THE DETERMINATION OF HUMAN URINARY ALDOSTERONE BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

by

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ABSTRACT

THE DETERMINATION OF HUMAN URINARY ALDOSTERONE BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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The clinical significance of the determination of steroid hormones and their metabolites in urine or in plasma is a means of evaluating adreno-cortical functions. The importance of measuring aldosterone in a clinical laboratory lies in aiding the physician in the diagnosis and treatment of patients with excessive secretion of aldosterone.

Measurement of aldosterone presents a technical challenge because of the minute quantities of aldosterone normally present in body fluids.

The purpose of this investigation was to develop a prototype method for the determination of human urinary aldosterone by high-pressure liquid chromatography (HPLC).

The author proceeded to devise an HPLC method for routine clinical evaluation of aldosterone by attempting to eliminate as many of the tedious, manual time-consuming, extraction and separation steps as possible.

The preliminary steps of this procedure are modifications of the gas-liquid chromatography methods as described by Zack, et al[33] and Bravo[34]

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The specimens for the analyses were 24-h urine collections.

Extraction, purification and separation, and finally quantification are carried out on duplicate 100-mL aliquots of a 24-h urine specimen.

By varying the parameters of reverse-phase HPLC, a method for the determination of human urinary aldosterone was developed.

The chromatograms recorded, of pure aldosterone standard and pure steroid standards which might interfere with aldosterone determination, are evidence that good resolution and symmetrical peaks can be obtained using reverse-phase HPLC.

Chromatograms of urine extracts were obtained, also, which showed a peak with an unresolved shoulder, at the same t_R as was obtained with pure aldosterone.

The loss of aldosterone was determined in triplicate by adding no aldosterone, 5 g, 26.7 g, and 53.4 g of pure aldosterone stock standard respectively, to 100-mL aliquots of 2x's glass-distilled water. Each 100-mL aliquot of water was then treated as a urine specimen, following all the required steps. The percent recovery was 64-71%.

Some modification of the procedure developed, plus further experimentation by varying HPLC parameters should produce a simple, rapid determination of urinary aldosterone by HPLC.

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation and thanks to Dr. Leonard B. Spiegel, my research advisor, for his help and guidance with this research project.

I wish also to express my appreciation and thanks to Dr. Peter W. Von Ostwalden and Dr. Francis W. Smith, for reading the manuscripts and for their many helpful suggestions.

Special thanks to my children and Dr. Gus Mavrigian for encouraging me to complete this research.

A very special thank you to my daughter, Mary Komara for reproducing the chromatograms and for typing the manuscript.

DEDICATION

This work is dedicated to the memory of my husband, John J. Komara, a unique human being, who while he lived, through his kindness, gentleness and understanding, was a constant source of strength and encouragement to me.

To my children, John David, James, Victor, Veronica, Matthew and Mary for their support and encouragement.

To my parents, Matej and Mary Hudak, from whom I inherited my strong Slovak ancestry, the strong desire to search for the truth and from whom I learned the values of hard work and self-discipline.

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LIST OF SYMBOLS

SYMBOL	DEFINITION
DOC	11-deoxycorticosterone
ACTH	Adrenocorticotropic Hormone
μg	Microgram
h	Hour
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NAD^+	Nicotinamide adenine dinucleotide (oxidized form)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
N	Normality
ng	Nanogram
mL	Milliliter
24-h	twenty-four hour
HPLC	High-Pressure Liquid Chromatography
TLC	Thin-layer chromatography
DMSO	Dimethylsulfoxide
t_R	Retention time
DHEA	Dehydroepiandrosterone
GLC	Gas-Liquid Chromatography
AUFS	Absorbance Units Full Scale
psig	pounds per square inch gauge
APA	Aldosterone-producing adreno-cortical adenoma
RIA	Radioimmunoassay

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CHAPTER I

INTRODUCTION

Aldosterone is a steroid hormone secreted by the adrenal cortex, a ductless gland of the endocrine system. Steroids are biologically active organic compounds that resemble cholesterol chemically and that contain a hydrogenated cyclopentanophenanthrene ring system. Hormones are chemical compounds formed in the ductless glands of the endocrine system, secreted into the blood or tissue fluids and transported to other parts of the body where they act upon target tissues or organs. The glands of the endocrine system exert their activity as an integrated system rather than as separate glands; impairment of the function of one gland may cause changes in function or response of other glands.

The endocrine system consists of the following glands: anterior and posterior pituitary, pineal body, thyroid, parathyroid, thymus, adrenal cortex and medulla, pancreatic islets of Langerhans, and gonads (testes, ovaries), and the placenta. (See Figure 1).[1]

One of the major functions of hormones is to maintain a dynamic steady state of the chemical composition of plasma and interstitial and intracellular fluids for the life and growth of the organism. Hormones act as regulators of

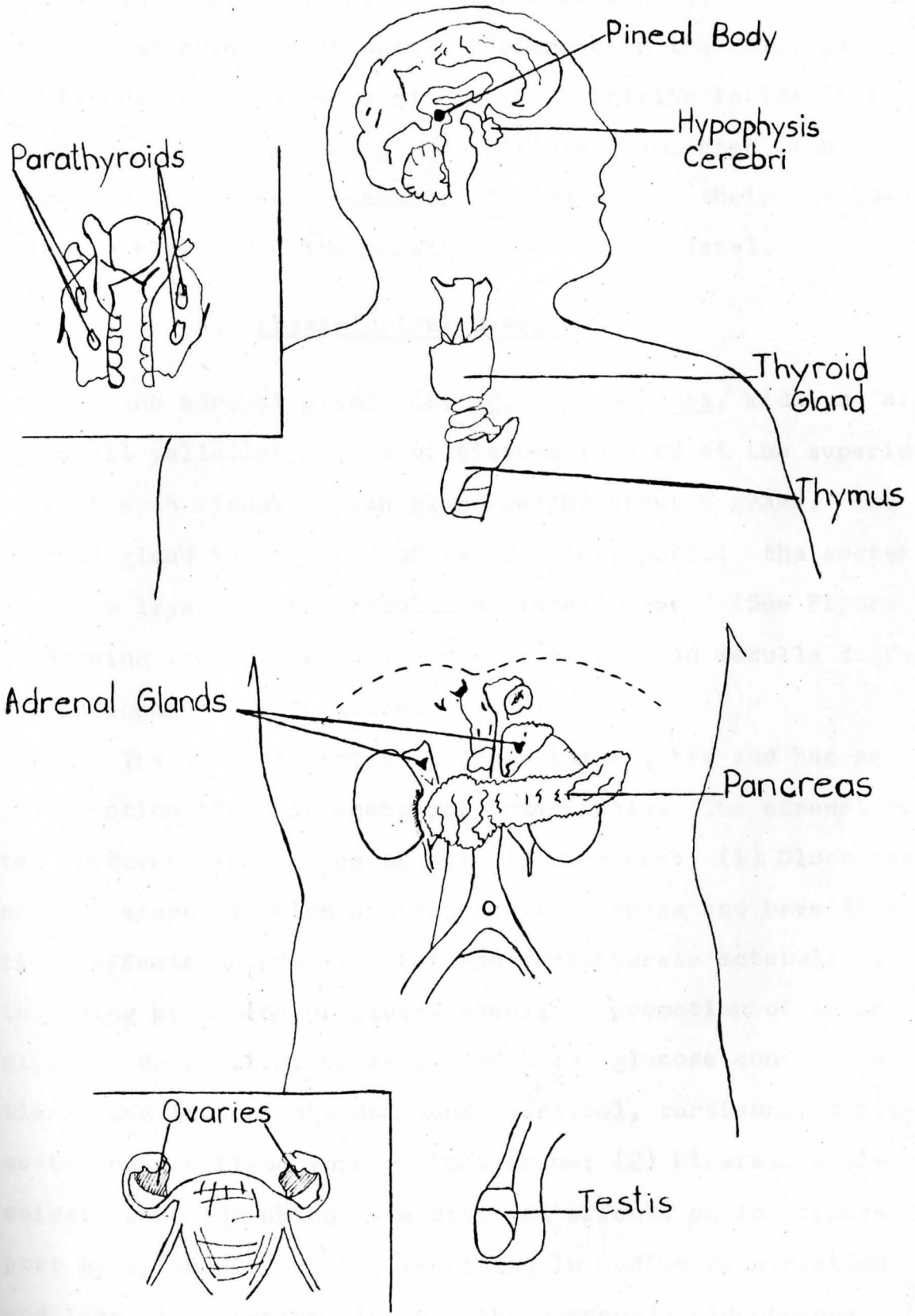


Figure 1. Endocrine glands. (Approximate location in man.)[1]

metabolism, reproduction, and stress responses.[2]

Endocrine measurements performed in the clinical laboratory are a valuable aid to the clinician in identification of certain pathologic conditions associated with increased or decreased amounts of hormones or their metabolites in blood or urine relative to a normal level.

A. Physiological Background

The adrenal glands (L. ad, near + renes, kidneys) are two small yellowish masses of tissues located at the superior pole of each kidney. Each gland weighs about 6 grams. The adrenal gland is composed of two distinct parts: the cortex or outer layer and the medulla or inner layer. (See Figure 2, drawing from Figure 28-5).[3] The cortex and medulla differ in development and function.

The adrenal cortex is essential to life and has as its function the maintenance of homeostasis. The adrenal cortex produces three types of steroid hormones: (1) Glucocorticoids: steroids which protect against stress and have distinct effects on protein, fat and carbohydrate metabolism, including promotion of gluconeogenesis, promotion of liver glycogen deposition, elevation of blood glucose concentrations, and include the compounds cortisol, cortisone, corticosterone and 11-dehydrocorticosterone; (2) Mineralocorticoids: steroids which have distinct effects on ion transport by epithelial cells, resulting in sodium conservation and loss of potassium, include the compounds aldosterone

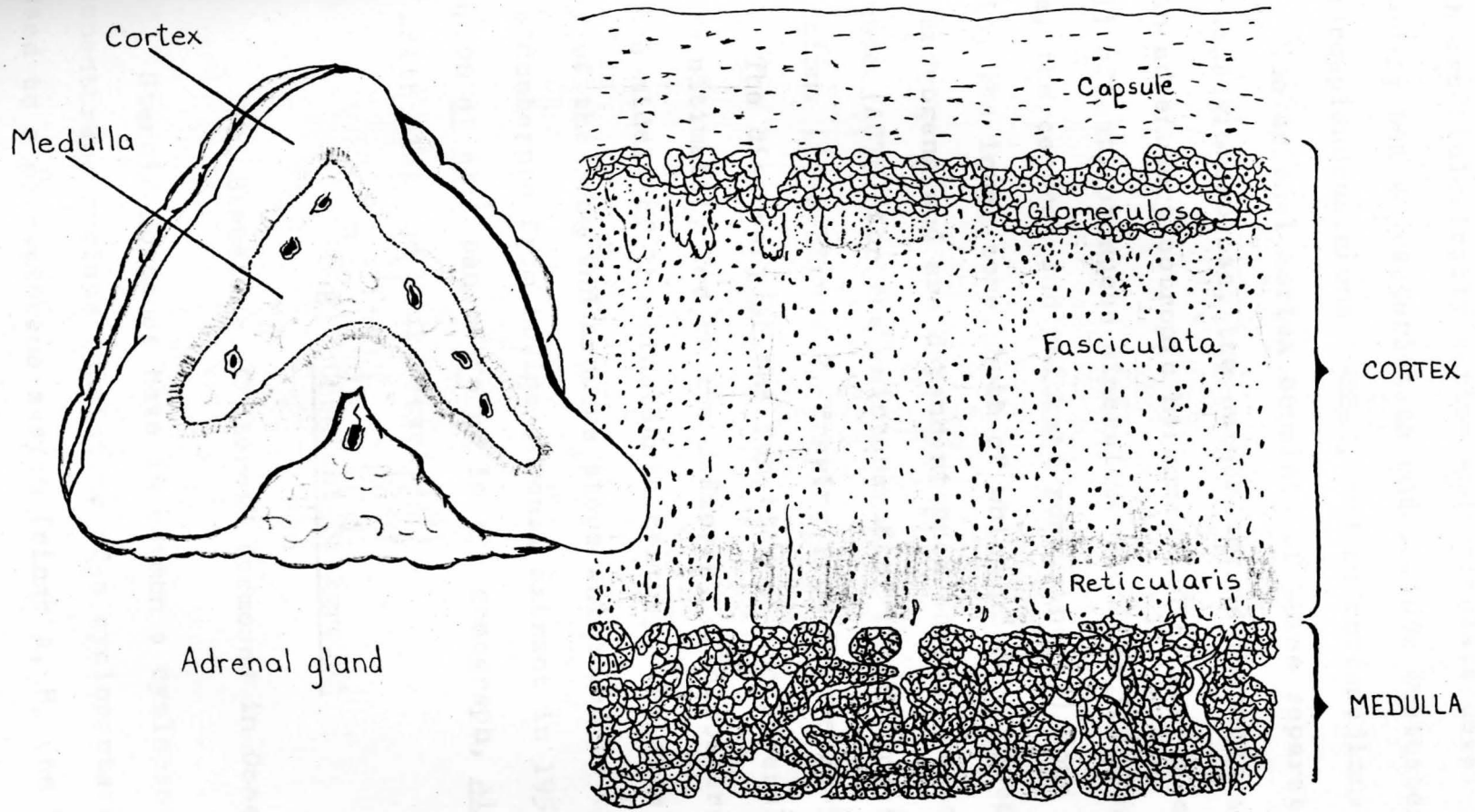


Figure 2. Microscopic structure of adrenal gland.[3]

and 11-deoxycorticosterone (DOC); (3) Androgens: steroids which are biologically active and stimulate development of secondary sex characteristics and include testosterone, dehydroepiandrosterone (DHEA), and androstenedione.[4,5]

The adrenal cortex consists of three separate zones: (1) Zona glomerulosa, the outermost and thinnest zone, upon which adrenocorticotropic hormones (ACTH) exerts only a small effect, produces mineralocorticoids; (2) Zona fasciculata, the central and thickest zone, and (3) Zona reticularis, the inner zone, both of which produce glucocorticoids and androgens and are dependent upon adrenocorticotropic hormone (ACTH) for the maintenance of their structure and function. (See Figure 2, drawing from Figure 28-5).[3]

The discovery of the function of the adrenal cortex which ultimately led to the discovery of aldosterone began in the middle of the nineteenth century. An excellent summary of the long and arduous steps which led to the discovery of aldosterone from bovine adrenal extract in 1953 by S. A. Simpson et al,[6] can be found in the monograph, Aldosterone, by Edith Glaz and Paul Vecsei.[7]

