coil volume, 2.45 mL; reagent flowrate, 1.0 mL/min.

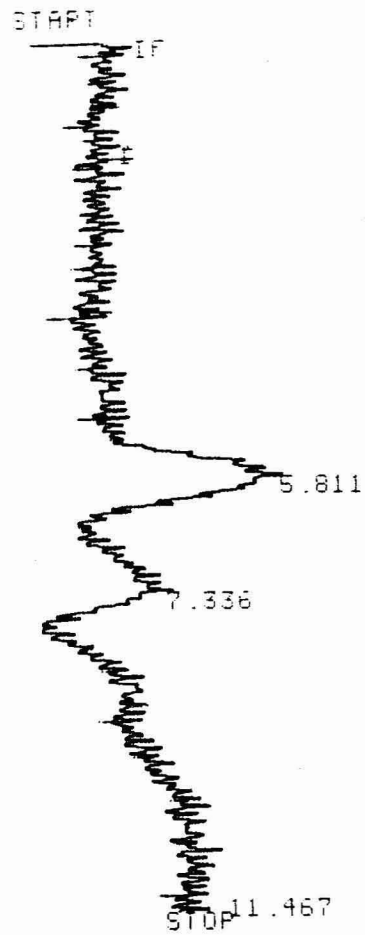


Figure 26: Chromatogram of a spiked urine specimen with standard aldosterone (200 ng) after hydrolysis and extracted with cyclohexyl cartridge only; applied potential, 0.22V; 5 mM CBP, 0.05 M Na_2HPO_4; pH 11.2; coil volume, 2.45 mL; reagent flowrate, 1.0 mL/min.

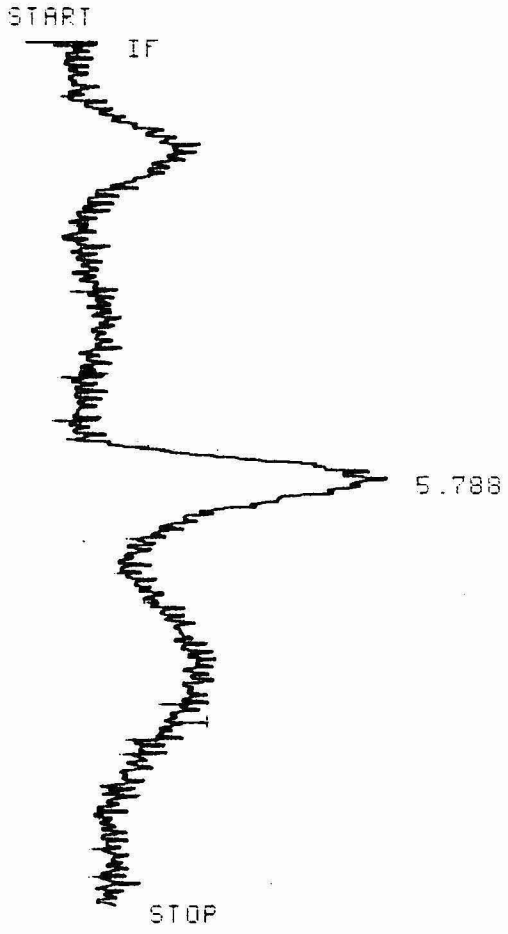


Figure 27: Chromatogram of an unspiked urine specimen;
applied potential, 0.22V; 5 mM CBP, 0.05 M Na_2HPO_4;
pH 11.2; coil volume, 2.45 mL; reagent flowrate, 1.0 mL/min.

