SOLID PHASE EXTRACTION OF ALDOSTERONE AND ANALYSIS USING AMPEROMETRIC DETECTION

by

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Solid Phase Extraction of Aldosterone
And Analysis Using
Amperometric Detection

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Abstract

A method has been investigated to detect aldosterone in serum and urine using solid phase extraction, high performance liquid chromatography, and amperometric detection. The procedure circumvents the hazards and disposal expense of the radioisotopic waste generated by the use of radioimmunoassay, and the large amounts of organic solvents necessary for liquid-liquid extraction of the hormone by classical methods of analysis.

Random first morning urine samples were acid hydrolysed, and serum was untreated. Aldosterone was successfully recovered from spiked samples using cyclohexyl solid phase extraction in a procedure that was inexpensive, rapid, and easy. Separation of the aldosterone from interferences was accomplished using HPLC on a reversed phase (C_{18}) column. Detection was accomplished using a thin-layer electrochemical cell via the redox reaction of copper (I) bis-phenathroline, following the reduction of the copper (II) form of this complex by the aldehyde functional group of aldosterone in a heated post-column reactor bath. This method proved to be very specific for the separation of aldosterone from interferences in complex biological matrices.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to my research advisor, Dr. James Mike, for his help and guidance with this research project, and for his advice, sense of humor, and encouragement during my years at Youngstown State University. I would also like to thank Dr. Daryl Mincey and Dr. Timothy Wagner for reading this manuscript, and for their helpful suggestions.

DEDICATION

This work is dedicated to my husband John, whose patience, cooperation, and support have been absolutely invaluable to me for eighteen years.

To my beautiful daughters Emily and Anna, for the gift of their continued affection despite my preoccupation with work and school.

To my sister, Paula Fitzgibbons Snyder, for her years of encouragement and faith in me that have made me stand taller, work harder, and smile.

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LIST OF ABBREVIATIONS

HPLC	High Performance Liquid Chromatography
ACTH	Adrenocorticotropic hormone
ATP	Adenosine triphosphate
nmol	Nanomole
ng	Nanogram
dL	Deciliter
pg	Picogram
mL	Milliliter
ug	Microgram
CBG	Corticosteroid binding globulin
THALD	Tetrahydroaldosterone
um	Micrometer
cm	Centimeter
mm	Millimeter
PEEK	Polyetheretherketone
SPE	Solid phase extraction
uL	Microliter
mg	Milligram
RIA	Radioimmunoassay
GC/MS	Gas Chromatography/Mass Spectrometry
NRC	Nuclear Regulatory Commission

ABEI	Aminobutylethylisoluminol
hr	Hour
CBP	Copper bis-phenanthroline
PCR	Post column reactor

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CHAPTER I

INTRODUCTION

A. The Biochemistry of Aldosterone:

1. Definition and Structure of Aldosterone.

Aldosterone is a corticosteroid hormone that presents analytical challenges. This twenty-one carbon steroid is found in minute quantities in biological fluids and maintains equilibrium among three known structural isomers (1). Secretion of this hormone by the adrenal cortex is intricately regulated in healthy individuals by complex mechanisms that are responsive to the intake of fluid, the concentration of sodium and potassium ions, as well as variations in physical activity (2). Aldosterone secretion also varies diurnally in normal subjects, with the highest plasma concentrations measured at about the time of awakening, and the lowest circulating levels just after onset of sleep (3). These hourly and daily fluctuations make clinical interpretation of levels obtained by random sampling difficult. The scientific community, however, remains interested in methods of aldosterone

measurement because of the link this hormone has to hypertension, which may contribute to diseases that are a leading cause of morbidity and mortality.

Aldosterone (11β-21-dihydroxy-3,20-dioxopregn-4-en-18-al) is the most potent of the mineral corticoid hormones, and regulates fluid electrolyte balance by promoting the retention of sodium ions, and the excretion of potassium ions and hydrogen ions (4). Because of its effect on these electrolytes, this corticosteroid has an important impact upon the regulation of fluid volume and on blood pressure (5).

The basic nucleus of the aldosterone molecule, and of all steroid hormones, is the hydrophobic cyclopentaneperhydrophenathrene ring system as shown in Figure 1 below. Small changes in the types of chemical substituents that are attached to this nucleus translate into large changes in steroid hormonal function.

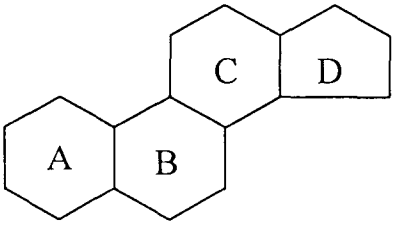

Figure 1. Cyclopentaneperhydrophenanthrene Ring (6)

Functional groups that are attached to the carbons numbered 10, 13, and 17 are especially significant for hormonal specificity. The steroid nucleus is numbered in a scheme illustrated in Figure 2.

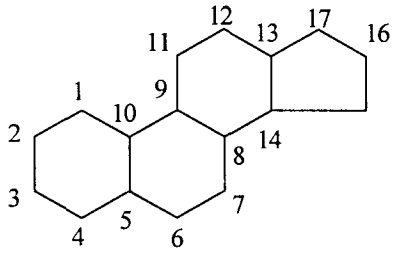

Figure 2. Numbering scheme of steroid nucleus. (7)

2. Biosynthesis

The aldosterone molecule is unique among the steroids because it alone possesses an aldehyde group as a substituent in one of its structural isomers. This aldehyde group is located at carbon 18, as shown below, in Figure 3.

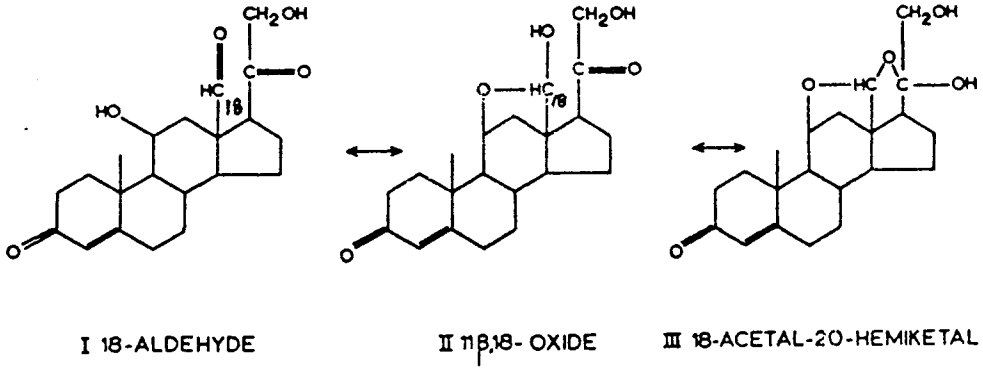

Figure 3. Structural Isomers of Aldosterone (8)

Aldosterone was first isolated in pure form by Simpson, Tait, Wettstein and Neher in 1953, and the chemical structure of the molecule was elucidated in 1954 by the same group (9).

Aldosterone is secreted by cells of the outer layer (zona glomerulosa) of the adrenal cortex. A major accepted pathway of biosynthesis of aldosterone is presented in Figure 4, depicting intermediates and associated enzymes.

Cholesterol desmolase (CYP11A), 21-hydroxylase (CYP21), and aldosterone synthase (CYP11B2) are all heme-containing, membrane-bound enzymes called cytochromes P450. These enzymes accept electrons from NADPH through associated proteins and utilize molecular oxygen to hydroxylate or otherwise oxidize substrates. Aldosterone synthase (CYP11B2) alone mediates the last three stages of aldosterone biosynthesis (10).

3β-hydroxysteroid dehydrogenase is the enzyme responsible for the conversion of pregnenolone to progesterone, and is a short chain dehydrogenase that removes a hydrogen from the 3β hydroxyl group to reduce NAD^+ to NADH (11).

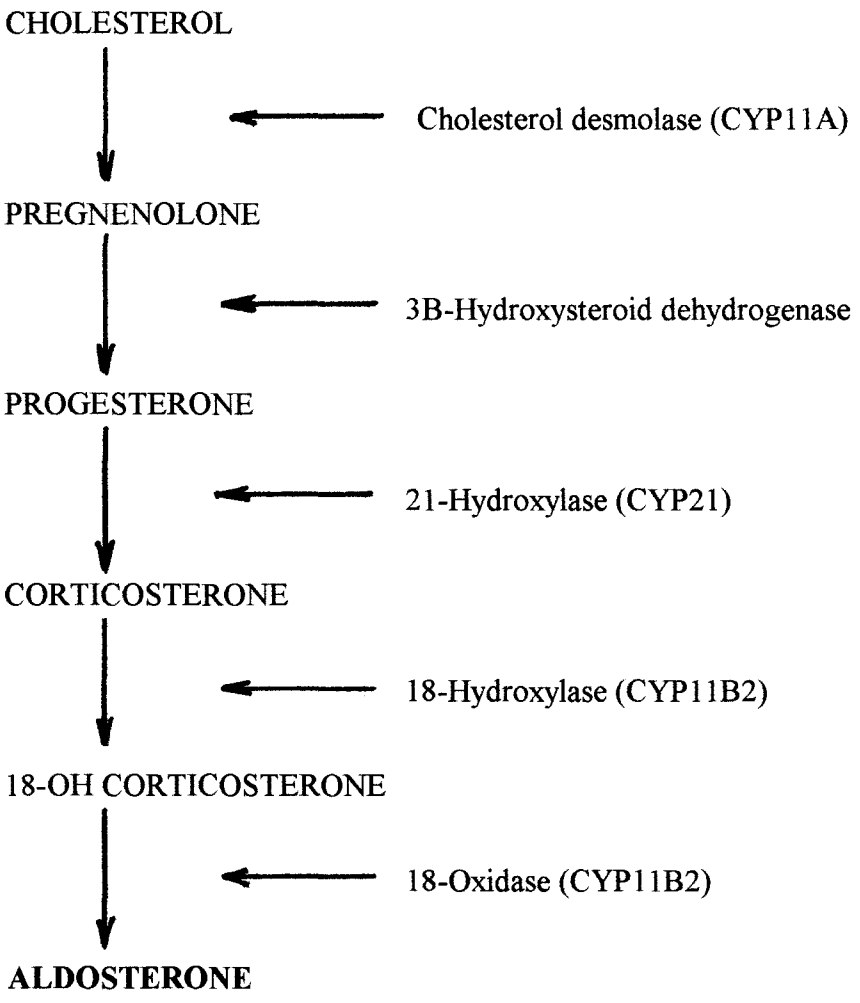

Figure 4: Intermediates and Enzymes of Aldosterone Biosynthesis (12).

The associated structures of this pathway are shown in Figure 5.

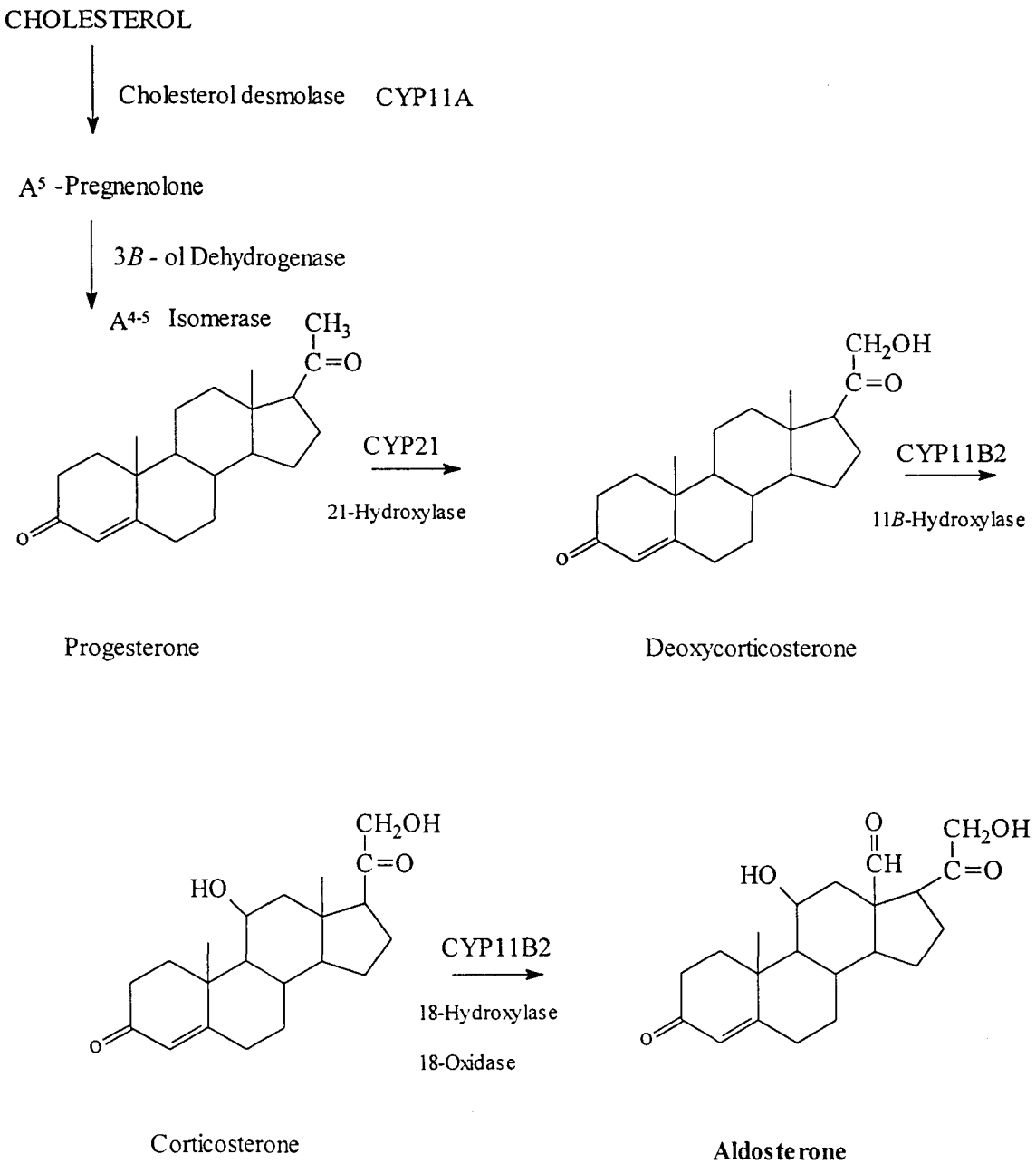

Figure 5. Aldosterone Biosynthesis

The first three steps in aldosterone biosynthesis are identical to the biosynthesis of cortisol, a glucocorticoid that influences both carbohydrate metabolism and immune response. However, only the zona glomerulosa of the adrenal cortex contains the18-oxidase capability of CYP11B2 for aldosterone synthesis (13), while lacking the 17-hydroxylase needed for cortisol production.

3. Regulation of Aldosterone Secretion

The factors that regulate blood aldosterone biosynthesis levels include the potassium ion concentration of extracellular fluid, the renin-angiotensin system, the quantity of body sodium ions and adrenocorticotropic hormone (ACTH) (14).

The major physiological regulator of aldosterone secretion in humans is the renin-angiotensin system. Renin is a proteolytic enzyme produced in the kidney when a renal cellular mass called the juxtaglomerular apparatus senses a decrease in afferent arteriole perfusion pressure. This may occur in a variety of pathological conditions, including shock, hemorrhage, and cardiac insufficiency. Renin enzymatically converts a liver protein called angiotensinogen to angiotensin I.

As angiotensin I (a decapeptide) passes with circulating blood through the lungs, it is transformed by angiotensin-converting enzyme to angiotensin II (15). Angiotensin II

binds to membrane receptors in the zona glomerulosa of the adrenal gland and sets in motion a cascade of enzymatic reactions to activate aldosterone biosynthesis (16). Angiotensin II also binds to receptors on the smooth muscle linings of blood vessels and causes vasoconstriction, which is at least as important in regulation of blood pressure as its role in stimulating aldosterone production.

A schematic representation of the renin-angiotensin-aldosterone system is depicted in Figure 6. This interconnecting system of hormones, oligopeptides, enzymes, electrolytes and fluid maintains a constant blood volume through the aldosterone induced reabsorption of sodium ions by the renal distal tubule when blood volume decreases. When blood volume is adequate, the renin-angiotensin system decreases aldosterone dependent sodium ion retention. An increase in plasma potassium ions results in an increase in aldosterone biosynthesis and secretion, while an increase in plasma sodium ions has the opposite effect (18).

4. Physiological Activity of Aldosterone

Cytoplasmic aldosterone receptors are found in the epithelial cells of the renal distal tubule collecting ducts, salivary glands and colon. These receptors are called Type I or mineralocorticoid receptors. There exists a high homology between the amino acid sequence of this Type I receptor, and the Type II glucocorticoid and Type III

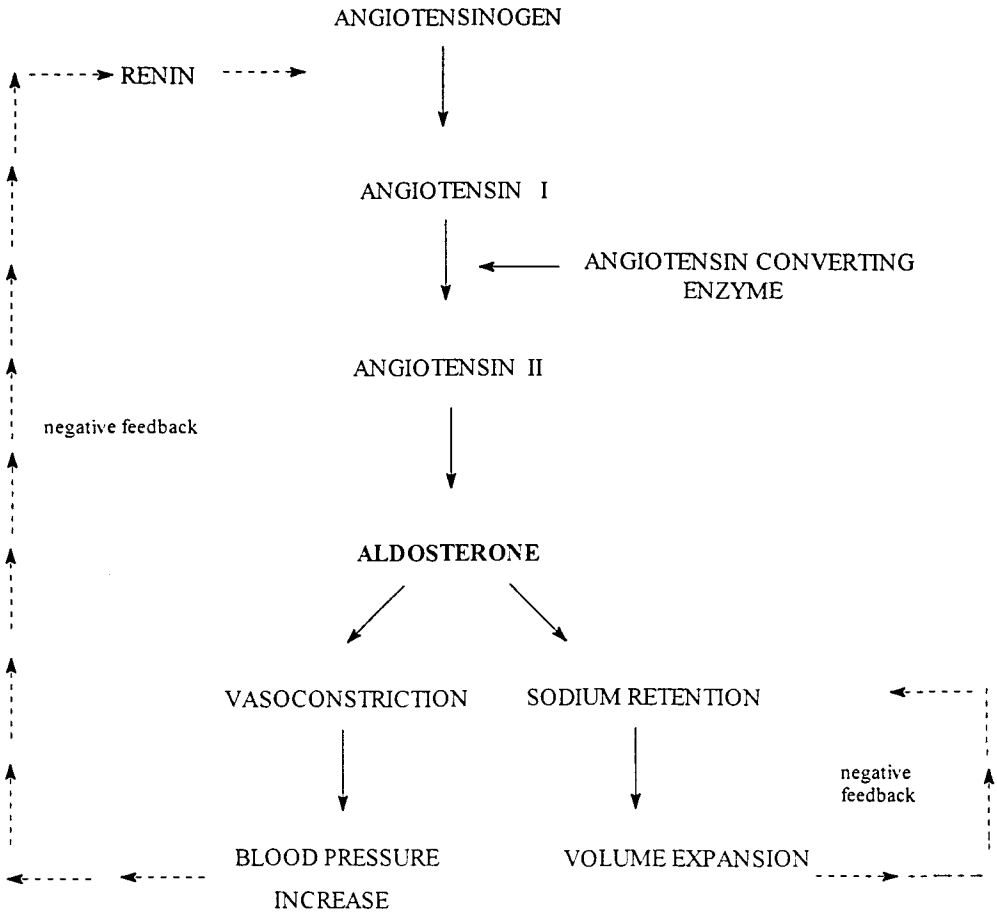

Figure 6. The Renin-Angiotensin-Aldosterone System (17)

progesterone receptors (19). Studies have determined that the glucocorticoid hormones hydrocortisone and corticosterone bind to the mineralocorticoid receptor with an affinity equal to that of aldosterone (20). These C-21 steroid hormones, like aldosterone, also possesses an 11β hydroxyl group. However, these corticosteroids lose their affinity for the mineralocorticoid receptor when the 11β hydroxyl is oxidized to a keto group. In aldosterone target tissues, the enzyme 11-hydroxysteroid dehydrogenase is associated with the receptor.

It has been postulated that 11-hydroxysteroid dehydrogenase protects aldosterone specificity by converting the 11β-hydroxyglucocorticoids into 11-ketosteroids, thus enabling only aldosterone dependent processes to be expressed. The 11β hydroxyl group of aldosterone is protected from oxidation as a result of hemi-acetal formation (21), and therefore is not affected by the enzyme "guarding" the hormone receptor. Recent studies have also shown that the mineralocorticoid receptor may also have a functional preference for aldosterone that is independent of the proposed 11β-hydroxysteroid dehydrogenase oxidation, and that this preference may be an intrinsic property of the receptor molecule itself (22).

The intracellular aldosterone-receptor complex binds to DNA and regulates the transcription of genes (23). It has been postulated that the effect of this gene transcription

is to increase the number of sodium ion channels that are open in the epithelial cells lining the gut, salivary glands, distal tubules, and collecting ducts. Potassium ion transport through distinct channels is increased as well. Aldosterone also increases the production of a Na^+/ K^+ - ATPase located in cell membranes (24). Other recent evidence shows that aldosterone may be involved in a methylation process of sodium channels that dramatically increases the channel activity (25).

5. Clinical Significance

In the human kidney, aldosterone promotes reabsorption of sodium ions, with bicarbonate and water, and enhances excretion of potassium ions and hydrogen ions. This hormone also facilitates sodium ion exchange for NH_4^+ in renal collecting duct cells (26).

Adults ingesting an average sodium diet (100-200 nmol/day) and maintaining an upright position for at least two hours have a serum aldosterone concentration of 5-30 ng/dL (50-300 pg/mL). A 24 hour urine collection from normal subjects contains approximately 3-19 μg of free and conjugated aldosterone (27). An average urine output of 1500 mL/day would thus produce a urine aldosterone output concentration of 2-13 ng/mL.