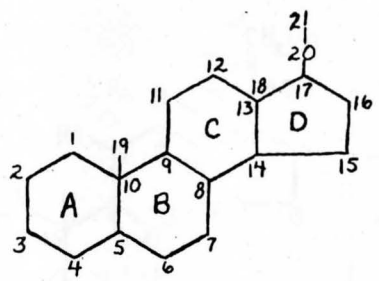
B. Chemical Background

1. Structure of Steroid Hormones in General

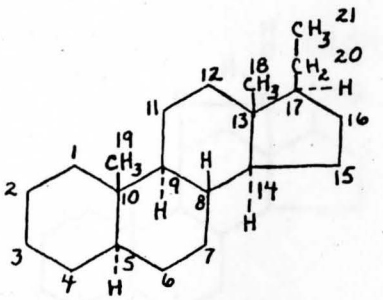
Steroid hormones have in common a cyclopentanoperhydrophenanthrene nucleus consisting of a cyclopentane ring (D) fused to a phenanthrene system (rings A, B, and C). The carbon atoms are identified by a standard numbering system,

by numbers 1 to 21. In the absence of functional groups, each carbon atom is bonded to hydrogen and/or carbon atoms. Unsaturated steroids have one or more double bonds. Angular methyl groups (18 and 19) are attached to carbon atoms 10 and 13. Substituent groups may be found in positions 3, 11, and 17. (See Figure 3).[8]

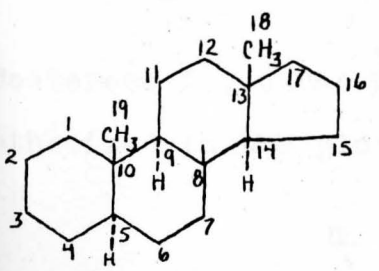
Steroid hormones may be classified and grouped according to the number of carbon atoms in the compounds; corticosteroids, including progesterones, have 21 carbon atoms; androgens, 19 carbon atoms; and estrogens, 18, carbon atoms. The parent hydrocarbon of each--respectively, pregnane, androstane, estrane--is used as the basis for systematic naming. (See Figure 3).[8] A detailed discussion on nomenclature can be found elsewhere.[9]



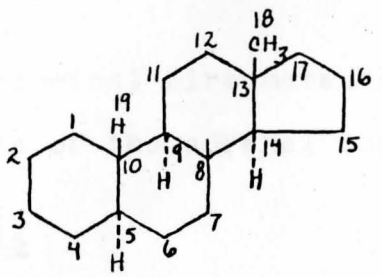
Steroid nucleus



5α-pregnane



5α-androstane

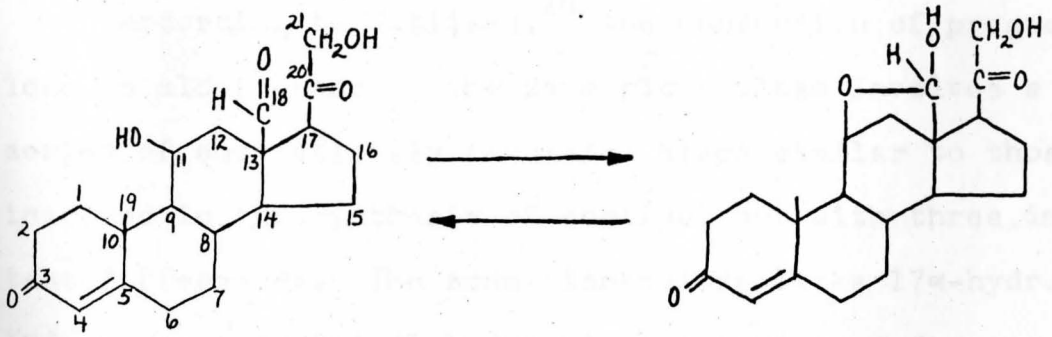


5α-estrane

Figure 3. Basic nucleus of steroid compounds.[8]

2. Aldosterone

Aldosterone, 11β,21-Dihydroxy-3,20-dioxopregnen-4-en-18-al, is the major and most potent mineralocorticoid that regulates electrolyte and water metabolism in the body. Aldosterone was crystallized from adrenal extracts by Tait, Simpson and their colleagues[10] in 1953 and, in 1954, they were successful in establishing the chemical formula. It was found that aldosterone, unlike other known corticosteroids, uniquely possessed an aldehyde group ($-C\overset{H}{\leq O}$) in position eighteen instead of the usual ($-CH_3$) group. Aldosterone was shown to have the following tautomeric structure with the equilibrium favoring the hemiacetal form:[11]



Aldehyde Hemiacetal

Aldosterone is, under normal physiological circumstances, synthesized in the glomerulosa cells of the adrenal cortex.

C. Biosynthesis

The major corticosteroids--viz., cortisol, corticosterone, and aldosterone in man--are secreted by the adrenals

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at the rate of approximately 10 to 30 mg, 2 to 4 mg, and 300 to 400 μg/24-h, respectively.[12]

According to Tietz,[13] the use of radioactive acetic acid and cholesterol for the study of in vivo and in vitro steroidogenesis has produced radioactive steroid hormones, lending support to the concept that both compounds are precursors of steroid hormones. The adrenal cortex is able to synthesize cholesterol as well as to remove it from the circulation. This cholesterol is then available for conversion into steroid hormones. Conversion of cholesterol into steroid hormones involves many enzymatically governed chemical reactions.

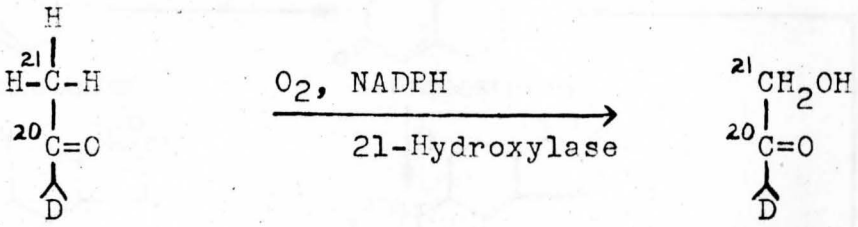
The principal steps involved in the major biosynthetic pathways for adrenal steroids may be seen in figure 4.[14]

According to Williams,[14] the conversion of pregnenolone to aldosterone by the zona glomerulosa involves a series of enzymatically regulated steps similar to those involved in the synthesis of cortisol but with three important differences. The zona glomerulosa lacks 17α-hydroxylase and proceeds to the 21-hydroxylase reaction, undergoes 11β-hydroxylation, and some of this steroid is acted on by 18-hydroxylase to form 18-hydroxycorticosterone, and then by 18-hydroxy-steroid dehydrogenase to form aldosterone. The enzyme, 18-hydroxysteroid dehydrogenase, is found only in the zona glomerulosa, and this is the reason that only the zona glomerulosa has the capacity to synthesize aldosterone.

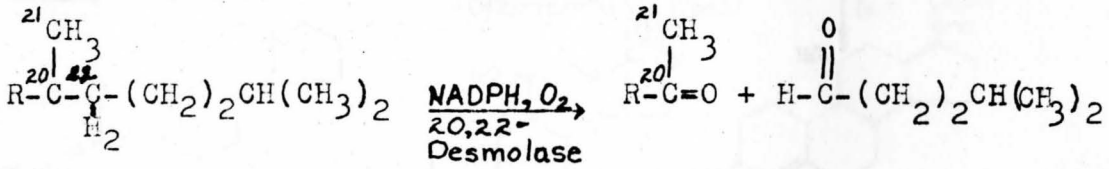
Tietz[15] broadly classified the different enzymes parti-

cipating in steroidogenesis into the following functional groups.

1. Hydroxylases. These enzymes catalyze the substitution of the hydroxyl group (-OH) for hydrogen (-H) and require besides oxygen, NADPH as cofactor. The reaction is irreversible:

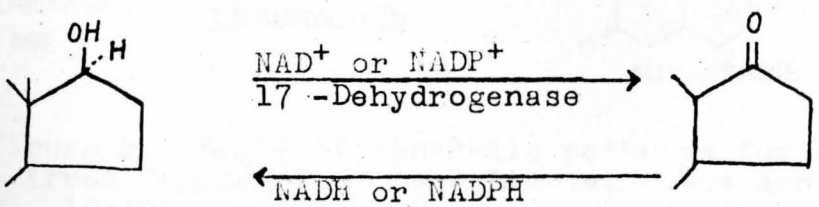


2. Desmolases. These enzymes split off the side chain, and again oxygen is required and NADPH is the required cofactor:



Cholesterol Pregnenolone Isocaproic aldehyde

3. Dehydrogenases. These enzymes catalyze the transfer of hydrogen (oxidation and reduction) and require as cofactor either NAD^+ or $NADP^+$ (oxidized or reduced form, depending on the direction of the reaction). The reaction is usually reversible:



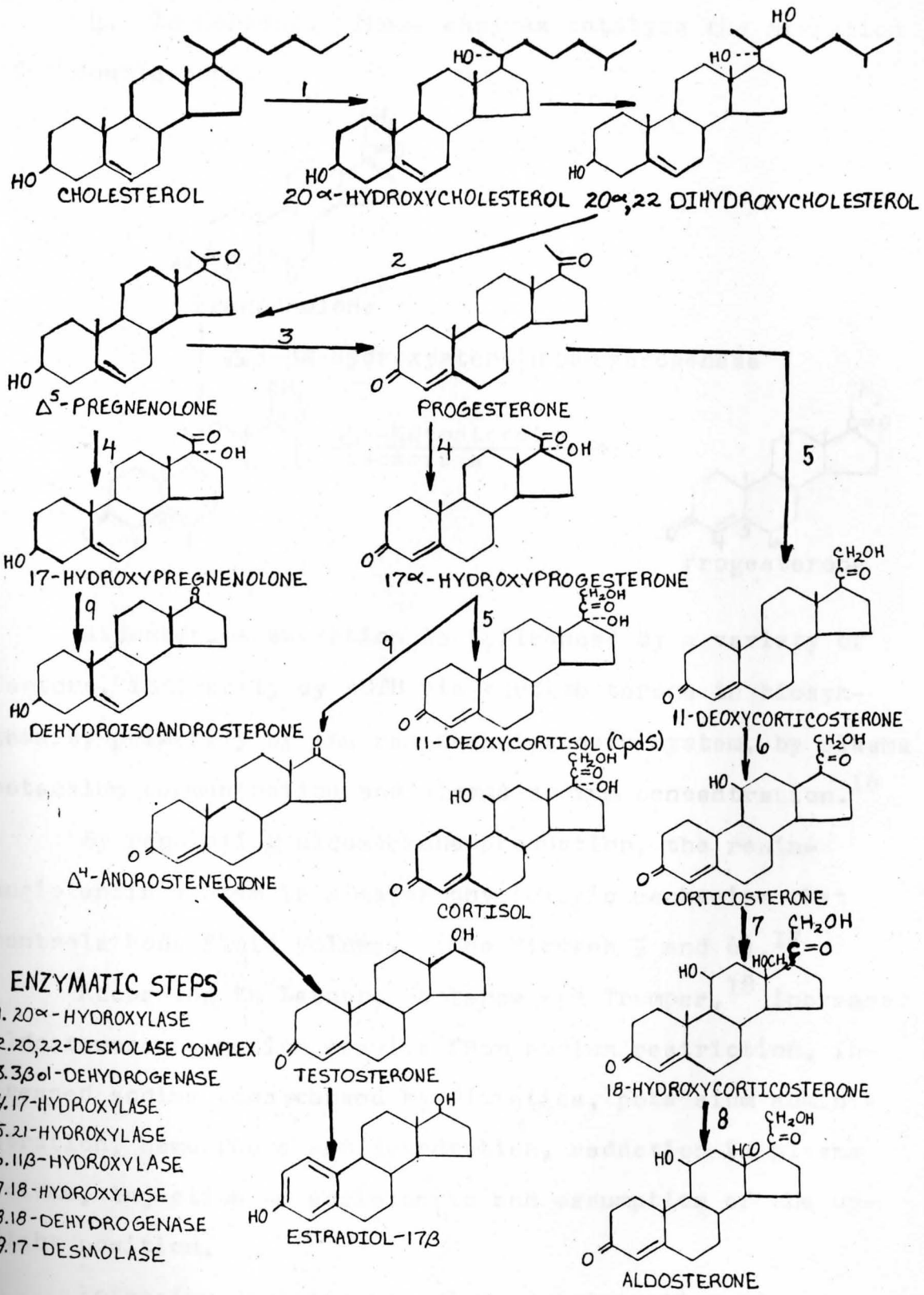
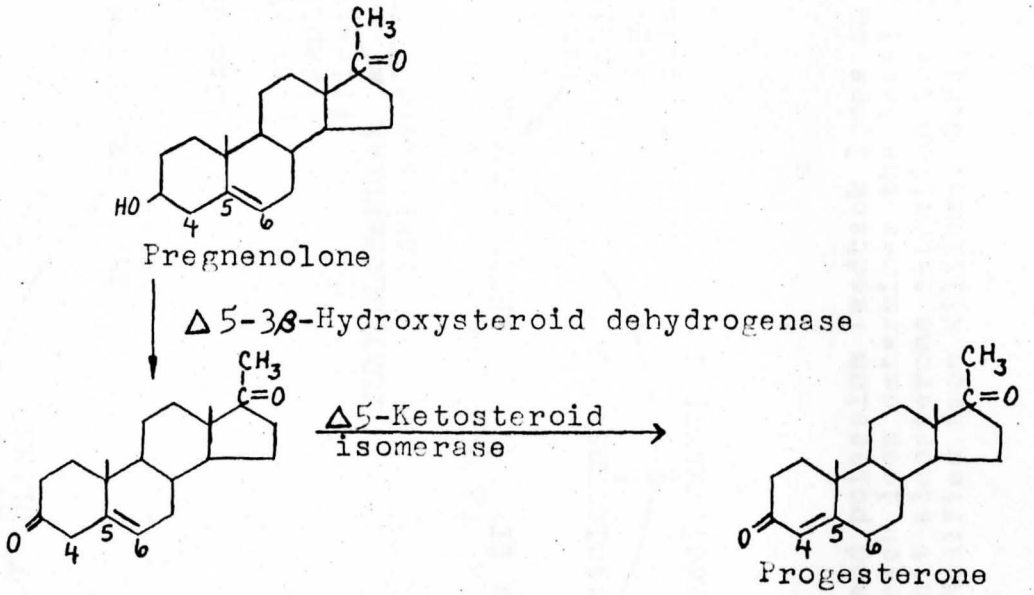


Figure 4. Major biosynthetic pathways for adrenal steroids, (From Temple, T.E., and Liddle, G.W.: Ann. Rev. Pharmacol. 10:199, 1970).[14]

4. Isomerases. These enzymes catalyze the migration of a double bond.



Aldosterone secretion is influenced by a variety of factors, indirectly by ACTH via corticosterone in biosynthesis, primarily by the renin-angiotensin system, by plasma potassium concentration and plasma sodium concentration.[16]

By regulating aldosterone production, the renin-angiotensin system is a major physiologic mechanism that controls body fluid volume. (See Figures 5 and 6).[17]

According to Latner, Cantarow and Trumper,[18] increased aldosterone secretion results from sodium restriction, increased sodium loss caused by diuretics, potassium administration, hemorrhage and dehydration, reduction in plasma volume, injection of angiotensin and assumption of the upright position.