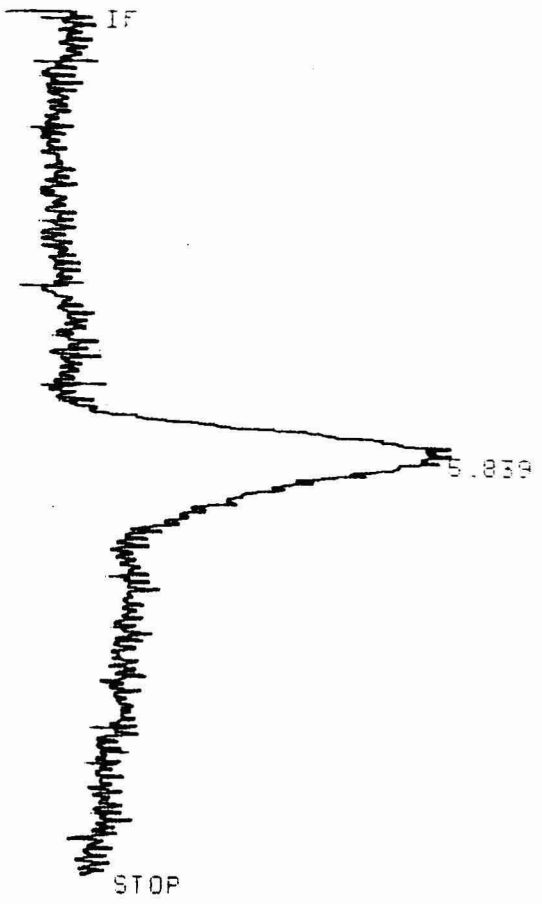


Figure 28: Chromatogram of a spiked urine specimen with standard aldosterone (200 ng) after hydrolysis and extracted with cyclohexyl and silica cartridges; applied potential, 0.22V; 5 mM CBP, 0.05 M Na_2HPO_4; pH 11.2; coil volume, 2.45 mL; reagent flowrate, 1.0 mL/min.

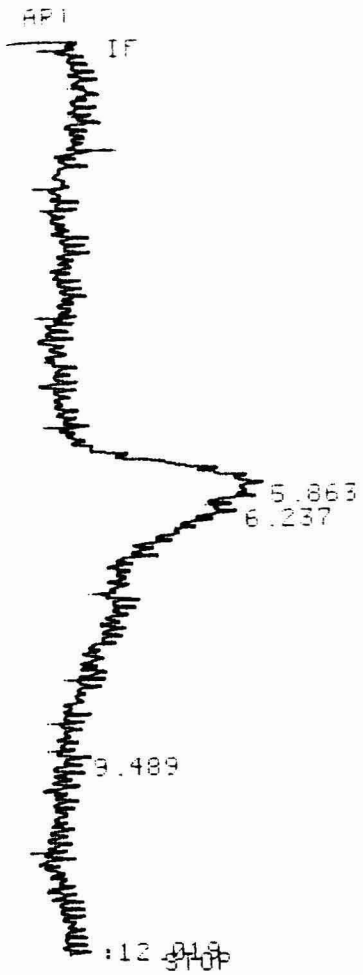


Figure 29: Chromatogram of a spiked urine specimen with standard aldosterone (200 ng) before hydrolysis and extracted with cyclohexyl and silica cartridges; applied potential, 0.22V; 5 mM CBP, 0.05 M Na_2HPO_4; pH 11.2; coil volume, 2.45 mL; reagent flowrate, 1.0 mL/min.

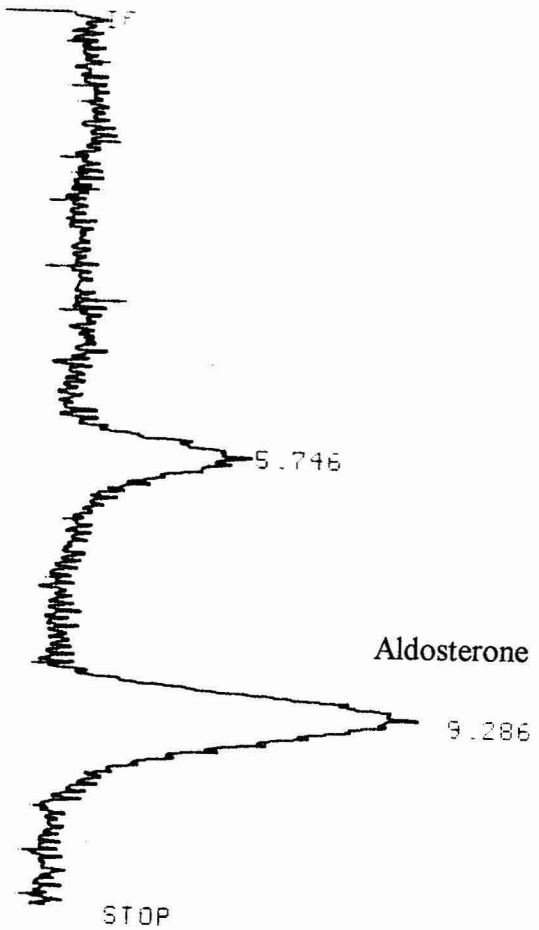


Figure 30: Chromatogram of pure Standard Aldosterone (200 ng)

applied potential, 0.22V;

reagent, 5 mM CBP, 0.05 M Na_2HPO_4; pH 11.2;

coil volume, 2.45 mL; reagent flowrate, 1.0 mL/min.

