THE USE OF SEP-PAK^{R} CARTRIDGES IN ALDOSTERONE DETERMINATIONS USING HIGH-PRESSURE LIQUID CHROMATOGRAPHY

by

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Submitted in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the

Chemistry

Program

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YOUNGSTOWN STATE UNIVERSITY

June, 1982

ABSTRACT

THE USE OF SEP-PAK^{R} CARTRIDGES IN ALDOSTERONE DETERMINATIONS USING HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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Aldosterone is a steroid which is essential to life. Because of its clinical significance, it is important that an accurate method be utilized when assaying its level in urine. Most clinical laboratories have used RIA procedures when analyzing urines for this steroid. One of the non-isotope procedures investigated for the determination of aldosterone is high-pressure liquid chromatography. Prior to the actual quantification of this steroid by HPLC, it must be extracted from the urine and hydrolyzed. This has usually been accomplished by tedious, time-consuming methods.

The purpose of this research was to devise a procedure which would quickly and completely extract aldosterone from urine specimens, while at the same time, eliminating interferring compounds. This study utilized Waters Associates, Inc. Sep-Pak silica and C_{18} cartridges. By utilizing these cartridges in sequence, undesirable components, such as urinary pigments can be removed. Recovery of aldosterone standard added to unhydrolyzed random urine was 53-73%.

Operating parameters include a reverse-phase C_{18} column and aqueous acetonitrile as the mobile phase.

Difficulties arose after acid hydrolysis of the urine sample was completed. There appears to be some type of problem occurring which prevents the extraction of aldosterone by these Sep-Pak cartridges under the particular operating parameters used. Suggestions for dealing with this problem are discussed.

ACKNOWLEDGEMENTS

I would like to express my sincerest appreciation to Dr. Leonard B. Spiegel, my research advisor, for all his valuable guidance and encouragement throughout this research. I also wish to thank Dr. Daryl Mincey and Dr. Richard C. Phillips for the time they contributed in reading this manuscript and for all their helpful suggestions.

I would like to thank Michael Gergel for his help in acquainting me with HPLC. And finally, special thanks to Dr. Nestor A. Stychno for the encouragement he offered me throughout this research.

DEDICATION

All the time and effort which was put into this research is dedicated to my husband Bruce, my sons Jeffery, Matthew and Bruce Daniel, my parents Fillipo and Vincenzina and my sisters Judy, Debbie and my brother Tony.

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LIST OF SYMBOLS

SYMBOL	DEFINITION
ACTH	Adrenocorticotropic Hormone
ATP	Adenosine Triphosphate
dL	Deciliter
DNA	Deoxyribonucleic Acid
GLC	Gas-Liquid Chromatography
HPLC	High Performance(Pressure) Liquid Chromatography
k'	Capacity factor
ng	nanogram
N	Theoretical plates
RIA	Radioimmunoassay
R_s	Resolution
t_R	Retention time
V_R	Retention volume
µg	Microgram
24 h	24 hour

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CHAPTER I

INTRODUCTION

A. Biochemistry of Aldosterone

The adrenal glands, one located on the top of each kidney, have the function of secreting numerous steroids. These steroids, which have the cyclopentanoperhydrophenanthrene ring (See Figure 1)[1] as a part of their chemical structure, can be further subdivided into 3 groups: the glucocorticoids, the mineralcorticoids and sex hormones.[2]

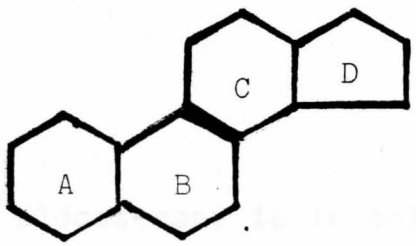


Figure 1.-- Cyclopentanoperhydrophenanthrene Ring[1]

Of the mineralcorticoids, whose main function is the control of electrolyte and fluid balance, aldosterone (CAS Registry Number [52-39-1])[3] is the most active. For a more in depth discussion on the adrenal cortex, the

reader is directed to various texts on biochemistry and physiology.[2,4]

The structure of aldosterone (Δ^4-Pregnen-18-al-11β , 21, diol-3,20-dione-(11➔18)-lactol)[5] was first elucidated in 1954 by Simpson, Tait, Wettstein, Neher, vonEuw, Schindler and Reichstein[6], and can be seen in Figure 2.[4]

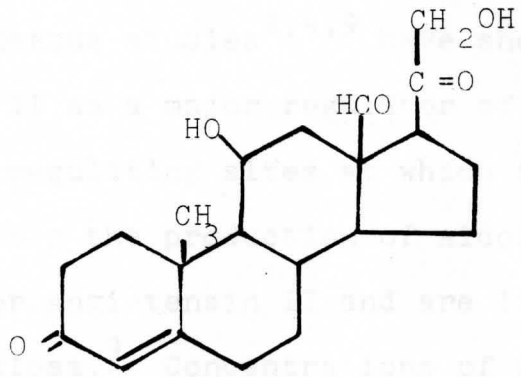


Figure 2.-- Aldosterone[4]

When aldosterone is in solution, there exists an equilibrium between the aldehyde structure and the hemiacetal. (See Figure 3)[4,7] The hemiacetal form is favored.

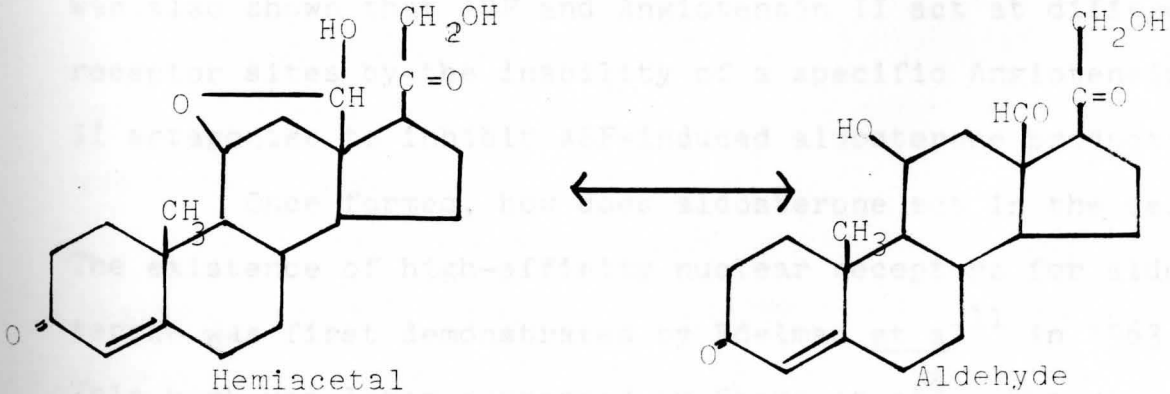


Figure 3.-- The two equilibrium forms of aldosterone.[4,7]

B. Mode of Action

Aldosterone is secreted by the zona glomerulosa of the outer layer of the adrenal gland known as the cortex.[2] Its secretion is regulated by numerous factors, including ACTH, somatotropin, plasma sodium and potassium concentrations and probably the most influential, the renin-angiotensin system.[4,8]

Numerous studies[2,4,9] have shown the importance of Angiotensin II as a major regulator of aldosterone secretion. The primary regulating sites at which the Renin-Angiotensin system controls the production of aldosterone are cellular receptors for Angiotensin II and are located in the adrenal zona glomerulosa.[9] Concentrations of Angiotensin II as low as 3×10^{-11} M elicit significant increases in aldosterone production.

Studies by Bravo *et al*[10] have demonstrated the presence in urine of a glycoprotein fraction called ASF (Aldosterone Stimulating Factor). It is thought that its site of action might be at the stage where cholesterol is converted to pregnenolone or of the latter substance to progesterone. It was also shown that ASF and Angiotensin II act at different receptor sites by the inability of a specific Angiotensin II antagonist to inhibit ASF-induced aldosterone production.

Once formed, how does aldosterone act in the cell? The existence of high-affinity nuclear receptors for aldosterone was first demonstrated by Edelman *et al*[11] in 1963. This work was later supported by Sharp *et al*[8]. The receptors

appear to be proteins.[8] It has been determined that the binding sites are present in target tissue (including kidney, salivary, sweat glands and the colon), but absent from non-target tissue.[8]

Other experiments have shown that with no aldosterone present, the receptors can be located in the cytoplasm of the cell, but on binding with the steroid, the resulting aldosterone-receptor complex attaches to the nuclear chromatin.[11] This induces the synthesis of proteins (AIP, Aldosterone-Induced Proteins) in the target cells.[12] The participation of DNA in chromatin acceptor activity has also been demonstrated.[12] Figure 4 shows a representation of a mineralcorticoid target cell.[8]

There are 3 possible theories as to the functions of AIP. One postulation is called the "Sodium Pump" Theory.[12] The term "sodium pump" refers to the energy-requiring mechanism which ejects sodium ions from the cell against an opposing gradient while simultaneously effecting the reentry of potassium ions.[4] The theory states that the sodium pump is directly activated by aldosterone. The steroid may serve to either increase the number of pumps or activate those which already exist.[12] At the present, there is not enough evidence to support this idea.

The second theory, known as the "Metabolic Theory" suggests that the supply of ATP is regulated by AIP.[8,12] It was demonstrated that aldosterone-mediated changes in sodium transport depend on an acetyl Co A precursor. The

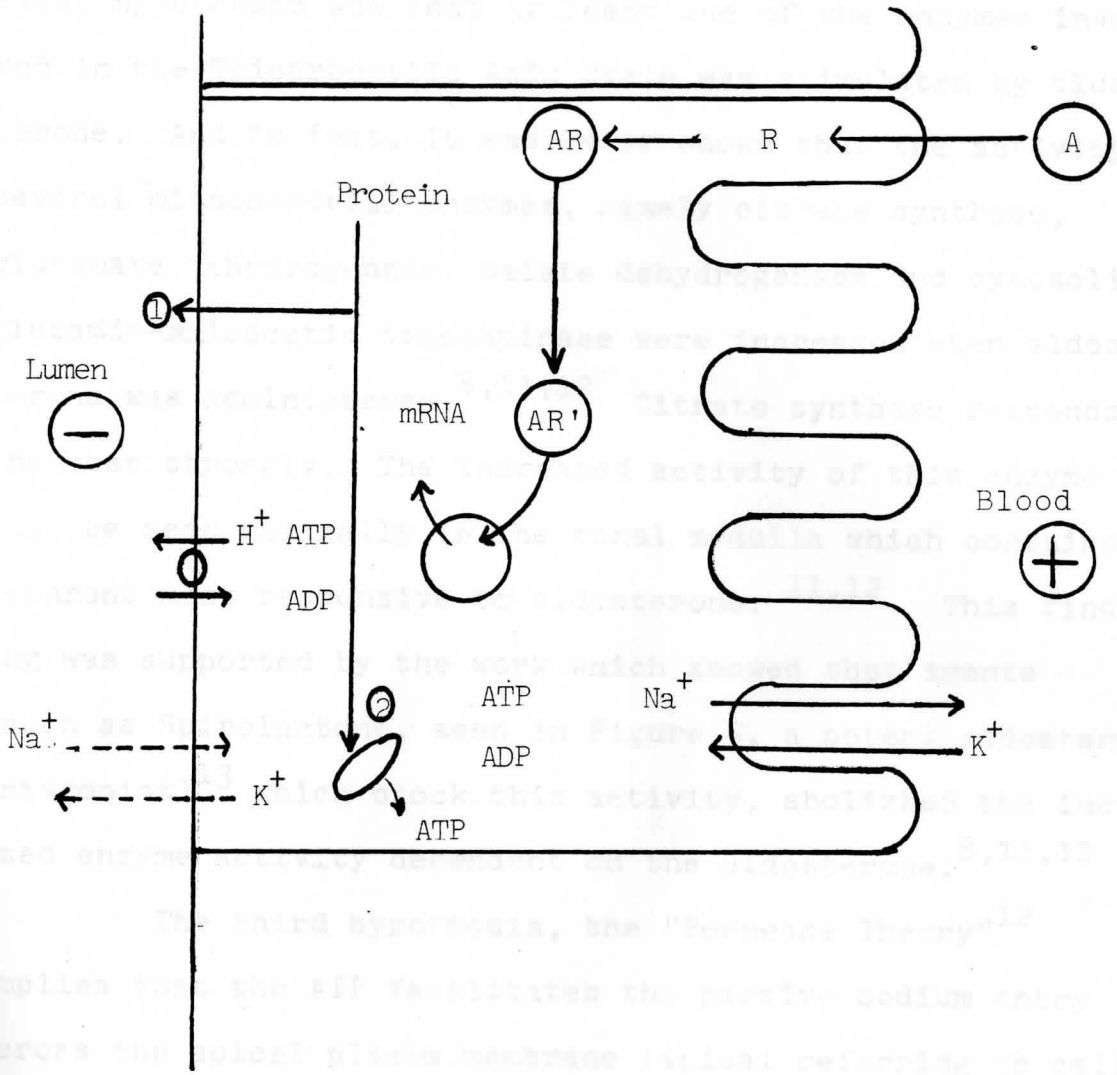


Figure 4.-- A schematic representation of a mineralcorticoid target cell. In this model, aldosterone (A) enters the cell and binds to a cytoplasmic receptor (R). This complex attaches to chromatin, initiating a cascade of events including mRNA and protein synthesis.[8]

first hypothesis was that at least one of the enzymes involved in the Tricarboxylic Acid Cycle was stimulated by aldosterone. And in fact, it was later shown that the activity of several mitochondrial enzymes, namely citrate synthase, glutamate dehydrogenase, malate dehydrogenase and cytosolic glutamic-oxaloacetic transaminase were increased when aldosterone was administered.[8,11,12] Citrate synthase responds the most strongly. The increased activity of this enzyme can be seen maximally in the renal medulla which contains nephrons most responsive to aldosterone.[11,12] This finding was supported by the work which showed that agents (such as Spirolactone, seen in Figure 5, a potent aldosterone antagonist)[13] which block this activity, abolished the increased enzyme activity dependent on the aldosterone.[8,11,12]

The third hypothesis, the "Permease Theory"[12] implies that the AIP facilitates the passive sodium entry across the apical plasma membrane (apical referring to cells located in the apex, or bottom tip of the gland)[14], thereby allowing greater access of sodium to pump sites.[8,11,12] Studies done with Amphotericin B (structure shown in Figure 6)[13], an antibiotic, supports this postulation.[8]

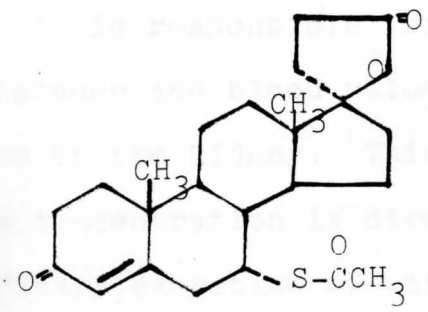


Figure 5.-- Spirolactone[13]

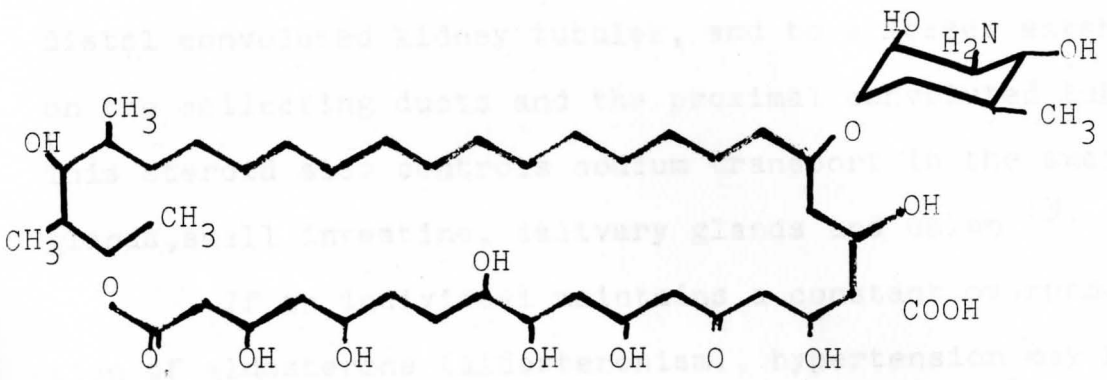


Figure 6.-- Amphotericin B[13]

Other work involving isolated toad bladder cells incubated with aldosterone were found to contain more sodium than paired controls. There have been several recent studies which support this " Permease Theory ".[8,11,12] However, more detailed analysis, involving proteolipid synthesis and changes in divalent ion content of the apical membrane must be made before a final determination as to the validity of this hypothesis can be made.