Potassium depletion, sodium administration, increase in plasma volume and assumption of the horizontal position

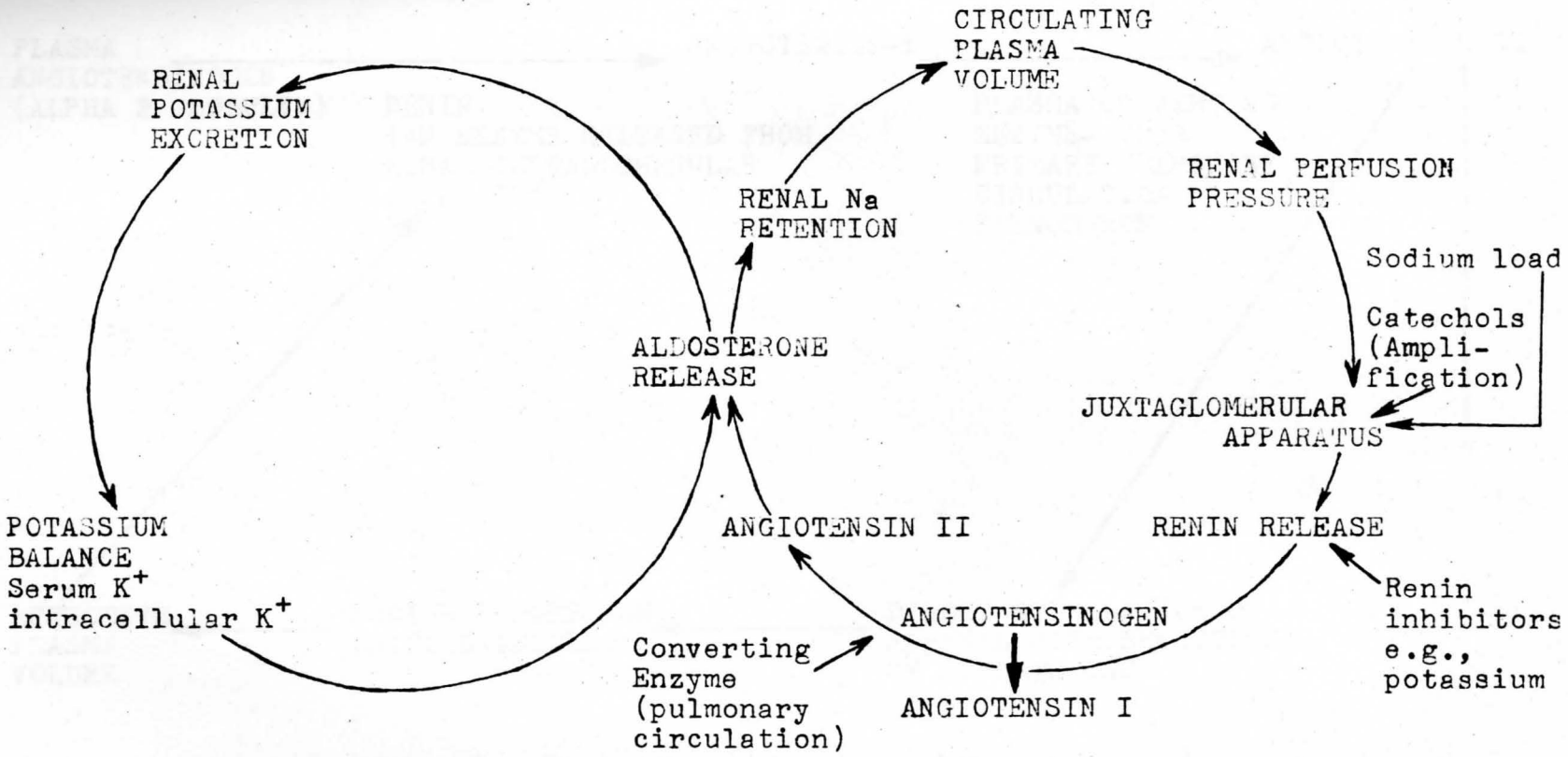


Figure 5. The interrelationship of the volume and potassium feedback loops on aldosterone secretion. Integration of signals from each loop determines the level of aldosterone secretion. Heparin and heparinoids inhibit aldosterone secretion through cytochemical alterations in the zona glomerulosa. (Modified from Williams, G.H., and Dluhy, R.G.: Amer. J. Med. 53:603, 1972.)[16]

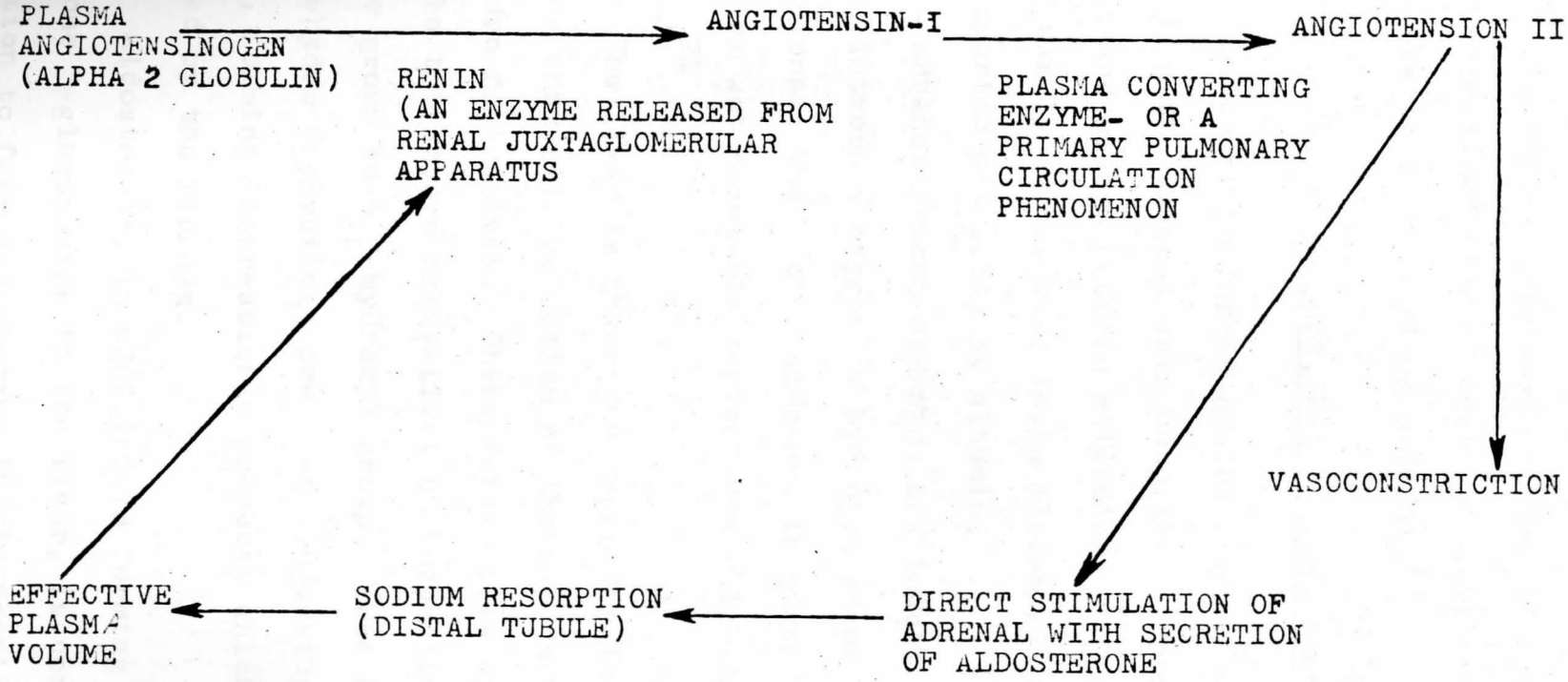


Figure 5. The renin-angiotensin system and aldosterone.[16]

results in decreased aldosterone secretion.

The renin-angiotensin system is believed to be the predominant mediator of changes in aldosterone secretion in response to these various stimuli.[19]

D. Transport, Metabolism, Excretion

Aldosterone formed by the adrenal cortex is quickly bound in the adrenal vein onto the surface of red blood cells and plasma protein molecules.[20] Aldosterone binds only weakly to transcortin, a plasma glycoprotein and is transported primarily by albumin.

Adrenocortical steroids are inactivated by enzymes which introduce oxygen or hydrogen atoms at one or more positions. These compounds are then conjugated in the liver to form water-soluble derivatives which are excreted in the urine.[21]

The liver is the major organ for inactivating steroids by the enzymatic reduction of the 4-5 double bond to form the dihydro derivatives. These derivatives are quickly reduced to the tetrahydro derivatives by enzymatic reduction of the 3-oxo group to a 3-hydroxyl group. (See Figure 7.)[22] The tetrahydro derivatives are then conjugated with glucuronic acid, forming water-soluble products which are quickly excreted by the kidneys.

Aldosterone, in addition to forming tetrahydroaldosterone 3-glucuronide in the liver, is conjugated at the 18 position to form aldosterone 18-glucuronide both in the liver

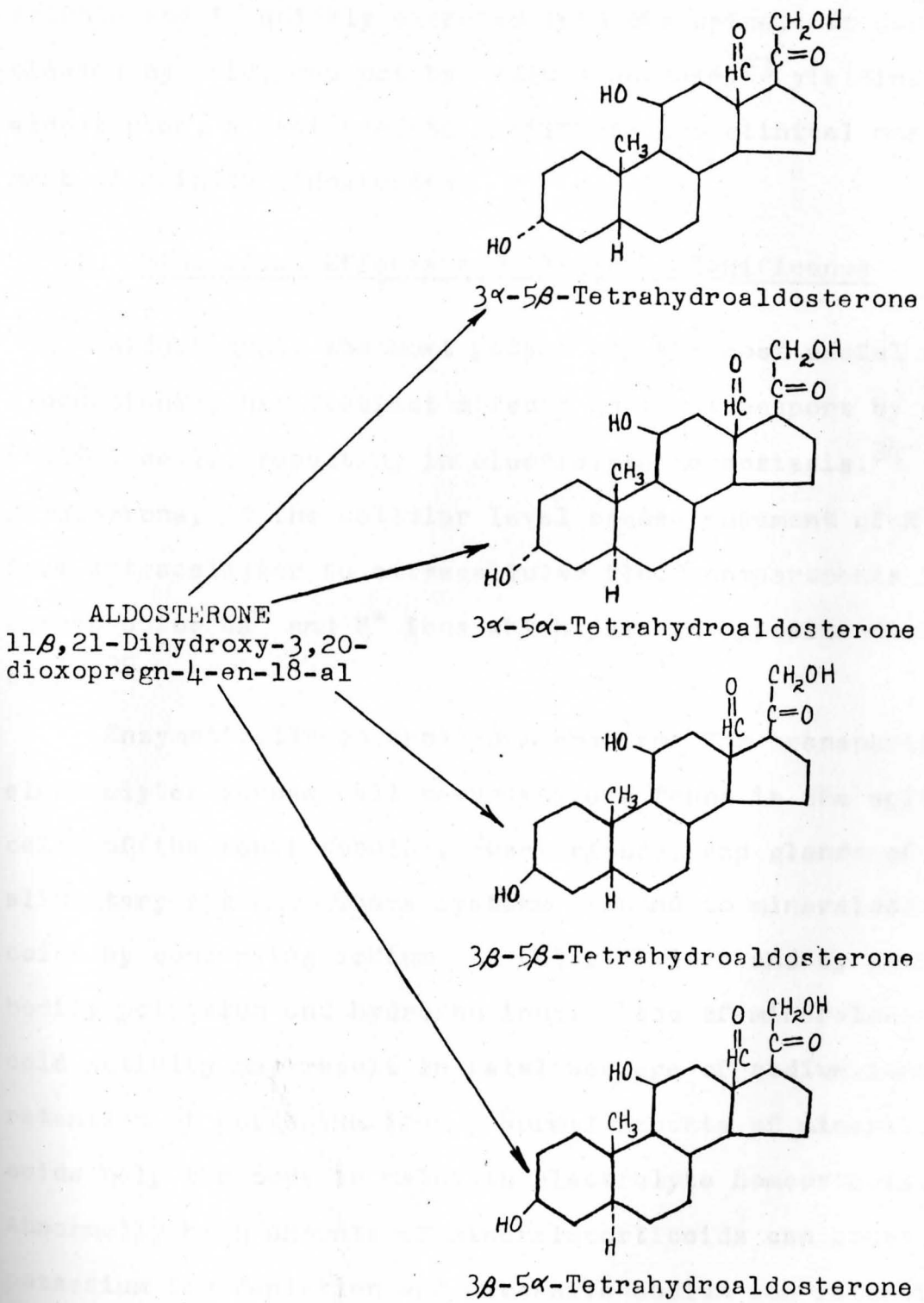


Figure 7. The chief urinary metabolites of aldosterone.[22]

and in the kidneys. Aldosterone 18-glucuronide is very water-soluble and is quickly excreted into the urine. It can be cleaved by acid, but not by β-glucuronidase,[23] yielding free aldosterone, a fact used to facilitate the clinical measurement of urinary aldosterone.

E. Biological Effects and Clinical Significance

Aldosterone, the most potent and the most useful mineralocorticoid, has distinct effects on ion transport by epithelial cells, resulting in electrolyte homeostasis.[24] Aldosterone, at the cellular level causes movement of K^+ ions from intracellular to extracellular fluid compartments in exchange for Na^+ and H^+ ions which pass in opposite directions.[25]

Enzymatically controlled mechanisms for transporting electrolytes across cell membranes are found in the epithelial cells of the renal tubules, sweat glands, and glands of the alimentary system. These systems respond to mineralocorticoids by conserving sodium and chloride ions and by excreting bodily potassium and hydrogen ions. Loss of mineralocorticoid activity may result in fatal wastage of sodium ions and retention of potassium ions. Normal amounts of mineralocorticoids help the body to maintain electrolyte homeostasis. Abnormally high amounts of mineralocorticoids can cause potassium ion depletion and excessive sodium ion retention, leading to edema, hypertension, and suppression of renal production of renin.[24]

Some of the alterations in serum electrolyte in the hypo-- and hyperadrenal cortical states are illustrated in figure 8.[26] Healthy subjects on a normal sodium diet and in a supine position for 2 hours have an aldosterone concentration in the range of 1 to 7 ng/100 mL of plasma; after standing for 4 hours the aldosterone values range from 3 to 28 ng/100 mL of plasma.[27]

Elevated urinary levels are found in primary aldosteronism (Conn's Syndrome) and secondary aldosteronism.

The clinical features of primary aldosteronism[28] include hypertension, muscular pains, polyuria, cramps, weakness and tetany. Laboratory test results include low plasma potassium with slightly elevated sodium, alkalemia (increased pH of the blood, irrespective of changes in the blood bicarbonate), suppressed plasma renin activity, and nonsuppressible aldosterone secretion. The majority of patients with Conn's syndrome have an aldosterone-producing adrenocortical adenoma (APA), about 20 percent may have nodular hyperplasia and very few had been reported to have carcinoma of the adrenal cortex.

Secondary aldosteronism, clinical and physiological conditions in which aldosterone levels are high in the abscence of APA include, nephrosis and cardiac failure with sodium retention, cirrhosis of the liver with ascites, pregnancy increasing to term, potassium loading, and salt depletion affecting extracellular fluid compartments.