An increased concentration of aldosterone and its metabolites in serum and urine is termed aldosteronism. The symptoms of aldosterone hypersecretion include hypertension, low serum potassium levels, metabolic alkalosis, and low serum renin activity (28).

The conditions associated with excess aldosterone are generally divided into two categories. The first, primary aldosteronism, refers to a pathology within the adrenal gland itself. This lesion could be an adrenocortical adenoma (tumor) in one or both glands, bilateral adrenal hyperplasia, or, rarely, adrenal carcinoma.

Secondary aldosteronism refers to overproduction of aldosterone in response to pathology outside the adrenal gland. Any condition that reduces perfusion pressure of the kidneys, such as heart failure, nephrosis, and cirrhosis, will stimulate renin and aldosterone secretion. Pregnancy also increases the production of aldosterone and renin (29).

Decreased aldosterone synthesis may express itself in increased serum potassium levels, decreased serum sodium, mild or moderate fluid volume depletion, acidosis, and hypotension (30). The causes of hypoaldosteronism are varied. Addison's disease is characterized by the diffuse destruction of adrenal cortex tissue and concomitant decrease in all corticosteroids. Inherited biosynthetic deficiencies of aldosterone also occur and vary in severity. The most common enzyme deficiency is 21-hydroxylase deficiency of cortisol biosynthesis, and results in insufficient production of aldosterone in two-thirds of

known cases, with an incidence of 1 in 10-15,000 births (31). Symptoms are hyponatremia, hyperkalemia, and hyperplasia of the adrenal cortex. The individuals affected are termed "salt wasters". Other congenital disorders of aldosterone biosynthesis involve deficiencies in aldosterone synthase and cholesterol desmolase (32). Recently, hyposecretion of aldosterone has been noted as one of the complications of acquired immunodeficiency syndrome (33).

6. Aldosterone Transport and Metabolism

When secreted into the peripheral circulation, aldosterone binds with low affinity to plasma albumin and corticosteroid binding globulin (CBG), and diffuses rapidly into the liver. Injections of ^{3}H aldosterone have a circulatory half-life of only about fifteen minutes (34). Clearance of aldosterone from the circulation is reduced in liver disease. Cirrhosis reduces hepatic blood flow and diverts blood away from hepatic sinuses (35).

Aldosterone, being derived from cholesterol, is a lipophilic compound. Metabolism of this hormone in the liver results in very water soluble conjugates. Most of the conjugates of these polar metabolites that are excreted into bile and urine have very little hormonal activity (36).

Glucuronides are the most common hydrophilic aldosterone conjugates produced in the liver. They are derivatives of glucuronic acid and aldosterone, in which the two

moieties are joined by an acetal linkage. This linkage may have either an α or β configuration and the glucuronic acid ring may be five or six-membered. These glucuronides are stable as free acids, but have also been obtained as salts (37).

Before conjugation of aldosterone to a glucuronide, the liver often reduces the double bond at C_4, and the 3-oxo group, to produce a tetrahydro-derivative (3α, 11β, 21-trihydroxy-20-oxo-5β-pregnan-18-al) known as tetrahydroaldosterone (THALD). This is conjugated as the 3-glucuronide and is a major metabolite of aldosterone, accounting for 20-35% of aldosterone production (38). This THALD conjugate can be hydrolysed readily by mammalian β-glucuronidase.

Both the liver and kidney metabolize aldosterone to an 18-glucuronide, which accounts for 7-12% of aldosterone metabolites (39). Also known as the 3-oxo conjugate because of the intact ketone group at C-3, this metabolite can be hydrolysed at pH 1 at room temperature to aldosterone and a mixture of glucuronic acid and glucurolactone. This 18-glucuronide of aldosterone is <u>not</u> readily hydrolysed by mammalian or bacterial β–glucuronidase (40). Figure 7 shows the structures of the two major metabolites of aldosterone. Several other minor pathways of aldosterone metabolism occur which produce small quantities of tetrahydroderivatives. In all of the aldosterone metabolites,

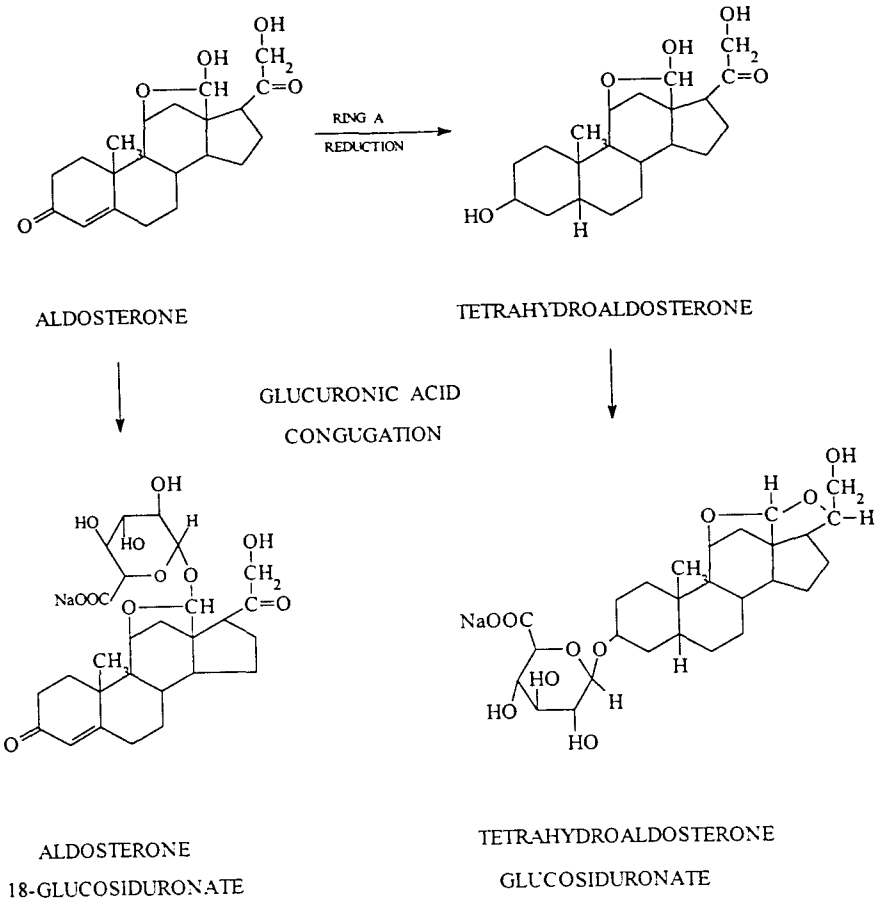

Figure 7. Major Urinary Metabolites of Aldosterone

the 11,18 hemiacetal system appears to remain intact. Only about 1% of secreted aldosterone is excreted in free form in human urine.

About 20% of these metabolites that are produced by the liver are excreted in bile and into the intestines, and thus do not appear in urine (41).

B. Analytical Methods.

1. Liquid Chromotography

Liquid chromatography is an analytical technique used for the separation of components in a mixture. When analytes are present together in a homogenous phase, a second phase needs to be added or formed so that some analytes preferentially move onto one phase while others move onto the second. Liquid chromatography involves a stationary phase and a mobile phase. A mixture of analytes is introduced onto a liquid mobile phase and carried through the stationary phase. The components of the mixture partition between the mobile and the stationary phases, and depending on their attraction to, or repulsion by these phases, they migrate at different rates through the analytical system. The components of the mixture thus have different retention times on the stationary phase. The narrow zone of sample that is initially introduced into the mobile phase emerges from the system as bands of components.

There are several different types of liquid chromatography in common use today, which depend on physical properties such as size, solubility, and charge for the separation of a mixture's components. These types of liquid chromatography can be classified by their physical configurations as either column or planar chromatography (42). Planar chromatography is often called thin layer chromatography, for the stationary phase can be a flat piece of paper, a thin layer of solid coated onto glass,or plaster sheets. Capillary action and/or gravity moves the mobile phase through the stationary phase.

Thin layer chromatography is still very useful in qualitative work, but the bulk of liquid chromatography for quantitative analysis is presently done using column chromatography. As its name suggests, the stationary phase utilized in column chromatography is held within a column through which the liquid mobile phase flows.

2. High Performance Liquid Chromotography

A very widely used type of liquid column chromatography is known as High Performance Liquid Chromatography (HPLC). A block diagram of such a system is shown in Figure 8.

Stationary phases of very small particle size (5-10 μm) are packed into columns that are 10-25 cm long, with internal diameters of 4-6 mm. There has recently been a

toward even shorter or narrower columns in order to decrease analysis time, and to conserve solvent (43).

A pump capable of operating at moderately high pressure is necessary to maintain an adequate flow rate through the tightly packed small particles of this stationary phase.

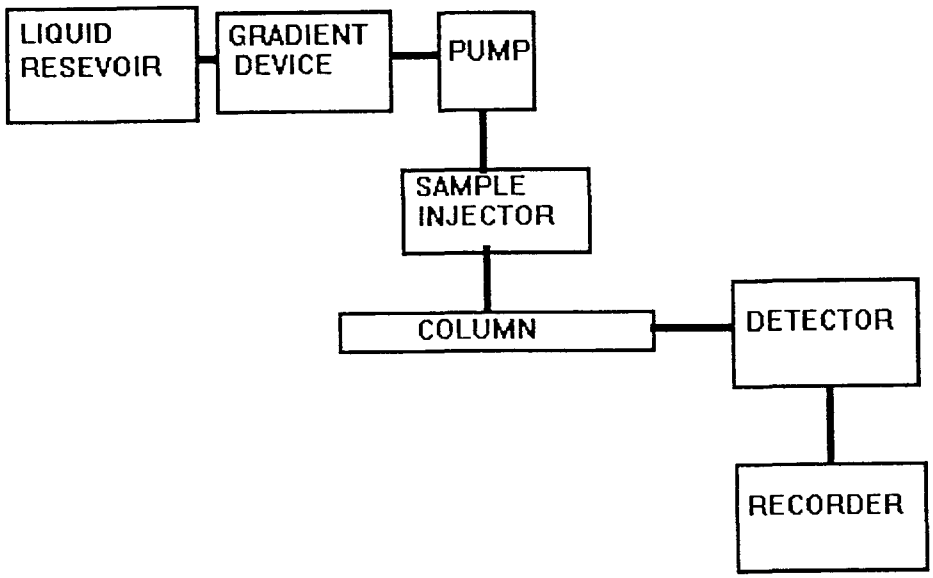

Figure 8. Block Diagram of a Liquid Chromatograph

Mobile phases are typically held in reservoirs with filtered inlets to prevent intake of minute contaminating particles which could damage seals within the pump, or clog the inlet of the column. Most HPLC systems have solvent programming or gradient devices

which direct the mixing of different solvents before they enter the pump, and have the pumping capacity to specify the solvent flow rate. Chromatographic runs which use only one solvent mix are termed isocratic elutions. Gradient elutions are chromatographic runs in which the composition of the mobile phases changes with time.

Many HPLC systems use a reciprocating piston pump with ruby ball inlet and outlet check valves. Multiple pump heads are often set to deliver solvent to the system in such a way that pulsation of flow is minimized (44). Samples can be injected into the flow stream of the mobile phase in various ways. Direct injections can be made onto the column head by syringe through a septum. More commonly, a sample injection valve introduces measured amounts of sample through a loop into the mobile phase as it flows onto the column. When samples are to be injected repetitively, auto samplers are often used to transfer sample from sealed vials to an automatic loop injector (45).

3. Chromatography Phases

Early chromatographic methods used columns packed with silica or alumina adsorbents, and used nonpolar solvent mixes to elute sample mixtures. Liquid chromatographic methods are classified as normal phase when the stationary phase is polar and the mobile phase relatively nonpolar. Methods in which the stationary phase is

nonpolar and the mobile phase is relatively polar are called reverse phase liquid chromatography.

In many reverse phase HPLC systems used today for the analysis of organic substances, the stationary phase packing of the column has a polymer or silica base onto which organic material is covalently bonded. Increasing numbers of organic molecules are now being bonded to stationary phase by column manufacturers. Manufacturers use silanization reactions to attach alkyl, cyano, amino, phenyl, or other functional groups to the free hydroxyl groups of silica particles (46). A typical reaction is shown below, in Figure 9.

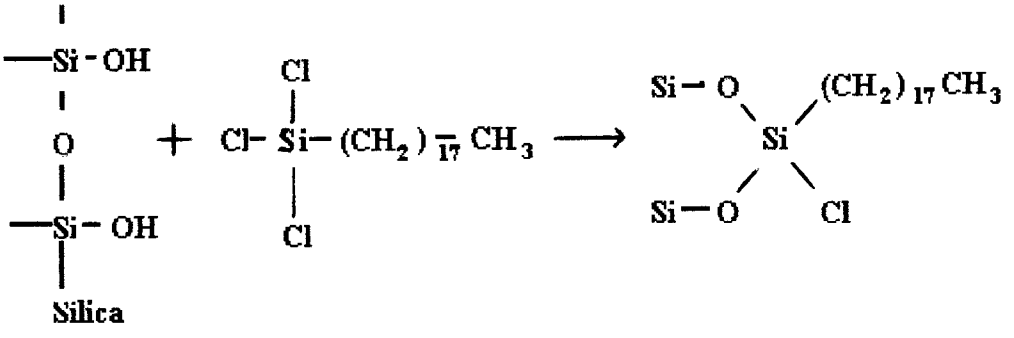

Figure 9. Attachment of Alkyl Groups to Silica Backbone of Solid Phase

Following these silanization reactions, the silica particles are covered with a film of bonded functional groups that has the properties of both a liquid and a solid (47). Any exposed free hydroxyl groups that remain on the surface of the silica can be "end-capped" by reacting them with trimethylchlorosilane, in a process that is in principle much like that of laboratory glassware silanization.

The mobile phase flowing over the stationary phase must be immiscible to the film on the solid particles. Solutes sweeping past the stationary phase in the mobile phase partition into and out of this film and distribute themselves according to their relative solubilities. In reverse phase HPLC, solutes that are relatively polar will be less soluble on the nonpolar film of the stationary phase and will travel through the column faster than a nonpolar solutes, which will be more soluble on this film.

4. Chromatography Parameters

After passing out of the column, these separated bands of solutes may be collected individually for further analysis. More commonly, a detector is used to monitor the effluent flowing from the column. The detector measures some physical property of that volume of mobile phase that is carrying separated bands of solutes from the column. The detector produces an electrical signal that is proportional to the amount of solute detected. This electrical signal is recorded by a data processor, or less commonly, a strip chart

recorder. A chromatogram is a plot of this generated signal versus the volume of mobile phase eluting from the column. Since most systems are run at a constant flow rate, *(e.g.*, one milliliter per minute), a chromatogram can also be thought of as a plot of detector signal versus time. As bands of solute mobile phase flow through the detector, a recording of the increase and decrease of signal versus time (t) becomes the familiar peaks that are present on the chromatographic baseline.

In order to describe chromatographic separations, a variety of terms are used. It is important to understand that there is a quantity of mobile phase in a column called "dead volume" or "void volume" (V_m) filling up the spaces between the stationary phase particles. V_m is the volume of mobile phase needed to elute a solute that has no attraction for the stationary phase. On a chromatogram, V_m may be indicated by a baseline disturbance of varying size, often called the "solvent front". When the solute partitions between the mobile and stationary phase, it is being retained, and the volume of mobile phase needed to elute it from the column is the retention volume, V_r. At constant flow rates, $V = t \times F$, and V_m and V_r can be referred to as t_o and t_r.

The capacity factor, or partition ratio (k'), can be described by a number of

theoretical equations, or measured directly from a chromatogram, as follows:

$$k' = \frac{t_r - t_o}{t_o} \qquad (48)$$

where t_r is the time in minutes for the species of interest to be eluted from the column, and t_o is the time in minutes needed to elute a solute that has no attraction for the stationary phase. This capacity factor provides a quantitative way to describe where substances elute relative to an unretained solute. Analyte peaks often exhibit their best chromatographic behavior when k' has a value between one and ten. When values of k' are too small, the analyte may be indistinguishable (unresolved) from the solvent front. When k' is too large, the chromatographic run time may be excessive.

As retention time increases, intra- and extra-column dispersion often widen solute bands. This band broadening widens peaks on a chromatogram, which makes their accurate measurement more difficult. Narrow, nonoverlapping bands of solute eluting off a chromatography column are translated into sharp narrow peaks on a chromatogram. Band broadening increases the incidence of overlap, and may cause unresolved peaks on a chromatogram. It is generally the objective of any chromatography method to obtain narrow,easily measurable, resolved peaks of the analytes of interest on a chromatogram. The degree to which two peaks are separated from one another can be described

quantitatively (49). Some of the parameters used in these calculations are illustrated in figure 10.

Resolution (Rs) of adjacent peaks on a chromatogram can be calculated with the equation:

$$R_s = \frac{2\,(t_r - t_o)}{w_1 \quad w_2}$$

where t_r is the retention time and w is the baseline width of each chromatographic peak. The numerical value of Rs will be equal to 1.5 as two peaks become just baseline resolved.

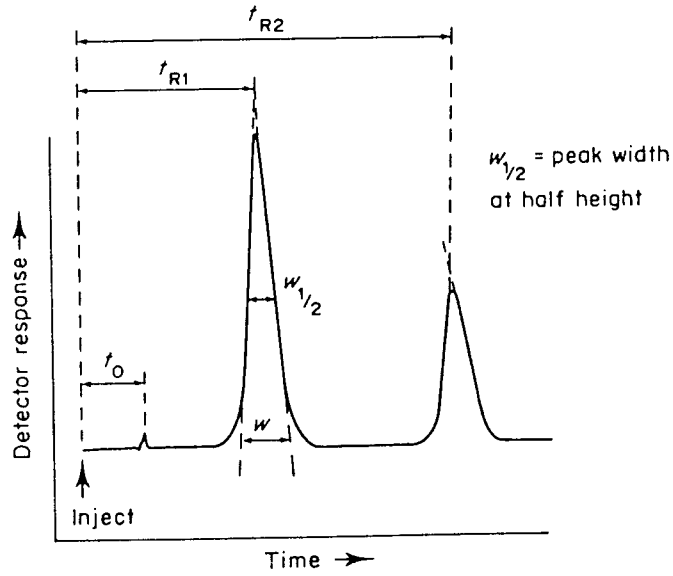

Figure 10. Retention and Dispersion Measurements (50).

The separation factor α, (sometimes called the selectivity factor) may also be used to describe how well two chromatographic peaks are separated from each other. This parameter may also be calculated from direct measurements of the chromatogram as

follows:

$$\alpha = \frac{k'_2}{k'_1} = \frac{t_r2 - t_r0}{t_r1 - t_r0} \qquad (51)$$

A separation factor of 1 indicates that peaks are coincidental. Increasing values of α that are greater than 1 indicate greater resolution of peaks on a chromatogram.

The parameters termed plate number (N), and plate height (H), describe the efficiency of chromatography columns in producing narrow bands of solutes in column eluate, and can also be calculated from a chromatogram.

The following equation is used when the baseline width of a peak is easily measurable:

$$N = 16\left(\frac{t_r}{w}\right)^2 \qquad (52)$$

Because baseline width of especially unresolved peaks is sometimes difficult to estimate, $w_{1/2}$, the width at peak half-height, may also be incorporated into an equation that numerically describes column efficiency, as below:

$$N = 5.54\left(\frac{t_r}{w_{1/2}}\right)^2$$

The theoretical plate height,(H) is calculated as:

$$H = \frac{L}{N}$$

where "L" is the length of the chromatograph column (53).

Improving separations of analytes in a chromatography system involves the consideration of several variables. The most important changeable features in such a system are temperature, and the natures of the mobile and stationary phases (54).

Although the variety of stationary phases available to chromatographers is rapidly increasing, column parameters such as particle size, internal diameter, length, and type of solid phase are much more expensive to adjust in an existing system than simple changes in mobile phase composition. Solvent selection, relative percentages of each solvent in the mix, buffers, additives, and flow rates are easily modified in any attempt to optimize a separation.

Temperature effects have historically been ignored in HPLC systems, but should be considered when problems arise. Many HPLC systems available today include controlled temperature compartments to house the analytical column portion of the apparatus. The interaction of an analyte and the column stationary phase is a function of the free energy gained or lost in the process, and this relationship to temperature can be seen in the following equation:

$$\ln k' = -\Delta H / RT + \Delta S / R + \ln \phi$$

where k' is the retention factor of the analyte of interest, ΔH is the enthalpy change for the retention process, and R is the molar gas constant. ΔS is the change in entropy, and ϕ is the phase ratio for the chromatography column of the system used (55).

5. Detection Systems

The detection of solute bands exiting from the column in the mobile phase can be accomplished in a variety of ways. Detectors that are selective for certain physical properties possessed by the solute alone as it travels through the detector in the mobile phase are appropriately named selective detectors. These include UV-visible absorption, conductivity, amperometric and fluorescence detectors. In contrast, bulk property detectors measure properties shared by both solute and mobile phase. The refractive index detector, for example, relies upon the difference between the refractive index of pure solvent and that of a mixture of solvent and solute.

The sensitivity, selectivity, accuracy, and precision of these different detectors are of interest when considering a particular analytical problem. The sensitivity of a detector is defined as the ratio of the detector response to a given sample concentration (56). The slope of a graph of analyte concentration plotted against detector signal can be used to compare the sensitivity of individual detectors for a particular analyte. The most sensitive

detector is that which produces the largest signal to noise ratio for a given concentration of analyte.

The selectivity of a detector is described as its capacity to detect only particular types of compounds that share a physical or chemical property. This property, for example, could be fluorescence in the presence of a derivatizing reagent, absorption at one specific wavelength in UV-visible detectors, or oxidation at a controlled potential in electrochemical detectors.

6. Electrochenical Detectors

Electrochemical detectors are considered selective detectors because they exploit the ability of some analytes to be oxidized or reduced at specific potentials relative to a reference electrode. These detectors can be designed to employ various electrochemical principles such as conductimetry, coulometry, polarography, and amperometry.