C. Clinical and Biological significance

Aldosterone has biological functions which are vital to survival. It is responsible for maintaining a constant electrolyte balance and blood volume by increasing reabsorption of sodium by the kidney. This is due to the fact that plasma sodium concentration is directly proportional to blood volume and urinary excretion of water. At the same time,

potassium ion excretion is increased.[13]

The action of aldosterone is primarily on the distal convoluted kidney tubules, and to a lesser extent, on the collecting ducts and the proximal convoluted tubules.[2,13] This steroid also controls sodium transport in the sweat glands, small intestine, salivary glands and colon.[13]

If an individual maintains a constant overproduction of aldosterone (aldosteronism), hypertension may result.[1]

Aldosterone can be determined in biological fluids. The principal metabolites of aldosterone found in the urine are tetrahydroaldosterone and aldosterone-18-glucuronic acid.[15,16] Free aldosterone makes up about 3% of the total urinary aldosterone.[16]

By performing aldosterone assays, a clinician may diagnose Conn's Syndrome or primary hyperaldosteronism, a dysfunction of the adrenal gland which causes increased aldosterone secretion. Overproduction may also be a result of extraadrenal disease, or secondary aldosteronism.

By correlating the aldosterone level with clinical symptoms, the physician may then be able to treat the disorder.

D. Current Methods of Analysis

The most widely accepted method in the clinical laboratory for plasma or urine aldosterone determinations is radioimmunoassay (RIA).[1] By injecting rabbits with aldosterone-3-carboxy-methoxime-18,21-diacetate coupled

with bovine albumin, a highly specific antisera to this steroid has been developed.[1,17] The aldosterone-conjugate can then be radioactively labeled with ^{3}H at carbons 1 and 2 of the aldosterone molecule, and then used in the steroid analysis.[17] The methods utilizing tritiated aldosterone do not lend themselves well to the routine clinical laboratory, however, because most laboratories do not possess liquid scintillation counters.

Recently, a method has been developed by Diagnostic Products which uses aldosterone tagged with radioactive iodine (^{125}I) instead of tritiated aldosterone.[18,19] When questioned about the method of antibody formation and aldosterone iodination, the manufacturer would not divulge the information, claiming it was a trade secret. With this method, competition between endogenous aldosterone and radioactive labeled aldosterone for an aldosterone-specific antibody takes place. The free aldosterone is separated from the bound portion by using a substance such as dextran coated charcoal. The radioactivity in either the bound or unbound portion is counted and the counts per minute are compared to known standards.[16,18]

The normal aldosterone values found by this method are as follows: plasma (standing position)- 4-31 ng/dL[18]

urine- 6-25 µg/24 h[18]

Studies were done on the precision of this method.[19] Within-the-run and run-to-run precision values for plasma and urine are shown in Table 1.[19]

TABLE 1.

PRECISION FOR THE ANALYSIS OF SERUM AND URINE ALDOSTERONE BY THE USE OF IODINATED ALDOSTERONE

	n*	Mean	SD#	CV@
Within-run				
Serum	20	121.0	6.8	5.7
Urine	19	12.8	0.4	3.4
Run-to-Run				
Serum	26	38.8	6.5	16.8
Urine	10	159.9	20.8	13.0

*n= number of samples analyzed

#SD= Standard Deviation

@CV%= Coefficient of Variation (Relative SD)

Recovery of aldosterone showed 102.2% recovery for serum and 104.0% for urine, which is excellent.[19]

This method still has certain drawbacks. Two extraction steps of the urine are necessary prior to analysis, with one extraction requiring mechanical rotation for 10 minutes and the second requiring mechanical rotation for 60 minutes. In addition, the hydrolysis step requires a 24 h incubation period at room temperature.

E. Statement of the Problem

Because of the important role aldosterone plays in electrolyte balance, and hence, maintenance of normal blood pressure. it is extremely desirable that a rapid, accurate method for its determination be developed.

As previously stated, the routine laboratory can measure this mineralcorticoid by RIA. However, in most of the procedures utilized, the sample must first be extracted to remove interfering substances. This preliminary step is certainly an undesirable feature in that it is time-consuming. In addition, the extraction may not always remove undesirable compounds, so that cross-reactivity between the aldosterone antibody and non-aldosterone components occurs. This would result in falsely elevated aldosterone levels.

Due to this, other research has been undertaken to find a faster, more accurate methodology for aldosterone determination. Recently, there has been work in the area of High Performance (or Pressure) Liquid Chromatography (HPLC). When this has been used, results have been extremely promising. However, most methods still require tedious extraction procedures.

It is the objective of this research to remove interfering urinary substances, namely, urinary pigments, from samples prior to the HPLC determination of aldosterone. This was accomplished by the use of Water Associates C_{18} and silica Sep-Pak cartridges. The quantification of urinary aldosterone levels was also investigated.

F. High Performance Liquid Chromatography

When referring to liquid chromatography, one is speaking of a chromatographic technique in which the moving or

mobile phase is a liquid. The mobile phase passes through a column which contains immobile packing material coated with a liquid known as the stationary phase.[20]

The components (solutes) of interest are dissolved in a liquid compatible with the mobile phase and are then placed at the top of the chromatographic column. Because of the small particles used for the stationary phase, high pressure must be applied to the column to move the sample mixture through. While flowing through the column, the sample is separated into its component parts. The extent of separation depends on the interaction which occurs between the solutes, the mobile phase and the stationary phase.

One of the characteristic features in a chromatographic separation is differential migration. That is, the different components of a sample move through the column at different rates in order for separation to occur. This is the result of the distribution of the sample molecules between the mobile phase and the stationary phase. The more time a sample molecule spends in the stationary phase, the longer it will take to move though the column.[21]

Another important feature in a separation is the spreading of the sample molecules along the column. As a component moves through the column, there is a difference in the migration rate of the individual molecules caused by physical processes. These physical processes include the following:1) eddy diffusion, which is due to the multiple flow paths that the solvent follows between different particles within the column.

2) mobile phase mass transfer refers to the concept that those sample molecules traveling the closest to the edge of the

flowpath travel slower than those molecules which travel down the center of the flowpath

3) stagnant mobile-phase mass transfer refers to the nonmoving mobile phase contained within the pores of the stationary phase which gives a degree of undesirable band broadening

4) longitudinal diffusion refers to the tendency of sample molecules to diffuse randomly in all directions.[21]

The sample molecules eventually travel the length of the column from which they are carried to the detector, where a signal is sent to the recorder and results in a chromatogram. (See Figure 7)[21] The sample elutes from the column in such a manner that it forms an approximate bell-shaped curve on the chromatogram. Each compound leaves the column at a characteristic time which can be used in its identification. The retention time (t_R) is a measure of time from when the sample was first injected onto the column to the time when the band maximum leaves the column.[21] This can be seen on the strip chart recorder. A typical chromatogram shows nearly complete separation or resolution between the peaks. The resolution (R_s) of two adjacent bands is given by the equation:[21]

$$R_s = \frac{(t_2 - t_1)}{\frac{1}{2}(t_{w_1} + t_{w_2})} \qquad (1)$$

where t_1 qnd t_2 refer to the t_R values of bands 1 and 2;

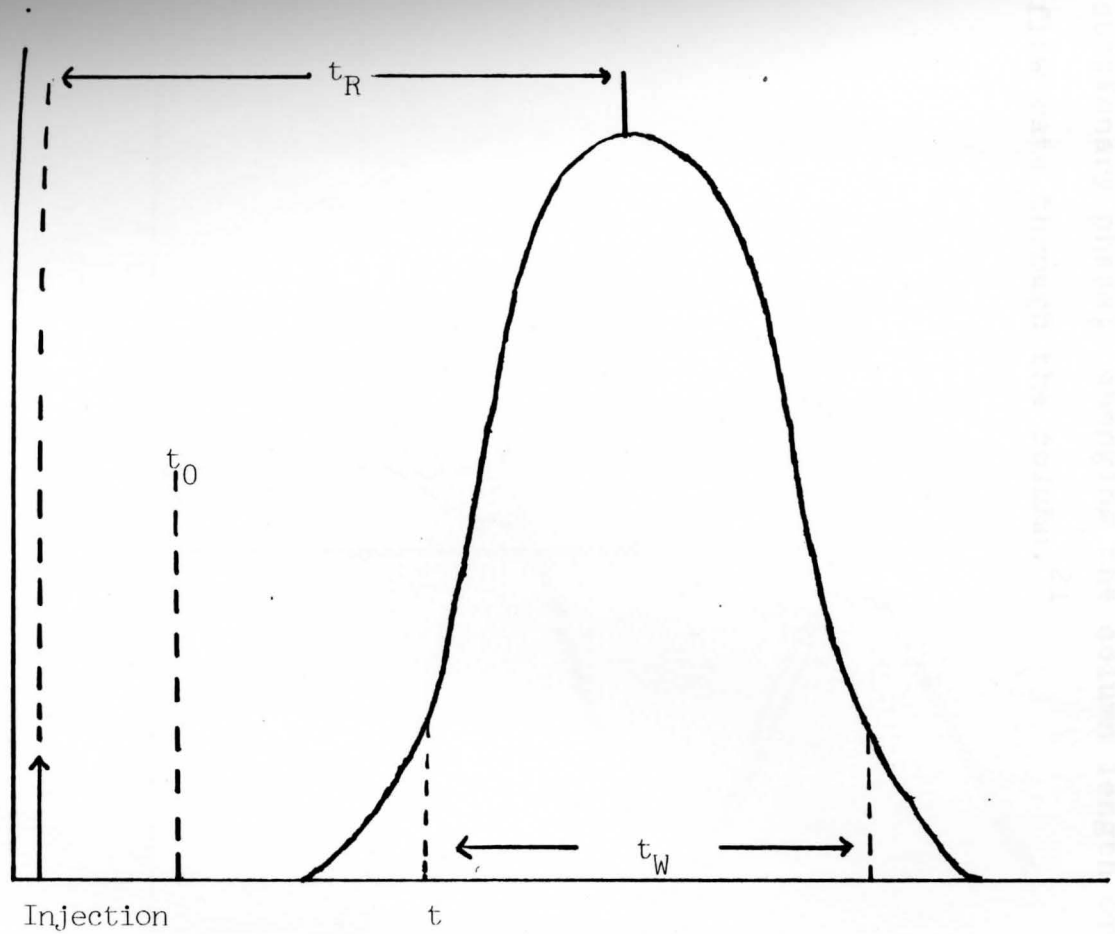


Figure 7.-- Typical chromatogram[21]

t_{w_1} and t_{w_2} are their band widths, which are measured at the points of inflection. (See Figure 8)[21] Better separation is indicated by larger R_s values while smaller R_s values are found with poorer separation. Changes in resolution can be achieved in several different ways. These include changing the composition of the mobile phase and/or stationary phase; changing the column length or the solvent flow rate through the column.[21]

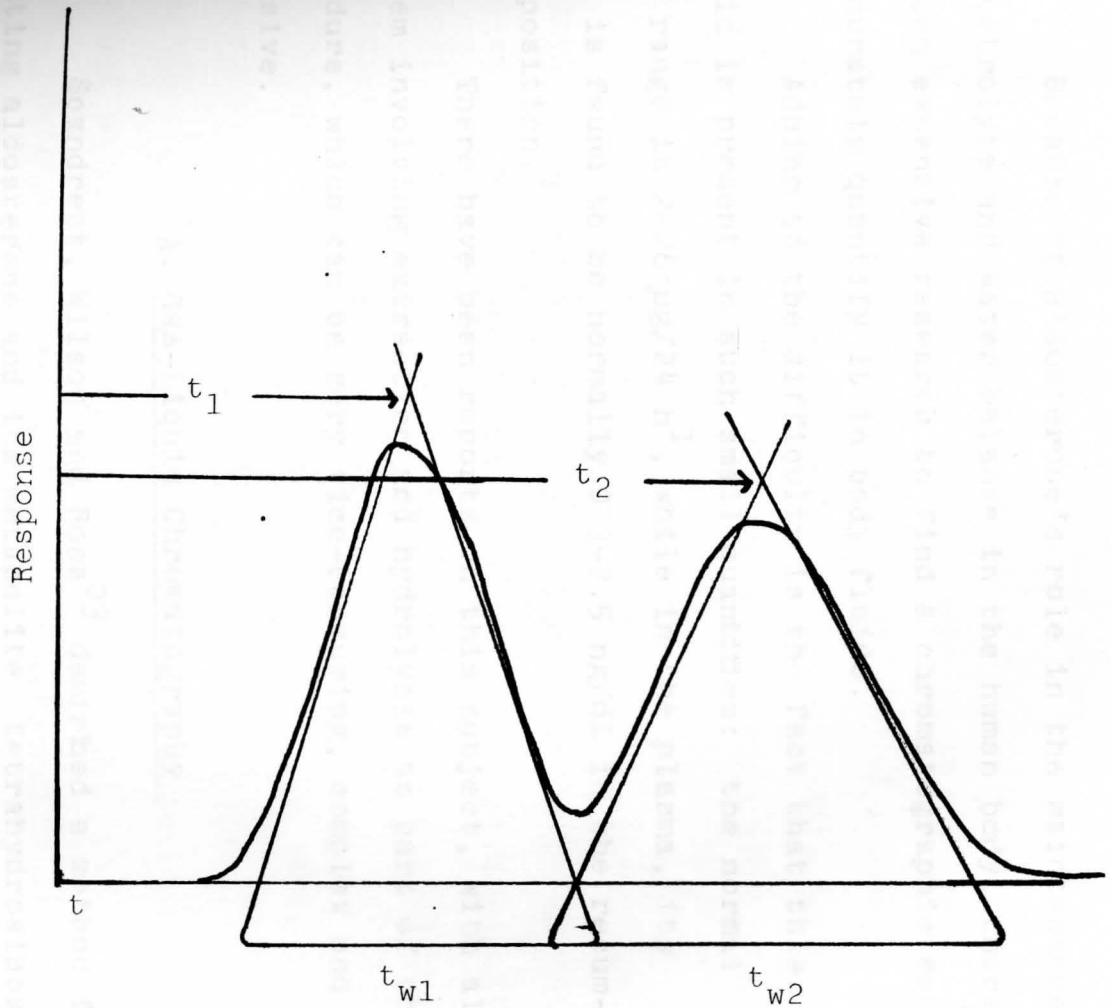


Figure 8.-- Resolution in LC[21]

CHAPTER II

REVIEW OF THE LITERATURE

Because of aldosterone's role in the maintenance of electrolyte and water balance in the human body, there has been extensive research to find a chromatographic method to accurately quantify it in body fluids.

Adding to the difficulty is the fact that this steroid is present in such small quantities: the normal urine range is 2-26 μg/24 h[1], while in the plasma, its level is found to be normally 0.3-2.5 ng/dL in the recumbent position.[1]

There have been reports on this subject, with all of them involving extraction and hydrolysis as part of the procedure, which can be very time-consuming, complex and expensive.

A. Gas-Liquid Chromatography

Scandrett, Wilson and Ross[23] described a method for isolating aldosterone and its metabolite tetrahydroaldosterone from both plasma and urine. The samples are extracted with Amberlite XAD-2 resin, followed by further purification by liquid chromatography with LH-20 resin columns. The step prior to GLC analysis involves separation by thin layer chromatography, a procedure which causes minimal damage and

structural alteration to the steroids. Heptafluorbutyryl gamma-lactone derivatives of tetrahydroaldosterone and aldosterone were formed and a chromatogram by GLC obtained.

The recovery for this method, as measured by added radioactivity was 51.5 ±7.4% for aldosterone and 48.7± 8.3% for tetrahydroaldosterone. The low recovery is indicative of a need for further investigation into this procedure, to find out exactly where the aldosterone is being lost.

Another procedure for evaluation of urinary steroids is described by Shackleton and Whitney.[24] This method incorporates Sep-Pak C_{18} cartridges for extraction of urinary steroids.

Steroid conjugates from the Sep-Pak extraction of unhydrolyzed urine were hydrolyzed enzymatically and the freed steroids were extracted. Methyloxime-trimethylsilyl derivatives of the steroids were prepared and gas chromatographic separation was performed.

This method was compared to Amberlite XAD-2 extractions and also to ethyl acetate extractions. The Amberlite XAD-2 extractions gave far poorer recoveries than did both the Sep-Pak and ethyl acetate extractions.

Recovery studies for Sep-Pak extraction of enzyme-hydrolyzed urines, each containing a known amount of tritiated aldosterone metabolite, showed excellent results.

With the first mL of methanol passed through the cartridge, 95 - 99 % (n=6) of the aldosterone was recovered.

Graef, Furuya and Nishikaze[15], in a time-consuming procedure, used Amberlite XAD-2 for extraction of urinary steroids, followed by enzymatic hydrolysis of conjugates with β-glucuronidase. Further purification is obtained by extraction with various organic solvents, including methylene chloride. This is followed by thin layer chromatography, after which, the area containing aldosterone is scraped off and extracted. Gas-liquid chromatography is performed. The per-cent recovery for this method is 76 ± 6 (n=8).