Decreased aldosterone levels are found in patients with hypoaldosteronism, hypomineralocorticism. Selective

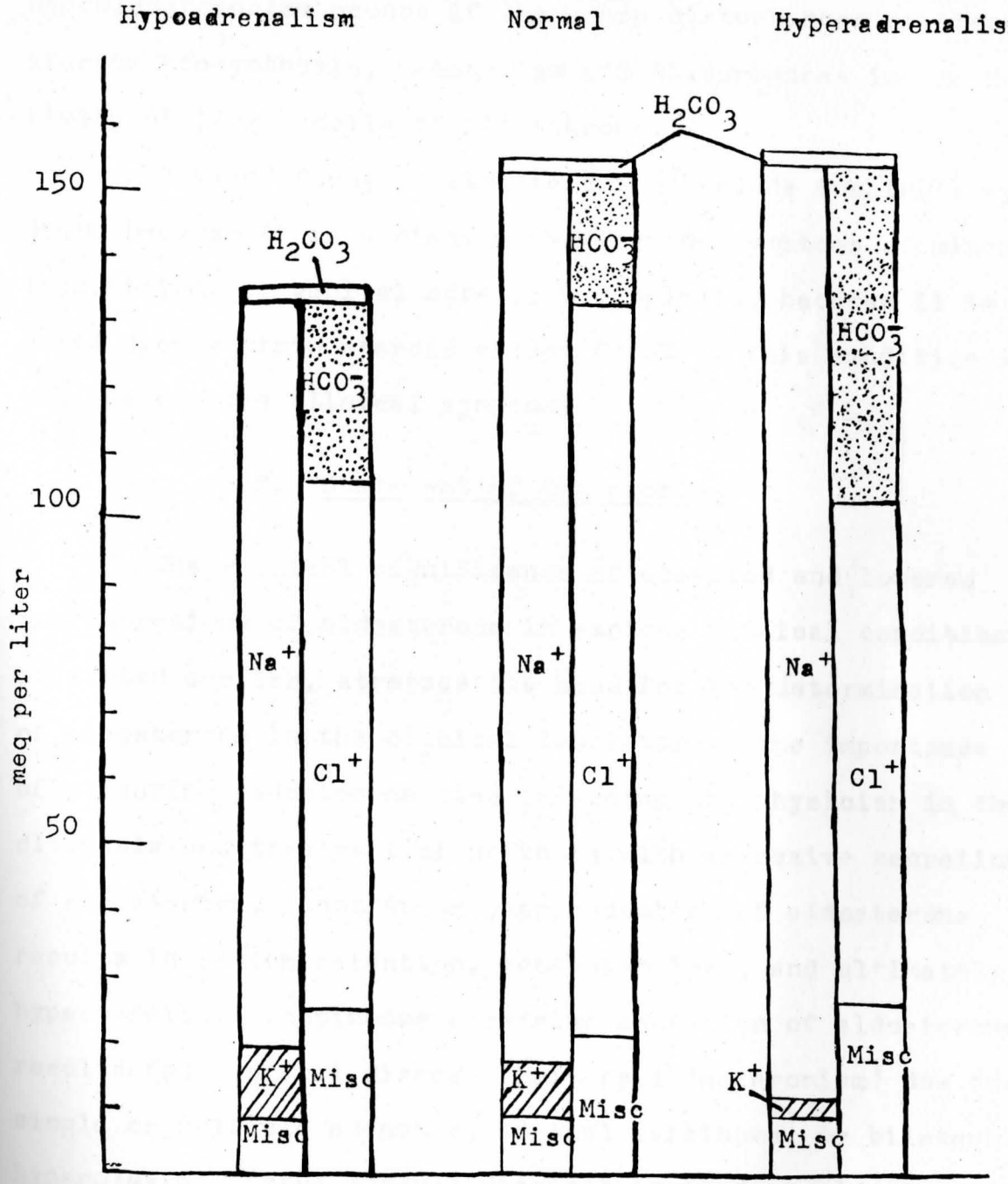


Figure 8. Serum electrolytes in hypoadrenal cortical and hyperadrenal cortical states in man.[26]

hypoaldosteronism occurs if there are disturbances of aldosterone biosynthesis, metabolism and disturbances in the reactivity of target cells to aldosterone.[29]

"Mixed" forms of aldosteronism include Cushing's syndrome because it is a disease showing the symptoms of mineralocorticism; congenital adrenal hyperplasia, because it is based upon certain steroid values found in this condition as well as on some clinical symptoms.[30]

F. Statement of the Problem

The clinical significance of elevated and lowered concentrations of aldosterone in various clinical conditions presented earlier, stresses the need for the determination of aldosterone in the clinical laboratory. The importance of measuring aldosterone lies in aiding the physician in the diagnosis and treatment of patients with excessive secretion of aldosterone. Continuous overproduction of aldosterone results in sodium retention, potassium loss, and ultimately hypertension. Continuous excessive secretion of aldosterone results from adrenal disease (primary aldosteronism) due to single or multiple adenomas, adrenal carcinoma, or bilateral hyperplasia. Extra-adrenal diseases (secondary aldosteronism), such as congestive cardiac failure, the nephrotic syndrome, or cirrhosis with ascites, are pathological conditions in which the rise in aldosterone secretion, is secondarily induced by pathogenic factors, either chronic or acute, which act upon the sensitive regulatory mechanisms of aldosterone

production.

The specimen for analysis is usually a 24-h urine collection because it reflects diurnal variations and any temporary changes that may occur in the patient, plus the aldosterone concentration is greater in urine than in plasma.

Urinary aldosterone is excreted in microgram quantities per 24-h output, therefore, requires special procedures capable of detecting such small quantities with reasonable accuracy. Methods for measuring urinary aldosterone are of the most complex and demanding nature. They are time-consuming, laborious, complex, expensive, and because they require special technical skills and instruments, are not suitable for routine analysis in clinical laboratories. The methods based on the double-isotope derivative formation[31] are extremely time-consuming, complex, and expensive, therefore, their application has been limited to use in specialized and research laboratories. A radioimmunoassay[31] was developed and appears to offer a suitable and reliable method for a routine laboratory.

The purpose of this investigation was to develop a new method for the determination of human urinary aldosterone utilizing high pressure liquid chromatography. After reading about various methods for aldosterone estimation and the numerous problems connected with them, the task of developing a new, rapid and simple method appeared insurmountable.

The author attempted to devise a simple and rapid method of high pressure liquid chromatographic determination

of human urinary aldosterone for routine clinical evaluation by attempting to eliminate as many of the tedious, manual, time-consuming, extraction and separation steps as possible.

At the time this investigation was undertaken, no known method of human urinary aldosterone estimation by high pressure liquid chromatography had been developed. Since then, DeVries et al, demonstrated normal phase HPLC can be used to assay urinary aldosterone.[32]

The preliminary steps involved in the method used in this investigation are modifications of the gas-liquid chromatographic methods as described by Zack, Webster, and Cerniauskas,[33] and Bravo.[34]

CHAPTER II

REVIEW OF THE LITERATURE

The determination of steroid hormones and their metabolites in urine or in plasma provides a means of evaluating adreno-cortical functions.

Recent intense interest in the elevated amounts of aldosterone in various clinical conditions and also in the mode of control of the hormone produced a large number of methods of assaying aldosterone.

Measuremont of aldosterone presents a technical challenge because of the minute quantities of aldosterone normally present in body fluids.

All current methods for the determination of aldosterone in urine include the following general steps: hydrolysis, extraction, purification and separation, and final quantification.[35]

Hydrolysis. Aldosterone is excreted in the urine as free aldosterone (approximately 3%) along with the water-soluble conjugates, tetrahydroaldosterone 3-glucuronide and aldosterone 18-glucuronide. Hydrolysis of the water-soluble conjugates is necessary to obtain free aldosterone for estimation. Two methods of hydrolysis are acid hydrolysis and enzyme hydrolysis. In acid hydrolysis, an aliquot of a 24-h urine specimen is acidified to pH 1, by means of a specified

concentration of a mineral acid (hydrochloric or sulfuric) and allowed to stand 24 hours at room temperature. In enzyme hydrolysis, an aliquot of a 24-h urine is adjusted by the addition of a buffer to the optimal pH for the enzyme used. An adequate amount of the respective enzyme (β-glucuronidase to hydrolyze glucosiduronates, but not the 18-glucuronide,[36] and sulfatase to hydrolyze sulfate conjugates) is added, and the test specimen is incubated for 18 to 76 hours at a specified temperature (e.g., 37°C).

Extraction. The hydrolyzed urine is extracted with specified volumes of an immiscible organic solvent a specified number of times for maximum recovery of aldosterone from the urine. Aldosterone is slightly polar and for its extraction, relatively non-polar organic solvents such as methylene chloride and chloroform are most suitable.

Purification and Separation. The solvent partition method[35] is used for preliminary purification and separation of compounds of interest. The pooled organic solvent fractions containing aldosterone and other urinary impurities are treated with a basic solution (sodium bicarbonate, sodium carbonate) whereby, the strongly acidic components migrate into the aqueous layer to be discarded. Phenolic steroids are extracted with an aqueous solution of sodium hydroxide.

The method used for final quantification determines the need for further purification and separation. Some physical techniques for further purification and separation of the solvent extracts are paper and column partition chro-

matography, adsorption chromatography (column and thin layer), or gas chromatography (GC).

Quantification. Methods for the estimation of aldosterone in urine are: colorimetric, fluorometric, gas chromatographic, and radioisotope methods (including double isotope derivative formation, and radioimmunoassays). It should be re-emphasized that until the development of radioimmunoassays, the methods of estimation of aldosterone in urine and in plasma were not suitable for the routine clinical laboratory because of their complex nature.

A. Methods for the Estimation of Aldosterone

1. Neher-Wettstein Method

The Neher-Wettstein method[37] for the determination of urinary aldosterone 18-glucuronide was the first physicochemical method developed for the determination of aldosterone. It was the method most widely used until the nineteen sixties.

Hydrolysis, Extraction and Purification of the Extract:

An aliquot of a 24-h urine specimen is acidified to pH 1, by means of an inorganic acid (hydrochloric or sulfuric).

The acidified urine specimen is allowed to stand for 24 hours and is then extracted three times with about 1/5 volume of chloroform.

The organic solvent fractions are pooled and washed with 0.05 volume of cooled 0.1 N NaOH solution, then with 0.05 volume of distilled water. The washed chloroform extract is distilled in vacuo at 50° C under nitrogen or any other inert gas atmosphere.

The organic extract is further purified by chromatography on a silica gel column.

Next, the extract is chromatographed on wide strips of Whatman paper No. 1, in Zaffaroni's formamide-chloroform system (38° C, 30% formamide impregnation). Aldosterone migrates with cortisone in this system. After migration, the area containing cortisone and aldosterone is detected by means of an ultraviolet lamp (254 nm) and a fluorescent screen, also, by the use of pilot strips with aldosterone or cortisone developed parallel. A mixture of chloroform and methanol (8:2) is used to elute the area of paper containing aldosterone. The eluate is taken to dryness and rechromatographed in Bush's C system (formamide-chloroform) on 1.5 cm wide paper strips.

While the strips are dried at room temperature, a solution containing 90 parts of 2 N NaOH is added to 10 parts of a 0.1 percent solution of blue tetrazolium and the mixture is filtered immediately before use. The strips are then developed in this solution. Bluish-violet formazan spots appear within a short time. Red or blue tetrazolium oxidizes α-ketols, as well as aldoses and ketoses, and thereby is reduced to an intensely colored pigment called a diformazan.

The test is more sensitive than Fehling's or Tollen's tests for the above types of compounds.[38] Semiquantitative estimation is carried out using specified amounts of aldosterone reference standards run in parallel. The chromatogram is heated in a 90° C oven, for approximately 20 minutes. The chromatogram is removed from the oven and is exposed to ultraviolet light of 350-360 nm which produces with aldosterone a bright yellow fluorescence. A second semiquantitative determination can be made from this reaction which is characteristic of the Δ^4-3-keto structure ("soda fluorescence reaction" described by Bush in 1952).[39] The limit of sensitivity in soda fluorescence and blue tetrazolium reaction by semiquantitative visual estimation on the paper is approximately 1.0 μg. No percent recovery was given.

2. Double Isotope Derivative Method

The double isotope derivative method of Kliman and Peterson (1960)[40] seemed to be the method most widely employed for the estimation of aldosterone until the advent of radioimmunoassay. This method[41] depends on the acetylation of aldosterone with tritium--labeled acetic anhydride of known specific activity to permit recovery of an aldosterone derivative of specific activity. Addition of ^{14}C isotope-labeled aldosterone diacetate early in the procedure permits calculation of aldosterone concentration present initially. Before the final product can be measured, purification with three paper chromatographic systems, along with

an oxidation step is required. When working with labeled reagents, great care must be taken to check specificity and the $^{3}H/^{14}C$ ratio must be constant on the final chromatograms. Normal ranges at the Bio-Science Laboratories are between 2-26 μg per 24-h urine output. Patients should have adequate sodium intake and an acidified urine (pH 3-5) collection is required, to insure stability of aldosterone . The test procedure requires three weeks time for completion.

3. Radioimmunoassay For Aldosterone

According to Waldhausl, Haydl, and Frischauf,[42] all of the radioimmunoassay methods then reported used paper chromatography or time-consuming extraction procedures and oxidation steps for the estimation of aldosterone.

They developed a method for the determination of urinary aldosterone using Sephadex LH-20 chromatography and radioimmunoassay. Ten mL of urine were used for analysis. The purification of aldosterone consisted of hydrolysis at pH 1.0, methylene chloride extraction, and column chromatography on Sephadex LH-20. Recovery of added 4-^{14}C-D-aldosterone was 44.7$\pm$7.1 (2SD)% for 60 experiments. The extraction was followed by radioimmunoassay analysis of aldosterone using sheep antiserum versus aldosterone, i.e. anti-aldosterone serum diluted 1:250,000 with borate bovine serum albumin methiolate buffer (BBSAM). The sensitivity of the overall assay was 1.6 μg aldosterone, the accuracy 92$\pm$3.4%. The coefficient of variation within one assay was 14% for a

given sample (n=19) and 20% for multiple assays. Seventeen healthy individuals on an uncontrolled diet had aldosterone excretion values of 9.5±3.6 μg (SD) per 24 h. They processed 20 samples in a period of three working days excluding acid hydrolysis.

The Bio-Science Laboratories now have adopted the radioimmunoassay procedure of Drewes, Demetriou, and Pilleggi.[43] The method involves purification by extraction, followed by acid hydrolysis and reextraction, and chromatography to remove naturally occurring steroids in potentially interfering levels. Radioimmunoassay involves the specific binding of aldosterone in competition with ^{3}H-aldosterone to an anti-aldosterone antiserum produced in sheep. Normal range at Bio-Science Laboratories: 2-26 μg per 24 h. Results can be expected in approximately one week.[44] Walsh et al have described a non-chromatographic RIA procedure for urinary aldosterone.[45]

4. Gas-Liquid Chromatographic Determination Of Aldosterone In Human Urine

Leung and Griffiths[46] developed a method for the determination of human urinary aldosterone using Amberlite XAD-2 column chromatography for isolation of aldosterone from human urine and quantification by gas chromatography. An aliquot of a 24-h urine specimen is acid-hydrolyzed, then a standardized quantity of ^{14}C aldosterone, 3000 counts/min, is added to the urine. Aldosterone is eluted from the Amberlite XAD-2 column with methanol and purified by Silica-gel

thin-layer chromatography (TLC). A stable gamma-lactone derivative of aldosterone is formed by oxidation with periodic acid and the derivative is isolated by TLC. An aliquot is removed to measure the added radioactive aldosterone tracer and the remainder is quantificated in a gas-liquid chromatograph using a flame ionization detector. The column consisted of a 6'x1/8" diameter U-shaped glass tube packed with 3% SE-30 on 100/120 mesh gas chrom Q. The sensitivity of the method is approximately 1 μg/L of urine, and the precision is about 10% in the normal range. The accuracy is between 95-99% and allows sufficient reliability for clinical applications where the necessary equipment is available. Total recoveries of aldosterone may range from 55-70% but each sample is accurately monitored by the addition of 4-^{14}C aldosterone.