Amperometry was the technique employed in the detection of aldosterone in this particular research project and is a good choice when high sensitivity and excellent precision are as important as accuracy. Amperometric detectors utilized in liquid chromatography systems can be designed to pass column eluate through a thin layer cell in the presence of a working electrode, where the oxidation or reduction of an electroactive substance occurs. The volume of this thin layer cell can be very small.

The working electrode can be considered as an oxidizing or reducing agent in the flow stream. An electrode becomes a stronger oxidizing agent for solutes passing over it as its positive potential increases. When the electrode potential becomes more negative, the working electrode becomes a stronger reducing agent of analytes in the flow stream. A working electrode operated as an oxidizing agent collects the electrons released on its surface from the electroactive analyte. Electrolysis current, then, rises and falls as the analyte in the flow stream passes through the thin layer cell. This current is converted to voltage and sent to a recorder after amplification. At the recorder, it is the peaks on a chromatogram that mirror the rise and fall of electrolysis current (57). A representation of electron transfer at the surface of a thin-layer electrode is shown in Figure 11.

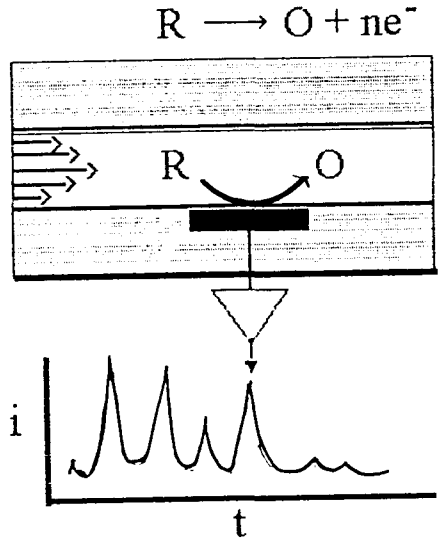

Figure 11. Electron Transfer at a Thin-layer Electrode Surface (58).

Because the surface of the working electrode is in the flow stream of the test solution, side oxidation-reduction reactions, adsorption of solutes to the electrode surface, ion exchange, and a variety of other chemical and physical processes affecting the electrode surface may occur (59). Light polishing of the electrode is necessary from time to time to achieve a reproducible and constant electrode surface activity, and especially when a decreased response to a known standard concentration of analyte is noted.

A three electrode cell is frequently employed in the amperometric detectors of HPLC systems. The reference electrode, often of Ag/AgCl or Hg_2Cl_2, is used to provide a stable potential against which the potential of the working electrode is measured. An auxiliary electrode is the current supporting partner of the working electrode. This third electrode performs the other half of the oxidation-reduction reaction on other electroactive substances in the flow stream of the detector cell. A device called a potentiostat maintains the working electrode at a constant potential. The electrochemical detector utilized in this study set the reference electrode at ground potential (60). Further details concerning the processes occurring at the electrode surfaces may be found elsewhere (61, 62).

An illustration of the three electrode electrochemical cell used for the bulk of this project is shown in Figure 12. The working electrode was made of glassy carbon, imbedded in a block made of polyetheretherketone (PEEK). The size of the thin layer cell

was determined by the thickness of the gaskets placed between the PEEK block and the stainless steel auxiliary electrode and reference electrode housing. The use of one 0.002" thin layer gasket in this project provided a detector cell volume of approximately 0.5 microliter (63).

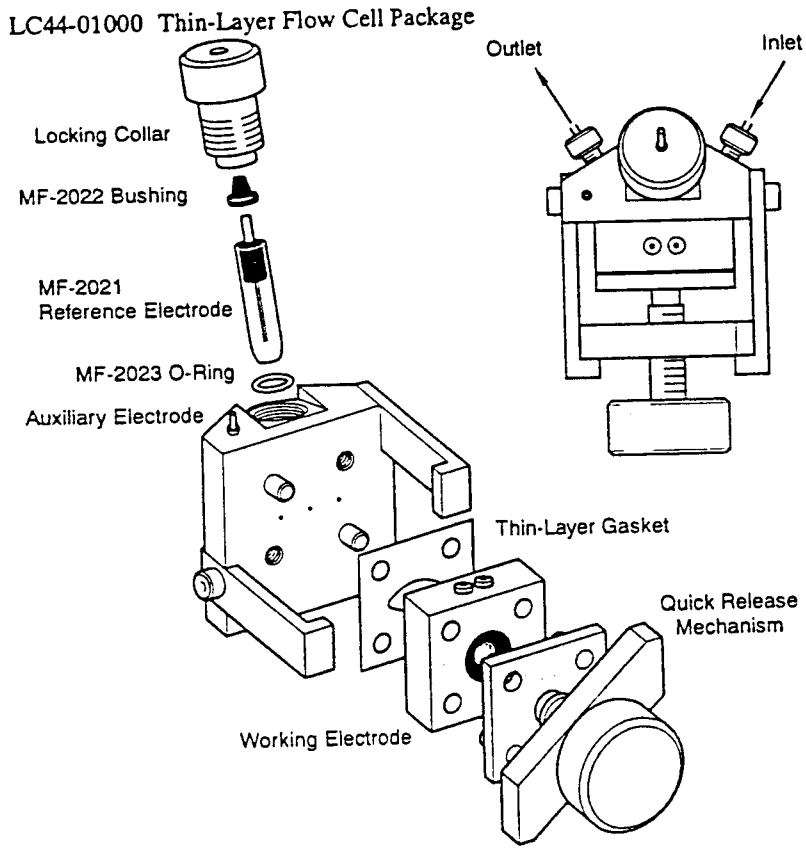

Figure 12. Thin-Layer Electrode Flow Cell Package (64).

7. Solid Phase Extraction

Qualitative and quantitative analysis by high performance liquid chromatography generally requires some type of sample preparation for cleanup purposes, trace enrichment, or transfer of analyte from a complex matrix to a column-compatible solvent.

Classical analytical extraction methods often involve liquid-liquid extraction in separatory funnels. These procedures are hampered by impure and wet extractions, phase emulsions, excessive analysis times, and the use of relatively large amounts of solvent (65).

These liquid-liquid extraction methods are sometimes used in conjunction with other classical preparation techniques, including distillation, filtration, centrifugation, and precipitation.

Solid phase extraction (SPE) cartridges used for the extraction and isolation of analytes became available in the late 1970's to circumvent some of the shortcomings of traditional methods (66).

Solid phase extraction is actually a form of low pressure liquid chromatography in which a liquid sample is passed through a cartridge or disc containing a specified weight (generally from 50 milligrams to several grams) of sorbent, or the solid phase. These disposable polypropylene discs or cartridges are available in various reservoir (column) volumes, with sorbent held in place by polyethylene or stainless steel frits. The sorbent retains analytes from a liquid sample passed through it much like an HPLC column.

The increasing availability of different types of SPE sorbents has paralleled that of traditional HPLC column packings. To extract the desired analytes from a sample, polar or nonpolar bonded phases, or ion exchange SPE cartridges can be selected to be used in

conjunction with solvents of suitable polarity or pH. SPE cartridges do not have the pH restrictions of HPLC column packings because they are disposable. Also, solvents that are potentially damaging to the silica backbone of the sorbent remain in contact with this solid phase for a relatively short time, and the small liquid volumes necessary for component extraction do not degrade the sorbent bed to an appreciable degree.

Generally, nonpolar sorbents are used to extract nonpolar analytes from aqueous solution, and ionic or polar solid phases are used to trap ionic or polar compounds of interest. Table 1 lists a few of the solid phases available for SPE and the types of compounds that these sorbents may be employed to extract.(67). The separation mechanism of these liquid-solid systems occur as a result of intermolecular attractions between the functional groups of the analyte molecules and the particular sorbent used.

The sorbent capacity of an SPE cartridge is defined as the total mass of a retained analyte per unit mass of sorbent, and has been estimated to be approximately two milligrams of analyte per gram of solid phase (68). However, interfering compounds present in the sample matrix may compete with the analyte for adsorption sites, and mass overload is possible if the weight of sorbent employed in the extraction of large amounts of sample is inadequate (69).

SOLID PHASE	SELECTIVITY
Non-polar	
Octadecyl (C18)	Non-polar organics from polar solution
Cyclohexyl	
Octyl (C8)	
Polar	
Cyanopropyl (CN)	Moderately polar or strongly polar organics
Aminopropyl	
Silica (Si)	
Ion Exchange	
Benzenesulfonylpropyl	Organic cations such as amines
Trimethylaminopropyl	Organic cations such as carboxylic acids

TABLE 1.
SELECTED SOLID PHASES
COMMONLY USED IN SPE

The minimum elution volume of an SPE cartridge is defined as a two bed volume of elution solvent, with bed volume being equal to 120 μL per 100 mg of sorbent (70).

Usually there are four discrete stages in any solid phase extraction. When using a nonpolar bonded phase, for example, to extract nonpolar analytes or from an aqueous solvent, the steps are as follows:

1. The SPE sorbent is conditioned by passing methanol, followed by water or buffer, through the sorbent.
2. The sample is passed through the sorbent, with desired compounds extracted onto the sorbent from a weak solution.
3) The sorbent is washed with a solvent that is selected to remove impurities.
4) The elution of analyte of interest from the SPE sorbent is done with a strong solvent.

When using reverse phase (nonpolar) sorbents, the terms "strong" and "weak" refer to the ability of a solvent to disrupt the hydrophobic interactions that hold a nonpolar compound to the sorbent. Thus water is a weak solvent in a reverse phase SPE or HPLC system. Figure 13 lists some selected solvents in order of increasing strength on reverse systems.

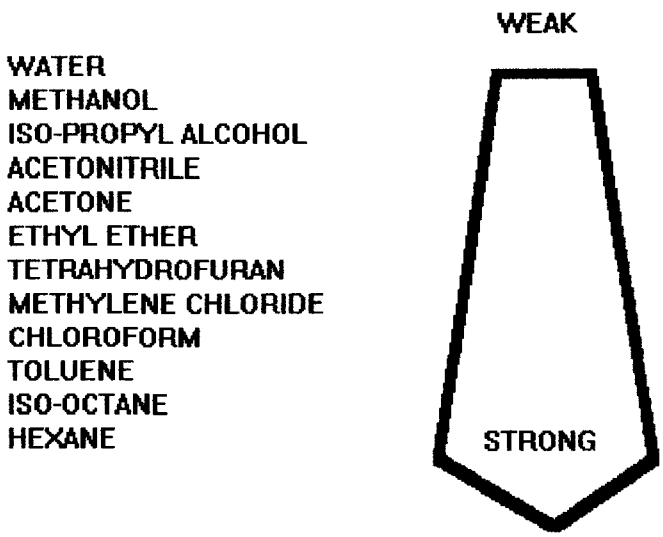

Figure 13. Solvent Strength in Reverse Phase Systems (71).

If the analyte of interest is more polar than the interferences in the sample matrix, a polar sorbent, such as silica, may be used to extract the analyte, and the sorbent is then washed with a relatively nonpolar solvent to remove unwanted components. The analyte to be isolated is then eluted from the solid phase with a polar solvent. This technique can

be thought of as a digital mechanism, *i.e.*, a substance is either "on" or "off" the sorbent in the cartridge.

8. SPE Method Development

Method development for a solid phase extraction of an analyte requires a careful review of a number of factors. Choosing the most appropriate sorbents and solvents for the task at hand requires information about the non-polar, polar and ionic functional groups of the molecule, as well as solubility and ionization data. The typical concentration of the analyte in the sample should be known. Sample matrix polarity and complexity also play an important part in the selection of an SPE strategy.

Initial SPE method development for a particular substance often involves screening a number of different sorbents in a particular category (*e.g.* nonpolar, polar, or ion exchange). To screen sorbents, a specific volume of standard analyte solution is passed through the selected SPE cartridges, and the effluents are collected and analyzed for the analyte. The sorbent offering the best retention will elute the least amount of analyte in this initial step. Following the choice of sorbent, it is then necessary to identify a solvent which effects a maximum elution of the retained analyte from the sorbent. The most effective elution solvent will contain the highest concentration of analyte with the fewest interferences (72).

If interfering substances are present in the sample matrix, the most efficient wash procedures must be identified. This may be achieved by applying standard analyte solution to a series of identical SPE cartridges. Next, a serial dilution of the most effective elution solvent is prepared. These serial dilutions, beginning with the most dilute, are each passed through a separate cartridges. The effluent of each cartridge is analyzed for the substance of interest. The wash solution which is the most concentrated, *but has not yet eluted the analyte,* will often do the best job of pulling interferences off the sorbent, and leaving the analyte behind (73).

Some of the advantages of an optimized SPE method compared to classical extraction techniques are speed, reduction in solvent use, less handling of sample,and ease of automation. Recovery of analyte is sometimes low, however, and the initial replacement of classical methods for trace enrichment and sample cleanup with an SPE method could involve significant expense in capital cost and analyst training requirements (74).

CHAPTER II

STATEMENT OF THE PROBLEM

Hypertension contributes to morbidity and mortality in modern society, affecting at least 10 to 15% of the adult population of the world's industrialized countries. At least 20 million adults in the United States are afflicted with this disorder (75). Primary aldosteronism, which is a term describing various pathological lesions in the adrenal gland, is estimated to affect 1 to 8 percent of these hypertensives (76). This disorder is associated with increased aldosterone levels in serum and urine of affected patients. Screening of hypertensive individuals using an inexpensive and environmentally safe assay for increased levels of this hormone would be valuable for diagnosis and treatment.

Aldosterone is part of a group of naturally occurring low molecular weight compounds called steroids. There are an immense number of unique steroids and their metabolites of closely related structure and molecular weight present in body fluids. Such closely similar chemical structures have been historically difficult to separate and quantitate. Because aldosterone is present in such minute quantities in urine and serum, detection methods must be sensitive and specific. This hormone and its metabolites can

isomerize to different forms in solution, react with organic solvents, and bond with the free silanol groups of untreated glassware.

Presently, aldosterone is measured in clinical laboratories primarily by radioimmunoassay, and commercial kits are available from various manufacturers. Other methods in use include nonisotpoic enzyme immunoassay, a GC/MS isotope dilution method, and chemiluminescent immunoassay procedures. All of these methods require either a Nuclear Regulatory Commission (NRC) license, expensive reagent, or complex equipment. All radioimmunoassays necessarily involve the hazards and disposal expense of isotopic waste.

The purpose of this research project was to develop a simple, rapid method of solid phase extraction of aldosterone from biological fluids, to be used in conjunction with high performance liquid chromatography and sensitive amperometric detection. Only equipment, reagents, and supplies that would be within the budgets and expertise of most laboratories were used.

CHAPTER III

LITERATURE REVIEW

The determination of aldosterone and its metabolites in serum and urine samples provides a means to evaluate a variety of pathological conditions. At this writing, many clinical laboratories employ a radioimmunoassay (RIA) procedure for aldosterone measurement in biological fluids. The Coat-A-Count ^{R} procedure available in commercial kits typifies this type of assay (77). This procedure is a solid phase radioimmunoassay, in which ^{125}I-labeled aldosterone competes for a fixed time with aldosterone in the patient sample for sites on an aldosterone specific antibody. This antibody is immobilized on the walls of a polypropylene tube. The supernatant is then decanted from the tube, which terminates the competition between the labeled and the endogenous hormone. The antibody-bound fraction of the labeled aldosterone remains in the tube, and is counted when the tube is placed in a gamma counter. The number produced by this count converts by way of a calibration curve to a measure of the aldosterone native to the patient sample. Sensitivity is reported as 15 pg/mL, with very little crossreactivity to related steroids. Serum samples are run without pretreatment. Urine samples, however, require an

hydrolysis step with 3.2 N HCl, a 24-hour incubation in the dark at room temperature, and an ethyl acetate extraction.

The determination of aldosterone by a chemluminescent immunoassay is described, in the literature, as a process in which aminobutylethylisoluminol (ABEI) is used as a label (78). This also is a competitive assay. A mixture of buffered patient serum, antialdosterone antibody, aldosterone-carboxymethyloxime-ABEI, and paramagnetic particles coated with a second antibody is incubated for two hours at room temperature. The magnetic particles and bound material are washed free of interference and reincubated to release the bound label. The samples are placed in a luminometer, chemiluminescence is initiated, and data reduction performed to produce an aldosterone concentration from a standard curve. The minimal concentration of aldosterone that can be distinguished from the zero standard is 11pg/mL, with an excellent reported correlation to RIA methods.

Isotope dilution GC/MS has been proposed as a reference method for the assay of aldosterone in serum (79). In this determination, a derivative of aldosterone with heptafluorobutyric anhydride is obtained in the presence of acetone. The di-hepta-fluorobutyric ester of aldosterone is formed with an abundant molecular ion of m/z 734. An isotopically labeled internal standard is employed. Samples must be purified after derivatization by Sephadex LH-20 chromatography and C_8 reversed-phase HPLC before

GC/MS quantitation. Excellent interlaboratory agreement was achieved with a maximum discrepancy of 0.9% between testing facilities. Testing laboratories reported good between-day precision (relative standard deviations ranging between 1.2 and 2.2%), and a detection limit of 100 pg/mL.

A procedure for the quantitation of the tetrahydroaldosterone (THALD) metabolite in urine has been reported (Yoshitake *et al.*) that uses chemiluminescence and reversed-phase HPLC (80). Enzyme hydrolysis releases THALD from its conjugate. This metabolite is then isolated and concentrated with a Sephadex G-25M column and Bond-Elut® C_{18} cartridges. The isolate is then oxidized to form a glyoxal derivative, which in turn is converted into a chemiluminescent quinoxaline by a reaction with 4,5-diaminophthalhydrazide. The chemiluminescent quinoxaline so formed is separated on a reversed-phase column with isocratic elution, followed by chemiluminescence detection. The detection limit given is 220 pg/mL at a signal-to-noise ratio of 3.

A variation of the above method (Yamaguchi *et al.*) was employed by converting the glyoxal derivatives of THALD and cortisol into fluorescent quinoxalines by reaction with 1,2-diamino-4,5-methylenedioxybenzene. These quinoxalines are then separated on a reversed-phase column and detected fluorimetrically, with detection limits for THALD at 450 pg./mL urine with a signal-to-noise ratio of 3 (81).

The availability of highly specific anti-aldosterone antibodies for use in RIA has shifted the focus of recent aldosterone research methods away from the chromatography separation techniques that were previously necessary for aldosterone detection.

Prior to the development of the more specific RIA assays available today, HPLC was a useful tool in the separation of aldosterone from other steroids when used in tandem with the first available RIA methods. Schonesshofer *et al.* extracted urine with diethyl ether before subjecting samples to HPLC on a polar-coated silica column at 40 ^{o}C with a n-hexane-isopropanol gradient elution (82). UV detection was employed to determine the fraction of mobile phase that eluted aldosterone. This fraction was then collected and analyzed by RIA. Intra-assay variability in terms of the coefficient of variation was found to be 9.4% for aldosterone. Liquid-liquid extraction was also popular for sample clean-up. DeVries *et al.* hydrolysed the acid-labile conjugate of aldosterone in urine and extracted the samples with dichloromethane (83). This group employed several subsequent sample washes and purified with three separate thin layer chromatography (TLC) steps. Following this purification, samples were subjected to normal phase liquid chromatography and UV detection. Detection limits of 5 nmol/24 hr were claimed.

In another liquid-liquid extraction method for sample cleanup, isocratic reversed phase separation of ten polar adrenocortical steroid standards with UV detection alone

was reported by D'Agostino *et al.* (84). They used computer aided optimization of mobile phase to identify the ternary solvent system of methanol-tetrahydrofuran-water (22.4:4.3:73.3) that produced a resolved aldosterone standard peak. This solvent system was used to evaluate aldosterone-containing samples from adrenocortical tumors, where hormone levels were obviously greatly increased. Patient samples were extracted with ethyl acetate and separations were carried out on 150 x 5 mm. i.d. ODS columns at 45 ^{o}C. No attempts at quantitation of aldosterone were reported.

Research by Yoshitake *et al.* detailed the solid phase extraction cleanup procedure used to isolate THALD from hydrolysed spiked urine using gel filtration and C_{18} solid phase cartridges. Both fluorimetry following derivatization, and HPLC were employed to detect sample components. Recoveries of THALD were low, averaging 52.2 $\pm$ 4.0% (85).

Shackleton and Whitney employed C_{18} solid phase cartridges to prepare urine samples for GC analysis. 10 mL portions of both hydrolysed and unhydrolysed urine samples which were spiked with ^{3}H-labeled aldosterone were extracted with SPE cartridges and traditional methods. Recovery of free and conjugated (principally glucuronides) of aldosterone from urine were greater than or equal to 96 percent after C_{18} cartridge solid phase extraction and derivatization for GC analysis (86).

The research team of Brochu *et al.* extracted aldosterone from plasma and cell culture media with C_{18} SPE cartridges prior to a competitive RIA procedure. The extraction was necessary to prevent crossreactivity with cortisol and coticosterone. Recovery estimated using plasma samples spiked with ^{3}H-labeled aldosterone was 97% $\pm$2% ($x \pm SD$) ($n = 10$). Plasma samples assayed without SPE extraction procedure were found to greatly overestimate aldosterone concentration (87).

Risk *et al.* used C_2 strong anion exchange and strong cation exchange SPE columns in sequence to isolate aldosterone glucuronide and tetrahydroaldosterone glucuronide from urine before hydrolysis. After hydrolysis, the released steroids were isolated by LC or HPLC from other polar components and quantitated by RIA. Urine samples were obtained from patients who had been placed on a restricted salt-controlled diet for 4 days, at which time a 1.0 μCi[1,2-^{3}H] aldosterone solution was infused. Recovery of ^{3}H label in unprocessed urine was 100% ($n = 14$), but only 63.2% after the liquid chromatography step (88).

A thorough review of the literature has revealed no reports of solid phase extraction of aldosterone from biological fluids in conjunction with amperometric detection and HPLC.