B. High-Pressure Liquid Chromatography

DeVries, Popp-Snijders, De Kieviet and Akkerman-Faber[25] describe an HPLC procedure for urinary aldosterone employing acid hydrolysis, and extraction which is followed by further purification by thin layer chromatography. The aldosterone is quantified by normal-phase HPLC, using prednisolone as the internal standard.

The recovery for this study was 63 ± 9 % (n=215). The loss of aldosterone can be probably attributed to the 3 thin layer chromatography extractions. In comparing this method to GLC, the C.V. for the HPLC procedure was 16% (n=215) with a lower detection limit of 5 nmoles/24 h, with the C.V. for the GLC being 22% (n=71) and a lower detection limit of 10 nmoles/24h. The HPLC procedure was found to be more sensitive.

Schoneshofer and Dulce[26] use gradient elution in their HPLC purification of 19 urinary steroids, including aldosterone, prior to immunoassay. There were different parameters utilized in this particular research, totalling 7 different systems. The systems were composed of different column packings and solvents for gradient elution. With gradient reverse-phase chromatography, using methanol and water as the mobile phase, adequate separation of steroids was obtained. As the steroids elute from the column, they are fractionated by a time-regulated sample collector. The steroids are then quantitatively assayed by RIA. The authors do state that due to their low concentrations and strong interferences by non-specifically ultraviolet absorbing substances, urinary steroids other than cortisol and estriol are difficult to assay by HPLC alone.

Aldosterone is one of the steroids assayed in adrenocortical extracts by Ballerini and Chinol.[27] The HPLC system utilized a chloroform-methanol gradient during the normal phase analysis and water-acetonitrile gradient during the reverse-phase chromatography. Good separation of aldosterone from the other steroids was obtained. No recovery results were given. Though the authors do not give an extraction procedure, this method would probably be applicable to urine assays if interfering substances are removed prior to HPLC.

O'Hare, Nice, Magee-Brown and Bullman[28] were able to use a dioxane-water gradient to separate aldosterone from the other polar adrenal steroids. Reverse-phase chromatography,

with a 25 cm Zorbax-ODS column was used in their research.

Reardon, Caldarella and Canalis[29] describe a method for the analysis of serum cortisol and 11-deoxycortisol by reverse-phase liquid chromatography. Recovery was determined by the addition of 100 ng cortisol and 100 ng 11-deoxycortisol to the serum prior to extraction. The results were 96±3 % (n=9) and 105±1 % (n=17),respectively. A comparison study with RIA was also performed. There was good correlation between the two methods, with the exception of cortisol determinations after metyrapone stimulation. The concentrations determined by HPLC were 50-90% lower, possibly due to the lack of specificity of the RIA procedure. That is, the antisera in the RIA procedure may have been "cross-reacting" with substances other than the cortisol, thus giving falsely elevated results. This HPLC procedure did incorporate a methylene chloride extraction step.

For a previous review of literature on HPLC of urinary aldosterone, the reader is referred to Komara.[30]

CHAPTER III

MATERIALS AND APPARATUS

A. Solvents and Reagents

Distilled, deionized and filtered water, redistilled over potassium permanganate and NaOH, was used for the preparation of all reagents and solvent systems.

Solvents and reagents were prepared or used as described below.

Methanol (Fisher-Scientific Company, Fairlawn, New Jersey, HPLC grade), further purified prior to use by passing through a Waters Associates C_{18} Sep-Pak cartridge.

Methylene chloride, acetonitrile (Burdick and Jackson Laboratories, Inc., Muskegan, Michigan, 'distilled in glass'), used without further purification.

Dimethylsulfoxide (DMSO, J.T. Baker Company, Phillipsburg, New Jersey), was used without further purification.

Hydrochloric acid and nitric acid (Fisher-Scientific Company, Fairlawn, New Jersey, ACS grade); concentrated HNO_3 was used to make up a 6N HNO_3 solution; concentrated HCl was used to make up a 2N HCl solution.

Charcoal (Central Scientific Company), was dried by heating at 100° C overnight. It was used in the attempt to remove pigments from urine.

Sephadex LH-20 (Pharmacia Fine Chemicals, Piscataway, N.J.), was utilized in the urine extraction procedure.

Acenapthene and uracil (Eastman Kodak Co., Rochester, N.Y.) were used to determine the number of theoretical plates (N) in the C_{18} column.

Nitrogen and argon (Airco, Inc.,New York, N.Y.) were used to evaporate solvents from the samples.

Sodium hydroxide pellets (Mallinkrodt, Paris, Kentucky; Analytical Reagent) were used to prepare a 2N solution.

Drierite (W.A.Hammond Drierite Co., Xenia, Ohio) was used as the dessicant in the detector.

B. Steroids

The following stercids were obtained from Sigma Chemical Company (St. Louis, Missouri)

1. D-aldosterone (Δ^4-Pregnen-18-al-11β, 21-diol-3,20 dione-(11$\rightarrow$ 18)-lactol)[5] Absorption Maximum; 240nm[33]

2. Cortisol (11β, 17α, 21-Trihydroxypregn-4-ene-3,20-dione)[5] Absorption Maximum: 242 nm[33]

3. β-Estradiol (1,3,5 (10)-Estra-triene 3,17β-diol)[5] Absorption Maximum: 225 nm and 280 nm.[33]

4. Corticosterone (Δ^6-Pregnene-11β, 21-diol-3,20-dione)[5] Absorption Maximum: 240 nm.[33]

5. Estriol ($\Delta^{1,3,5\,(10)}$-Estratriene-3, 16α,17β-triol)[5] Absorption Maximum: 280 nm.[33]

6. Estrone ($\Delta^{1,3,5(10)}$-Estratriene-3-ol-17-one)[5] Absorption Maximum: 283-285 nm.[33]

7. Reichstein's Substance S (11-deoxy-17-hydroxy-corticosterone)[5] Absorption Maximum: 242 nm.[33]

8. 11-Deoxycorticosterone (21, Hydroxypregn-4-ene-3,20-dione)[34] Absorption MAximum: 240 nm.[33]

9. Tetrahydrocortisone (3α,17α,21 Trihydroxy-5β-pregnene-11,20-dione)[34]

10. 5-β-Pregnan-3α,20α-diol

11. Progesterone (4-Pregnene-3,20,dione)[5] Absorption Maximum: 240 nm.[33]

12. Prednisolone (11β,17α,21-Trihydroxy-$\Delta^{1,4}$-pregnadiene-3,20 dione)[5] Absorption Maximum; 242 nm.[33]

All steroids were stored in a dessicator at 5° C.

Stock solutions were prepared with pure methanol and stored in the freezer.

Crystalline aldosterone stock solution: 5mg/dL Methanol. All other stock steroid solutions contained 10 mg/dL.

C. Apparatus

Venoject tubes, borosilicate, silicone coated, 13 x 75 mm for collection of Sep-Pak eluate; Disposable scintillation vials, 20 mL capacity, used to hold samples (Kimble, Teruma, Maryland).

Ground glass equipment was used for the distillation of water.

Samples were dried in a Multi-Temp Block #2093 (Lab-Line Instruments, Inc.) with either argon or nitrogen, depending on availability, carried through a Silli-Vap™ 30

(Pierce, Rockford,Ill.)

High-pressure liquid chromatography was carried out with a Beckman, Model 110-A Pump, Isocratic Liquid Chromatograph, Model 330. (Beckman Instruments, Fullerton, California)

Sargent Welch, Model IP pH meter (Sargent-Welch Co., Cleveland,Ohio). The recorder used was also by Sargent-Welch, Model DSRG-2.

Microliter syringes (Hamilton), in 10 µL, 25µL, 50 µL and 100 µL sizes, were used to inject samples into the HPLC.

Column: A µBondapak C_{18} column with the dimensions, 3.9mm x 30 cm, is packed with octadecylsilane (functional group) which has µPorasil (very polar, fully porous, silica particles) as its inert support. The particle size is 10 microns.[24]

A guard column, packed with C_{18}/Corasil (37-50 µm particles) as its packing, was inserted between the sample valve and the µBondapak C_{18} column. Its function is to protect the analytical column by capturing components of the sample which are strongly retained and thus prevents contamination of the analytical column. The packing of the guard column was replaced at regular intervals. This is easily accomplished because the packing is of a pellicular nature and is easily tap-filled into the guard column.[21]

Sep-Pak cartridges, C_{18} and silica packings, (Waters Associates, Milford, Massachusetts) were used to extract aldosterone from urine samples.

Gelman filters (Ann Arbor, Michigan 48106) were used; a 25 mm filter with a 1.2 μm pore was utilized to remove particles from the solvents while a 13 mm filter with a .2 μm pore was used to remove particles from samples.

A 10 mL glass syringe was used to pass urine samples through Sep-Pak cartridges and Gelman filters prior to injection into the HPLC unit.

Altex-Hitachi, Model 110-1- Spectrophotometer was used as the detector. This is a variable wavelength detector.

CHAPTER IV

EXPERIMENTAL

A. Sep-Pak® Cartridges

Prior to this investigation, treatment of urine samples with various organic solvents was the principal means of aldosterone extraction. With the introduction of Sep-Pak cartridges, there appeared to be a new means for aldosterone extraction.[26] The Sep-Pak cartridges utilized in this research were prepacked with either silica or octadecylsilane (C_{18})-silica. With the use of these cartridges, one is able to isolate a narrower range of compounds than possible by extraction procedures alone.[35]

The Sep-Pak cartridges operate on the principles of liquid chromatography, enabling one to separate different compounds or different groups of compounds according to their differences in polarity.

The type of Sep-Pak employed will vary with the type of sample one is working with. One would use the Sep-Pak C_{18} cartridge when the sample is dissolved in an aqueous, polar solution, as opposed to the use of silica Sep-Pak when a non-polar organic solvent contains the dissolved constituent of interest.

To obtain maximum retention of the desired sample onto the Sep-Pak, one should introduce it onto the cartridge in a "weak solvent" as is practical. (See Figure 9). With C_{18} cartridges, this would mean water, whereas, with the silica Sep-Pak, one would use an organic solvent, such as methylene chloride.

Because of the nature of the packing material, non-polar compounds would elute before polar compounds when using silica Sep-Paks because the silica is highly polar, and has a higher affinity for such constituents. On the other hand, the non-polar characteristics of the C_{18} cartridges would allow for the elution of the polar compounds prior to those of a lesser polarity.

Waters Associates, Inc. have published a chart (See Figure 10) which demonstrates possible solvent-Sep-Pak combinations.[37]

Figure 11 shows the polarity spectrum of the Sep-Pak cartridges. Assuming that one knows the polarity of the compound of interest, one would prepare samples for analysis as follows:[35]

After placing the sample on the Sep-Pak C_{18} cartridge, compounds of high polarity would be eluted by washing with a polar solvent. One must choose the polar solvent which will not elute the compound of interest.

The next step involves the removal of the compound of interest by washing the cartridge with a less polar solvent. Extremely non-polar compounds remain on the cartridge.

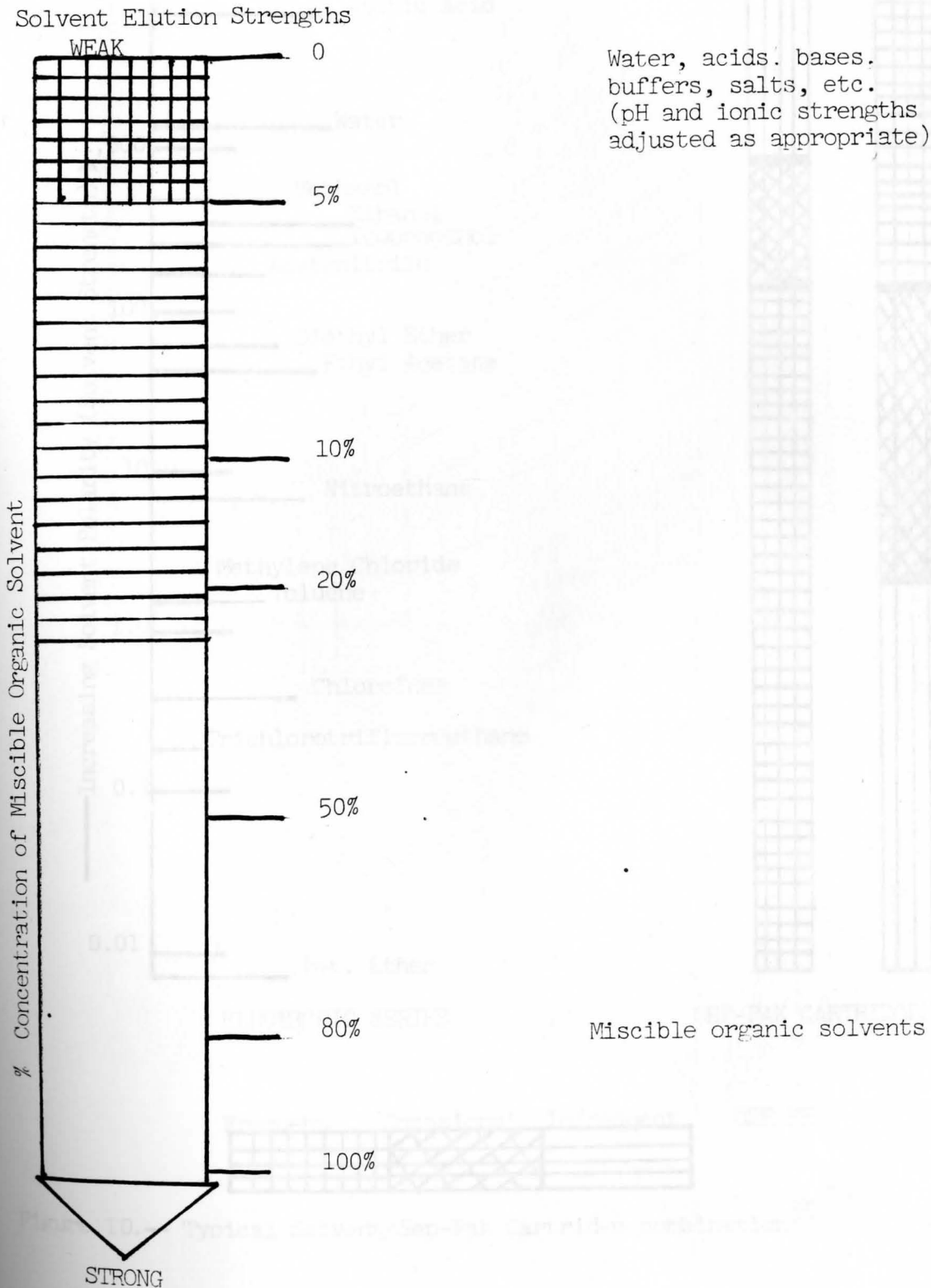


Figure 9 .-- Solvent Elution Guide for Sep-Pak C_{18} [34]

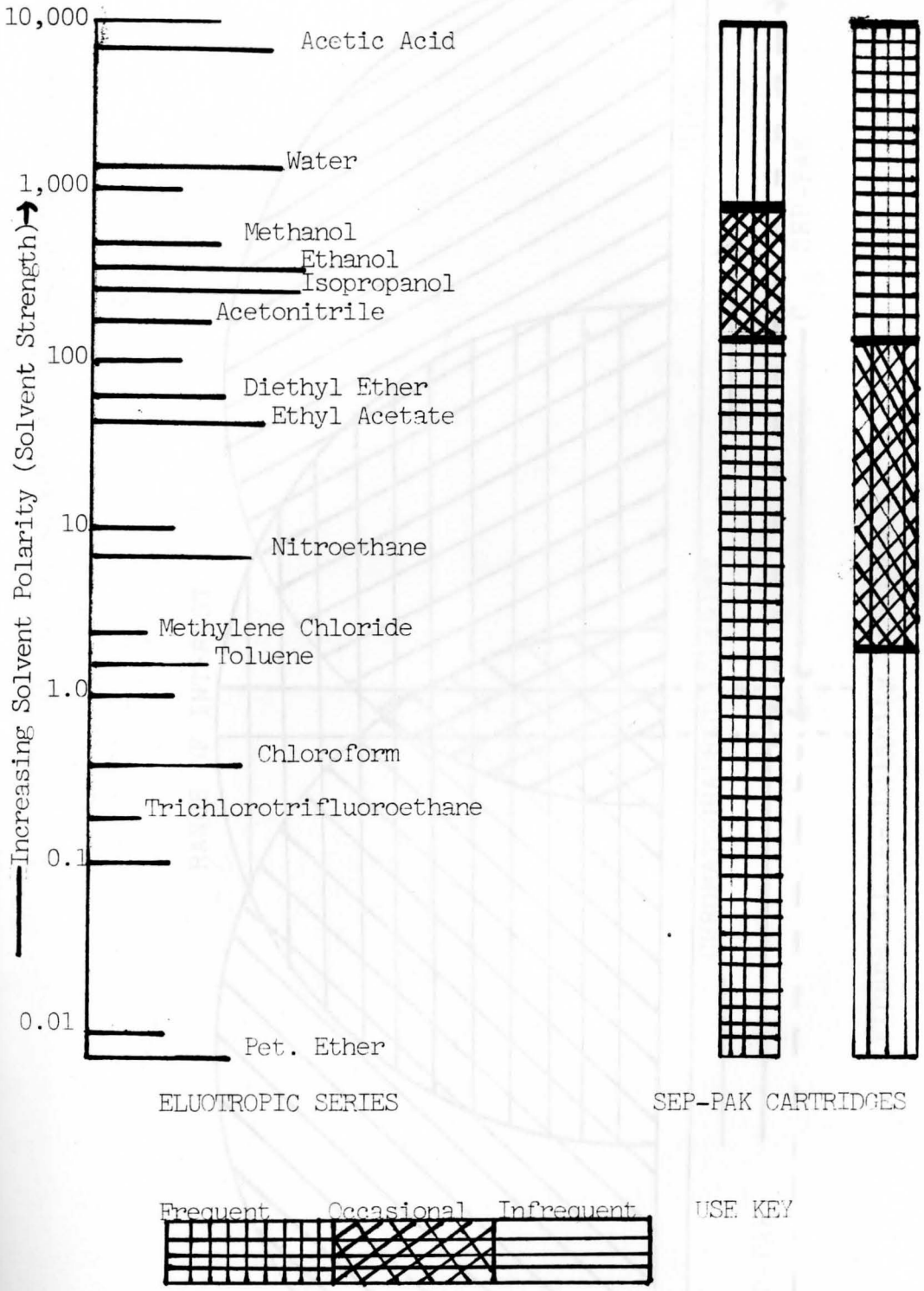


Figure 10.-- Typical Solvent/Sep-Pak Cartridge combination[35]