Zack, Webster and Cerniauskas[47] developed a quantitative gas-liquid chromatographic method for aldosterone in human urine. Their method involves acid hydrolysis of duplicate aliquots of a 24-h urine specimen, methylene chloride extraction of the urine followed by oxidation of aldosterone in the extract with periodic acid to aldosterone gamma-lactone. Gas chromatography is performed on a SE-52 column using temperature programming to elute aldosterone as a single peak. Aldosterone assays were performed on urines from twelve normal young adults using this procedure. The mean for this group ±1SD was 16.9±8.7 μg/24 h (corrected for recovery). The median was 14.8 μg/24 h. The 95% limits ±2S.D. for this

procedure is 0-34 μg/24 h. The range of the corrected values observed in this group is 5.5-33.0 μg/24 h. The average recovery was 84% of 5 μg of pure aldosterone added to 100 mL of water which was then subjected to this procedure.

5. High-Pressure Liquid Chromatography

It must be emphasized that at the time this research project was undertaken, no method for the determination of human urinary aldosterone using high-pressure liquid chromatography (HPLC) had been reported in the literature.

Siggia and Dishman[48] reported an analysis of steroid hormones using high-resolution liquid chromatography. In this report, they dealt with the development of column packings which have a high separating power for various polar and moderately polar steroids. Representative members of several classes of steroid hormones were separated on a variety of columns under different conditions. The effect of the solid support on the reversed phase liquid-liquid partition chromatography of these steroids was studied. Under a given set of chromatographic conditions and at a given inlet pressure, improved separations of synthetic mixtures of steroids were obtained using a combination of liquid-liquid partition and liquid -solid adsorption effects.

Wortmann, Schnabel and Touchstone[49] developed a method for the quantitative determination of corticosteroids from human serum by high-pressure liquid chromatography. In this method, cortisol, cortisone and aldosterone were sepa-

rated as a group. Human serum was subjected to a methylene chloride extraction procedure, followed by separation and quantification on reversed-phase column packing material, Sil-X-RP by high-pressure liquid chromatography. Sil-X-RP is the trade name for the column packing obtained from Perkin-Elmer, whose particles were coated with octadecyl dichlorosilane as the stationary phase. Radioactive cortisol was added to the blood prior to extraction and an aliquot counted in a liquid scintillation counter before sample injection into the chromatograph. This was done for the purpose of calculating % recovery of the extraction process. An average recovery of 86% resulted. Radioactive cortisol was injected with and without the biological material and the portion representing the cortisol peak was collected for the determination of the recovery from the liquid column. An average recovery of 93% was obtained. The collected radioactive and non-radioactive standards were identified by thin-layer and paper chromatography in different systems.

Thirty serum samples of healthy persons were analyzed using this method. The average value for the cortisol, cortisone, and aldosterone group was 16.31 μg per 100 mL of serum with a standard deviation of 2.10 and a standard error of 0.38. The average precision value representing one third from 10 mL of serum was 0.54 μg. The average standard deviation of all the triplicate determinations was 0.05, indicating good reproducibility and precision.

After the present research project was completed, the

one and only report found in the literature on the use of high-pressure liquid chromatography for the assay of human urinary aldosterone was that of DeVries, Popp-Snijders, DeKieviet and Akkerman-Faber.[32]

DeVries' method involves acid hydrolysis, methylene chloride extraction, washes with aqueous sodium hydroxide and saturated aqueous sodium chloride solutions, drying over sodium sulfate, filtration, evaporation and drying under nitrogen. The residue is dissolved in 2 mL of 70% methanol in water and washed with toluene. The washed solution is split into two equal parts, and 10 μg of aldosterone is added to one portion to correct for loss of material. The residue is dried under nitrogen and purified by TLC three times. The solvent systems are: (1) chloroform-methanol-water (90:10:0.8); (2) ethyl acetate-methanol-water (85:15:1); and (3) benzene-acetone-water (70:30:0.5) (twice in succession on the same thin-layer plate). Prednisolone (5-10 μg) is added to the eluate as internal standard and quantification of aldosterone is performed by normal phase HPLC on a 250x2.2 mm SI-10 MicroPak column (Silica gel, particle size 10 μm). The solvent used is 1.5% methanol in chloroform; flow-rate is 70 mL/h, which corresponds to a pressure drop of 1100 p.s.i.. The recovery was 63.1 $\pm$ 9.4% (n=215).

DeVries *et al*[32] compared their gas-liquid chromatography method with the HPLC method they had developed.

The results showed that for HPLC, the coefficient of variation (C.V.) was 16% (n=125), with a lower detection limit

of 1.80 μg/24 h (5 nmoles/24 h). Compared to GLC, C.V. was 22% (n=71), with a lower detection limit of 3.60 μg/24 h (10 nmoles /24 h). Normal values were 6.34 μg $\pm$ 4.54 μg/24 h (17.6 $\pm$ 12.6 nmoles/24 h) (n=15), range 1.80-16.2 μg/24 h (5-45 nmoles/24 h).

DeVries *et al*[32] are now using successfully HPLC in an aldosterone assay of human urine for the final separation and quantification.

O'Hare *et al*[50] separated a mixture of polar adrenal steroid standards including aldosterone using reversed-phase HPLC with dioxane-water gradient.

Ballerini *et al*[51] also showed that seven steroids in adrenocortical extracts including aldosterone could be separated by HPLC by using either a chloroform-methanol gradient on a 5 μm silica column or a water-acetonitrile gradient on a 10 μm C_{18} reversed-phase column. This work supports the work of O'Hare *et al*[50].

CHAPTER III

MATERIALS AND APPARATUS

A. Solvents and Reagents

All chemicals used were redistilled, reagent grade or of the highest purity available.

Redistilled deionized water was used for the preparation of all reagents and solvent systems.

Solvents and reagents were prepared or used as described below.

Methylene chloride, methanol, acetonitrile and acetone (Burdick and Jackson Laboratories, Inc., Muskegon, Michigan, 'distilled in glass', chromato-quality) were used without further purification.

Hydrochloric acid and nitric acid (Fischer Scientific Company, Fair Lawn, New Jersey, ACS reagent grade); concentrated hydrochloric acid was used as such and as a 6N HCl solution. Nitric acid was used to prepare a 6N HNO_3 solution for acid cleaning U.V. cells of the U.V. detector, tubing and stainless steel filters of the HPLC system.

Sodium bicarbonate, sodium dichromate, sodium sulfate (anhydrous), potassium hydrogen phthalate, dimethylsulfoxide (DMSO), toluene (J.T. Baker Chemical Company, Phillipsburg, New Jersey, 'Baker analyzed') were used without further purification.

Sulfuric acid (Matheson Scientific, Cleveland, Ohio, ACS reagent grade) along with sodium dichromate was used to prepare a "cleaning solution" for glassware.

Chloroform spectro-grade, ACS, (Eastman Kodak Company, Rochester, New York) was used without further purification.

Dichlorodimethylsilane (Eastman Organic Chemicals Distillation Products Industries, Rochester, New York) along with toluene was used to prepare a 2% dichlorodimethylsilane solution.

Prepurified nitrogen (Airco Inc., New York, New York) was used to dry extracted urine specimens.

B. Steroids

The following steroids (Sigma Chemical Company, St. Louis, Mo.,) were the purest quality obtainable, 95-98%.

1. D-Aldosterone (anhydrous). Δ4-Pregnen-18-al-11 , 21-diol-3, 20-dione. Absorption maximum: 240 nm.
2. Reichstein's "Substance S". 11-Desoxy-17-hydroxycorticosterone. Absorption maximum: 242 nm.
3. Deoxycorticosterone. 21-Hydroxypregn-4-ene 3, 20-dione. Absorption maximum: 242 nm.
4. Corticosterone. 11β, 21-Dihydroxypregn-4-ene-3, 20-dione. Absorption maximum: 240 nm.
5. Estradiol. β Estradiol; estra-1,3,5(10)-triene-3, 17β-diol. Absorption maxima: 225, 280 nm.
6. Estriol. Estra-1,3,5(10)-triene-3, 16α, 17β-triol. Absorption maximum: 280 nm.

7. Estrone. 3-Hydroxyestra-1,3,5(10)-triene-17-one. Absorption maximam: 283-285 nm.
8. Hydrocortisone. Cortisol; 11β, 17α, 21-trihydroxy-4-pregnene-3, 20-dione. (Applied Science Laboratories Inc., Houston, Texas).
9. Progesterone. Δ4-Pregnene-3, 20-dione. Absorption maximam: 240 nm. (Applied Science Laboratories Inc., Houston, Texas).

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

Stock solutions of steroids were prepared with methanol as the solvent just before using.

Crystalline aldosterone stock solution: 5 mg per 100 mL methanol. Aldosterone working solution: 1:5 dilution in methanol of aldosterone stock solution.

C. Apparatus

All steroid solutions were prepared from crystalline reagents weighed on a Mettler H20 balance, (Mettler Instrument Corporation, Princeton, New Jersey). All remaining solutions were prepared from reagents weighed on a Mettler H33 balance.

Twelve-, fifteen- and three-mL pyrex conical centrifuge tubes, plain and graduated, with and without stoppers as well as 15-mL round bottom centrifuge tubes were silanized with 2% dimethyldichlorosilane in toluene.

All glassware was chromic acid cleaned before silanization.

For suction filtration, a 500-mL Buchner Funnel, a 2000-mL suction flask, Whatman filter paper #541 and a water aspirator were used.

Water was purified by distilling an alkaline potassium permanganate solution using ground glass equipment.

A grid rack bar apparatus was constructed to hold sixteen 500-mL or 250-mL separatory funnels for extracting and washing 24-h urine specimens. (See Figure 9).

For evaporation of extracts, a rotary evaporator (Buchi Instruments) was used.

Extract drying was carried out with nitrogen forced on to the extracts through a Silli-Vap™ (Pierce, Rockford, Ill.) heated with a Multi-Temp-Blok (Lab-Line Instruments, Inc.,). (See Figure 10).

The Beckman Model 76 pH meter (Beckman Instruments, Inc.,) was used.

High-pressure liquid chromatography was carried out with the Waters Associates High-Pressure Liquid Chromatograph, (HPLC) Model ALC/GPC-502/401.

The modular characteristics of the Waters' HPLC system allows use of a wide range of options and accessories, purchased with the basic instruments or added later.

A schematic diagram showing the modules used for this research for varying HPLC parameters and optimizing resolution of desired components is shown in figure 11.[52]

The elements of this HPLC system were, Solvent Reservoirs, Magnetic Stirrers; initially: Septum, Valve/Loop sample injector; subsequently: U6K Injector, M6000 Pump A

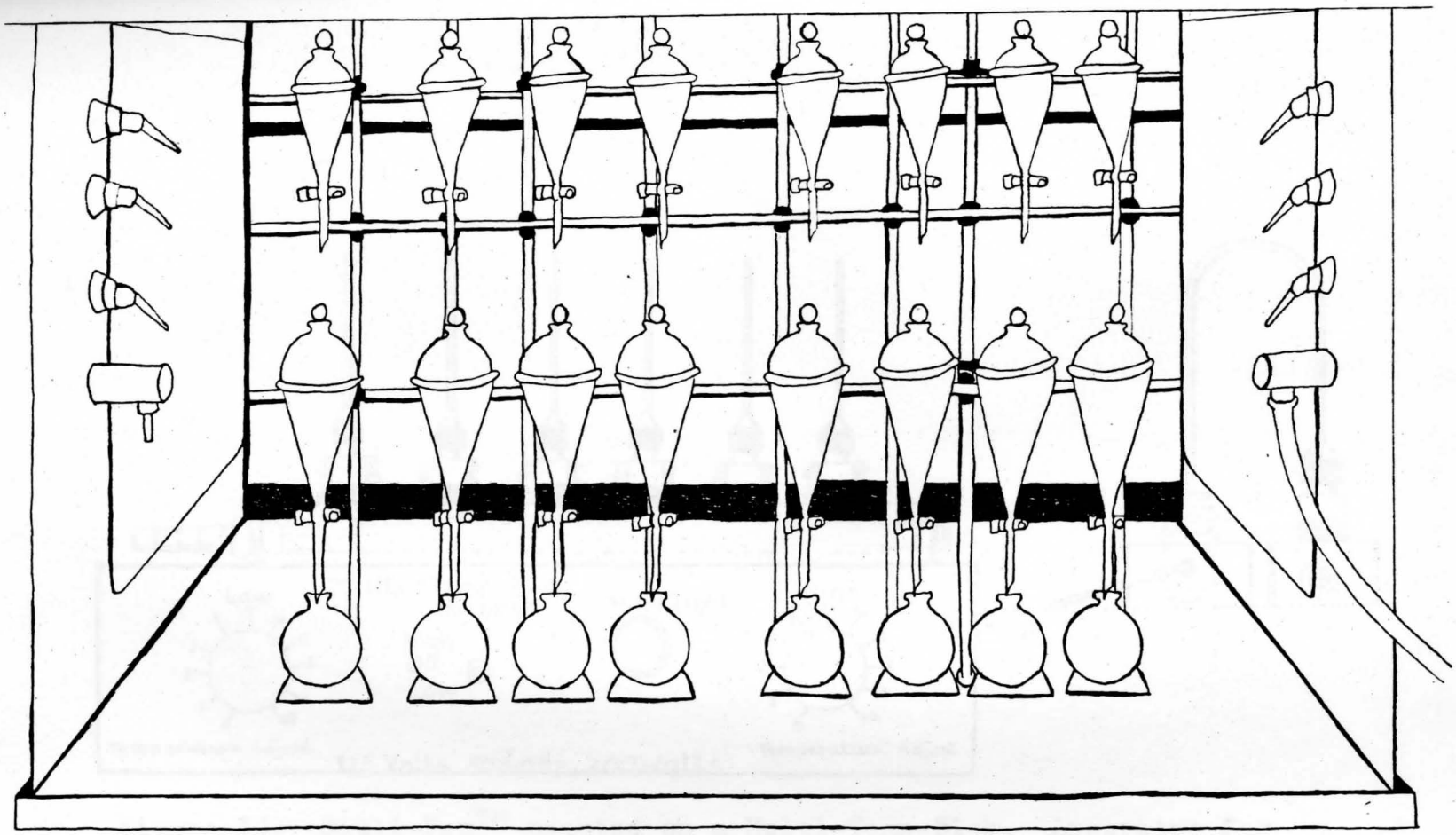


Figure 9. Grid rack bar apparatus constructed to hold separatory funnels for extractions and washings of 24-h urine specimens.