CHAPTER IV

MATERIALS AND METHODS

A. Steroids

The following steroids were of the purest quality available and were purchased from Sigma Chemical Co., St. Louis, MO ;

1). D-aldosterone (anhydrous) (11-*B*-21-dihydroxy-3,20-dioxopregn-4-en-18-al)

2). 3β-5β-tetrahydoaldosterone (3α,11β, 21-trihydroxy-20-oxo-5β-pregnan-18-al)

3). Bisnorcholenaldehyde (4-pregnene-20-carboxaldehyde-3-one).

All steroids were stored at room temperature in a dessicator, as directed by the supplier.

B. Reagents and Solutions

All reagents and solutions were prepared with double de-ionized, ultra-filtered water. Chemicals and solvents were of analytical-reagent grade, or of the highest purity available. The suppliers of all chemicals are listed below.

Methanol, dichloromethane, acetonitrile, and acetone were obtained from Fisher Scientific, Fairlawn, NJ, and used without further purification. Concentrated nitric acid (71%) was also obtained from this source. Concentrated hydrochloric acid (36.5-38.0 %) was purchased from VWR Scientific, Norwood, Ohio. Sodium hydroxide was obtained from EM Science, Gibbstown, NJ. Trimethylchlorosilane was purchased from Sigma Chemical, St. Louis, MO., and triethylamine was obtained from J.T. Baker, Philipsburgh, NJ. Propylene glycol antifreeze for the 95 ^{o}C bath was manufactured by Safe Brands, Omaha, NE. Sodium phosphate, anhydrous, dibasic, was purchased from General Chemical Division, Morristown, NJ. Copper sulfate pentahydrate was obtained from Mallinckrodt, Paris, KY, and 1,10 phenanthroline was purchase from GFS Chemicals, Columbus, Ohio. These were all used without further purification. Tanks of prepurified helium and nitrogen (Airco, Inc., New York, NY.) were used to degas the mobile phase and dry SPE eluates, respectively.

C. Solid Phase Extraction Cartridges.

Cyclohexyl and silica Mega Bond Elut® cartridges were purchased from Phenomenex, Torrance, CA. Sep-Pak C_{18} cartridges were procured from Waters Associates, Milford, MA. Maxi-Clean® C_{18} cartridges were purchased from Alltech, Deerfield, IL.

D. Equipment.

A Mettler H20 balance (Mettler Instrument, Princeton, NJ) was used to weigh all chemicals.

The HPLC used in this study was an IBM LC/9533; Ternary Gradient Liquid Chromatograph, manufactured by IBM Instruments, Inc., Danbury, CT. The analytical column was an Alltech Adsorbosphere 5μm reversed phase column (4.6 mm i.d., 250 mm). The C_6 and C_{18} guard columns were also acquired from Alltech Associates.

Teflon®, PTFE, and PEEK tubing, and the stainless steel nuts, ferrules, and connectors were obtained from Alltech Associates. Teflon®ferrules, and acetal resin unions and nuts were purchased from Upchurch Scientific, Oak Harbor, WA. Hamilton syringes were used to make all injections on the HPLC, which was equipped with a Rheodyne injection valve.

Analyte detection was accomplished using a BAS Model LC-44 Electrochemical Cell and a BAS Model CV-27 Potentiostat (Bioanalytical Systems, West Lafayette, IN). Chromatograms were produced on an HP 3396 Series II Integrator, manufactured by Hewlett Packard, Wilmington, DE.

Silanized 10 mL test tubes for drying SPE eluates were obtained from Becton Dickinson, Rutherford, NJ. Measurement of the pH of reagents and samples was

done with a Corning Model 340 pH Meter purchased from Corning Instruments, Corning, New York.

E. Copper (II) bis-Phenanthroline (CBP) Synthesis:

Copper (II) bis-Phenanthroline was synthesized by a method used by Hathaway *et. al.* to prepare bis (bipyridyl) copper complexes (89). Watanabe *et. al.* had utilized this method to prepare CBP for the reduction of divalent copper coupled to the oxidation of the aldehyde group of reducing sugars (90). For this synthesis, 0.02 moles of 1,10-phenanthroline was dissolved in 30 mL of HPLC grade methanol. This was added to a warm aqueous solution of 0.01 mole copper (II) sulfate pentahydrate in 30 mL of double deionized water. This mixture was refrigerated at 4 ^{o}C for an overnight period. The pale blue precipitate that was produced was vacuum filtered, washed with cold methanol and dried in a vacuum dessicator for 24 hours. The dry precipitate was then redissolved in 100 mL of warm double deionized water, and refrigerated overnight. Upon retrieval, the reprecipitated CBP was vacuum filtered, and dried again in a vacuum dessicator. The CBP made in this fashion was stored in a clean vial in a dessicator until used for reagent solution preparation. The molecular weight of the CBP was calculated as 591.95 a.m.u.

F. Reagent Solution Preparation:

Initial experiments for detecting aldosterone in standard solutions in this research were done using CBP crystals dissolved in an aqueous solution of 0.05 M Na_2HPO_4, which acted as the supporting electrolyte. No aldosterone standards were detected until the matrix of the supporting electrolyte was changed from water to a 50/50 mix of methanol and water.

Previous studies indicated that a 3mM CBP solution produced a maximum signal to noise ratio in the detection of aldosterone standards (91). In the present research project, it was found that a supporting electrolyte concentration of 0.02 M Na_2HPO_4 resulted in the best signal to noise ratio with the fewest problems in reagent line precipitation. The chemical composition of the precipitate was not determined in this study.

G. Stock Standards

A standard 50 ppm aldosterone solution was prepared by dissolving 5.0 mg of crystalline aldosterone in 100 mL of methanol to which 10 drops of 2% triethylamine had been added. Triethylamine was added to prevent methyl ketal formation between the aldehyde functional group of the steroid and the alcoholic solvent (92,93).

Tetrahydroaldosterone stock solution was prepared by dissolving 1.0 mg of crystalline THALD in 20 mL of methanol, to which 2 drops of 2% triethylamine were added. This was also a 50 ppm stock standard.

A 50 ppm solution of 4-pregnene-20-β-carboxaldehyde-3-one (Bisnorcholenaldehyde) was prepared by dissolving 50 mg of the pure crystalline compound in 100 mL of methanol, to which 10 drops of 2% triethylamine were added. Subsequently, 1 mL of this solution was diluted to 10 mL with pure methanol.

All glassware in contact with standard or working solutions was cleaned with chromic acid, and silanized. The stock standard solutions were stored at 4 ^{o}C when not in use. Working standards were made from stock standards on the day of use.

H. Specimen Collection and Storage.

Urine samples were collected in Teflon® containers as first morning samples from healthy volunteers. Specimens were stored in Teflon® at 4 ^{o}C without preservative and processed within eight hours of collection. Urine was centrifuged at 5000 x g at 4 ^{o}C immediately prior to processing in 50 mL plastic tubes. The supernatant was poured off, with cellular and amorphous debris adhering to the bottom of the tubes. Aliquot volumes were measured in a plastic graduated cylinder, and spiked with aldosterone standard if required.

Hydrolysis of endogenous acid-labile aldosterone glucuronide in the specimens was accomplished by adjusting the pH of the samples to pH 1.0 with concentrated HCl and incubating them in the dark for 22 hours at room temperature (22 ^{o}C). The pH was then readjusted to pH 6.0-7.0 with 6 M and 2 M NaOH. Spiking of samples with aldosterone stock solution was done both before and after readjustment to the normal physiological range. Unhydrolysed freshly collected specimens were also spiked with aldosterone standard.

Approximately 40 mL of blood was collected by venipuncture from a healthy volunteer into clean, silanized tubes. The samples were allowed to clot at room temperature. Specimens were then centrifuged at 2500 rpm to separate serum and cells. The serum was then taken off cells with a plastic pipette and frozen in Teflon® tubes at -20 ^{o}C until it was processed for analysis. Approval for the collection of urine and blood samples from volunteers was obtained from the Human Subjects Research Committee at Youngstown State University.

I. Sample Processing and Analysis

A 6 cc/1 g Mega Bond Bond Elut® cyclohexyl solid phase extraction cartridge was conditioned with 5 mL of methanol, and two 5 mL portions of water. A 20 mL urine sample was pulled through this solid phase by using a vacuum aspirator at a rate of 3-5 mL

per minute. The cartridge was sequentially washed with 5 mL water, 5 mL of 30 % acetone and another 5 mL water. Air was pulled through the cartridge by vacuum for 5 minutes to dry the solid phase. Hexane (4 mL) was pulled through the cartridge, followed by air drying using vacuum for 5 minutes. Elution of the cartridge was done with two 3 mL portions of a 90/10 mixture of CH_2Cl_2 / CH_3CN. The eluates were combined in a 10 mL silanized tube and dried at 22 ^{o}C under nitrogen. After drying, the residue was taken up in 200 μL of methanol, vortexed for 1 minute, and injected through a 20 μL loop onto the HPLC. The mobile phase and reagent flow rates were both set at 1 mL/min, with a post column reactor temperature of 95 ^{o}C, a cooling coil bath temperature of 4-6 ^{o}C, and a voltage of 0.35 V applied to the working electrode of the electrochemical detector.

Detection of the aldosterone in the column effluent depended on the post column oxidation of the aldosterone C18 aldehyde group by the divalent copper of the CBP , which was itself then reduced. The copper (I) in the flow stream was then oxidized in the electrochemical cell at the working electrode, and a current produced and detected. This current was transduced to voltage and sent to the integrator. The plotter sensitivity of the integrator at an attenuation of 1 was 2 mv full scale. For detection of aldosterone in samples and standards, the integrator attenuation was set at 3. A diagram of the experimental set-up is shown in Figure 14.

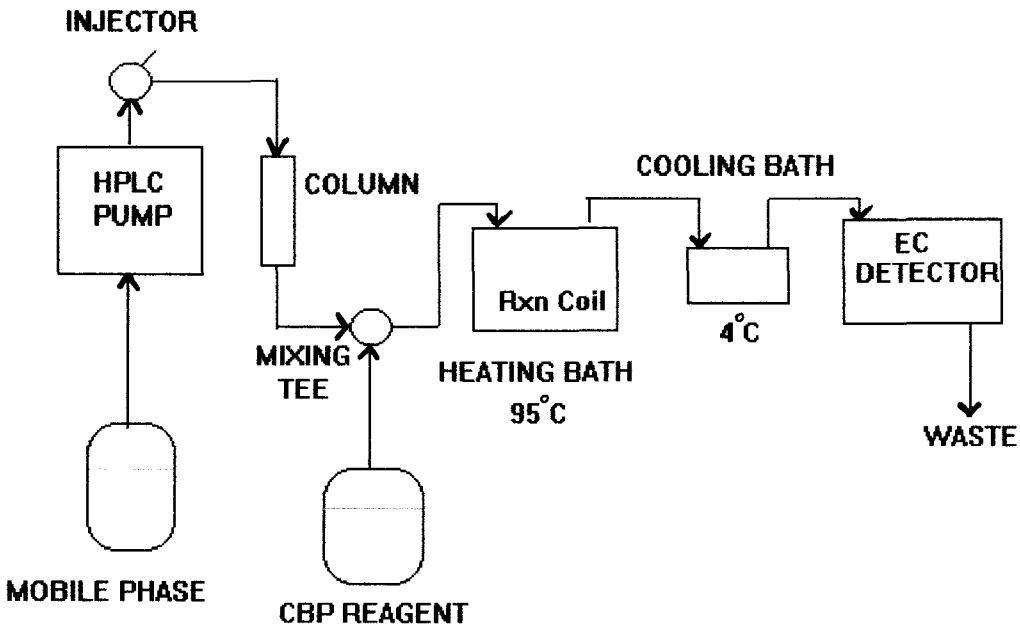

Figure 14. Diagram of Experimental Apparatus

The mobile phase was filtered at the inlet line with a stainless steel sinker frit of 2 μm porosity to keep particulate contaminants out of the HPLC pump, and to anchor the line to the bottom of the solvent reservoir. The mobile phase was degassed and overlaid with helium to prevent pump cavitation. An inlet filter was installed immediately after the injection port to remove particulate matter from the sample. This filter was also followed by a guard column placed before the analytical column to protect this column from sample

impurities and prolong its lifetime. Poly(tetrafluoroethylene) (PTFE) tubing of 0.5 mm i.d. was used to connect the column and reagent reservoir at the mixing tee. Post-column reactor and cooling coils of PTFE and PEEK were knotted and coiled in several different configurations during this project to achieve the maximum reagent and column effluent mixing, with a minimum of precipitate formation in the tubing.

The actual detection of aldosterone in spiked biological samples was accomplished with a PCR coil of 10 ft of 0.5 mm i.d. PEEK tubing tied in alternating overhand knots, connected in tandem with a second 10 ft of 0.5 mm i.d. tubing looped in a loose coil of 2 cm diameter. The post-column reactor bath contained propylene glycol heated to 95 ^{o}C. The total volume of the post-column reactor tubing was 1.2 mL. The cooling bath was fashioned from a 500 mL insulated thermos bottle filled with crushed ice and water, with temperature maintained at 4-6 ^{o}C. The cooling coil was constructed of 240 cm of 0.8 mm i.d. PTFE tubing, with a volume of 1.2 mL.

A backpressure regulator placed between the cooling coil and the detector was adjusted with enough pressure to keep the methanol fraction of the mixed reagent and column effluent from boiling in the heated post column reactor.

J. Methods

The amperometric detection method used was a modification of work done by Watanabe and Inoue in 1983 with reducing sugars (94). Reducing sugars and aldosterone possess an oxidizable aldehyde group. The redox reaction of copper bis (phenanthroline) (CBP) was coupled to the ability of the aldehyde group of the sugar to reduce the divalent CBP to a monovalent complex. This reaction took place in alkaline solution at high temperature in a post column reactor. Amperometric detection occurred as the CBP was reoxidized at a working glassy carbon electrode. The essential scheme of detection is shown in Figure 15.

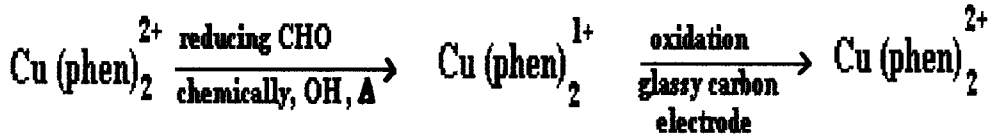

Figure 15. Redox Reaction of CBP for Aldehyde Detection.

Detection limits for glucose utilizing this method were 0.2 ng for glucose, making this method extremely sensitive. The applied potential used was low, at +75mv, presenting

a barrier against organic interferences, which have higher oxidation potentials. Few interferences allowed the authors to claim high selectivity also. Applications for serum and urine were given.

Previous research by Bose (95) demonstrated the application of this method in detection of aldosterone standards, although attempts to detect this hormone in extracted spiked urine samples were unsuccessful.

The urine solid phase extraction method used in this research was a modification of that developed by Schenck *et al.* (96). This group loaded 25 mL urine samples on cyclohexyl SPE cartridges and washed with water, 25% acetone, and hexane. Aldosterone was eluted with 90:10 mix of methylene chloride/acetonitrile. The eluate was then pulled through a silica SPE cartridge, washed with methylene chloride and acetonitrile, and eluted with methanol. The methanol eluate was taken to dryness and the residue was redissolved in methylene chloride. The sample was then injected on a silica HPLC column. A fraction corresponding to the retention time of aldosterone was collected, taken to dryness, redissolved in 25% acetonitrile/water containing prednisone as an internal standard, and injected on a C18 column. A UV detection system at 240 nm was employed. Recovery of aldosterone with this method was given at 74.5% $\pm$ 10%.

The intent of this research project was to simplify the above procedure, and to eliminate the use of multiple HPLC columns for the detection and quantification of aldosterone.

Serum samples were processed and extracted with variations of a method published by E. Merck (97). In this method, a 1 mL plasma sample was loaded on a C_{18} SPE cartridge, washed with water, and eluted with acetonitrile. The eluate was evaporated and the residue was reconstituted in 1 mL of mobile phase. This sample was then injected on a C_{18} reversed phase HPLC column with a mobile phase of acetonitrile/0.5 mM sodium acetate buffer. UV detection at 235 nm was also used to quantitate aldosterone.

CHAPTER V

RESULTS AND DISCUSSION

A. Optimization of Applied Voltage.

Prior to solid phase extraction studies of aldosterone in biological samples, an optimization of the system used to detect aldosterone standards was necessary. Previous studies with CBP in a supporting electrolyte of Na_2HPO_4 indicated that detection of aldosterone standards was possible at an applied voltage of 0.22 V (98). Because a different EC detector was employed in this research, a hydrodynamic voltammogram study was undertaken to determine the voltage at which the best signal to noise ratio could be obtained for aldosterone detection.

Figure 16 depicts a graph of the relationship between the applied voltage to the working electrode and the detector response. This graph displays the change in magnitude of the signal height, the noise height, and the value of the signal to noise ratio as the applied voltage was increased from 0.00 to 0.60 V.

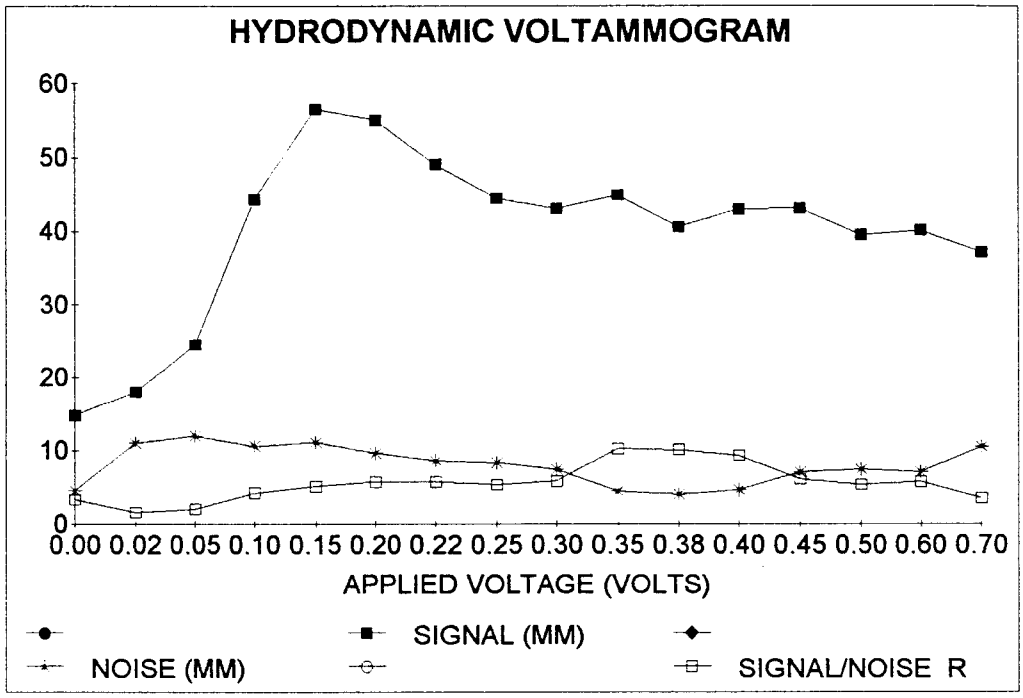

Figure 16. The Dependence of Detector Response on Applied Potential
Sample; Aldosterone Working Standard (200 ng)
Column: Alltech Adsorbosphere C_{18}, 5 μm, 4.6 mm i.d.
Injection Loop: 20 μL
Mobile Phase: Methanol/Water (55/45)
Reagent: 3 mM CBP in 0.05 M Na_2HPO_4
Reagent and Mobile Phase Flow Rates: 1 mL/min
Integrator Chart Speed: 1 cm/min
Integrator Attenuation: 3

A chromatogram illustrating the measurement of signal to noise is depicted in Figure 17. The signal produced by aldosterone at a retention time of 8.9 minutes was measured from the apex of the peak to the midpoint of the parallel lines enclosing the measured noise on the baseline. The largest detector signal for the 200 ng aldosterone injections was produced at an applied voltage of 0.15 volts. However, the largest signal to noise ratio was obtained at 0.35 volts, and this voltage was selected for all further studies in this project.

B. Supporting Electrolyte

A study was also undertaken to determine the optimal concentration of supporting electrolyte in the CBP reagent. The 3 mM CBP solutions of varying concentrations of Na_2HPO_4 were put on the system with a constant voltage of 0.35 V applied to the detector.

Triplicate 200 ng injections of aldosterone working standard were employed to determine which supporting electrolyte concentration produced the best signal to noise ratio at the detector. The results of this series of experiments is given in Table 2, with average values shown for the injections.