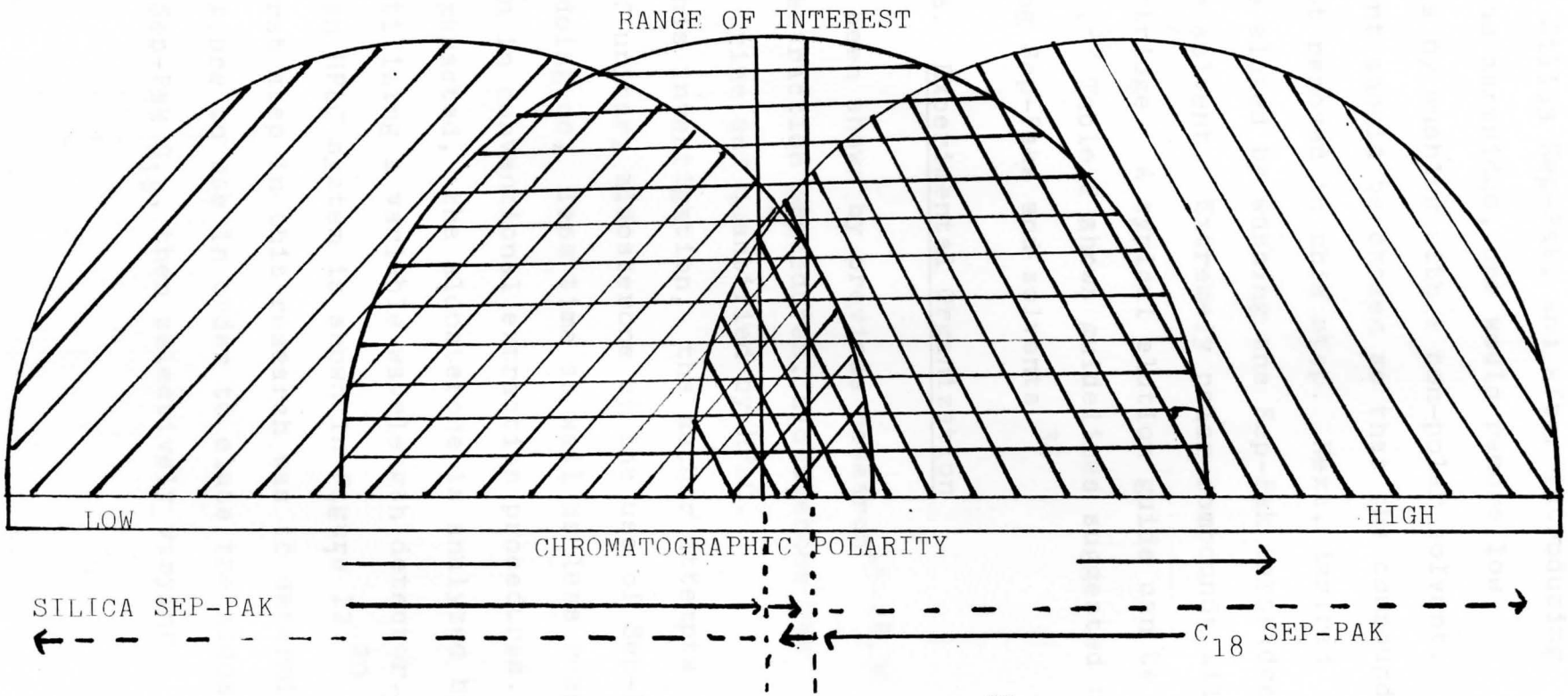


Figure 11. -- Polarity Spectrum[33]

When employing the Silica Sep-Pak, and after introducing the sample onto the cartridge, one would remove low polarity compounds by washing with a non-polar solvent. Again, the solvent should be chosen so that the compound of interest is not removed in this step. Next, desired components can be eluted by washing the Sep-Pak cartridge with a more polar solvent. Extremely polar compounds will remain on the cartridge. A typical elution guide can be seen in Figure 9.[35] Table 2 shows guidelines suggested by Waters in choosing Sep-Paks and solvents.[35]

B. Experimental Organization

It has been shown by previous research[25-28,30] that after long extraction procedures, aldosterone can be isolated from urine and quantified by HPLC.

With this investigation, the author attempts the extraction of urinary aldosterone by the use of Sep-Pak cartridges. In doing so, less time as well as less reagents are required than in conventional extraction procedures.

Once extracted, the aldosterone is analyzed by an HPLC system utilizing a variable wavelength detector. The basic setup for an HPLC system is shown in Figure 12.[20]

The first step in this research was to determine which solvent mixture to use in order to elute the aldosterone from the Sep-Pak C_{18}, then selectively varying

TABLE 2

SEP-PAK CARTRIDGE SEPARATION GUIDELINES [35]

Cartridge Packing	Silica	C_{18}
Packing Polarity	HIGH	LOW
Typical Solvent Polarity Range	Low to Medium	Medium to High
Typical Solvent	Hexane Chloroform Ethyl Acetate THF	Water Buffers Water/Methanol Water/Acetonitrile
Sample Elution Order	Least Polar Compounds First	Most Polar Sample Components First
Solvent Change Required to Elute Retained Compounds	Increase Solvent Strength	Decrease Solvent Strength

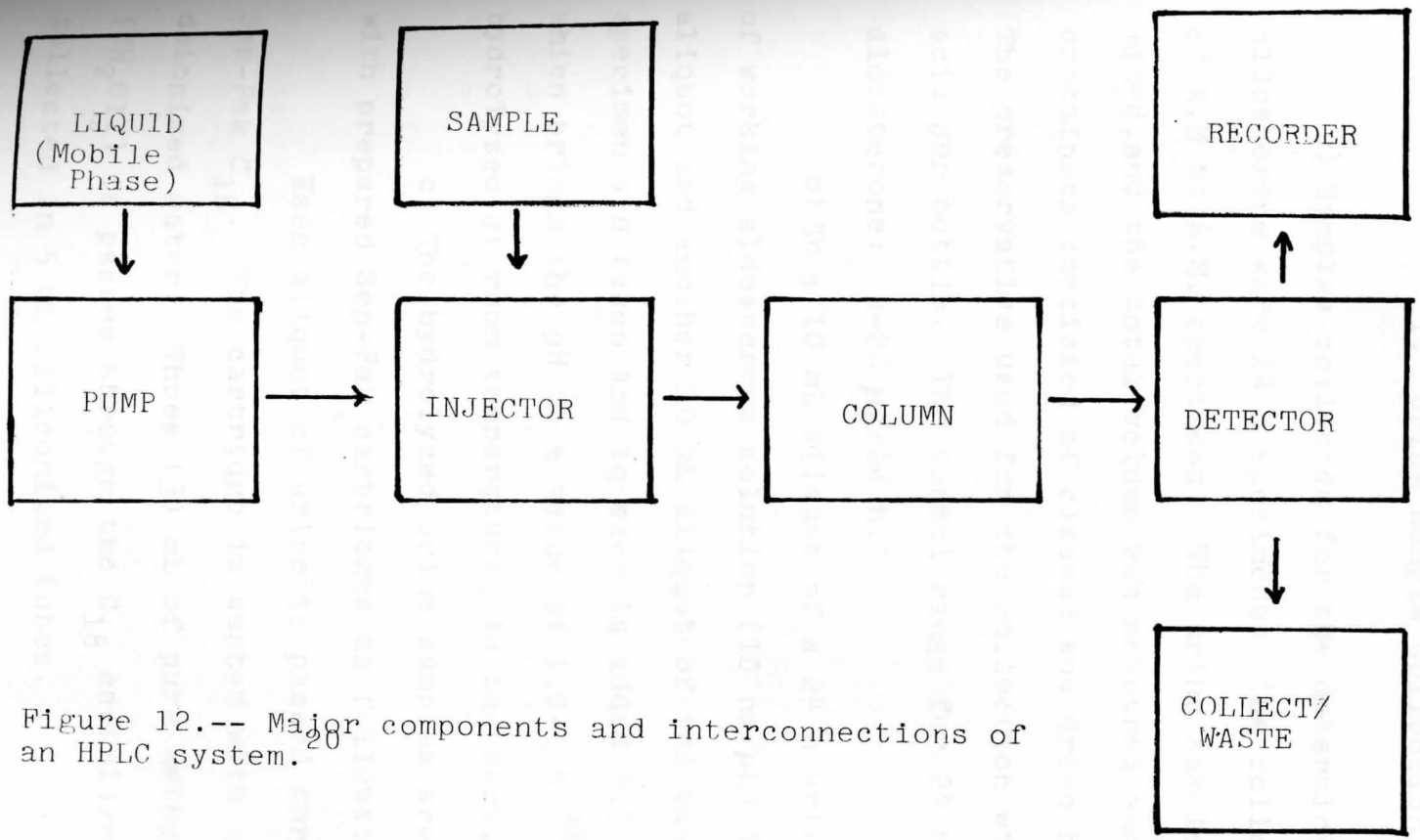


Figure 12.-- Major components and interconnections of an HPLC system.[20]

the composition of the eluting solvent in order to elute aldosterone without eluting other undesirable urinary components.

1. Urine Sample Collection

a) Samples collected for the determination of urinary aldosterone were 24 h specimens. The collections consisted of A.M to A.M. specimens. The urine samples were pooled, mixed, and the total volume was measured and recorded. The containers consisted of cleaned and dried brown glass bottles. The preservative used for the collection was 1 gm boric acid per bottle. The normal range for 24 h excretion of aldosterone: 2-26 µg/24 h.[1]

b) To a 10 mL aliquot of a 24 h urine specimen, 10 µL of working aldosterone solution (10 ng/µL) is added. This aliquot and another 10 mL aliquot of the same 24 h urine specimen are taken and to each is added 2.0 mL of 2 N HCl which brings the pH to a value of 1.0-1.5.[38] The samples are hydrolyzed at room temperature, in the dark, for 8 h.

c) The hydrolyzed urine samples are then extracted with prepared Sep-Pak cartridges as follows:

Each aliquot of urine is passed through a prepared Sep-Pak C_{18}. The cartridge is washed with 10 mL of distilled, deionized water. Three (3) mL of pure methylene chloride (CH_2Cl_2) is passes through the C_{18} cartridge. This eluant is collected in 5 mL siliconized tubes.

This eluant is then passed through a Sep-Pak silica

cartridge which has been previously washed with 15 mL of CH_2Cl_2. The solution which elutes through the Sep-Pak is collected in siliconized tubes. Three (3) additional mL of CH_2Cl_2 are passed through the Sep-Pak silica to ensure that all the aldosterone has been eluted.

d) The eluted sample is evaporated to dryness under nitrogen at 37°C.

e) The dried sample is reconstituted with 100 µL of acetonitrile-water (50:50,v/v).

f) The dissolved sample is injected into the HPLC chromatograph.

h) Detection of UV absorbing components is accomplished with a 240 nm detector coupled to a recorder.

C. Methods and Procedures

1. Estimation of Aldosterone in Urine

A method utilizing HPLC is used in the estimation of aldosterone. The amount of aldosterone present in the sample is based on peak area. The peak area is calculated by the following equation[21]:

$$\text{area} = \text{height} \times \text{width at } \tfrac{1}{2} \text{ height} \qquad (2)$$

The peak area is used to determine the concentration of aldosterone by interpolation from a standard curve.

CHAPTER V.

RESULTS AND DISCUSSION

With high-pressure liquid chromatography, it is important to determine how well the system is working, in particular, the column. One method of checking the efficiency of the column is by determining the capacity factor, k'. The k' value provides an estimate as to how well a solute is being retained by a column.[20] The k' value can be found by the equation[21]:

$$k' = \frac{t_R - t_0}{t_0} \qquad (3)$$

where t_R is the retention time of the sample and t_0 is the time for the mobile phase (or other unretained) molecules to move from one end of the column to the other.[21] Ideally, the k' value should lie between 1.5 and 5.0.[20] The capacity factor for this column was determined to be 4.3.

Another method used in determining column performance is by calculating the number of theoretical plates (N). The more plates in a column, the better the separation. N can be determined from the equation[22]:

$$N = 25\left(\frac{V_R}{W}\right)^2 \qquad (4)$$

where V_R is the retention volume and W is the peak width at 4.4% of the peak height. (See Figure 13)[22] The number of theoretical plates, N, determined for the column used ,were 4,389. Minimum number according to the manufacturer was 3,000. N was determined in the C_{18} column by utilizing .01 M solution of acenapthene as a marker and applying the

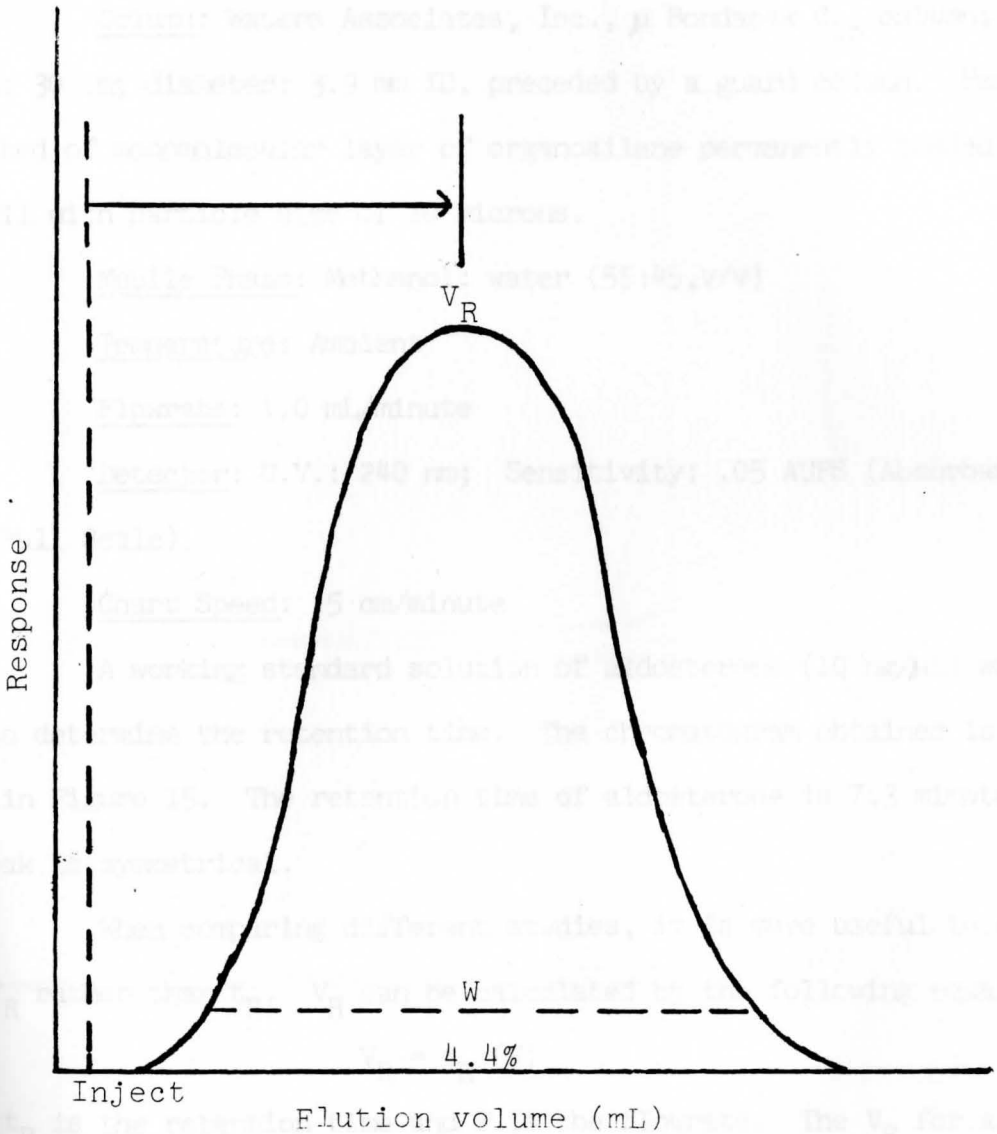


Figure 13.-- 5 6 Method[24]

5 σ method.[22] (Refer to Equation 4). The resulting chromatogram can be seen in Figure 14. Once N was determined, the retention times (t_R) of different standards could be determined. The parameters used were:

Column: Waters Associates, Inc., μ Bondapak C_{18} column; length: 30 cm; diameter: 3.9 mm ID, preceded by a guard column. Packing consisted of monomolecular layer of organosilane permanently bonded to μPorasil with particle size of 10 microns.