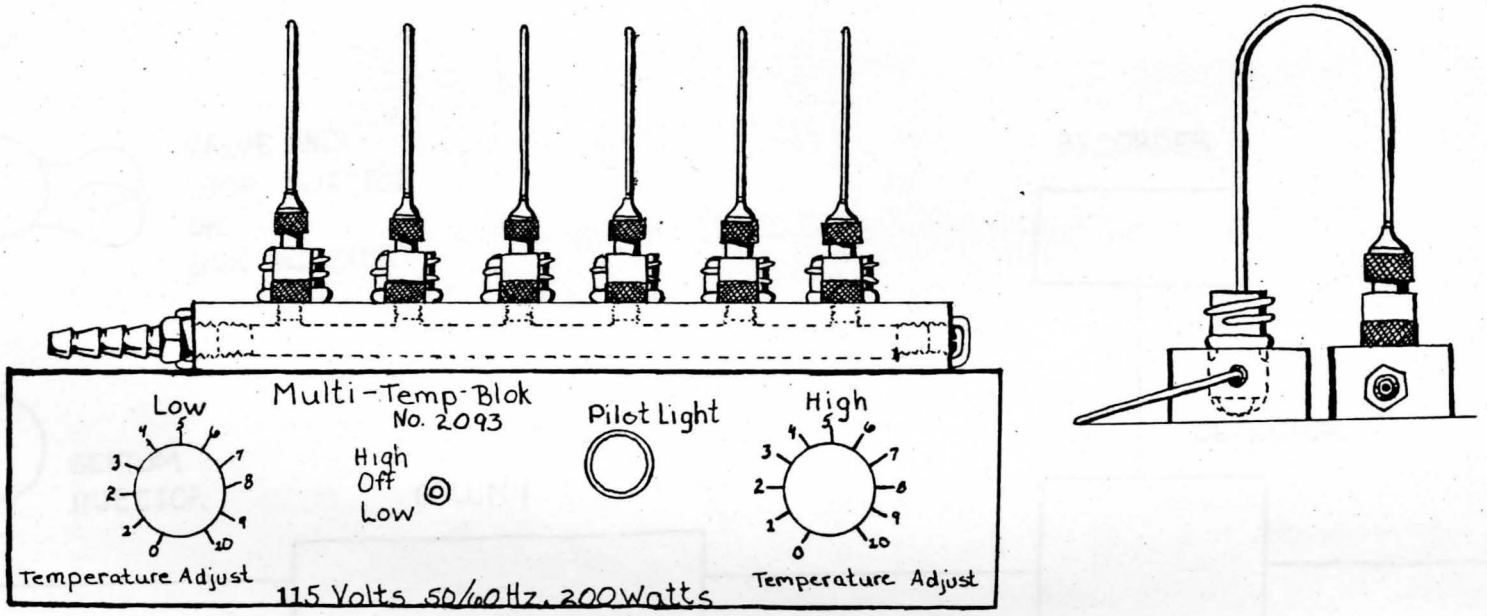


Figure 10. Silli-Vap™ mounted on a Multi-Temp-Blok. Apparatus for evaporation under N_2 atmosphere.

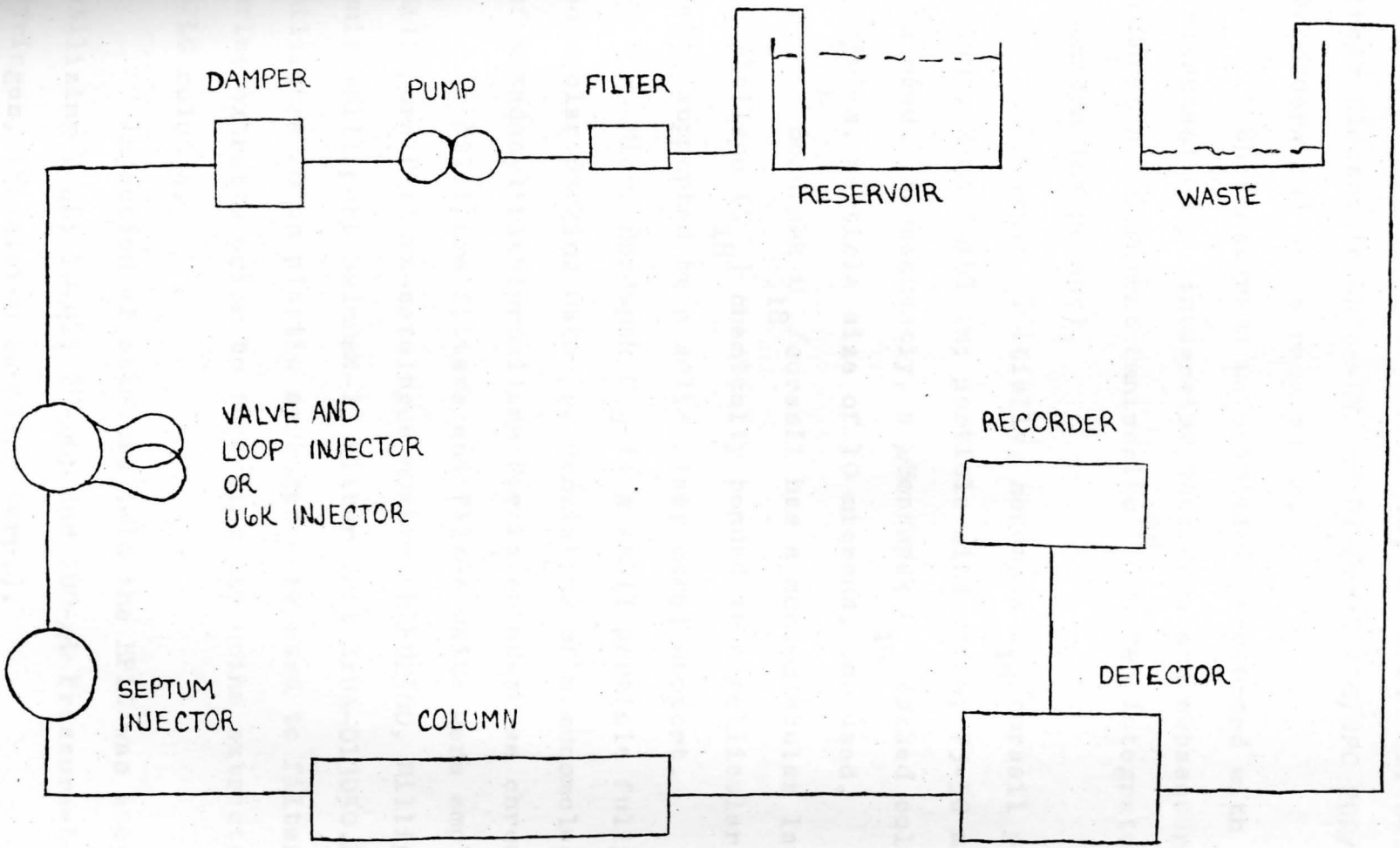


Figure 11. HPLC flow system diagram.[52]

and B and their Electronic Units, Columns, Dual Detectors and their Electronic Units-UV and RI-Model ALC/GPC 202/R401, recorders and waste reservoirs.

Chromatograms were initially recorded with a Linear Instrument Corp. Integrator Recorder, and subsequently with a Houston Instrument Omniscribe^{TM} Two Pen Integrator Recorder (Houston Instrument).

Columns: Initially, Bondapak C_{18}/corasil packed column, 2 mm IDx61 cm; particle size range; 73-50 microns, was used. Subsequently, a μBondapak C_{18} packed column, 4 mm IDx30 cm, particle size of 10 microns, was used.

Bondapak C_{18}/corasil has a monomolecular layer of octadecylsilane (C_{18}) chemically bonded to a pellicular (a porous crust supported by a solid glass core) support.

Micro Bondapak C_{18} is a small particle fully porous non-polar packing material consisting of a monomolecular layer of octadecyltrichlorosilane for reversed-phase chromatography.[47]

Millipore filters and filter units were employed. Millipore filters-catalogue number FHLP02500, Millipore filter units-Millipore Swinnex-13 Filter Unit SxHA-013050.45 -10324, Millipore 20 cc plastic syringes were used to filter the urine extracts prior to injecting the urine extracts onto the HPLC columns.

Injection of extracts into the HPLC was accomplished utilizing 1-μL; 10-μL; 25-μL; and 100-μL Pressure-Lok Liquid Syringes, (Precision Sampling Corp.).

Hamilton High-pressure Microliter Syringes were also used for the injections.

CHAPTER IV

EXPERIMENTAL

A. High-Pressure Liquid Chromatography

1. Introduction

In 1906, Michael Tswett, a Russian, developed a new separations technique which he termed "chromatography." Other men have slowly added to our knowledge of chromatography. In 1952 Martin and Synge were given the Nobel prize for their work done in paper chromatography. Historical milestones in liquid chromatography can be found elsewhere.[53]

Chromatography basically involves separations based on specific interactions between sample molecules and the stationary and moving phases.[54]

Liquid chromatography (LC) is a chromatographic process in which the moving phase is a liquid.[54] Since liquid chromatography separations are based on solubility,[55] sample components migrate through the chromatographic system only when they are in the mobile phase. Composition of the mobile phase is of prime importance in the separation.

In liquid chromatography, the mobile phase is noncompressible.[54] Because diffusion in the mobile phase is extremely low, high-pressure liquid chromatography is possible. Resistance to mass transfer in a liquid is very high.

Liquid chromatography is a solubility based phenomenon, therefore, factors such as temperature, vapor pressure, etc., affect the separation only by their effect on solubility and mobile phase viscosity.[55]

High-Pressure Liquid Chromatography is based on developments in several areas: equipment, special columns and column packings, technique, theory, high-pressure pumps and sensitive detectors.[56]

Selecting the proper solvent, packing and operating conditions usually results in good separations of sample components.

In most instances, good separations are achieved by matching the polarity of the sample and the packing, and using a solvent which has a noticeably different polarity from the packing.

Waters Associates[57] published a chart called the Eluotropic Series which correlates the approximate relative polarities of commonly used solvents with the relative polarities of the column packing materials. (See Figure 12.)[58]

General sample-solvent interactions as a function of polarity in HPLC[59] are shown in Figure 13.[58]

Waters Associates[59] have developed a general guide as a better basis for selecting the column packing if the nature and type of functional groups and molecular weight of the sample are known. (See Figure 14.)[59]

The characteristics a column must have to achieve good liquid chromatographic separations are: (1) Capacity to re-

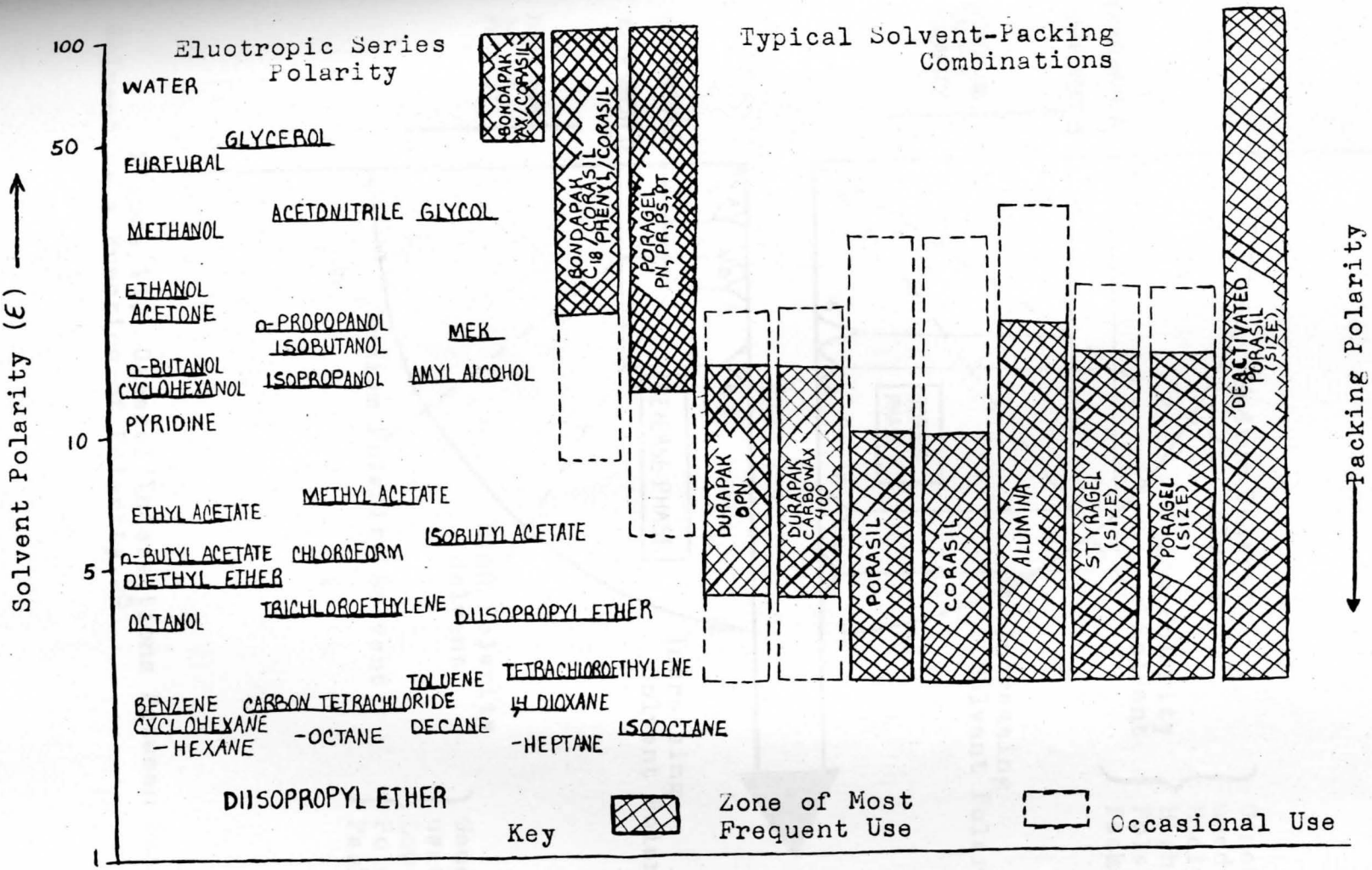


Figure 12. Eluotropic Series and Column Packings Polarities.[58]