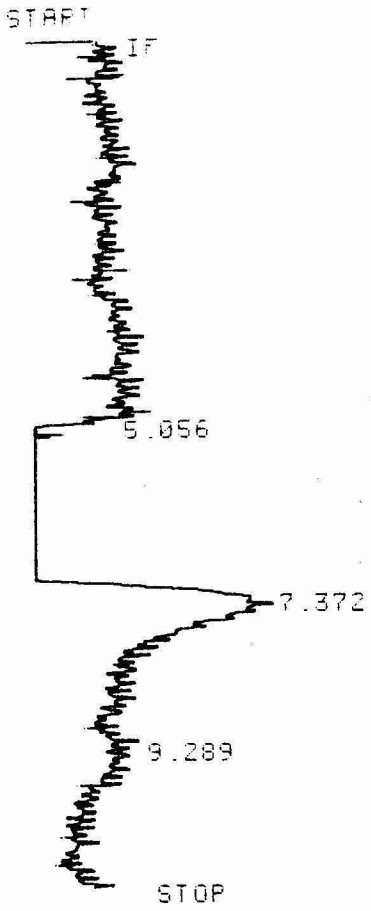


Figure 31: Chromatogram of Standard Aldosterone (200 ng) with few drops of 6 N HCl added; applied potential, 0.22V; reagent, 3 mM CBP, 0.05 M Na_2HPO_4; pH 11.2; coil volume, 2.45 mL; reagent flowrate, 1.0 mL/min.

chromatogram for the spiked specimen after hydrolysis had only one conspicuous peak at 5.8 minute (fig.28) whereas the spiked specimen before hydrolysis had two peaks at 5.9 and 6.2 minutes (fig.29).

After the chromatograms of the urine specimens, aldosterone (200 ng) stock solution was injected twice. During the second time, a few drops of 6.0 N HCl were added to the solution. The unspiked specimen of pure aldosterone produced the aldosterone peak at 9.3 min with another peak showing up at 5.7 min. The spiked specimen of aldosterone with hydrochloric acid produced a noticeable peak at 7.4 min (fig.30 and 31).

From the chromatograms, it is possible that the acidification of the urine to pH 1.0 has promoted the tautomerism of aldosterone (42). The retention time of 7.4 min might be an indication of this factor. Since no peak response at t_R 7.4 min was obtained for the urine specimens that were treated with silica cartridges, possible errors in the solid-phase extraction procedures might be present. The possibility of a glucose peak at 5.8 min was investigated by injecting a pure sample of glucose (200 ng).

CHAPTER VII

CONCLUSION

This research has shown that reversed phase HPLC along with amperometric detection can be utilized as a simple and faster technique for assaying aldosterone. The method has been found to be highly sensitive in the detection of aldosterone. The detection limit was 20 ng with the integrator attenuation at 6. A lower detection limit can be obtained by eliminating the background current due to impurities that might be present in the CBP reagent along with the pump noise. The results indicate the retention time of aldosterone to be 9.4 min.

No difficulties were encountered when pure aldosterone standards were used. Although the 24 hr. urine was collected and hydrolyzed with 6 N HCl, past and present studies have suggested the use of boric acid as the preservative with β-glucuronidase/ arylsulfatase as the hydrolyzing agent respectively (27,29). Previous studies by Bennett (43) have also been found to encounter the same problem with acid hydrolysis. Increasing the pH range to a more ideal range (i.e. 2-8) has been suggested by the same author.

Recent clinical procedures (44) have substituted hydrochloric acid with 50% acetic

acid to achieve a pH of between 2.0 and 4.0 for hydrolysis of aldosterone conjugates. The use of strong mineral acids, such as hydrochloric acid has also been forbidden in the above procedures.

The total hours of urine hydrolysis and its affect on aldosterone and it 18-glucuronide conjugate can be futher investigated. Although the urine extraction was simple and less time consuming, further research needs to be developed in that procedure. It has been suggested that washing the hydrated cyclohexyl column with pure hexane might have eluted the aldosterone into the waste and hence that step might be skipped.

No previous published literature regarding this method for the detection of aldosterone was found. A careful study and updating on the above mentioned suggestions, might make this procedure sensitive enough, that it can be used in the detection of aldosterone in human urine and serum samples.

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