Mobile Phase: Methanol: water (55:45,v/v)

Temperature: Ambient

Flowrate: 1.0 mL/minute

Detector: U.V.: 240 nm; Sensitivity: .05 AUFS (Absorbance Units Full Scale).

Chart Speed: .5 cm/minute

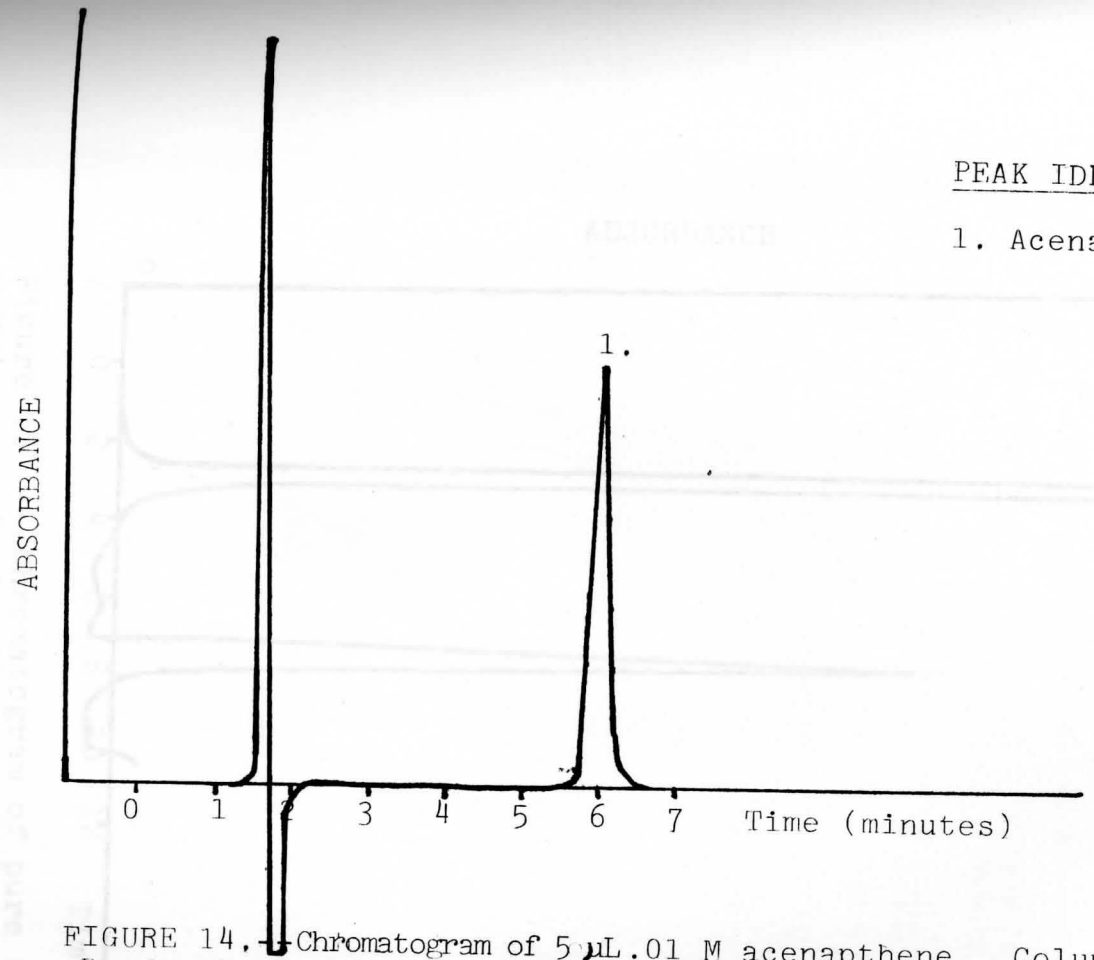
A working standard solution of aldosterone (10 ng/μL) was used to determine the retention time. The chromatogram obtained is shown in Figure 15. The retention time of aldosterone is 7.3 minutes and the peak is symmetrical.

When comparing different studies, it is more useful to use the term V_R rather than t_R. V_R can be calculated by the following equation[21]:

$$V_R = t_R (F) \qquad (5)$$

where t_R is the retention time and F is the flowrate. The V_R for aldosterone for the parameters utilized was 7.3 mL. This value is in agrrement with V_R values determined in previous studies.[30,37,38]

The retention times of various steroids which might interfere with the aldosterone determination are found in Table 3. The experimental parameters used were identical to those used for aldosterone.



PEAK IDENTITY

1. Acenapthene

FIGURE 14. Chromatogram of 5 µL .01 M acenapthene. Column packing, µBondapak C_{18}; Mobile phase : Acetonitrile-water (60:40,v/v); Temperature: Ambient; Flowrate: 2.5 mL/minute; Detector: UV (254nm) Sensitivity: 0.5 AUFS (Absorbance Units Full Scale); Chart Speed: 1 cm/minute.

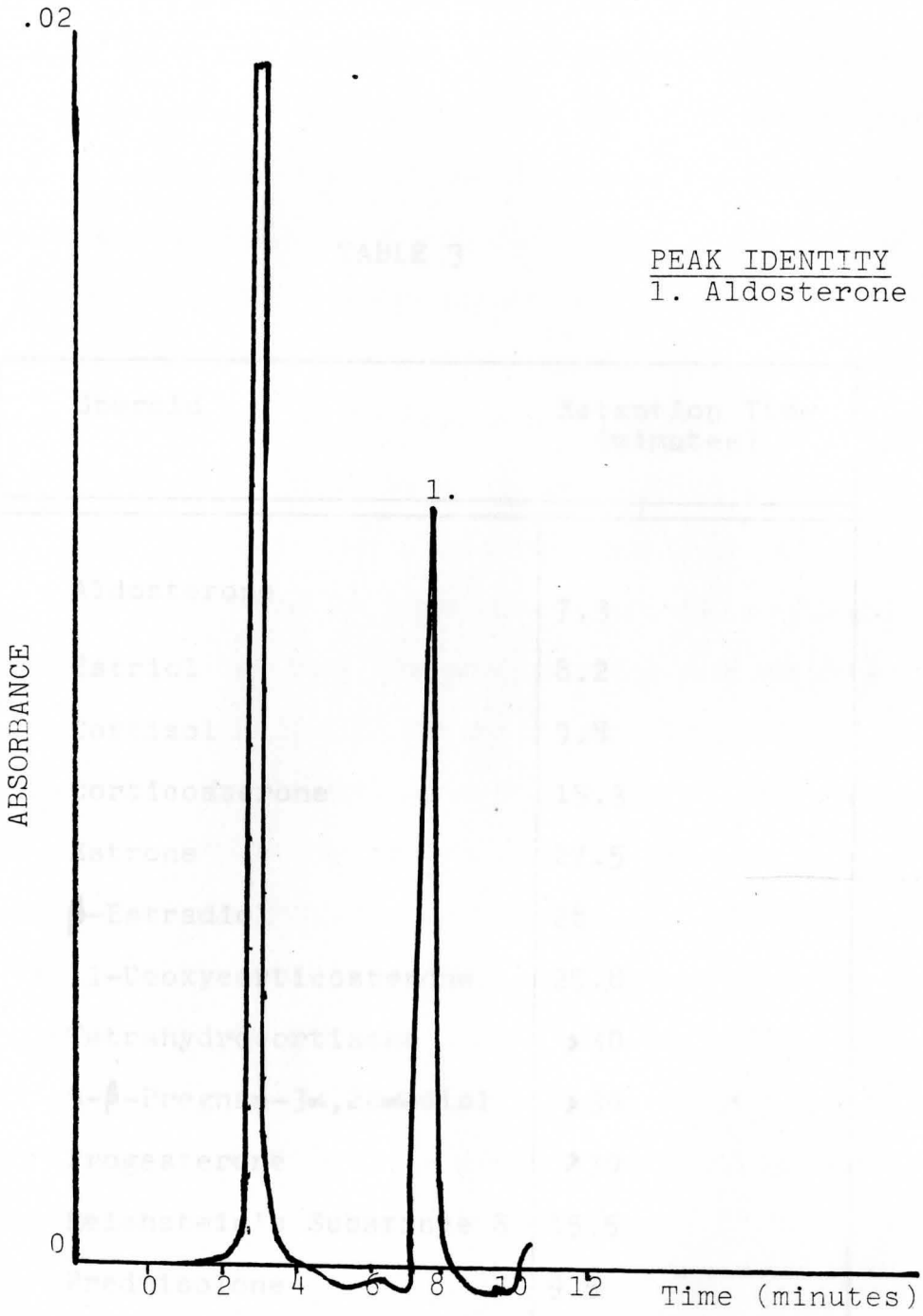


Figure 15 -- Chromatogram of pure aldosterone working standard (100 ng). Column: μBondapak C_{18}; Mobile phase: Methanol-water (55:45,v/v); Temperature: Ambient; Flowrate: 1.0 mL/minute; Detector: UV (240nm); Sensitivity: 0.02 AUFS; Chart Speed: .5 cm/min.

TABLE 3

Steroid	Retention Time (minutes)
Aldosterone	7.3
Estriol	8.2
Cortisol	9.8
Corticosterone	15.3
Estrone	27.5
β-Estradiol	28
11-Deoxycorticosterone	25.8
Tetrahydrocortisone	> 30
5-β-Pregnan-3α,20α-diol	> 30
Progesterone	> 30
Reichstein's Substance S	15.5
Prednisolone	9.5

Of the different steroids chromatographed, estriol was the only one which did not give good separation from aldosterone. However, by decreasing the flowrate to 0.5 mL/minute, good resolution was obtained. This is shown in Figure 16. Prednisolone was used as the internal standard for this research. It is a compound which is not normally found in urine; if present, it is due to exogenous origins. It can be separated from aldosterone.[24] The obtained t_R, using original operating parameters, was 9.5 minutes. The value (t_R for aldosterone/t_R for prednisolone) should be constant for every HPLC run if the column is functioning properly. For parameters utilized in this research, this value should be .77.

Once the desired retention times were determined, a standard curve for the aldsterone was run. The area under the peak was determined. The amounts used were 30,50,100,150,200 and 250 ng. Table 4 shows the areas for each amount. The standard curve for aldosterone is presented in Figure 17. The standard curve appears linear to about 150 ng aldosterone, which differs from Komara's[30] results, which were linear to 267 ng aldosterone. This could be due to problems encountered with the injector,including a damaged O-ring seal. The difference in wavelength used (240 nm vs 254 nm) could also be a contributing factor. When doing a determination on urine specimens, the standard curve will be used to calculate the amount of steroid present in the sample.

A. Use of Sep-Pak Cartridges

A problem encountered early in the research involved the methanol used. It appeared that upon evaporation of 2-3 mL of methanol,the residue contained a substance which interfered with the aldosterone peak. Through various procedures, it was found that the unidentified

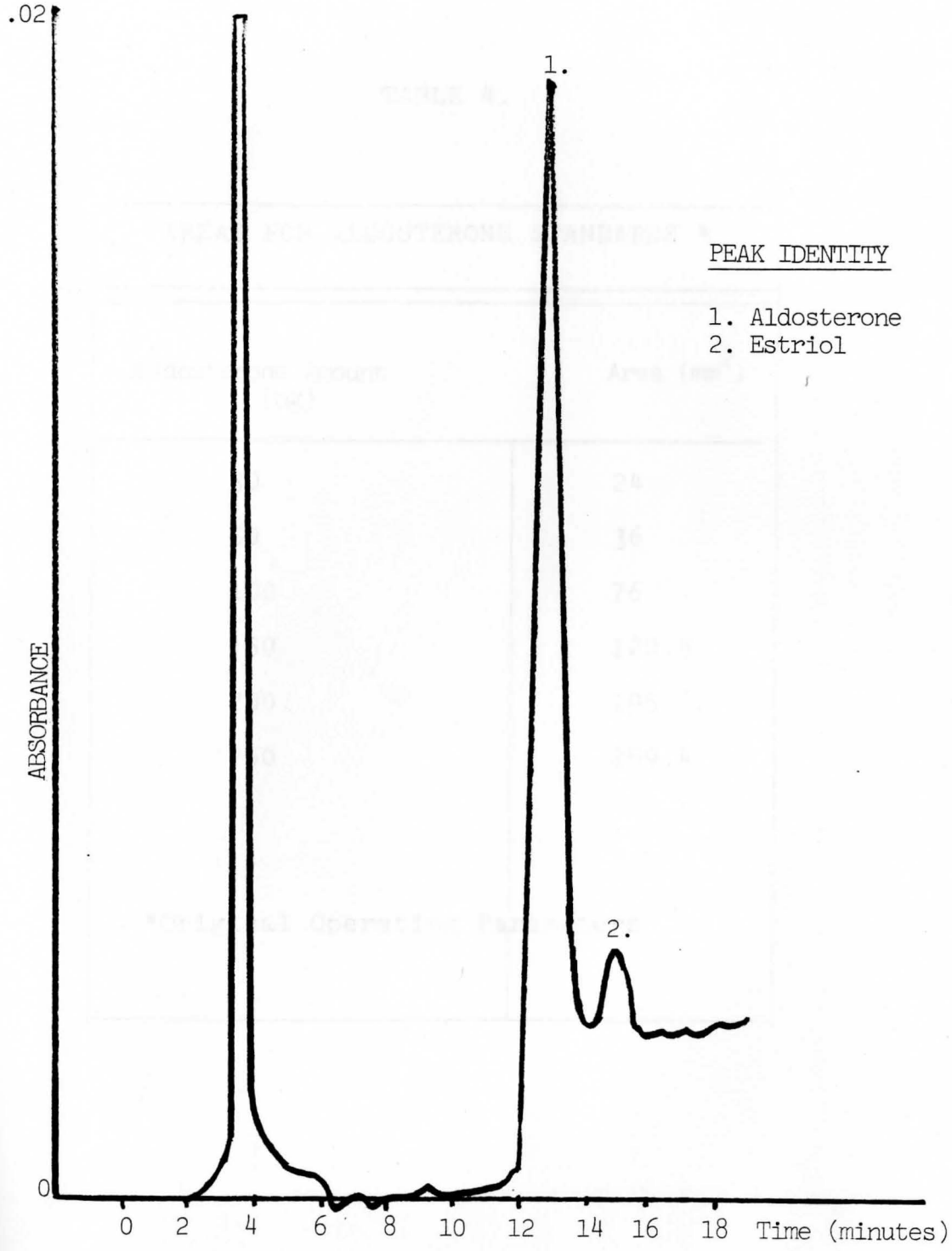


Figure 16.-- Chromatogram of 7 µL of 1:1 mixture of aldosterone and estriol. Column: µBondapak C_{18}; Mobile phase: Methanol-water (55:45,v/v); Temperature: Ambient; Flowrate: 0.5 mL/minute; Detector: UV (240 nm); Sensitivity: .02 AUFS; Chart Speed: 1 cm/minute.

TABLE 4.