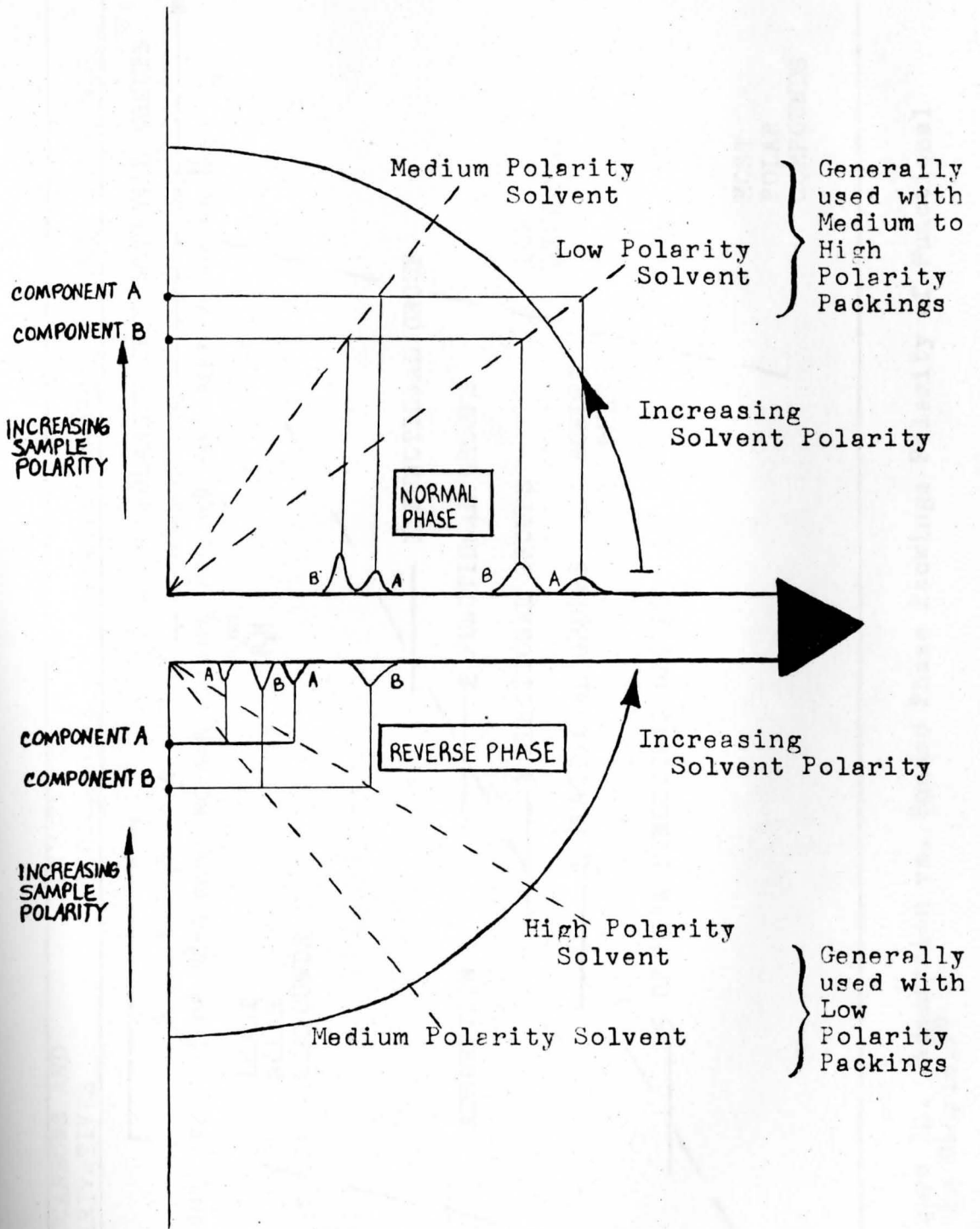


Figure 13. General Interactions Between Sample and Solvent as a Function of Polarity.[58]

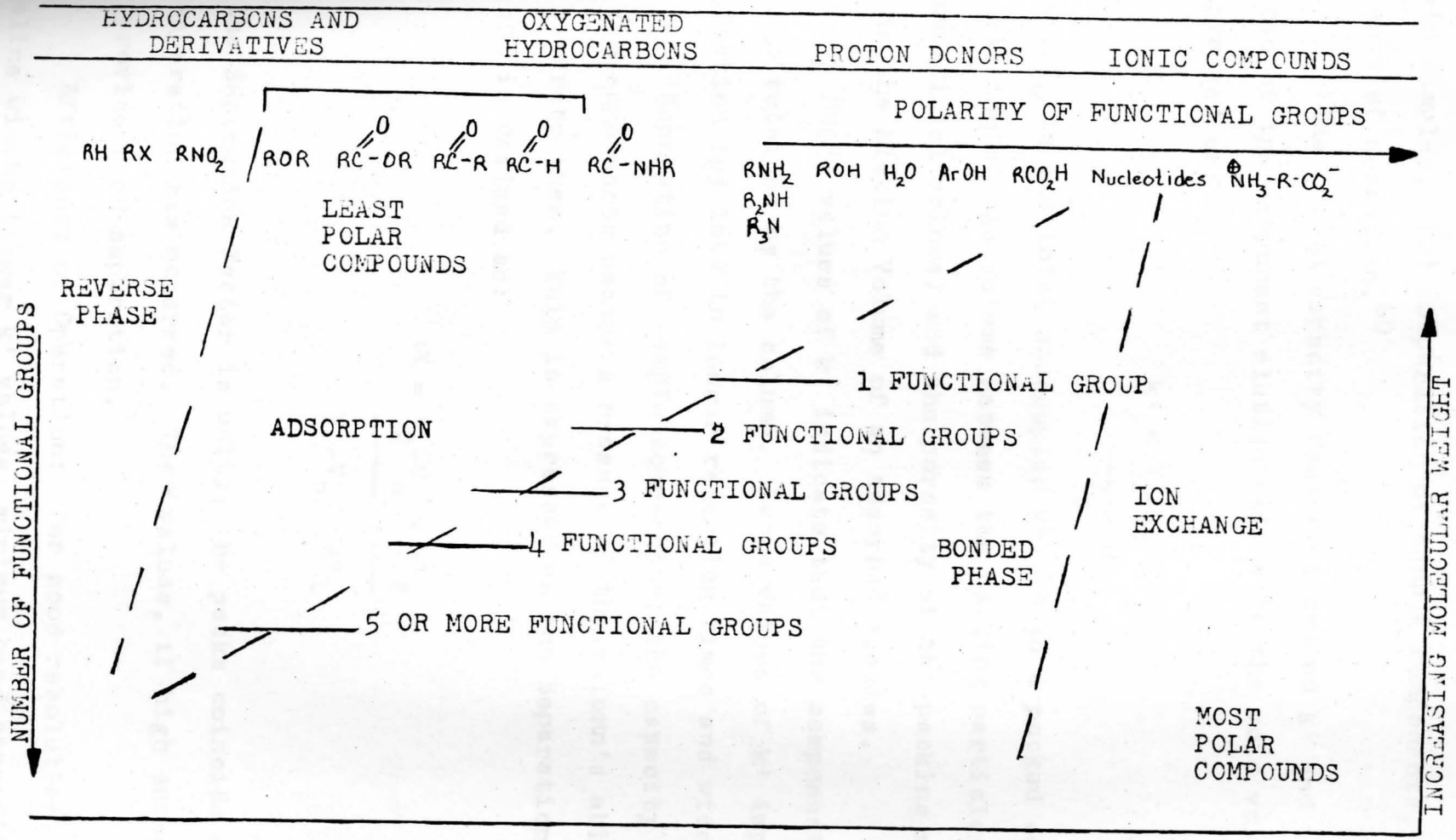


Figure 14. Adsorption vs. Bonded Phase Packings Polarity of Functional Groups in the Sample.[59]

tain samples, (2) Separation of sample components, (3) Efficiency of operation.[60]

The column capacity factor is called k' and is the ratio of the component elution volume to the void volume expressed as:

$$k' = \frac{V_1 - V_o}{V_o} \qquad (1)$$

where V_o is the total unoccupied volume in a packed column, consisting of the volume between the packing particles (the interstitial volume) and the porosity of the packing material. V_1 is the Elution Volume of an adsorbed species.

Small values of k' indicate that the components are little retained by the column. Large values of k' improve separation but lead to longer retention times and wider peaks.

Separation of sample components; the capacity factors of two components become a measure of the column's ability to separate them. This is expressed as the Separation Factor, α, and is defined as:

$$\alpha = \frac{V_2 - V_o}{V_1 - V_o} = \frac{k'_2}{k'_1} \qquad (2)$$

If the Separation Factor is unity, the peaks coincide and no separation has occurred. The α values, if high enough, may provide good separation.

Efficiency of Operation: for good resolution, narrow baseline widths (lower k' values, minimum band spreading) are

sought. The Theoretical Plate Number, N, an empirical measure of column efficiency, is expressed as:

$$N = 16\left(\frac{V}{W}\right)^2 \qquad (3)$$

Where $V=t_R$ x flow rate; W=peak width at baseline.

$$N = 16\left(\frac{t_R}{W}\right)^2 \qquad (4)$$

Where t_R=retention time; W=peak width at baseline.
The narrower the peak, the higher N, the more efficient is the column.

Another factor, Resolution R, is the total measure of component peak separation at their apexes and at their baselines. Components with R=1 are 98% resolved.

$$R = \frac{V_2 - V_1}{\frac{1}{2}(W_2 + W_1)} \qquad (5)$$

Resolution may also be expressed in terms of k', and N.

$$R = \frac{1}{4}\ \frac{(\alpha-1)}{\alpha}\ (\sqrt{N})\ \left(\frac{k'}{k'+1}\right) \qquad (6)$$

The three important characteristics of a chromatographic separation are: the Separation Factor--$\frac{\alpha-1}{\alpha}$, Efficiency--(N) and Capacity (Retention Time)--$\frac{k'}{k'+1}$.

Gradient elution is a technique of changing the relative concentrations of two mobile phase solvents during a chromatographic separation. Gradient elution is an operational tool for optimizing separation of many sample compo-

nents varying widely in retention volume during a single run.

Isocratic elution is a technique whereby elution of sample components is achieved with constant solvent composition during the chromatographic separation.

B. Experimental Organization

When this investigation was undertaken, no known report on the determination of urinary aldosterone by high-pressure liquid chromatography was found in the literature. Therefore, a prototype HPLC method for the determination of urinary aldosterone had to be developed. The author proceeded to accomplish this by varying the parameters of high-pressure liquid chromatography.

The flow system of this HPLC set-up consists of a reservoir, followed by a filter and a pumping system which contains a pulse damper. (See Figure 11.)[52] The solvent is pumped through the filter and pumping system to the valve and loop injector, then flows through the septum injector to the separating column and detector, then on to a waste reservoir.

The Waters Model 660 Solvent Programmer was used for performing both gradient elution and flow programming chromatography.

Solvent programming (gradient elution) is accomplished with the addition of a second pump.

Solvent programming is used for rapidly screening a number of specific solvent compositions to optimize operating

conditions for a new liquid chromatographic separation.[62]

1. Experimental Protocol

The preliminary steps for the determination of human urinary aldosterone by HPLC are modifications of the methods as described by Zack, Webster and Cerniauskas;[33] and by Bravo.[34]

The initial procedure involved:

a. Collection of 24-h urine specimens.

b. Measuring the volumes of filtered 24-h urine specimens.

c. To a 100-mL aliquot of a 24-h urine specimen, 0.5 mL of aldosterone stock standard (10 μg/mL) is added. This aliquot and a second 100 mL aliquot of the same 24-h urine specimen are taken and both are adjusted to a pH of 1.0 with concentrated hydrochloric acid and are allowed to stand for 24 h.

d. The hydrolyzed urine samples are transferred to 500-mL separatory funnels, extracted three times with 100-mL portions of methylene chloride, and finally with one 50-mL portion of methylene chloride.

e. The sample extracts are combined and these combined extracts of the sample and the sample with added aldosterone are evaporated separately using a rotary evaporator at 37° C to approximately 25 mL.

f. The concentrated extracts are transferred to 250-mL separatory funnels with four 10-mL portions of methylene

chloride, and then washed three times with 0.1 volume of 8% sodium bicarbonate and twice with 25-mL portions of glass-distilled water.

g. The concentrated extracts are transferred to 3.0-mL silanized conical centrifuge tubes and are taken to dryness under a stream of nitrogen at 35°-40° C.

h. The residues are dissolved with 1 mL of glass-distilled methanol taken from the draw-off valve of the HPLC system.

i. The dissolved sample is injected into the HPLC chromatograph.

j. Detection of UV absorbing components is accomplished with a 254-nm detector coupled to a recorder with electronic integrator.

C. Methods and Procedures

1. Urine Sample Collection

Urine specimens for the determination of urinary aldosterone were collected under most of the conditions outlined in a personal communication received from Dr. H.E. Thompson[63] of Youngstown Hospital Association (YHA), Department of Laboratories. They are as follows:

a. "Salt-loading." Salt loading was omitted. Reason: This investigator is not a medical doctor and by law is not permitted to prescribe any chemicals.

b. The 24-h urine collection: Usually A.M. to A.M. For example: At 7:30 A.M., patient voids and the urine is discarded. All urine subsequently voided in the next 24 hours is saved including the voiding at 24-h from

the start. The collection is pooled, mixed and total volume measured and recorded. An appropriate aliquot is saved with refrigeration until analyses have been completed.

c. The container: Thoroughly cleaned, 4 L organic solvent brown glass bottles.

d. "Preservative in the container for 24-h urine collections: YHA: 15 mL 6 N HCl;"

Note: There will be bacterial growth in urine specimens when held at room temperature without a suitable preservative.

e. Stated normal ranges for 24-h excretion of aldosterone. Bio-Science: 2-26 μg; Laboratory Medicine Data (used by YHA): 4-22 μg; Zack et al.,[33] M±2SD; 0-25 μg.

2. Estimation of Aldosterone in Samples

Estimation of aldosterone is performed using the HPLC system. This is coupled to an Omniscribe 2-Pen integrator recorder which recorded the chromatogram. Quantification of aldosterone is based on either peak height or total integration counts which is directly proportional to the mass of aldosterone injected.

CHAPTER V

RESULTS AND DISCUSSION

First, it was determined whether a chromatogram of the old stock aldosterone standard containing 47.2 μg/mL could be obtained.

The initial HPLC configuration, as described in Chapter Four, was used and the initial operating parameters were:

Sample: Aldosterone stock standard. Injected amount: 5 μL.

Column Length: 61 cm; outside diameter (O.D.): 3.18 mm; inside diameter (I.D.): 2 mm.

Packing: Bondapak C_{18}/Corasil Reverse-Phase. Particle size range: 37-50 microns.

Mobile Phase: Methanol:H_2O (50:50)

Temperature (Temp.): Reservoir-ambient; column-ambient; detector-ambient.

Flowrate: 0.5 mL/min.

Detector: UV; Wavelength: 254 nm; Sensitivity: 0.02 AUFS (Absorbance Units Full Scale).

Chart Speed: 0.25 in/min.

Amounts of aldosterone standard injected directly onto the column were 20 μL, 15 μL, 14 μL, 12 μL, 10 μL, and 5 μL, respectivey. It was determined that 5 μL injections of standards and samples would be the volume to inject, because most peaks obtained would remain on scale on the chromatogram.

The HPLC system and columns had been purchased a short time before this research was started.

The polarities of the sample and packing as well as the solvent are important to the separation process.

Waters Associates,[64] presented graphically an Eluotropic series and column packings polarities which is a useful means of rating the relative polarities of the solvents used. (See Figure 12.) Methanol and water were chosen as the solvent because of their medium to high polarities, and because aldosterone and other steroid standards are soluble in methanol-water mixtures.