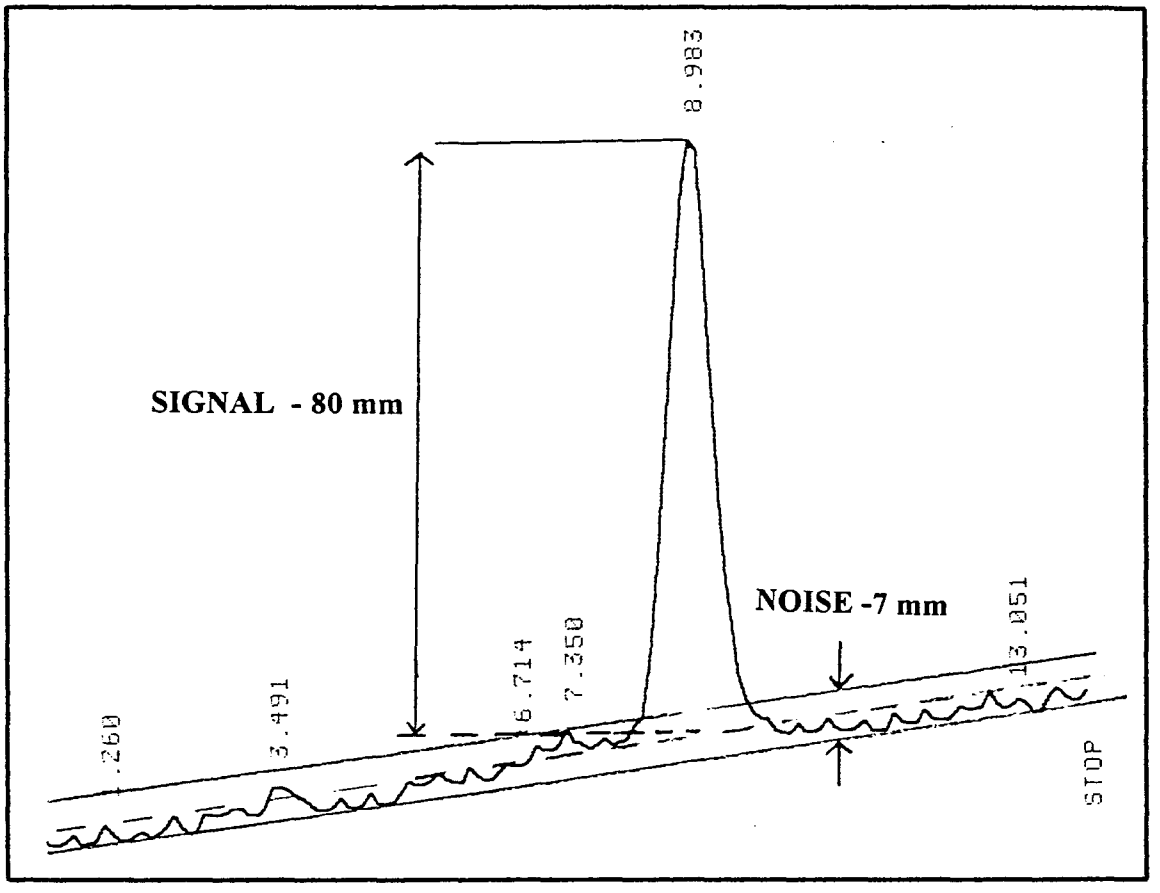

Figure 17. Measurement of Signal and Noise Height
Sample: 200 ng Aldosterone Standard
Reagent: 3 mM CBP in 0.02 M Na_2HPO_4
Mobile Phase: Methanol/Water (55/45)
Applied Voltage: 0.35 V

NA_2HPO_4 MOLARITY	SIGNAL (mm)	NOISE (mm)	SIGNAL/NOISE
0.01	76.9	6.8	11.3
0.02	99.0	7.0	14.1
0.03	104.9	9.7	10.8
0.04	115.9	8.0	14.5
0.05	122.4	7.5	16.3

Table 2. Signal and Noise Heights, and Signal to Noise Ratio for Changing Electrolyte Strength of CBP

The CBP reagent with a supporting electrolyte concentration of 0.05 M produced the highest signal to noise ratio for 200 ng aldosterone injections. However, buffer precipitation in the lines of the post-column reaction tubing blocked the reactor in every trial within one hour of pumping reagent of this Na_2HPO_4 concentration. Because an improved signal to noise ratio was observed as supporting electrolyte was increased from 0.01M to 0.02M sodium phosphate dibasic, the 0.02 M concentration was selected for further experiments as a compromise between detector sensitivity and line precipitation.

C. A Standard Curve of Aldosterone.

After the voltage and supporting electrolyte parameters for the aldosterone detection system were chosen, a standard plot of aldosterone was constructed. Injections of 20, 40, 60, 80, 100, 160, 200, and 334 ng were made in triplicate using the appropriate working standards and a 20 μL injection loop. All system parameters were unchanged from those used in the construction of the hydrodynamic voltammogram, with the exception of a supporting electrolyte strength of 0.02 M Na_2HPO_4. The attenuation of the integrator was set at 3. The results of this experiment are tabulated in Table 3 and display the averages of the signal peak area for each set of working standard injections.

Peak area was calculated by multiplying peak height times peak width at half-height, and areas are in square millimeters. This data is graphically presented in Figure 18. The standard curve appears linear at least up to 334 ng. This system had a lower detection limit of 60 ng aldosterone, with a signal to noise ratio of 2. A representative chromatogram is presented in Figure 19, for a 200 ng injection.

Aldosterone Injected (ng)	Peak Area
0	0.0
20	47.5
40	130.2
60	136.6
80	223.3
100	271.0
160	410.3
200	475.0
334	693.0

Table 3: Peak Areas of Aldosterone Standards
Column: Alltech Adsorbosphere C_{18}, 5 μm, 4.6 mm i.d.
Injection Loop: 20 μL
Mobile Phase: Methanol/Water (55/45)
Reagent: 3 mM CBP in 0.02 M Na_2HPO_4
Reagent and Mobile Phase Flow Rates: 1 mL/min
Integrator Chart Speed: 1 cm/min
Integrator Attenuation: 3

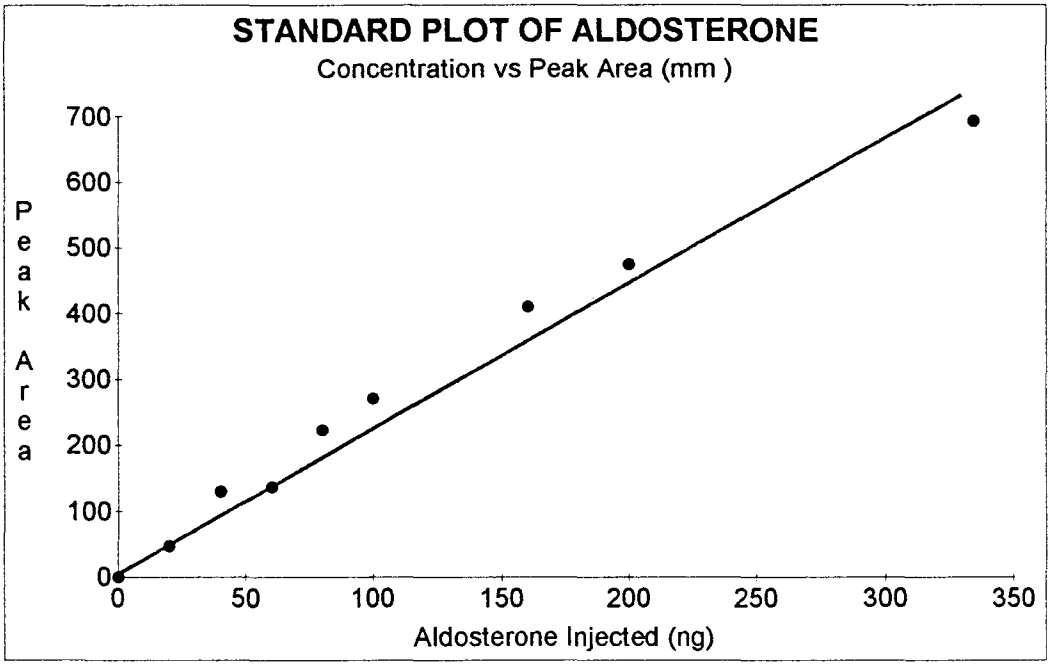

Figure 18. Aldosterone Standard Curve
Column: Alltech Adsorbosphere C_{18}, 5 μm, 4.6 mm i.d.
Injection Loop: 20 μL
Mobile Phase: Methanol/Water (55/45)
Reagent: 3 mM CBP in 0.02 M Na_2HPO_4
Reagent and Mobile Phase Flow Rates: 1 mL/min
Integrator Chart Speed: 1 cm/min
Integrator Attenuation: 3

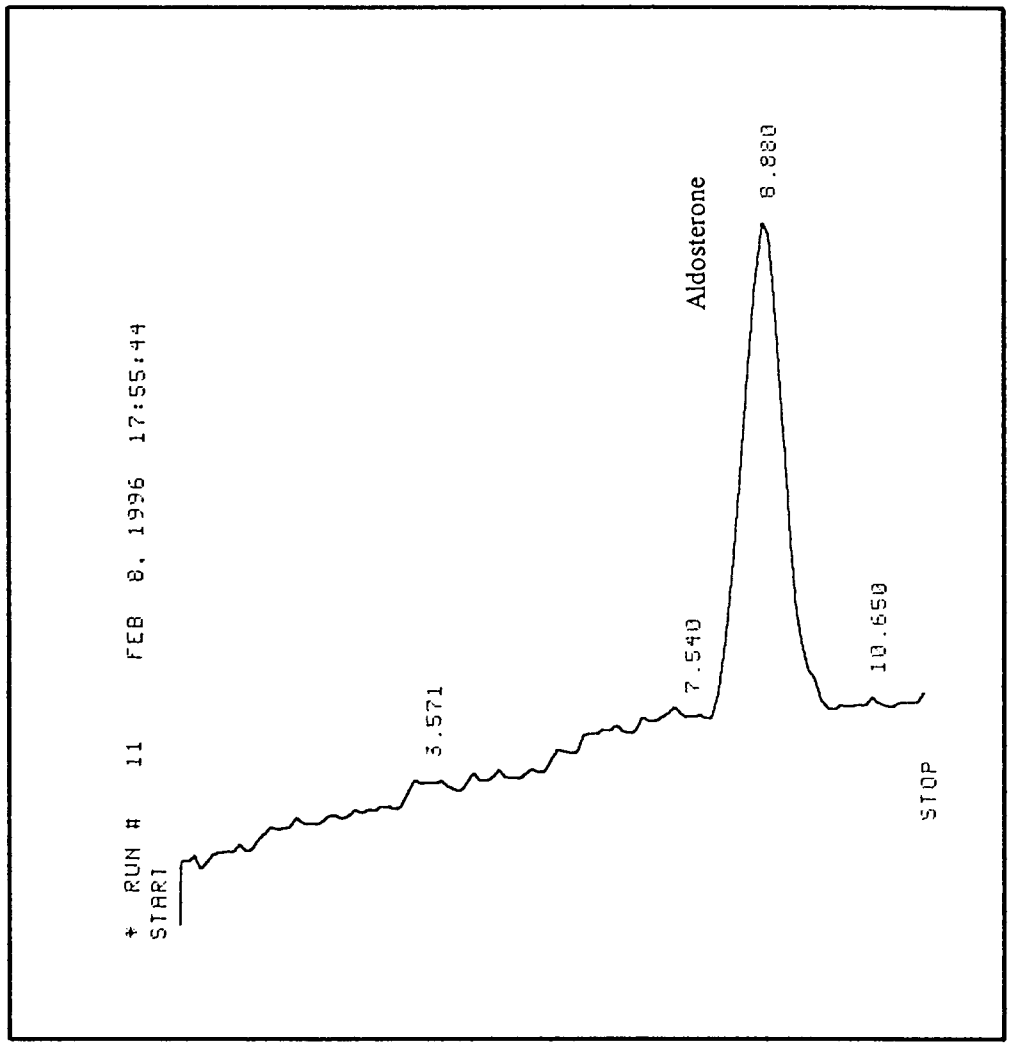

Figure 19. Chromatogram of 200 ng Aldosterone Standard
Mobile Phase: Methanol/Water (55/45)
Reagent: 3 mM CBP in 0.02 M Na_2HPO_4
Reagent and Mobile Phase Flow Rates: 1 mL/min
Applied Voltage: 0.35 V

D. Investigation of an Internal Standard

As the standard curve data was being collected, several 200 ng injections of 4-pregnene-20-carboxaldehyde-3-one (bisnorcholenaldehyde) were made. This compound has the structure shown in Figure 20. Because the molecule possesses a C21 aldehyde functional group, as well as a steroid nucleus that is quite similar to that of aldosterone, it was felt that the bisnorcholenaldehyde might be usable as an internal standard for aldosterone quantitation. However, when this steroid was injected at the operating conditions used to prepare the standard curve, no peak was apparent on the chromatogram for this substance between the injection time, and twenty-three minutes. An appropriate internal standard in chromatographic studies is a compound that elutes near, but is resolved from, the peak or peaks of interest (99). The average retention time of the injected aldosterone standards was 8.8 minutes. Therefore, it appeared that this ring compound was not suitable for further use in quantitation. It may be possible that bisnorcholenaldehyde is retained for a much longer period of time on the column because it has fewer polar functional groups than aldosterone.

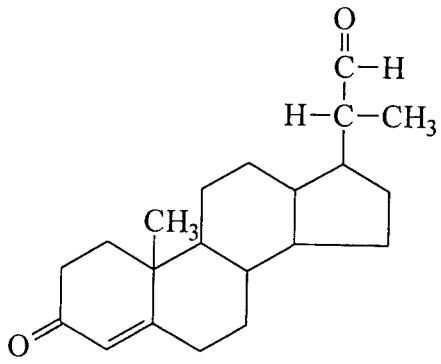

Figure 20. Bisnorcholenaldehyde.

E. Conformation of Post Column Reactor Tubing.

A study was undertaken to determine the effect on the aldosterone standard signal of the coil conformation of the post-column reactor tubing, under identical operating conditions. When the first 10 feet of 0.05 mm i.d. post-column reactor tubing was knotted in alternating overhand knots, a signal for a 200 ng aldosterone standard measured 99.8 mm in height with a signal to noise ratio of 13. The signal produced when this length of tubing was replaced with 10 feet of 0.05 mm i.d. unknotted tubing was only 65.5 mm in height with a signal to noise ratio of 9.2. However, the precipitate formed in the tubing of the post-column reactor with each day's use was extremely difficult to remove from

knotted tubing, and it was decided to continue studies with unknotted tubing in the 95 ^{o}C bath.

As standard curve data was being collected for a plot, the within-day retention time precision was also determined. Using the operating conditions as outlined previously, the mean retention time of the aldosterone peak was 8.8 minutes + 0.1 (n = 20).

Because the compound tetrahydroaldosterone is the most abundant aldosterone metabolite found in urine, a 200 ng injection of a THALD standard was made at the above operating conditions to check for possible interferences with the aldosterone standard. Figure 21 is a chromatogram of one of these injections. The average retention time for THALD was 11.9 minutes, and it was therefore not expected to overlap with that of aldosterone, should it also be present in appreciable quantities in biological samples.

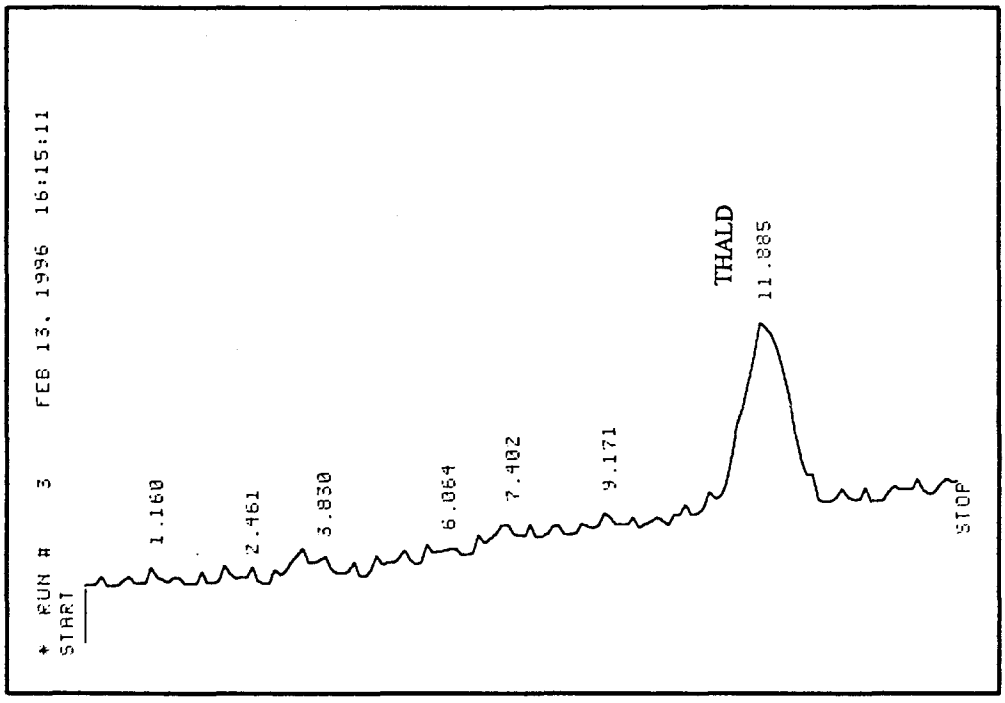

Figure 21: Chromatogram of 200 ng THALD Standard
Mobile Phase: Methanol/Water (55/45)
Reagent: 3 mM CBP in 0.02 M Na_2HPO_4
Reagent and Mobile Phase Flow Rates: 1 mL/min
Applied Voltage: 0.35 V

F. Solid Phase Extraction of Urine

Unhydrolysed urine samples were processed to test the mechanics and usefulness of the cyclohexyl cartridge SPE procedure outlined in the experimental protocol of Chapter IV. The 20 mL aliquots of urine were spiked with 100 μL of the 50 μg/mL stock aldosterone standard. This represented a 250 ng/mL concentration, or approximately 10 times the normal physiological range for this hormone in human subjects. If all aldosterone that was spiked into the sample had been recovered from the solid phase extraction, the residue remaining after drying the final eluate under nitrogen would have contained 5 μg of the steroid. After this residue was taken up in 100 μL of 0.1% triethylamine in methanol, a 20 μL injector loop would have injected 1,000 ng to the column. Presented in Figure 22 is a chromatogram of the 200 ng aldosterone standard injected immediately before the extracted, reconstituted urine sample. The peak corresponding to aldosterone is noted at 9.487 minutes. A chromatogram of the urine injection is shown in Figure 23. The peak corresponding to the retention time of aldosterone was noted as such at 9.442 minutes on the chromatogram. An injection of 200 ng of aldosterone standard immediately following the injection of urine sample produced a peak at 9.379 minutes. Other peaks on the chromatogram of the extracted urine sample are unidentified, although the peak at 2.995

minutes is probably the solvent front of the sample. The standard plot of aldosterone was used to estimate the recovery of the extraction process. The area of the peak corresponding to the retention time of the aldosterone in the sample is equivalent to an injection of 162 ng of pure standard. This represents only 16.2 % of the 1000 ng that would have been detected if all the aldosterone spiked into the sample had been recovered. The air drying of the cartridges by vacuum may have caused the air oxidation of the aldehyde functional group that is needed for aldosterone detection with this experimental procedure. The vigorous vortexing that was necessary to reconstitute the dry residue of the extract for injection on the HPLC could also have contributed to air oxidation of the hormone. Losses of analyte could have occurred if the aldosterone was washed off the cyclohexyl cartridge with the 30 % acetone wash, or was retained on the cartridge during the elution with the 90/10 mixture of CH_2CL_2/ CH_3CN. It is also possible that the some of the hormone was never fully partitioned into the solid phase as the sample was pulled through under vacuum.

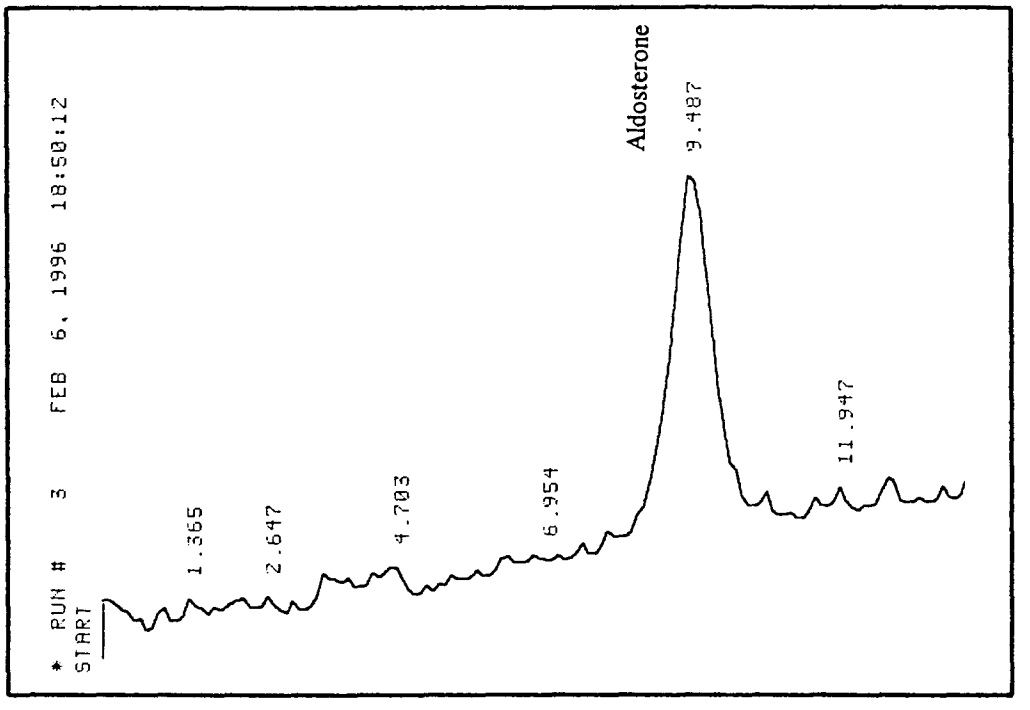

Figure 22. Chromatogram of 200 ng Aldosterone Standard
Mobile Phase: Methanol/Water (55/45)
Reagent: 3 mM CBP in 0.02 M Na_2HPO_4
Reagent and Mobile Phase Flow Rates: 1 mL/min
Applied Voltage: 0.35 V

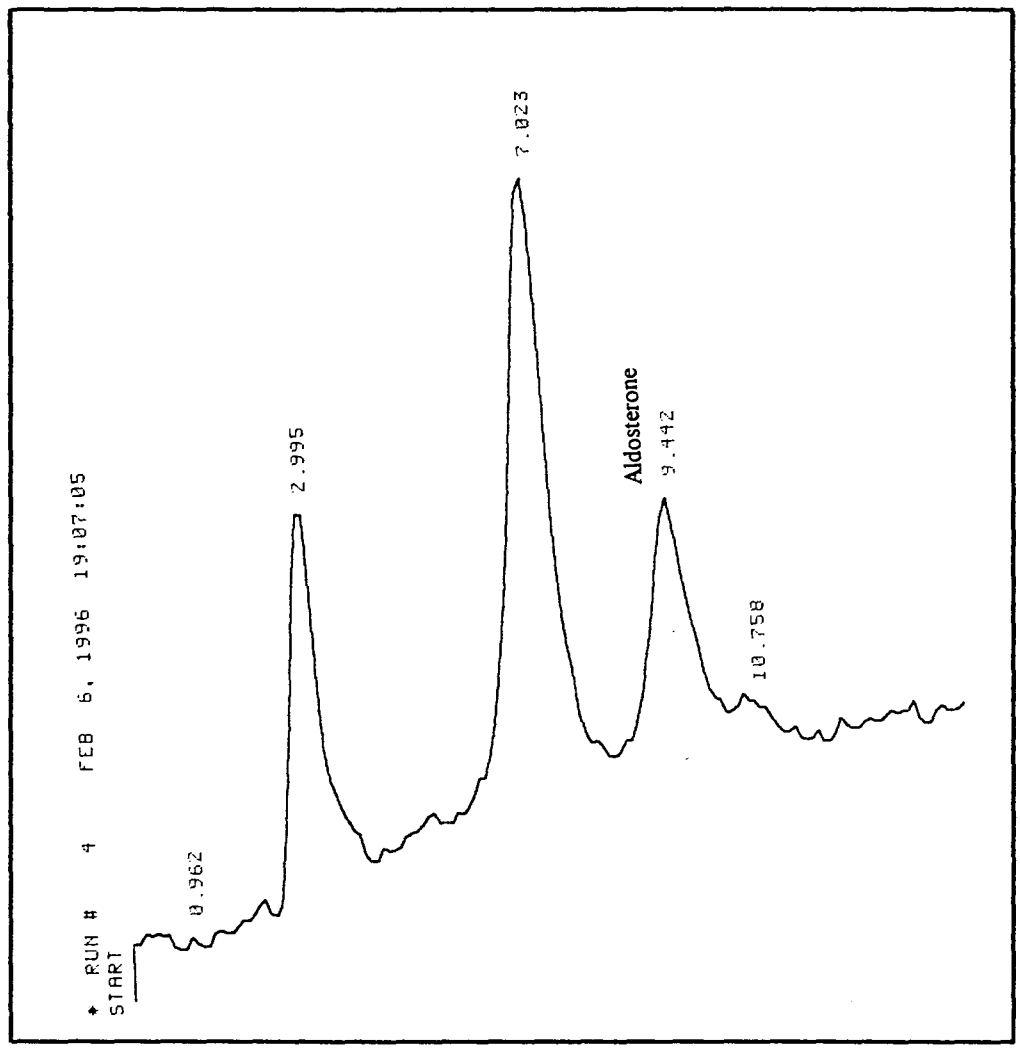

Figure 23. Chromatogram of 20 mL Unhydrolysed, Spiked Urine After Cyclohexyl SPE
Mobile Phase: Methanol/Water (55/45)
Reagent: 3 mM CBP in 0.02 M Na_2HPO_4
Reagent and Mobile Phase Flow Rates: 1 mL/min
Applied Voltage: 0.35 V

The large unidentified peak present on the chromatogram at 7.023 minutes of this unhydrolysed 20 mL urine specimen was well resolved from the aldosterone peak. It was expected, however, that as larger volumes of sample were extracted, this peak could interfere with aldosterone detection. In order to remove this more polar substance from the sample, a silica 6cc/1g SPE cartridge was used in tandem with the cyclohexyl 6 cc/1 g cartridge to extract 20 mL of unhydrolysed urine sample that also had been spiked with 5 μg of aldosterone.