AREAS FOR ALDOSTERONE STANDARDS *	
Aldosterone Amount (ng)	Area (mm^2)
30	24
50	36
100	76
150	120.5
200	195
250	259.4

*Original Operating Parameters

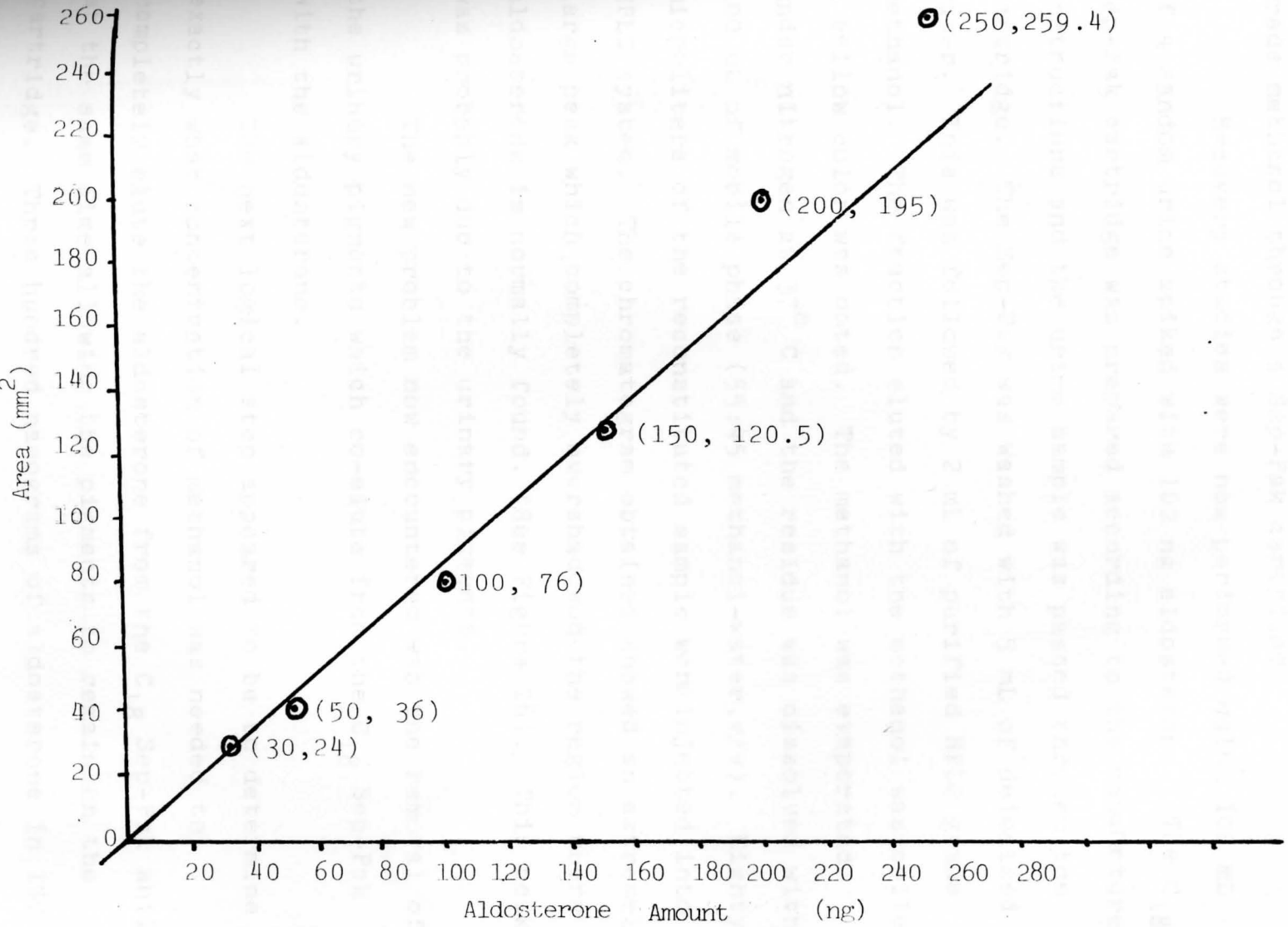


FIGURE 17.-- ALDOSTERONE STANDARD CURVE. Column: μBondapak C_{18}; Mobile phase: 55% aqueous methanol; Flowrate: 1.0 mL/minute; Detector: UV (240 nm) Sensitivity: .05 AUFS; Chart Speed: .05 cm/minute.

interfering substance could be removed by passing the HPLC grade methanol through a Sep-Pak cartridge.

Recovery studies were now performed using 100 mL of a random urine spiked with 100 ng aldosterone. The C_{18} Sep-Pak cartridge was prepared according to the manufacturer's instructions and the urine sample was passed through the cartridge. The Sep-Pak was washed with 5 mL of deionized water. This was followed by 2 mL of purified HPLC grade methanol. The fraction eluted with the methanol was collected. A yellow color was noted. The methanol was evaporated under nitrogen at 37° C and the residue was dissolved with 100 uL of mobile phase (55:45 methanol-water,v/v). Eighty microliters of the reconstituted sample were injected into the HPLC system. The chromatogram obtained showed an extremely large peak which completely overshadowed the region where aldosterone is normally found. (See Figure 18). This peak was probably due to the urinary pigments.

The new problem now encountered was the removal of the urinary pigments which co-elute from the C_{18} Sep-Pak with the aldosterone.

The next logical step appeared to be to determine exactly what concentration of methanol was needed to completely elute the aldosterone from the C_{18} Sep-Pak while at the same time allowing the pigments to remain on the cartridge. Three hundred nanograms of aldosterone in 100 mL of deionized water were passed through a prepared C_{18} Sep-Pak. The same cartridge was then serially eluted

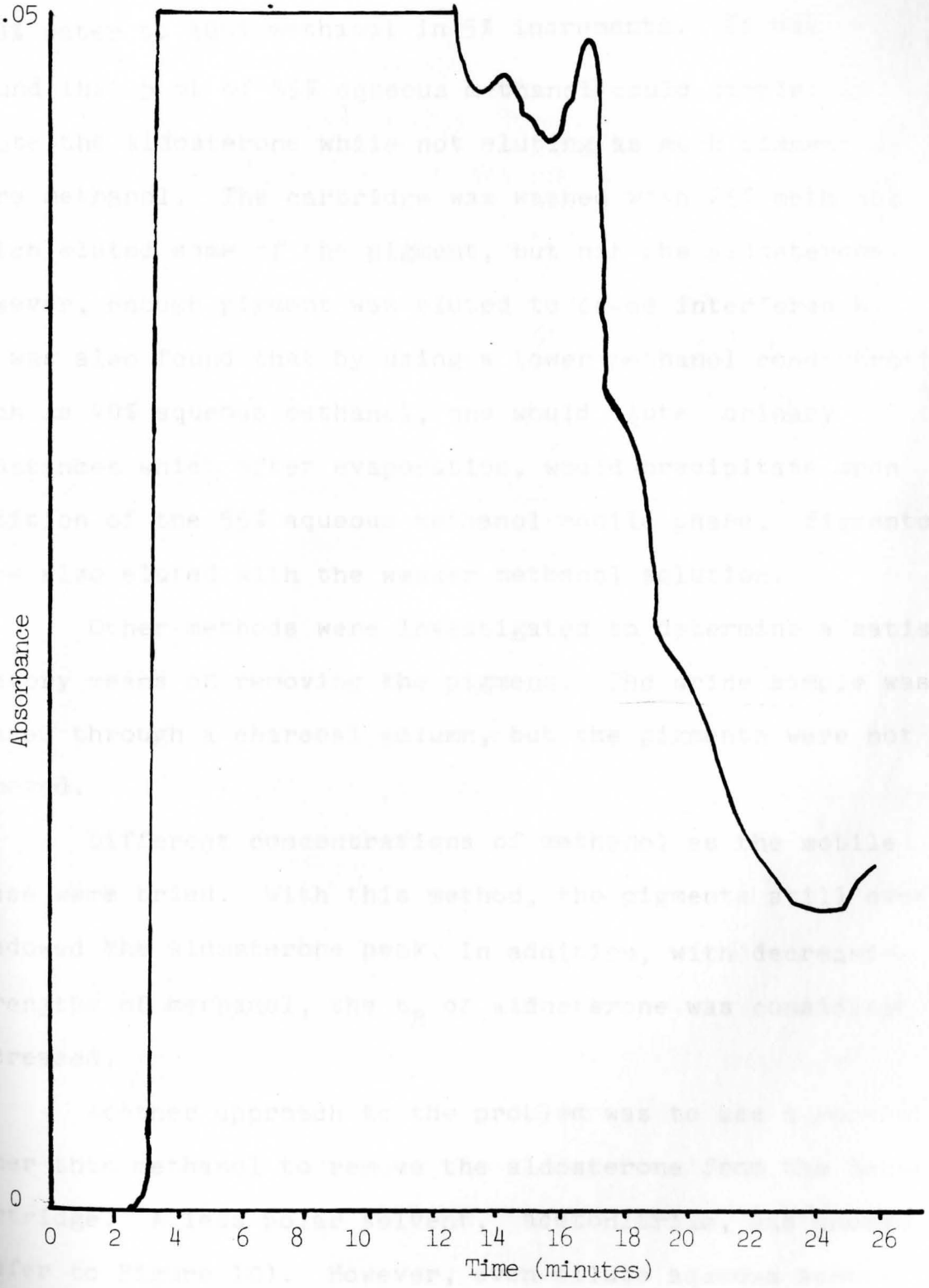


FIGURE 18.-- Chromatogram of C_{18} Sep-Pak treated urine. Column: μBondapak C_{18}; Mobile phase: Methanol-water (55:45,v/v); Flowrate: 1.0 mL/minute; Detector: UV (240 nm); Sensitivity: .05AUFS; Chart Speed: 05 cm/minute.

by varying concentrations of methanol-water, ranging from 100% water to 100% methanol in 5% increments. It was found that 5 mL of 55% aqueous methanol could completely elute the aldosterone while not eluting as much pigment as pure methanol. The cartridge was washed with 25% methanol which eluted some of the pigment, but not the aldosterone. However, enough pigment was eluted to cause interference. It was also found that by using a lower methanol concentration, such as 40% aqueous methanol, one would elute urinary substances which after evaporation, would precipitate upon addition of the 55% aqueous methanol mobile phase. Pigments were also eluted with the weaker methanol solution.

Other methods were investigated to determine a satisfactory means of removing the pigment. The urine sample was passed through a charcoal column, but the pigments were not removed.

Different concentrations of methanol as the mobile phase were tried. With this method, the pigments still overshadowed the aldosterone peak. In addition, with decreasing strengths of methanol, the t_R of aldosterone was considerably increased.

Another approach to the problem was to use a solvent other than methanol to remove the aldosterone from the Sep-Pak cartridge. A less polar solvent, acetonitrile, was chosen. (Refer to Figure 10). However, even dilute aqueous acetonitrile eluted sufficient pigment to cause interference with the chromatogram. Another less polar solvent, methylene

chloride, was used in the same fashion. Again, some pigment was removed from the cartridge so as to completely obliterate the aldosterone peak when chromatographed.

Another method for obtaining resolution is to use a different type of column packing. It was decided that an attempt would be made to separate pigments from aldosterone by utilizing a phenyl column. μBondapak phenyl has been used in steroidal applications.[41] This packing is made by chemically bonding a phenyl group to μPorasil at 10 % by weight. It is a reverse-phase packing. It is used when samples have strong polar groups.

The phenyl column was positioned prior to the C_{18} column, thereby increasing N and hopefully producing separation. This procedure did not accomplish the resolution of the aldosterone and pigment peaks.

Sephadex LH-20 was used in the attempt to remove urinary pigment prior to HPLC. Two slurries were prepared, using pure methanol in one and pure methylene chloride as the solvent for the other. Again, in both instances, resolution between aldosterone and urinary pigments was not obtained.

Waters Associates produces silica Sep-Pak in addition to C_{18} Sep-Pak. It was decided to attempt to remove the urine pigments by incorporating the silica Sep-Pak into the extraction procedure.

Ten (10) mL of acid hydrolyzed random urine was passed through a prepared C_{18} Sep-Pak, which was then washed with 10 mL of water. Then pure methanol was passed through the mini - column. The eluant was collected in a siliconized tube and in turn was passed

through a silica Sep-Pak. The pigments have a greater affinity for the methanol than for the silica packing and hence are not removed from the methanol eluent.

Elution was attempted using a different solvent, pure methylene chloride. Aldosterone is very soluble in methylene chloride. The silica Sep-Pak was prepared by passing 15 mL of methylene chloride through it, as suggested by the Waters Company. The procedure undertaken was as follows: 10 mL of hydrolyzed urine was passed through a prepared C_{18} Sep-Pak. It was washed with 10 mL of water. This was followed by the addition of 3 mL of HPLC grade methylene chloride. Two layers of solvents result, with the aqueous solution overlaying the methylene chloride. Care must be taken to remove all of the top layer before passing the lower, organic layer through the silica Sep-Pak because water can inactivate the silica packing. When the water-free eluent was passed through a silica Sep-Pak, the resulting solution was pigment-free. Hence, the pigments have a greater affinity for the silica than for the methylene chloride.

Once it was recognized that the interfering pigments could be removed, the same procedure as desribed was performed again. This time, however, 2 urine samples were used, one spiked with 250 ng of aldosterone and the other unspiked. The resulting samples were evaporated under N_2 at 37°C and reconstituted with 100 µL of mobile phase. The operating parameters were those originally used. It was discovered that

upon performing HPLC on the samples, there was an extremely large peak eluting at the same time as the aldosterone. When an evaporated residue of methylene chloride was reconstituted with mobile phase, which was then chromatographed, this large peak was present. The peak was determined to be caused by a methylene chloride contaminant.

The next step undertaken was to change the mobile phase so that good separation could be obtained. Instead of the 55% methanol, 50:50 acetonitrile-water was used. Acetonitrile is less polar than methanol. Due to this, the aldosterone should be eluted at a different time than the methylene chloride contaminant, and upon experimentation, this was found to be the case. The rest of the operating parameters remained the same. Under these conditions, the t_R of aldosterone was found to be approximately 3.8 minutes. There was no interference from the methylene chloride contaminant. (See Figure 19). However, it was found that with this mobile phase, there was poor resolution between aldosterone and prednisolone, such that the prednisolone could no longer be used as the internal standard. A new internal standard utilized would have to have a polarity similar to aldosterone; it addition, it should not normally be found in human urine. The internal standard should also absorb in the ultraviolet region. Most importantly, it must have a distinctive peak from aldosterone when chromatographed.

A recovery study was undertaken to determine the efficiency of the system. Two hundred nanograms of aldosterone were added to 10 mL of water. This was passed through a

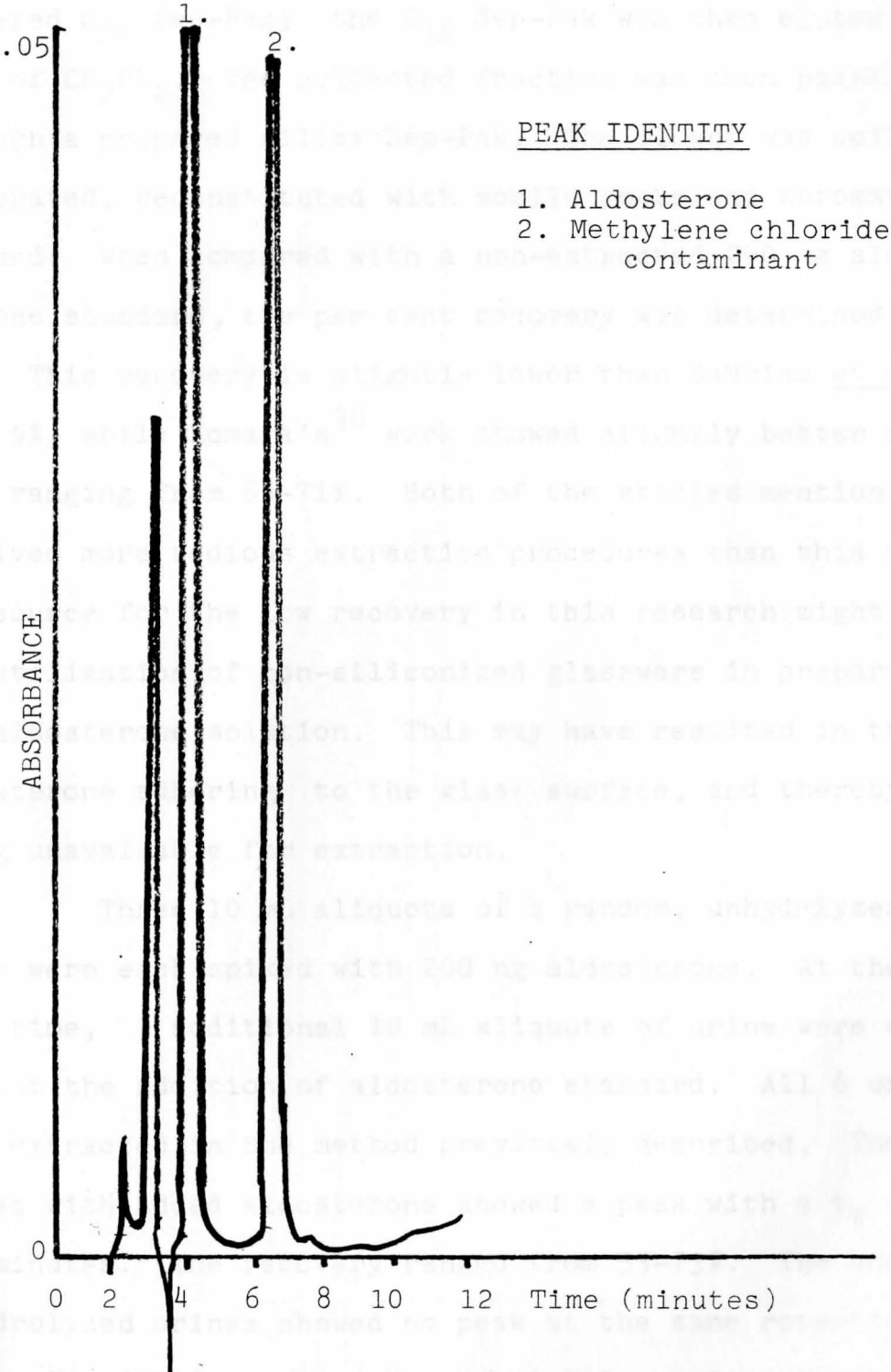


Figure 19.-- Chromatogram of aldosterone eluted with methylene chloride. Column: μBondapak C_{18}; Mobile phase: Acetonitrile-water (50:50,v/v); Flowrate: 1.0 mL/minute; Detector: UV (240 nm); Sensitivity: .05 AUFS; Chart Speed: .5 cm/minute.

prepared C_{18} Sep-Pak; the C_{18} Sep-Pak was then eluted with 3 mL of CH_2Cl_2. The collected fraction was then passed through a prepared silica Sep-Pak. The eluent was collected, evaporated, reconstituted with mobile phase and chromatographed. When compared with a non-extracted 200 ng aldosterone standard, the per cent recovery was determined to be 59%. This recovery is slightly lower than DeVries et al[25], 63 ± 9%, while Komara's[30] work showed slightly better recoveries, ranging from 64-71%. Both of the studies mentioned involved more tedious extraction procedures than this research. One source for the low recovery in this research might be the utilization of non-siliconized glassware in preparing the aldosterone solution. This may have resulted in the aldosterone adhering to the glass surface, and thereby, being unavailable for extraction.

Three 10 mL aliquots of a random, unhydrolyzed urine were each spiked with 200 ng aldosterone. At the same time, 3 additional 10 mL aliquots of urine were extracted without the addition of aldosterone standard. All 6 urines were extracted in the method previously described. The 3 urines with added aldosterone showed a peak with a t_R of 4.1 minutes. The recovery ranged from 53-73%. The unspiked, unhydrolyzed urines showed no peak at the same retention time. In addition, there was absolutely no interference from the urinary pigments with this method. (See Figures 20 and 21). By combining the sequential utilization of C_{18} and silica Sep-Pak with appropriate eluting solvents, one is able

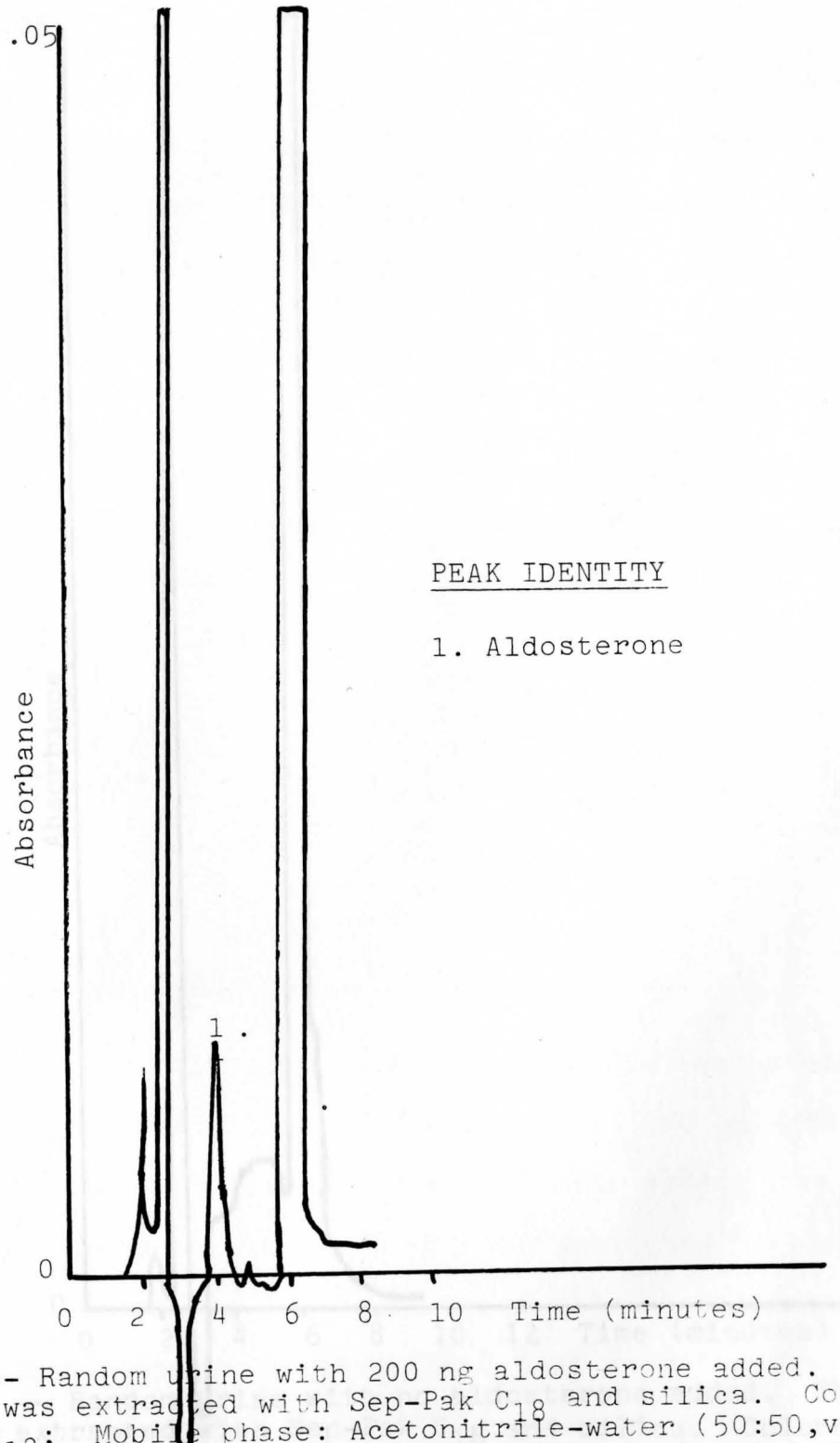


FIGURE 20.-- Random urine with 200 ng aldosterone added. The sample was extracted with Sep-Pak C_{18} and silica. Column: μBondapak C_{18}; Mobile phase: Acetonitrile-water (50:50,v/v); Flowrate: 1.0mL/minute; Detector: UV (240 nm); Sensitivity: .05 AUFS; Chart Speed: 05 cm/minute.

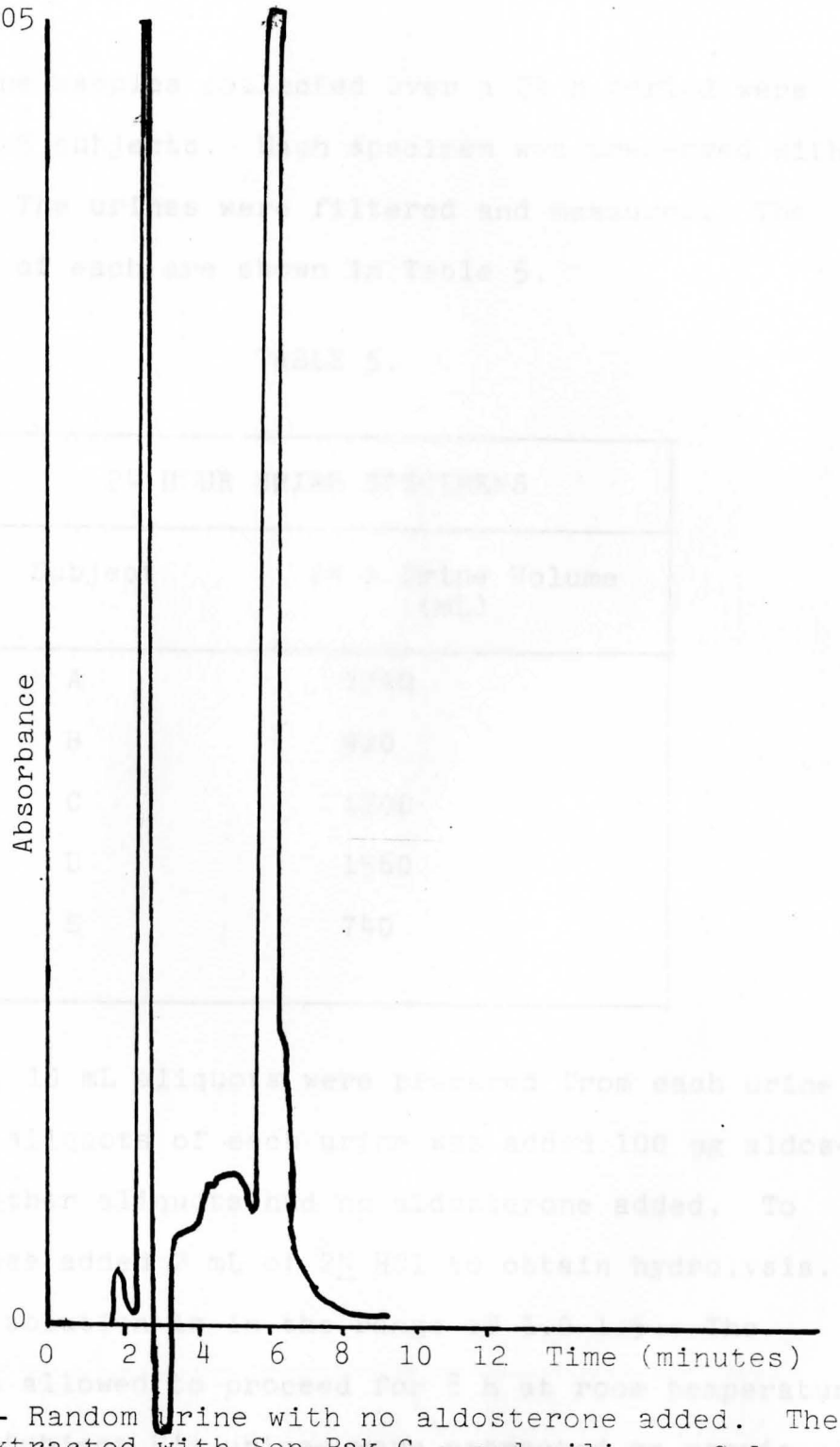


FIGURE 21. -- Random urine with no aldosterone added. The sample was extracted with Sep-Pak C_{18} and silica. Column: μBondapak C_{18}; Mobile phase: Acetonitrile-water (50:50,v/v); Flowrate: 1.0 mL/minute; Detector: UV (240 nm); Sensitivity: .05 AUFS; Chart Speed: 0.5 cm/minute.

to remove any contaminating pigments with minimum time and effort.

Urine samples collected over a 24 h period were obtained from 5 subjects. Each specimen was preserved with 1 gm borate. The urines were filtered and measured. The total volumes of each are shown in Table 5.

TABLE 5.

24 HOUR URINE SPECIMENS	
Subject	24 h Urine Volume (mL)
A	1760
B	920
C	1200
D	1560
E	740

Two, 10 mL aliquots were prepared from each urine. To one of the aliquots of each urine was added 100 ng aldosterone. The other aliquots had no aldosterone added. To each aliquot was added 2 mL of 2N HCl to obtain hydrolysis. The pH of the solution is in the range of 1.0-1.5. The hydrolysis was allowed to proceed for 8 h at room temperature. Subject D and Subject E's urines were extracted as previously described. There was no interference from urinary pigments. (See Figures 22-25). Neither spiked nor unspiked hydrolyzed urine samples exhibited an aldosterone peak (t_R = 4.1 minutes).

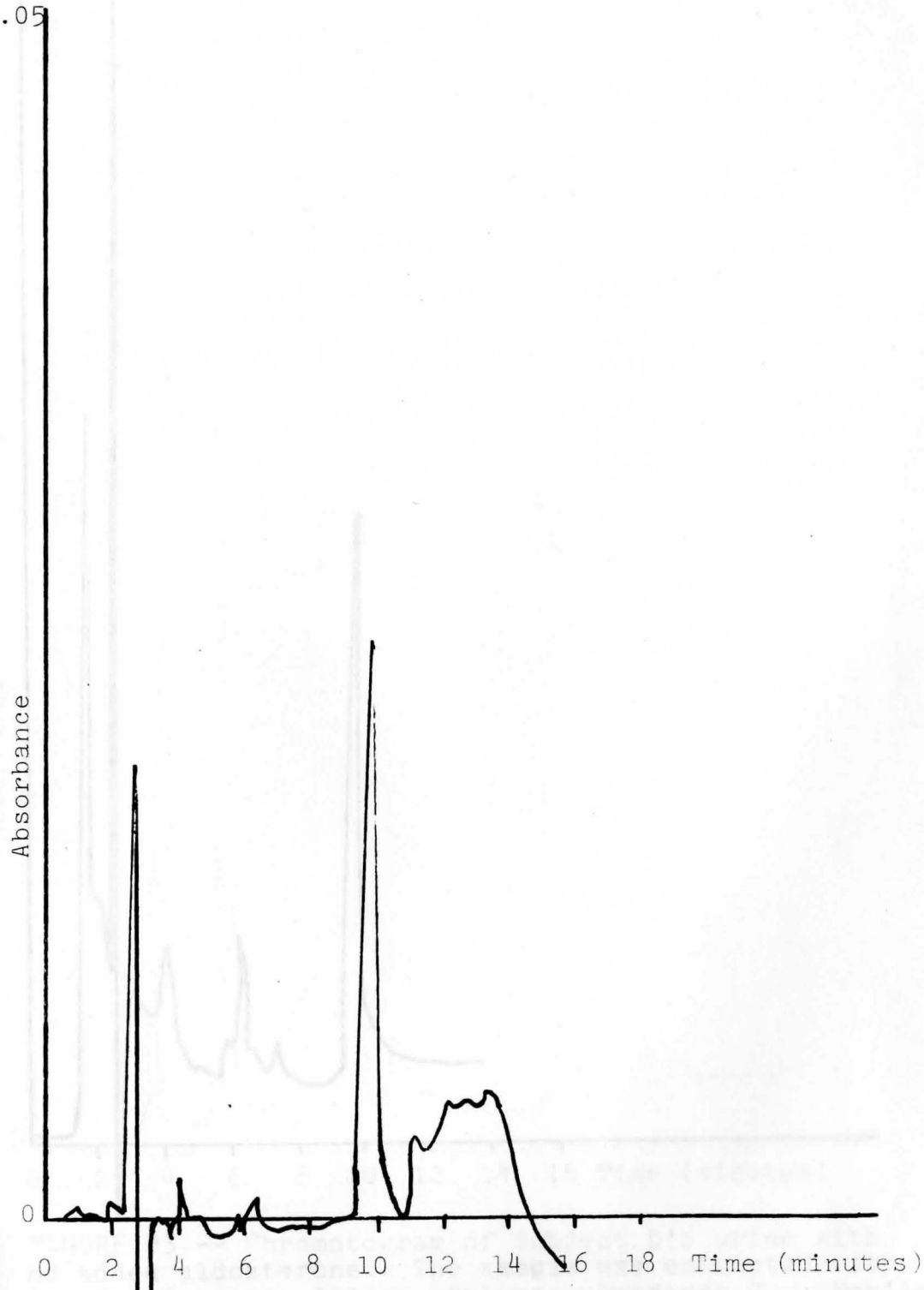


FIGURE 22.-- Subject D's urine with 100 ng aldosterone added. The sample was extracted with Sep-Pak C_{18} and silica. Column: μBondapak C_{18}; Mobile phase: Acetonitrile water (50:50,v/v); Flowrate: 1.0 mL/minute; Detector: UV (240 nm); Sensitivity: .05 AUFS; Chart Speed: .5cm/minute.