The silica cartridge was first conditioned with 6 mL of methylene chloride. The dry eluate from the cyclohexyl SPE extraction was reconstituted in 6 mL methylene chloride. This reconstituted eluate was then pulled through the silica cartridge using vacuum. The cartridge was then washed with 3 mL of methylene chloride, followed by two 3 mL washes of acetonitrile. The silica was eluted with three 2 mL aliquots of methanol, and the eluate was dried at room temperature under nitrogen. After reconstitution with 200 μL of 0.1% triethylamine, the sample was injected on the HPLC. This chromatogram is shown in Figure 24. A large solvent front was apparent at 3.198 minutes, as well as a small, sharp contaminant peak at 7.656 minutes. Although the possible interfering peak was greatly diminished, the peak corresponding to the retention time of aldosterone was missing.

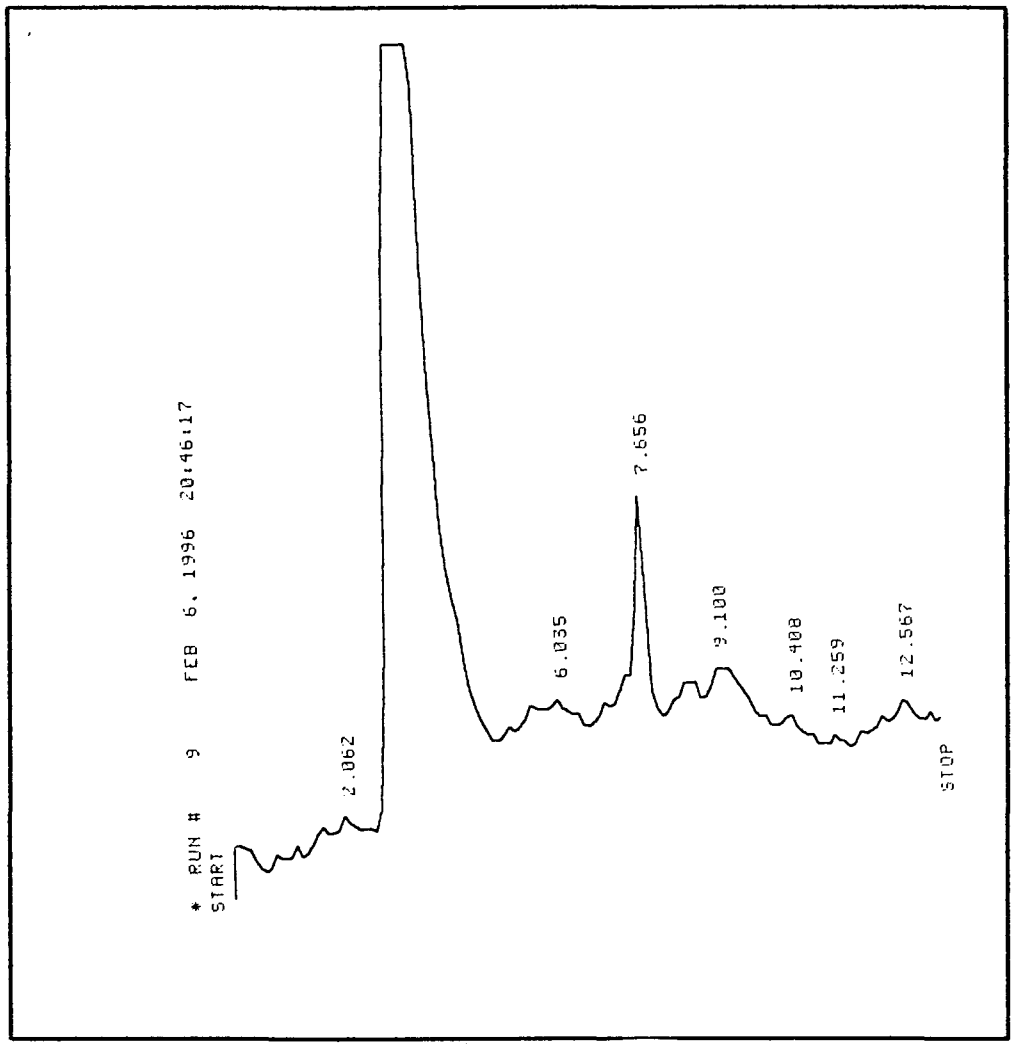

Figure 24. Chromatogram of 20 mL Spiked, Unhydrolysed Urine After Cyclohexyl and Silica SPE
Mobile Phase: Methanol/Water (55/45)
Reagent: 3 mM CBP in 0.02 M Na_2HPO_4
Reagent and Mobile Phase Flow Rates: 1 mL/min
Applied Voltage: 0.35 V

G. Estimation of Loss of Analyte During Hydrolysis

The 6 cc/1 g cyclohexyl cartridge extraction procedure produced encouraging results in the extraction of aldosterone from spiked urine samples, even though recoveries were low. Although the addition of a silica cartridge to the SPE strategy in the above described experiment cleaned up the interference from the sample, the analyte was not recovered. It was therefore decided to investigate the solid phase extraction of hydrolysed urine samples using only cyclohexyl cartridges if samples of less than or equal to 20 mL were processed.

A recovery study of urine spiked with aldosterone standard, both before and after acid hydrolysis, was next attempted. A 100 mL morning urine sample from a healthy volunteer was centrifuged at 5000 x g, and decanted. A 20 mL aliquot of this sample was spiked with 3 μg of aldosterone. This portion of the sample, and three unspiked 20 mL aliquots, were adjusted to pH 1.0 with 6N HCl. These were placed in a dark cupboard for 22 hours at room temperature (22 ^{o}C). When retrieved, one of the unspiked aliquots was then spiked with 3 μg aldosterone. All urine aliquots were then adjusted to pH 6.0 with 6 N and 3 N sodium hydroxide.

The cyclohexyl cartridges were conditioned with methanol and water as previously described, and samples were pulled through using vacuum at a flow rate of 2 mL/min. Wash and elution solvents were pulled through at rate of 1 mL/min. The dried eluates were reconstituted with 200 μL of 0.1% triethylamine, and then injected on the HPLC. The system parameters were as follows:

Column: Alltech Adsorbosphere C_{18}, 5μm, 250 mm, 4.6 mm i.d.
Mobile phase: Methanol / Water (55/45)
Reagent: 3 mM CBP in 0.02 M Na_2HPO_4
Mobile phase and reagent flow rates: 1 mL/min.
PCR volume: 0.6 mL.
Applied voltage: 0.35 V.
Chart speed: 1 cm/min Attenuation: 3.

A 200 ng aldosterone standard was injected before each extracted urine sample. An average retention time of 8.9 + 0.1 minutes for six injections of standards was noted. An injection of the extract of the urine aliquot spiked with 3μg aldosterone prior to hydrolysis yielded a relatively clean chromatogram. This chromatogram is presented in Figure 25. A peak corresponding to the retention time of the aldosterone standard was observed at 8.841 minutes, although a shifting

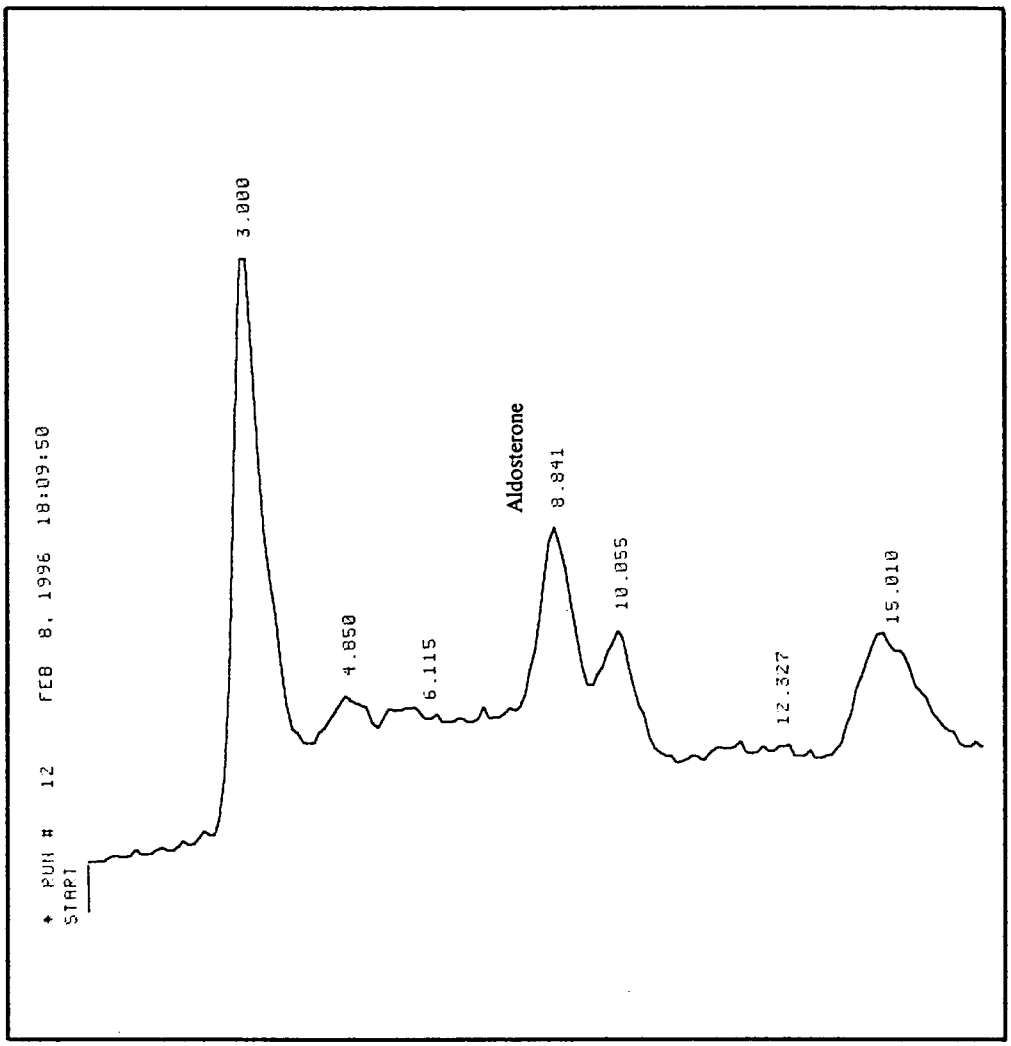

Figure 25. Chromatogram of 20 mL Urine
Spiked With 3 μg Aldosterone Prehydrolysis
SPE: Cyclohexyl 1 g/6 cc
Mobile Phase: Methanol/Water (55/45)
Reagent: 3 mM CBP in 0.02 M Na_2HPO_4
Reagent and Mobile Phase Flow Rates: 1 mL/min
Applied Voltage: 0.35 V

baseline and much noise were present. The height of the peak was 32.5 mm, yielding a peak area of 195 mm^2 at a signal to noise ratio of 7, with an integrator attenuation of 3.

The urine sample spiked with 3 μg aldosterone post-hydrolysis also yielded a peak corresponding to the retention time of aldosterone. In this case, the peak was twice as high as that of the sample spiked pre-hydrolysis with an equal amount of standard. This peak, shown on Figure 26, at a retention time of 8.817 minutes, had a signal height of 66.0 mm, with an area of 396 mm^2. It appeared that losses of 50% of the analyte occurred when urine samples were subjected to acid hydrolysis. Plugging of the post-column reactor tubing prevented replication of this experiment with extracts prepared from the original sample.

An unspiked urine sample extracted in a manner identical to the two previously processed samples revealed no peak at the retention time of aldosterone standards that was distinguishable from baseline noise, although several broad, wide peaks were observed on the chromatogram. This chromatogram is presented in Figure 27. It is possible that the strong acid conditions necessary to hydrolyze the aldosterone glucuronide, could have affected the aldehyde functional group of the endogenous aldosterone as the hormone-glucuronide linkage was split. It is more probable,however, that detection limits were not low enough to detect this analyte in only 20 mL of sample.

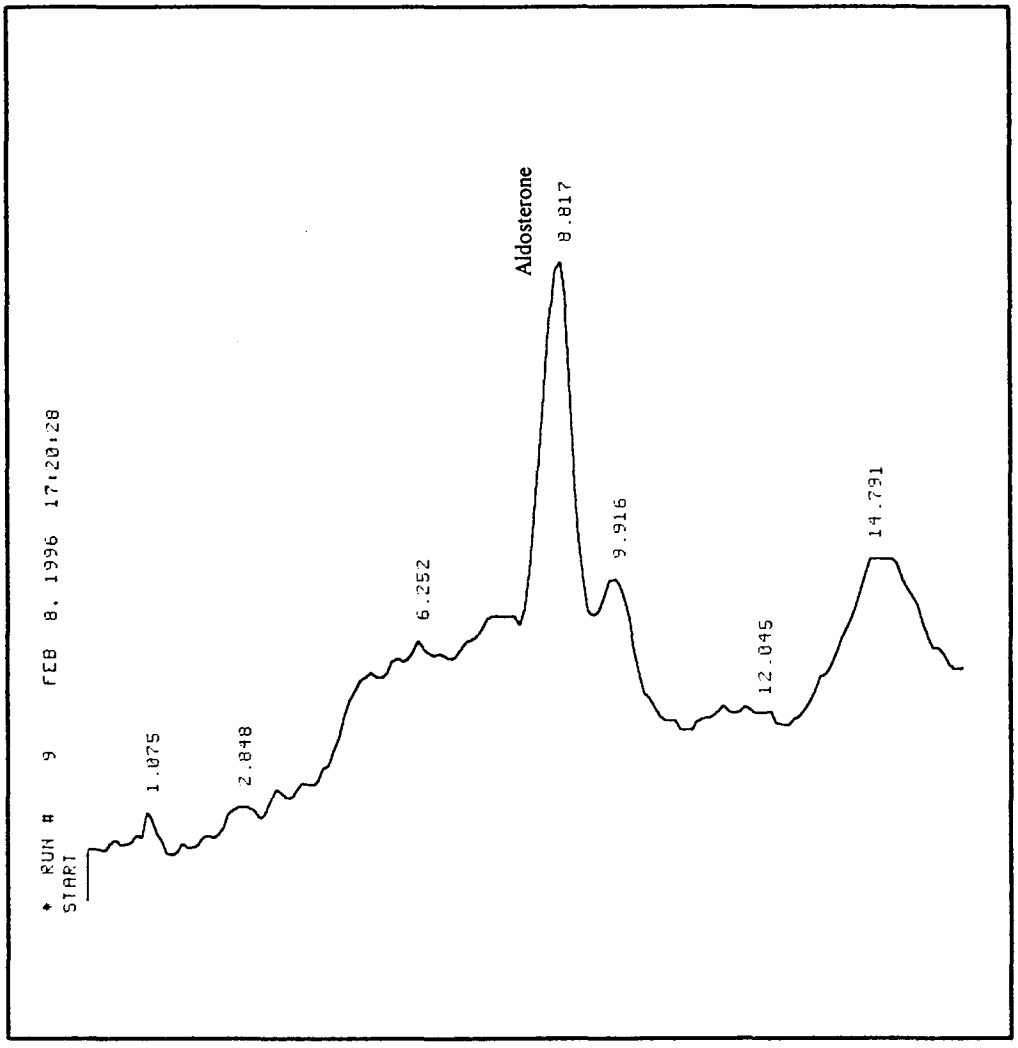

Figure 26. Chromatogram of 20 mL Urine Spiked
With 3 mg Aldosterone Posthydrolysis
SPE: Cyclohexyl 1 g/ 6 cc
Mobile Phase: Methanol/Water (55/45)
Reagent: 3 mM CBP in 0.02 M Na_2HPO_4
Reagent and Mobile Phase Flow Rates: 1 mL/min
Applied Voltage: 0.35 V

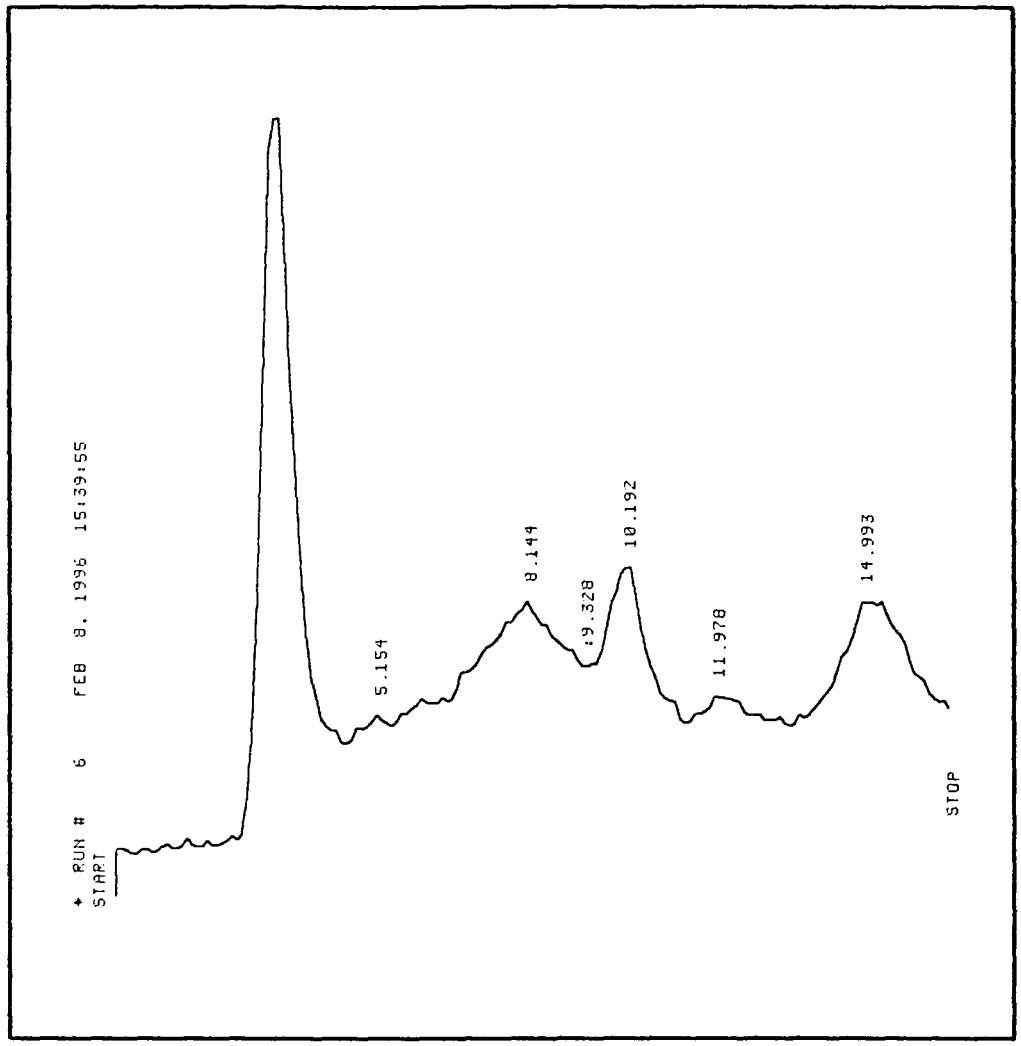

Figure 27. Chromatogram of 20 mL Unspiked, Hydrolysed Urine
SPE: Cyclohexyl 1 g/6 cc
Mobile Phase: Methanol/Water (55/45)
Reagent: 3 mM CBP in 0.02 M Na_2HPO_4
Reagent and Mobile Phase Flow Rates: 1 mL/min
Applied Voltage: 0.35 V

To investigate the effect of increasing the sample volume in order detect aldosterone in the sample, two 100 mL aliquots of a fresh, centrifuged urine sample were hydrolysed as previously described, and readjusted to pH 7.0. These samples were extracted with both the cyclohexyl and silica SPE cartridges in tandem. One of the dried eluates was reconstituted for injection with 100 μL of 0.1 % triethylamine in methanol, while the other eluate was taken up in 200 μL of the same solvent. All column, mobile phase, detector, and reagent parameters were identical to the previous experiments in which 20 mL samples were analyzed.

The residue of the extracted sample that was reconstituted in 100 μL of solvent was centrifuged to separate liquid from particulate matter, and injected on the HPLC. The resulting chromatogram is presented in Figure 28. Broad, off-scale peaks extending from the solvent front, completely obscured that portion of the chromatogram at the expected retention time of aldosterone. It appeared that the reconstitution volume of the dried residue may have been too small.

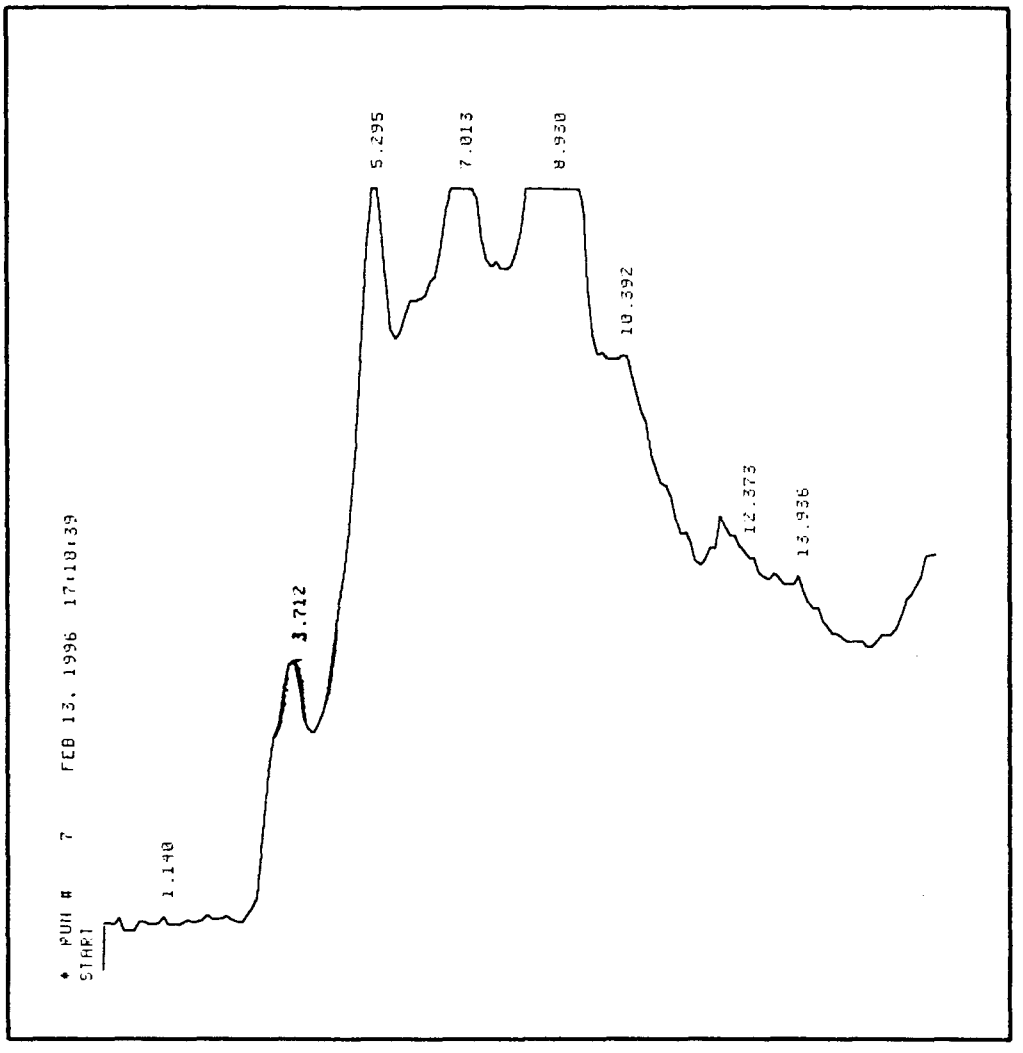

Figure 28. Chromatogram of 100 mL Unspiked , Hydrolysed Urine.
SPE: Cyclohexyl and Silica
Reconstitution Volume: 100 µL
Mobile Phase: Methanol/Water (55/45)
Reagent: 3 mM CBP in 0.02 M Na_2HPO_4
Reagent and Mobile Phase Flow Rates: 1 mL/min
Applied Voltage: 0.35 V

Next, the extracted sample that had been reconstituted in 200 μL of solvent was injected on the system. The chromatogram that was recorded is shown in Figure 29.

A large, off-scale peak at 8.882 minutes was noted. However, the mean retention time of the standard aldosterone injections made immediately before and after these sample injections was 9.2 + 0.1 min (n = 3). Without collecting the fraction of column effluent between 8 and 10 minutes after injection of this sample ,and subjecting the effluent to further analysis, the peak at 8.882 minutes can not be identified with certainty as aldosterone.

H. Alteration of Mobile Phase Composition.

The possibility of resolving the aldosterone peak from interferences near the solvent front was also investigated. Because a change in mobile phase composition is an inexpensive means of altering the retention time of an analyte, the 55/45 methanol/water mobile phase was replaced with solvent mixes containing larger percentages of water. The steroid nucleus is hydrophobic, and a larger percentage of water in the mobile phase of reversed phase systems was expected to increase the retention time of aldosterone, and separate the analyte peak from the more polar interferences.

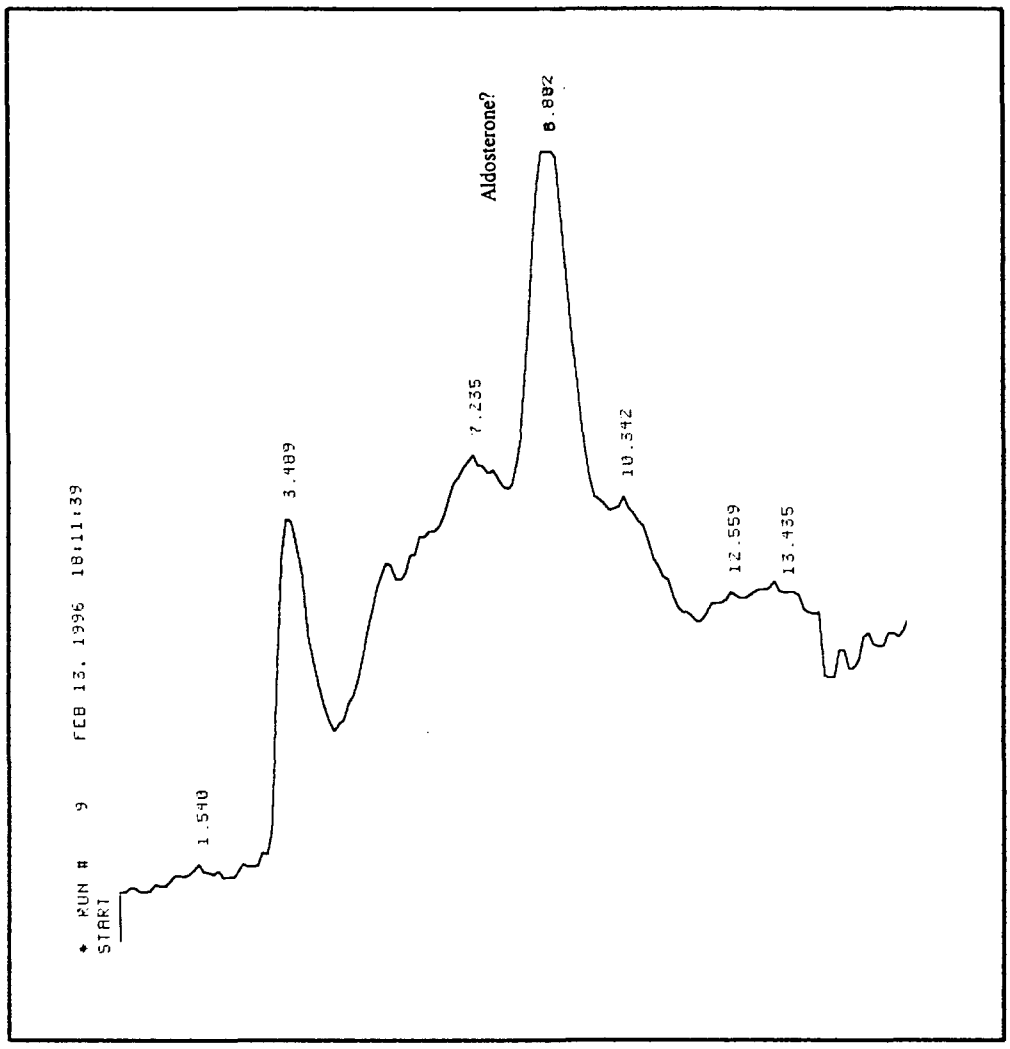

Figure 29. Chromatogram of 100 mL Unspiked, Hydrolysed Urine
SPE: Cyclohexyl and Silica
Reconstitution Volume: 200 µL
Mobile Phase: Methanol/Water (55/45)
Reagent: 3 mM CBP in 0.02 M Na_2HPO_4
Reagent and Mobile Phase Flow Rates: 1 mL/min
Applied Voltage: 0.35 V

The results of this change in mobile phase composition are presented in Table 4, and shown graphically in Figure 30.

Methanol in Mobile Phase (%)	Retention Time (min)	Peak Height (mm)	Peak Width (mm)
55.0	9.0	75.0	7.0
50.0	13.5	63.5	11.5
40.0	33.9	46.5	20.0

Table 4: Effects of Change in Mobile Phase Composition on Aldosterone Chromatography
Column: Alltech Adsorbosphere C_{18}, 5 μm, 4.6 mm i.d.
Injection Loop: 20 μL
Mobile Phase: Methanol/Water
Reagent: 3 mM CBP in 0.02 M Na_2HPO_4
Reagent and Mobile Phase Flow Rates: 1 mL/min
Integrator Chart Speed: 1 cm/min
Integrator Attenuation: 3

As the percentage of water rose, and the percentage of methanol fell in the mobile phase, the retention time of the aldosterone standard peak increased, the peak width increased, and peak height decreased. A change in the mobile phase of this

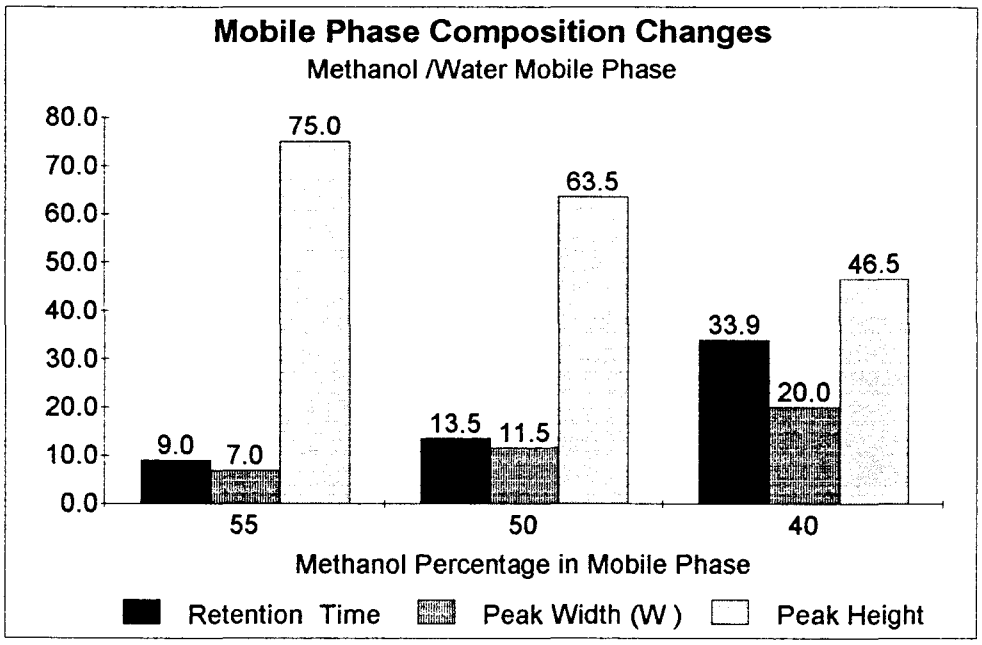

Figure 30. Effects of Change in Mobile Phase Composition on Aldosterone Chromatography
Column: Alltech Adsorbosphere C_{18}, 5 μm, 4.6 mm i.d.
Injection Loop: 20 μL
Mobile Phase: Methanol/Water
Reagent: 3 mM CBP in 0.02 M Na_2HPO_4
Reagent and Mobile Phase Flow Rates: 1 mL/min
Integrator Chart Speed: 1 cm/min
Integrator Attenuation: 3

analytical system to include a larger percentage of water would have then produced a shorter, broader analyte peak on an already noisy baseline. Therefore, no change in mobile phase composition was adopted for the injection of extracts of urine samples.

I. Investigation of C18 SPE of Aldosterone.

Various methods exist in the literature for the use of C18 solid phase extraction cartridges to isolate steroids from biological fluids (100-107). Most of these techniques are used to clean up samples prior to radioimmunoassay. After aldosterone was successfully extracted in this research project from 20 mL spiked urine samples with cyclohexyl cartridges, it was attempted to transfer the same method to 300 mg C_{18} SPE cartridges. Hydrolysed 10 mL urine samples that were spiked post-hydrolysis to 200 ng/ml, were prepared as described previously. Maxi-Clean® 300 mg, C_{18} cartridges were conditioned with 2 mL of methanol, and two 5 ml aliquots of water. The 10 mL samples were pulled through the solid phase, and the cartridges were then washed with 5 mL of water, 30 % acetone, and a final 5 mL of water. The elution was done with two 4mL aliquots of methylene chloride. This eluate was then dried at room temperature under nitrogen, and taken up in 150 µL of 0.02% triethylamine in methanol.

Next the sample was injected on the HPLC, with parameters of the detection system unchanged from previous experiments. On all chromatograms of urine samples, a solvent front was apparent at 3.4 minutes, but no other peaks were distinguishable from baseline noise. Figure 31 is a representative chromatogram of an injection of 10 mL spiked sample. Figure 32 is a chromatogram of the extract of 10 mL unspiked urine. A sample derived from 100 mL, unspiked, urine that was processed in the same manner on Maxi-Clean® C_{18} cartridges, is presented in Figure 33.

No aldosterone peak was observed on the chromatograms of either the spiked or unspiked 10 mL urine samples that were extracted with C18 cartridges. Increasing the sample volume did not result in isolation of the analyte, as hoped, for the interferences in the sample matrix were so concentrated by this procedure that the area corresponding to the retention time of aldosterone was obscured.

J. Solid Phase Extraction of Serum

An experiment was done in which solid phase extraction cartridges were used to isolate aldosterone from spiked serum samples. A five mL sample of centrifuged serum was spiked with 7.5 μg aldosterone to a level of 1.5 μg/mL. Sep-Pak C_{18} cartridges were conditioned with methanol and water. One mL of spiked serum was pulled through each of these three cartridges. After washing with 5 mL water, one cartridge was

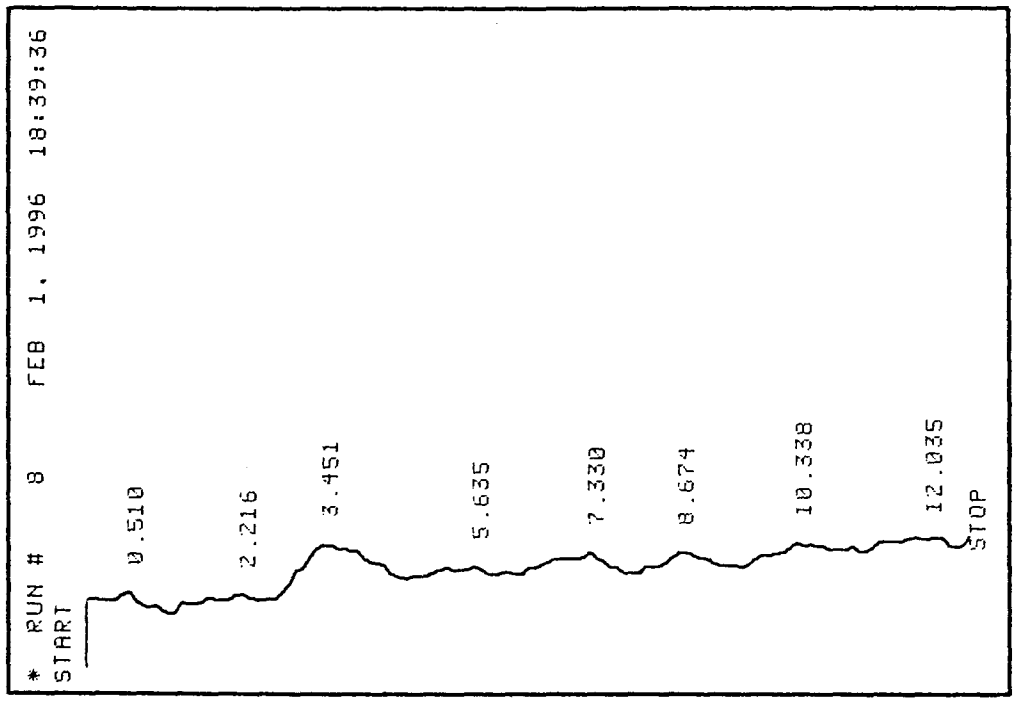

Figure 31. Chromatogram of 10 mL Urine
Spiked With 2 μg Aldosterone
SPE: 300 mg C_{18} Cartridge
Mobile Phase: Methanol/Water (55/45)
Reagent: 3 mM CBP in 0.02 M Na_2HPO_4
Reagent and Mobile Phase Flow Rates: 1 mL/min
Applied Voltage: 0.35 V

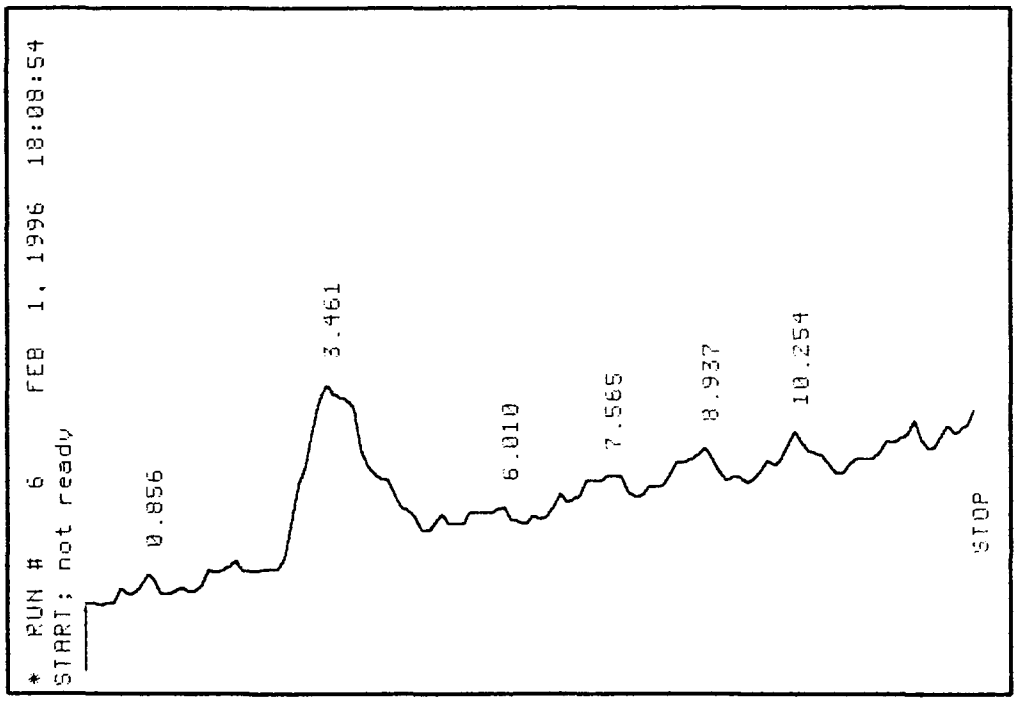

Figure 32. Chromatogram of 10 mL Unspiked Urine
SPE: 300 mg C_{18} Cartridge
Mobile Phase: Methanol/Water (55/45)
Reagent: 3 mM CBP in 0.02 M Na_2HPO_4
Reagent and Mobile Phase Flow Rates: 1 mL/min
Applied Voltage: 0.35 V