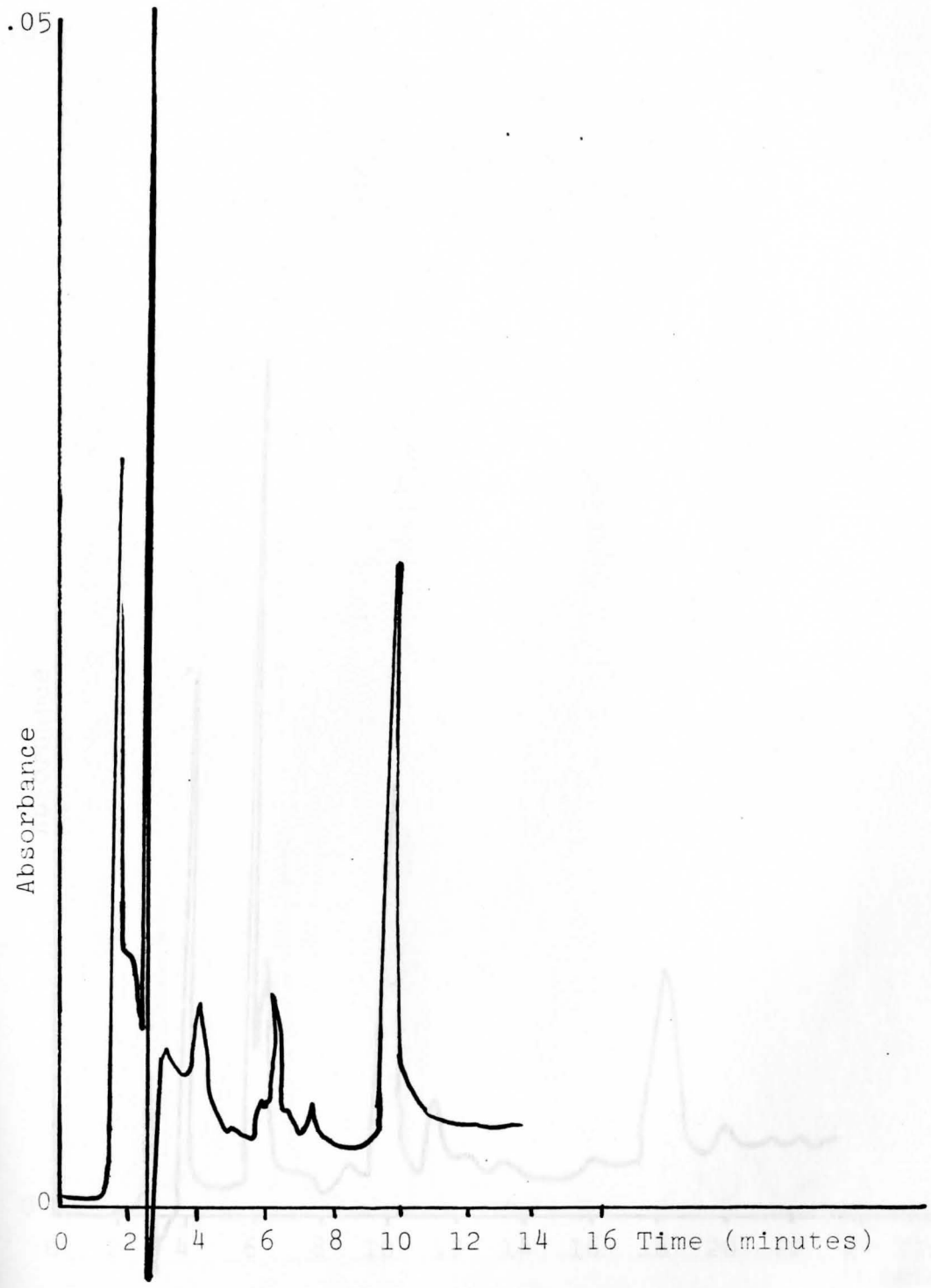


FIGURE 23.-- Chromatogram of Subject D's urine with no added aldosterone. The sample was extracted with Sep-Pak C_{18} and silica. Column: μBondapak C_{18}; Mobile phase: Acetonitrile-water (50:50,v/v); Flowrate: 1.0 mL/ minute; Detector: UV (240 nm); Sensitivity: .05 AUFS; Chart speed; .5cm/minute.

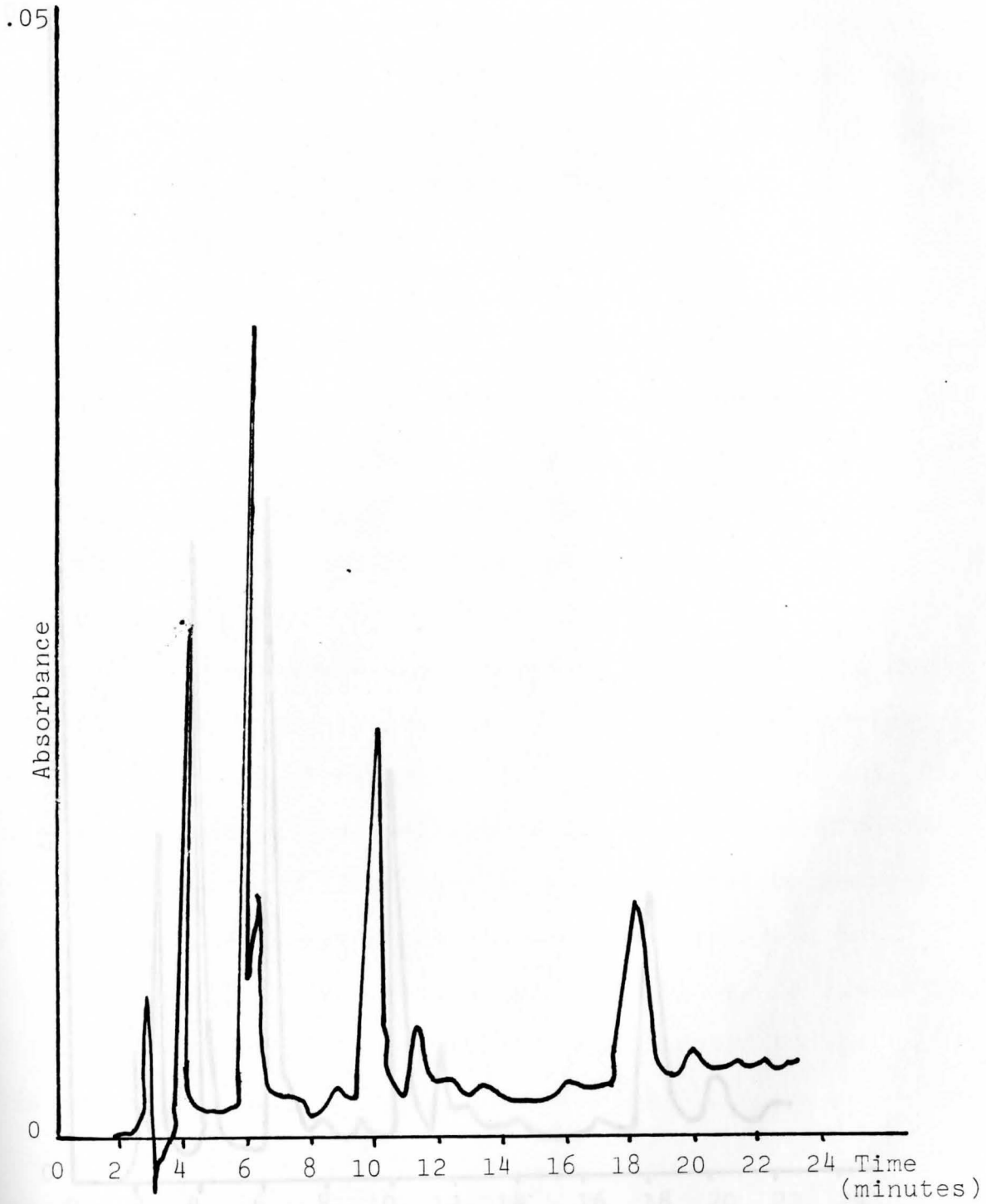


FIGURE 24.-- Chromatogram of Subject E's urine with 100 ng aldosterone added. The sample was extracted with Sep-Pak C_{18} and silica. Column: μBondapak C_{18}; Mobile phase: Acetonitrile-water (50:50,v/v); Flowrate:1.0 mL/minute; Detector: UV (240 nm); Sensitivity: .05 AUFS; Chart Speed: .5 cm/minute.

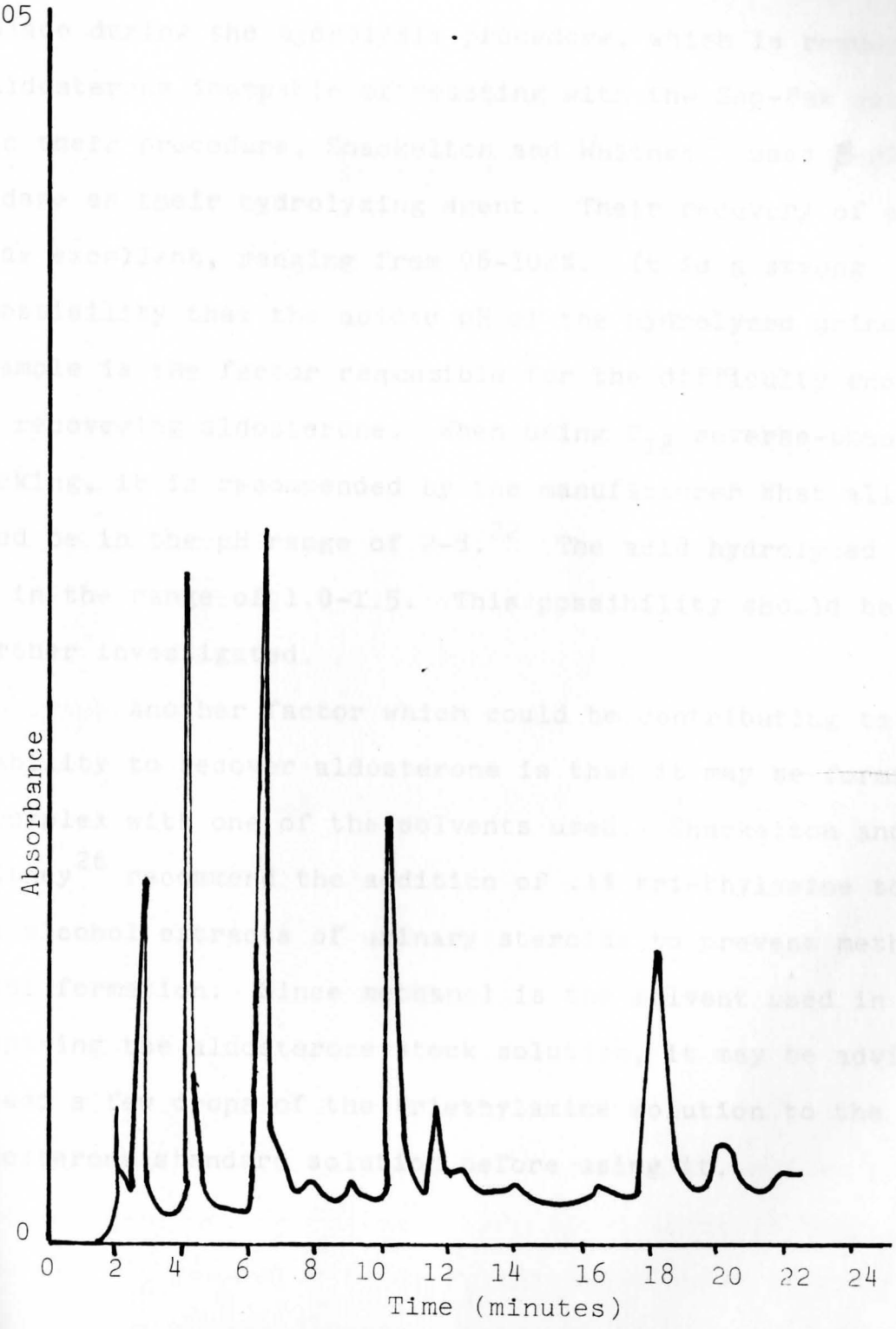


FIGURE 25.-- Chromatogram of Subject E's urine with no aldosterone added. The sample was extracted with Sep-Pak C_{18} and silica. Column: μBondapak C_{18}; Mobile phase: Acetonitrile-water (50:50,v/v); Flowrate: 1.0 mL/minute; Detector: UV (240 nm); Sensitivity: .05 AUFS; Chart Speed: .5 cm/minute.

Apparently, some unknown chemical reaction is taking place during the hydrolysis procedure, which is rendering the aldosterone incapable of reacting with the Sep-Pak cartridge. In their procedure, Shackelton and Whitney[26] used β-glucuronidase as their hydrolyzing agent. Their recovery of aldosterone was excellent, ranging from 96-102%. It is a strong possibility that the acidic pH of the hydrolyzed urine sample is the factor responsible for the difficulty encountered in recovering aldosterone. When using C_{18} reverse-phase packing, it is recommended by the manufacturer that all solutions used be in the pH range of 2-8.[22] The acid hydrolyzed urine is in the range of 1.0-1.5. This possibility should be further investigated.

Another factor which could be contributing to the inability to recover aldosterone is that it may be forming a complex with one of the solvents used. Shackelton and Whitney[26] recommend the addition of .1% triethylamine to all alcohol extracts of urinary steroids to prevent methyl ketal formation. Since methanol is the solvent used in preparing the aldosterone stock solution, it may be advisable to add a few drops of the triethylamine solution to the aldosterone standard solution before using it.

CHAPTER VI

CONCLUSIONS AND SUGGESTIONS FOR FURTHER RESEARCH

This investigation produced a simple and quick method for the removal of urinary pigments. With this procedure, one is able to produce an aldosterone peak when spiked, unhydrolyzed urine is extracted with C_{18} and silica Sep-Paks. However, the operating parameters utilized, pH of the system in particular, do not allow for the quantification of the aldosterone level. By increasing the pH to a more ideal range (i.e. 2-8), it is felt that more favorable results can be obtained.

A. Suggestions for Further Research

It is really difficult to determine exactly what is happening to the aldosterone in the urine during the hydrolysis, especially with the use of only a UV detector.

Perhaps aldosterone tagged with radioactive ^{3}H might be incorporated into future research. By utilizing labeled aldosterone, one might be able to determine whether or not the aldosterone in the acid-hydrolyzed urine is being held by the C_{18} Sep-Pak. Perhaps, because of some structural alteration during the hydrolysis, it is being bound by the silica Sep-Pak and thereby, not being eluted at all.

Another factor to be considered is the method by which hydrolysis of the urinary steroid conjugate is obtained. Acid hydrolysis was used in this research, and it was after this step in the procedure that difficulties arose in recovering aldosterone. DeVries et al[25] mention in their paper the appearance of a shoulder on the aldosterone peak of a urine extract. They attribute it to the effects of TLC on the aldosterone, stating it might be due to the shift in equilibrium between the tautomers of aldosterone. Perhaps it is due to the hydrolysis of the urine sample. If this is so, it is possible that the resulting tautomer would react differently, as in the case of the Sep-Pak extraction. Another means of hydrolyzing the urine sample, such as enzymatic hydrolysis, might be investigated. Whitney and Shackelton[24] use β-glucuronidase as their hydrolyzing agent, as do Scandrett et al[23]. Graef et al[15], however, report that incubation with β-glucuronidase results in steroid destruction, especially with long incubation periods.

If this would fail to resolve the problem, another factor to take into consideration is utilization of a different means of detection. It has been shown by previous work[24] that urinary aldosterone can be eluted from a C_{18} Sep-Pak cartridge using 2 mL of pure methanol. The only drawback with this is that urinary pigments are also eluted. If detection other than UV were employed, such as electrochemical or fluorometric, then perhaps, the interference by the non-aldosterone urinary substances could be eliminated.

Whigham [40] has utilized a technique where aldosterone is first oxidized, then analyzed fluorometrically by combining it with dilute sulfuric acid followed by the addition of methanol containing ferric chloride. It has also been suggested that steroids in general can be monitored by fluorescence after reacting it with Dansyl hydrazine.[41]

Another approach to determining aldosterone levels would be to utilize a specimen which would contain fewer interfering substances than urine, but which would simultaneously give information as to the in vivo production and metabolism of aldosterone. McVie et al[42] have done aldosterone analysis on human saliva. They demonstrated that there is good correlation between salivary aldosterone concentrations and urinary aldosterone excretion.

Another factor to be considered in the HPLC analysis of aldosterone is the effect of mobile phase. The DuPont Company has devised an automated instrument which allows one to improve chromatographic separation.[43] This 4-solvent system allows one to systematically optimize the composition of the mobile phase to achieve improved resolution of sample components. The solvents are chosen on the basis of their relative strength as proton acceptors, proton donors or dipole interaction. To find the ideal mobile phase composition, seven preliminary chromatographic experiments are run. By using 7 different mixtures of the 4 solvents, the best chromatographic resolution is obtained.[44]

Fast et al[45] have developed an optimization procedure which involves flow rate, column temperature and gradient

shape. This research utilized a variable-size simplex search algorithim. Simplex methods are those " in which a series of experiments is set up such that the reaction conditions for a given experiment are dictated by the results of the preceding experiments in that series."[45] The variables investigated were chosen because they affect separationby their effect on theoretical plates and capacity factor.

Another variable which can be investigated is the solvent which is used to elute interfering substances from the Sep-Pak cartridge prior to eluting aldosterone. In their work, involving a C_{18} mini-column, Zief et al[46] used acetone-water (20:80) to remove interfering substances from urine samples prior to HPLC. If undesirable components could be removed from the Sep-Pak cartridges in this manner, perhaps, the aldosterone could then be eluted with pure methanol.

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