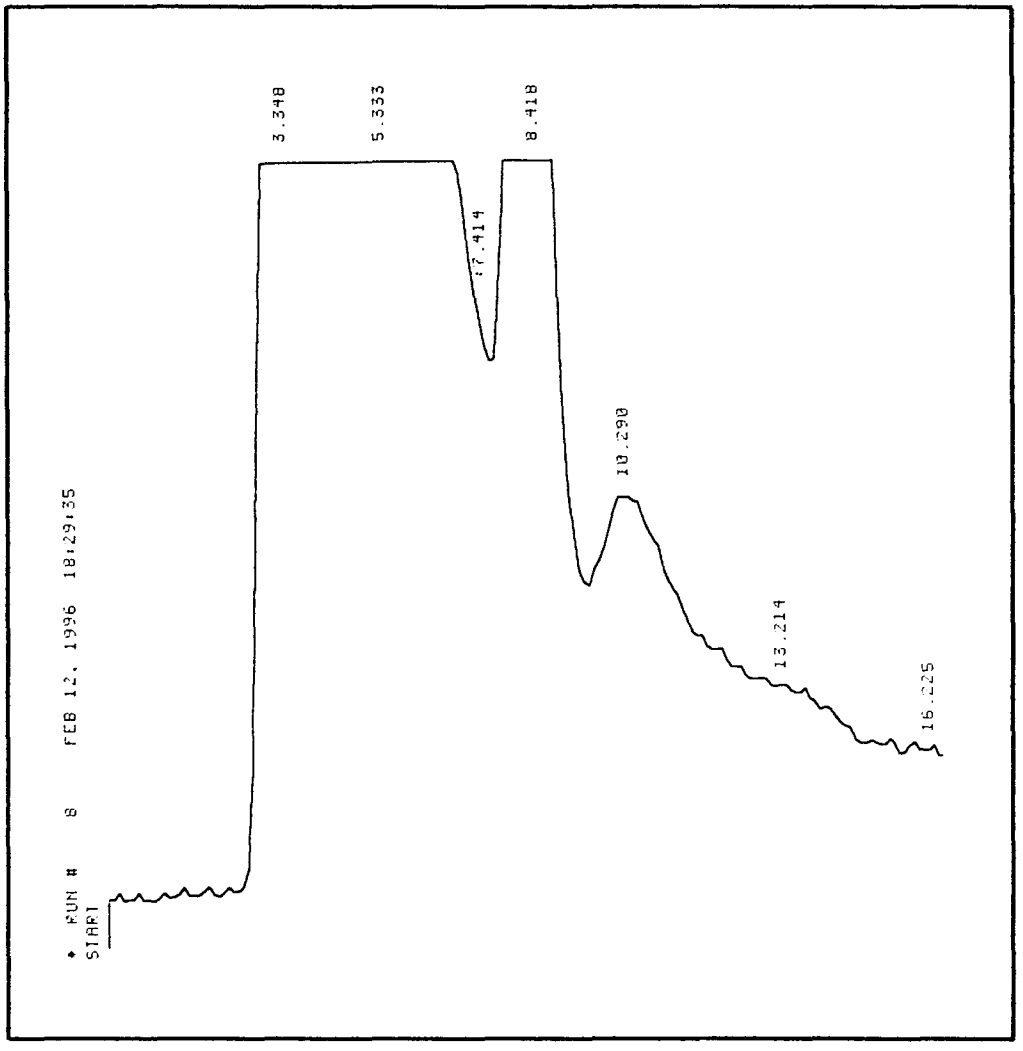

Figure 33. Chromatogram of 100 mL Unspiked Urine
SPE: 300 mg C_{18} Cartridge
Mobile Phase: Methanol/Water (55/45)
Reagent: 3 mM CBP in 0.02 M Na_2HPO_4
Reagent and Mobile Phase Flow Rates: 1 mL/min
Applied Voltage: 0.35 V

eluted with 1 mL 0.1 % triethylamine in methanol, the next cartridge with 1 mL acetonitrile, and the last with 1 mL methylene chloride. All eluates were dried under nitrogen and reconstituted in 200 μL 0.1 % triethyamine in methanol before injection on the HPLC. All system parameters were unchanged from the conditions used to construct the standard curve, with the exception of the mobile phase. A 50/50 methanol/water mobile phase was used to increase the resolution of any aldosterone peaks detected from interferences appearing near the solvent front.

Figure 34 is a chromatogram of a 400 ng aldosterone standard injected under the these conditions. The aldosterone peak was observed at 12.593 minutes. An extremely noisy baseline at integrator attenuation 3 reflected the cycling pressure fluctuations occurring on the HPLC pump at this time.

Figure 35 is a chromatogram of the serum extract that was eluted with acetonitrile. Although noise was again extreme, a large peak was apparent at 12.501 minutes that matched the retention time of the aldosterone standard. Figure 36 is a chromatogram of the serum sample eluted with acetonitrile. Again, a large peak appeared at the retention time of the aldosterone standard. Figure 37 is a chromatogram of the serum eluted with methylene chloride. A very small peak appeared amid noise at the retention time of aldosterone.

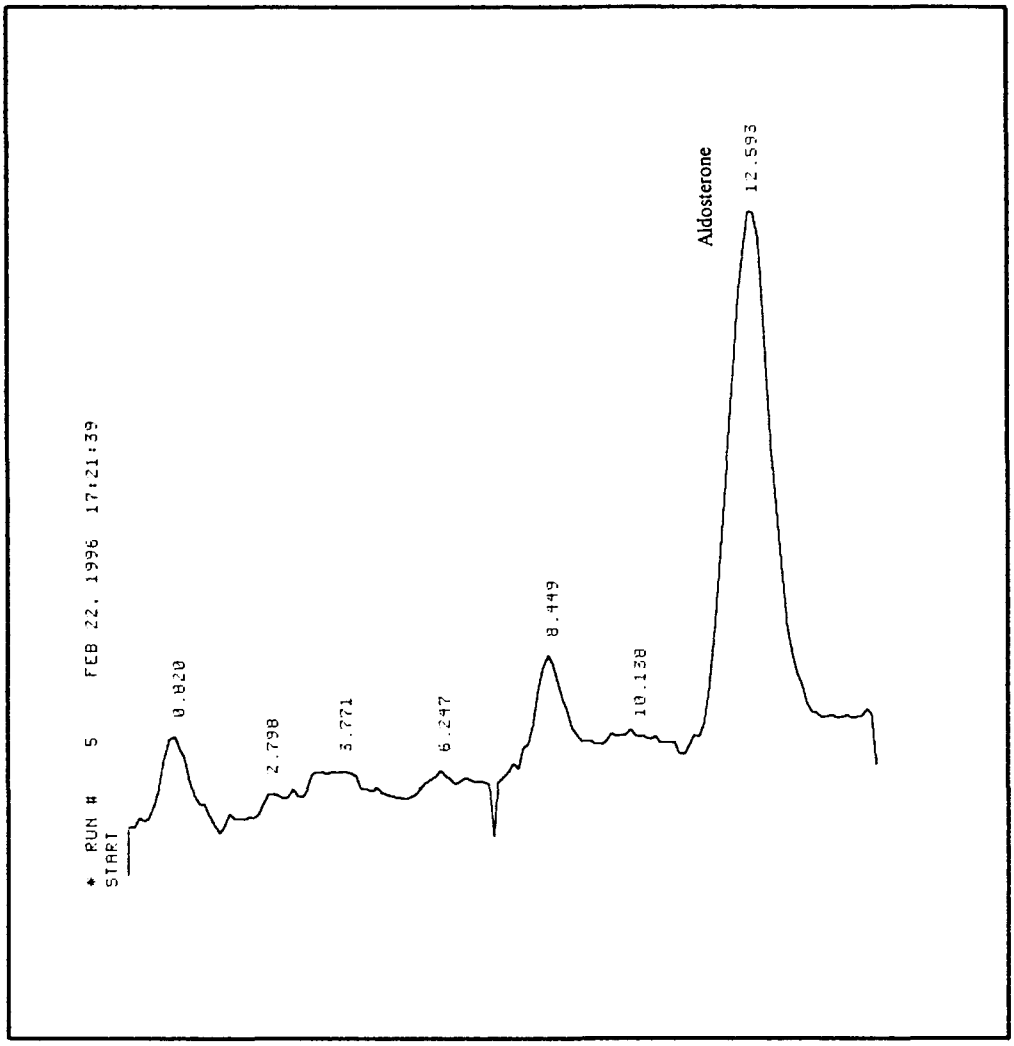

Figure 34. Chromatogram of a 400 ng Aldosterone Standard
Mobile Phase: Methanol/Water (50/50)
Reagent: 3 mM CBP in 0.02 M Na_2HPO_4
Reagent and Mobile Phase Flow Rates: 1 mL/min
Applied Voltage: 0.35 V

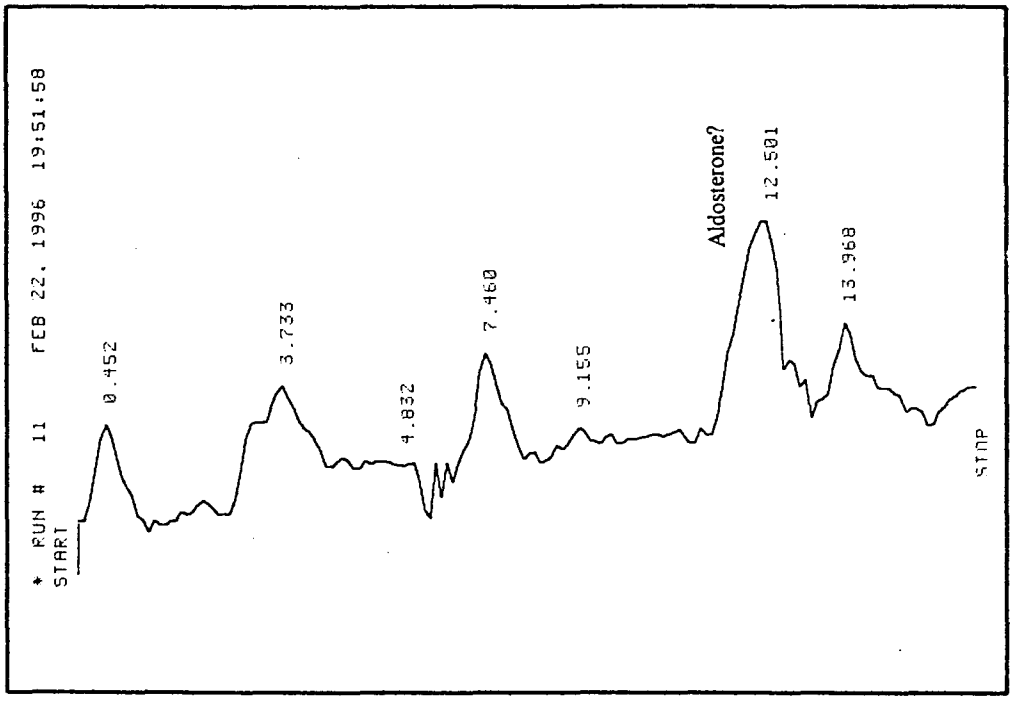

Figure 35. Chromatogram of Spiked Serum
Elution Solvent: Methanol
Mobile Phase: Methanol/Water (50/50)
Reagent: 3 mM CBP in 0.02 M Na_2HPO_4
Reagent and Mobile Phase Flow Rates: 1 mL/min
Applied Voltage: 0.35 V

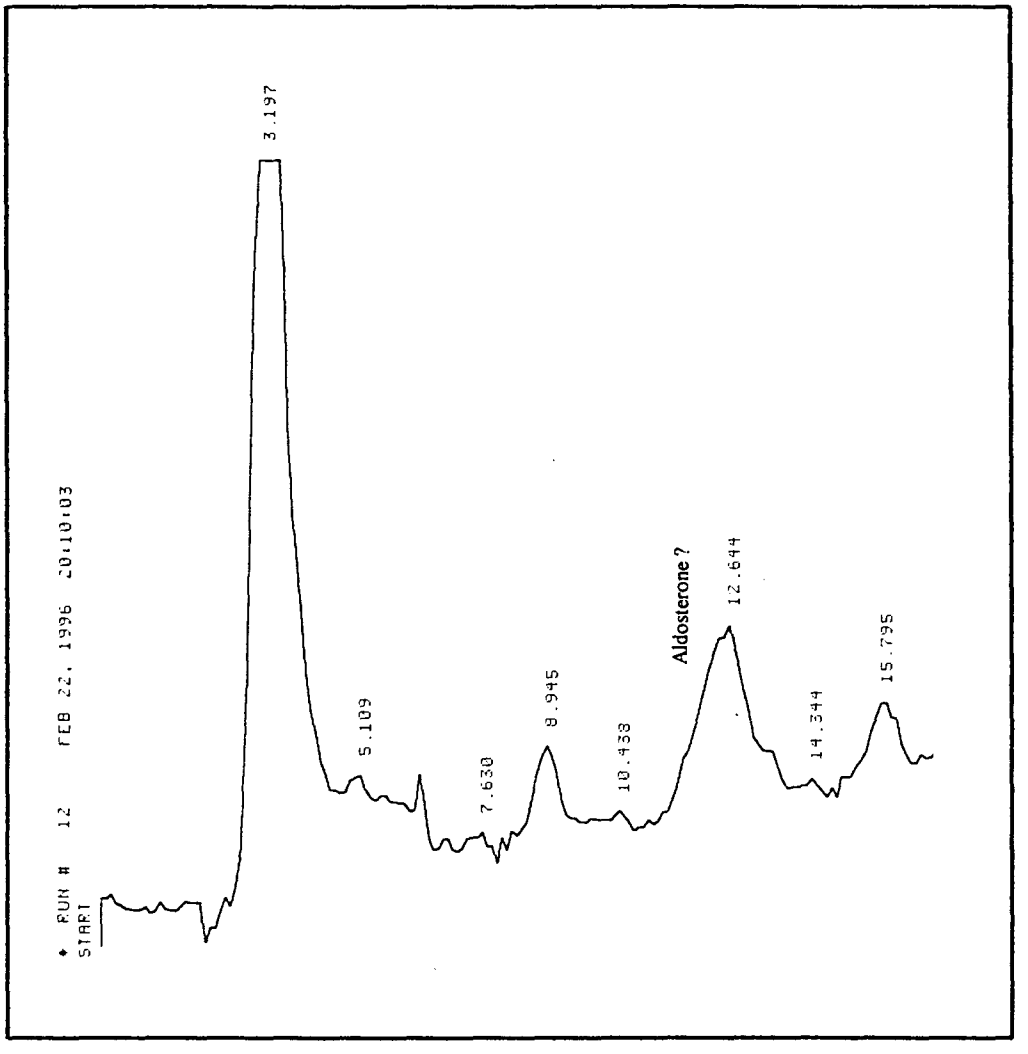

Figure 36. Chromatogram of Spiked Serum
Elution Solvent: Acetonitrile
Mobile Phase: Methanol/Water (50/50)
Reagent: 3 mM CBP in 0.02 M Na_2HPO_4
Reagent and Mobile Phase Flow Rates: 1 mL/min
Applied Voltage: 0.35 V

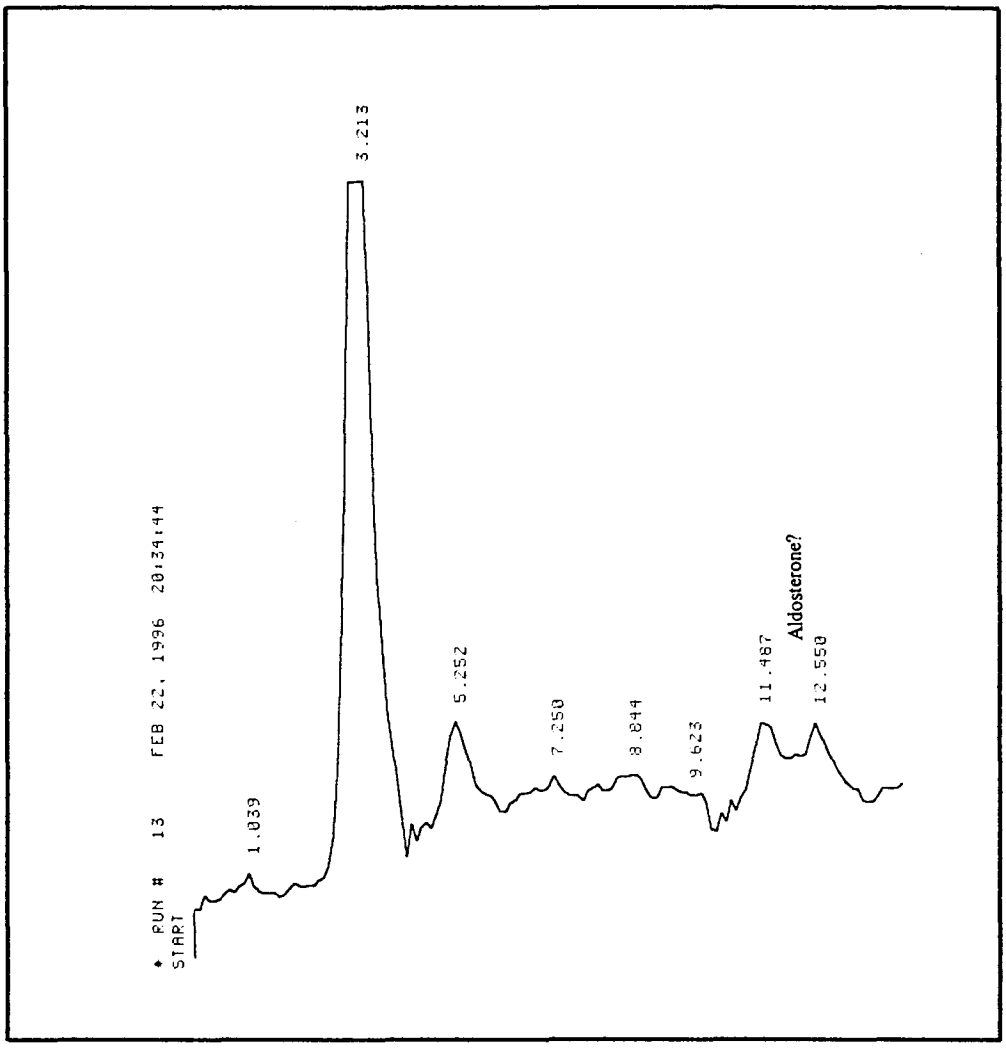

Figure 37. Chromatogram of Spiked Serum
Elution Solvent: Methylene Chloride
Mobile Phase: Methanol/Water (50/50)
Reagent: 3 mM CBP in 0.02 M Na_2HPO_4
Reagent and Mobile Phase Flow Rates: 1 mL/min
Applied Voltage: 0.35 V

Quantitation of aldosterone in these serum samples was not attempted due to instrumentation difficulties. However, it appears that at least some of the analyte spiked into the samples was extracted by the C18 cartridges, and that both methanol and acetonitrile can be used as elution solvents.

CHAPTER VI
CONCLUSION

This research project has shown that it was possible to use solid phase extraction to recover aldosterone from biological samples for amperometric detection, following HPLC. The method was found to be sufficiently selective to detect this analyte as an isolated chromatographic peak in spiked urine samples when volumes of up to 20 mL of urine were processed. Aldosterone from spiked serum samples may also be detected amperometrically following solid phase extraction and HPLC. The retention time of the hormone recovered from the urine and serum samples matches that of aldosterone standards within 0.1 minutes. The cyclohexyl solid phase extraction method is simple, rapid, and inexpensive. At this writing, the 60 ng detection limit of the system used, at an integrator attenuation of 3, does not appear to be low enough for the analyte to be detected in unspiked samples. However, if noise on the chromatographic baseline could be eliminated, the method has excellent potential for hormone quantitation. The assay is also practical because the method investigated in this study requires only reagents, supplies, and equipment that are readily available in most university, hospital, and research laboratories.

The formation of precipitate in the lines of the post column reactor was the major obstacle in achieving a lower detection limit. When concentrations of supporting electrolyte in the copper (II) bis-phenanthroline reagent were greater than 0.02 M Na_2HPO_4, the best signal to noise ratios were demonstrated. A knotted open tubular post column reactor also resulted in better signal to noise ratios for detection of aldosterone than unknotted tubing. In both cases, however, the post column reactor tubing became plugged within one hour of system run time, in contrast to the customary 4-5 hr. of run time possible with lower electrolyte strength and straight PCR tubing. After each run, it was necessary to disassemble all post column reactor tubing and flush with 1N nitric acid, followed by a lengthy rinsing procedure to remove any residual acid that might damage the working electrode. When a 1:1 mixture of CBP reagent and mobile phase was heated to 95 ^{o}C in a test tube, the solution became faintly hazy at 1 minute. An unheated control tube remained clear.

Reagent pump noise was kept to a minimum by periodically passivating the system with 6N nitric acid. When an upward baseline drift appeared on the chromatograms, making signal to noise measurement difficult, all stainless steel fittings were replaced

with Teflon® or PTFE. When the signal height of the injected aldosterone standards began to diminish, the thin layer working electrode was polished.

Fluctuations in pressure occurred on the HPLC as the methanol and water were combined by the ternary gradient pump for the specified mobile phase mixtures. To remedy this problem, all mobile phases were premixed and placed in one reservoir. This eliminated some baseline noise. After several months of use, however, the HPLC displayed leaking under the pump heads, extreme cycling pressure fluctuations, and an inability of the pump to deliver at flow rates greater than 3 mL/min. These problems indicated that the pump piston seals needed to be replaced before baseline noise could be diminished, and the detection limits improved.

Acid hydrolysis of spiked urine samples appeared to be responsible for a loss of up to 50% of the analyte. A focus of future investigation could be concerned with the effect that the acid conditions have on the aldehyde functional group and the steroid nucleus of the aldosterone molecule.

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APPENDIX

Documentation of Approval from

Human Subjects Research Committee

Youngstown State University / Youngstown, Ohio 44555-3091
Dean of Graduate Studies
(216) 742-3091

October 11, 1995

Ms. Barbara S. Saulitis
Department of Chemistry
UNIVERSITY

Dear Ms. Saulitis:

The Human Subjects Research Committee has reviewed and approved your project, "Amperometric Detection of Aldosterone in Liquid Chromatography," (HSRC#96-8).

We wish you well in this study.

Sincerely,

Peter J. Kasvinsky
Dean of Graduate Studies

c: J. Mike, Chemistry
S. Ellyson, Chair, HSRC