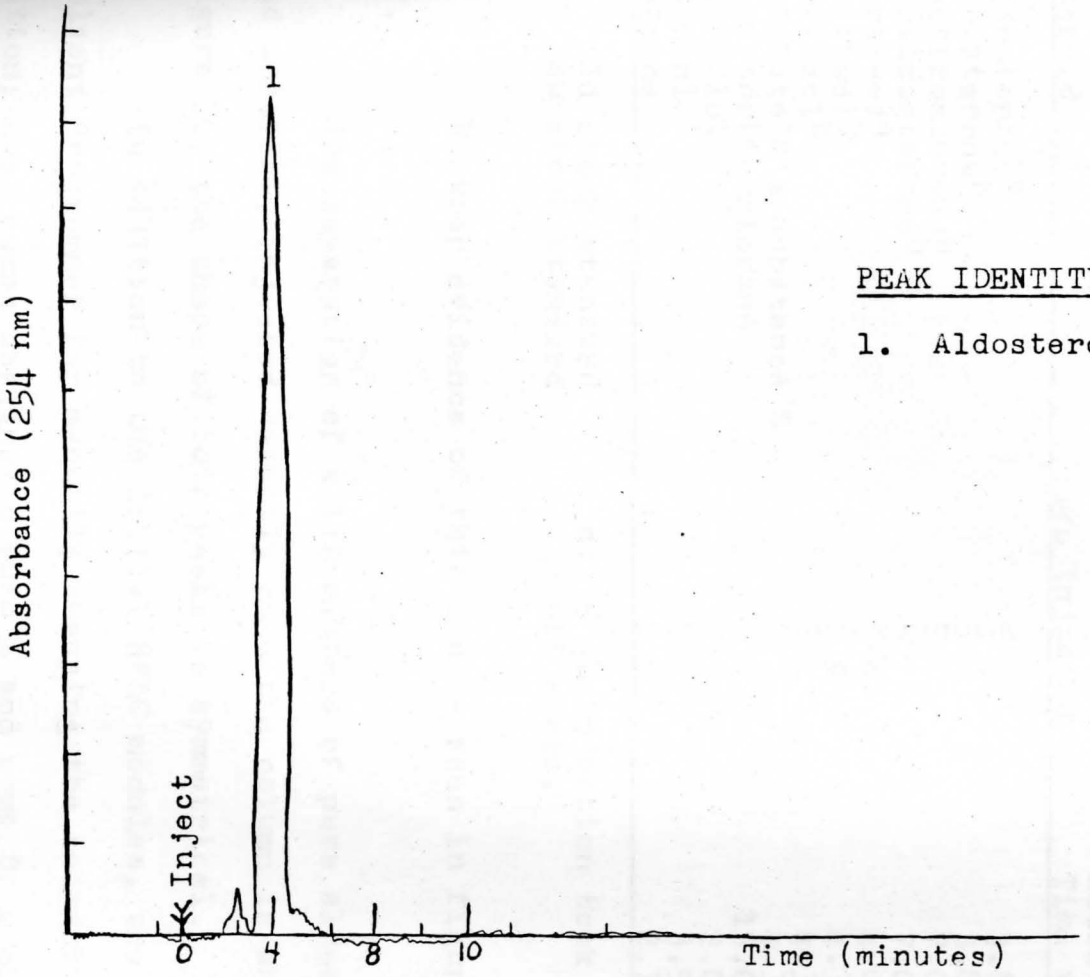
The chromatogram obtained of a 5 μL injection of pure aldosterone stock standard is shown in figure 15.

The shape of the aldosterone peak is symmetrical and the retention time is 3.8 minutes.

Chromatograms of the steroid standards, for example, of aldosterone and the steroids that might interfere with aldosterone determination were recorded utilizing the initial HPLC configuration as described in the experimental organization.

Table I shows the retention times obtained from the chromatograms recorded of the old and new pure stock aldosterone standards. Similarly, the retention times of other steroids that might interfere with aldosterone determination were obtained from their respective recorded chromatograms.

Symmetrical peaks of all the steroids were obtained on the chromatograms recorded.



PEAK IDENTITY

1. Aldosterone

Figure 15. Chromatogram of pure aldosterone stock standard. Column packing, Bondapak C_{18} Corasil Reverse-Phase; Mobile phase, $CH_3OH:H_2O$ (50:50); Temperature, ambient; Flowrate, 0.5 mL/min.; Detector, UV (254 nm); Sensitivity, 0.02 AUFS; Chart speed, 0.25 in/min.

The chromatograms recorded are evidence that pure aldosterone and pure steroids that might interfere with aldosterone determination can be obtained with good resolution using the HPLC system and the operational conditions described above.

TABLE I

THE RETENTION TIMES OF PURE STANDARDS

Steroid	μL Injected		Retention Time (min.)
Aldosterone[a]	5		3.8
Aldosterone[b]	5		3.8
Corticosterone[a]	5		7.0
Corticosterone[b]	5		7.0
Cortisol[a]	5	c	4.0
Cortisol[b]	5	c	4.3
Cortisol[b]	3		4.3
Reichstein's Substance S	5		4.3
Deoxycorticosterone	5		15.0
Estradiol	5		2.0
Estriol	5		3.5
Estrone	5		2.13

a: Old stock standard
b: New stock standard
c: 5 μL injection took peak off scale.

Further evidence of this can be seen in figure 16 and 17.

The separation of a 1:2 mixture of pure aldosterone and cortisol, injected directly onto the column is shown in figure 16; the shape of both peaks is symmetrical.

In addition to the initial HPLC modules, the M-660 Solvent Programmer for manually changing the solvent composition; dual pumps designated pump A and pump B; μBondapak C_{18} reverse-phase column and the Linear Instruments integrator recorder were used.

The retention times for aldosterone and cortisol in this mixture now are 9.8 minutes for aldosterone and 12.5 minutes for cortisol, respectively. The reason for the longer retention times for aldosterone and cortisol in the mixture is the decrease in particle size of the column packing.

The change in the flowrate to 1 mL/min caused the need for a pressure adjustment. In order to prevent a pump malfunction, the pressure cannot exceed 2000 psig maximum.

The separation of a 1:2 mixture of pure aldosterone and cortisol injected directly onto the column, and using CH_3CN:H_2O (24:76), is shown in figure 17; the shape of both peaks is symmetrical. The retention times for aldosterone and cortisol in this solvent mixture now are 14.7 minutes for aldosterone and 18.1 minutes for cortisol, respectively. The reason for the longer retention times for aldosterone and cortisol in the mixture is the mobile phase polarity increased due to the greater percent of water.

The reason for the change in the mobile phase CH_3CN:H_2O (24:76) was to decrease the pressure to prevent the HPLC down-time experienced with CH_3OH:H_2O (50:50), and also, to prevent pump malfunction.

However, in a busy clinical laboratory, the safety of personnel has to be considered, therefore, with methanol, being the less hazardous of the two reagents, the CH_3OH:H_2O solvent was chosen. The filters on the pump and column had to be cleaned more frequently. A column can become plugged by particulate matter in the sample and/or solvent building

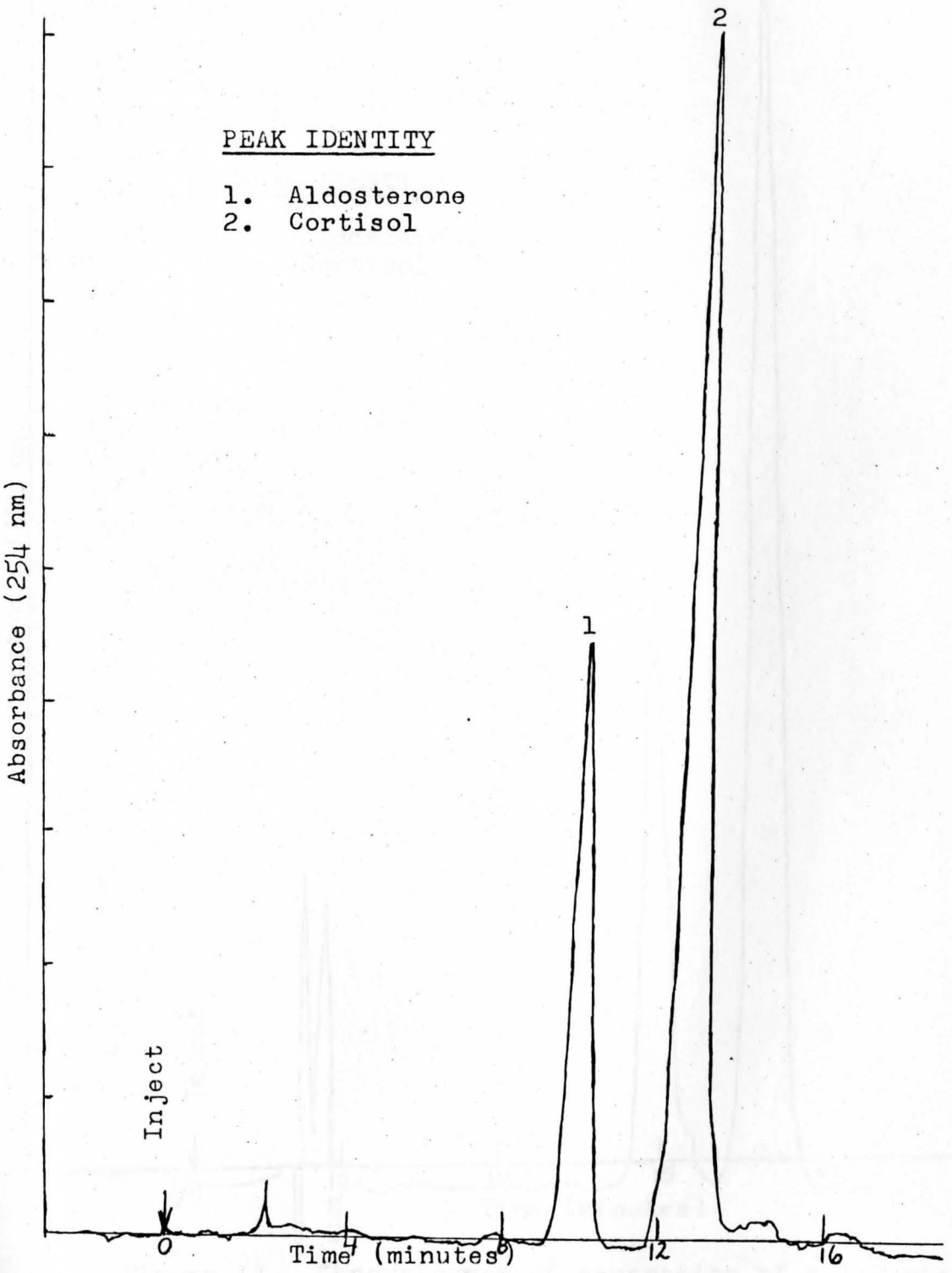


Figure 16. Chromatogram of separation of pure aldosterone (1) and cortisol (2) (1:2) mixture. Column packing, μBondapak C_{18} Reverse-Phase; Mobile phase, $CH_3OH:H_2O$ (50:50); Temperature, ambient: Flowrate, 1 mL/min.; Detector UV (254 nm); Sensitivity, 0.02 AUFS; Chart speed, 0.25 in/min.; Integrator, 3000 counts/min.

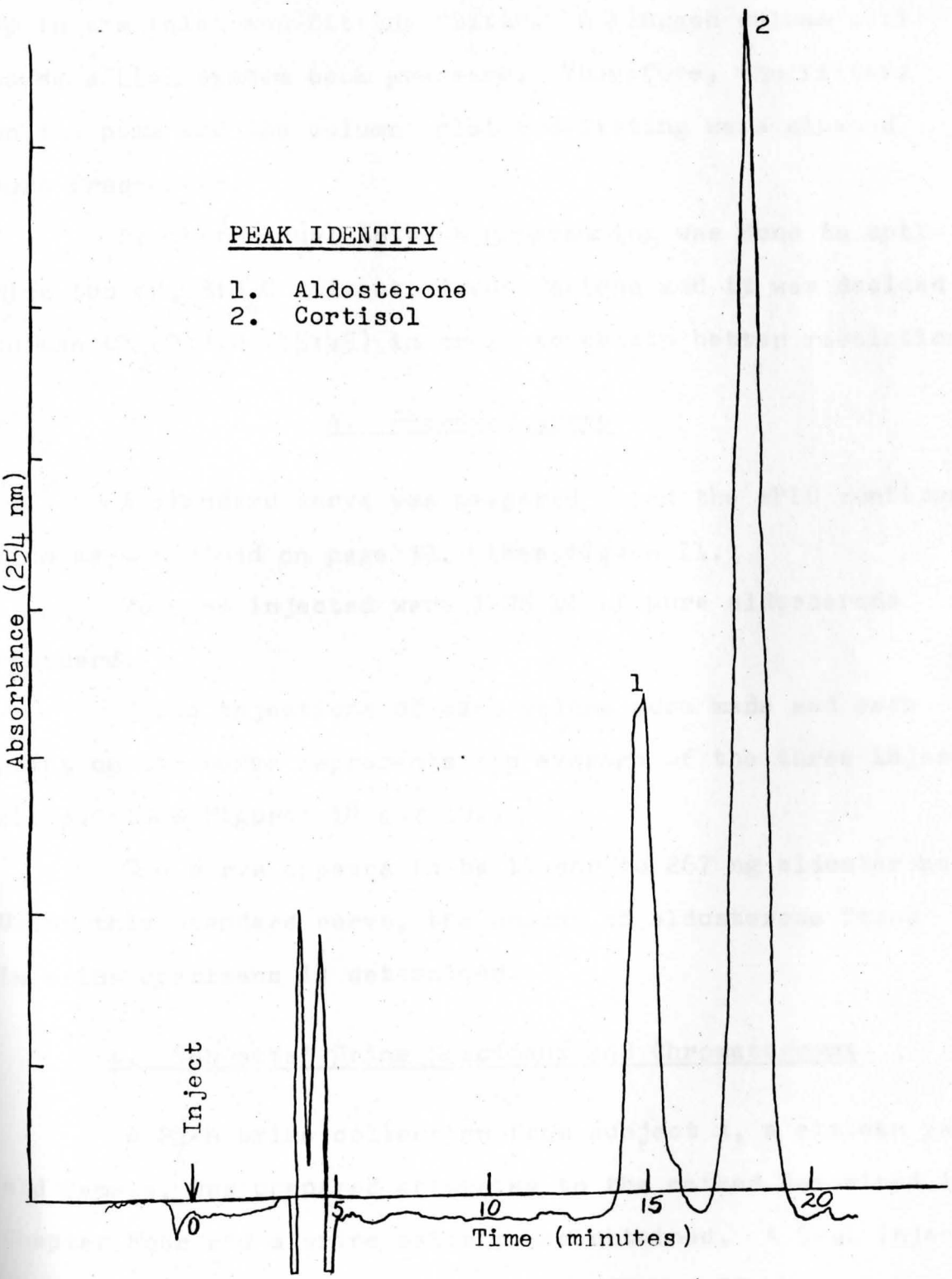


Figure 17. Chromatogram of separation of a 1:2 mixture of pure aldosterone and cortisol. Column packing; μBondapak C_{18} Reverse-Phase; Mobile phase, $CH_3CN:H_2O$ (24:76); Temperature, ambient; Flowrate, 1 mL/min.; Detector, UV (254 nm); Sensitivity, 0.02 AUFS; Chart speed, 0.2 in/min.; Integrator Amplitude, low.

up in the inlet end-fitting filter. A plugged column will cause a high system back pressure. Therefore, the filters on the pump and the column inlet end-fitting were cleaned more frequently.

Further manual solvent programming was done to optimize the $CH_3OH:H_2O$ solvent concentrations and it was decided to use $CH_3OH:H_2O$ (55:45) in order to obtain better resolution.

A. Standard Curve

A standard curve was prepared using the HPLC configuration as described on page 37. (See Figure 11.)

Volumes injected were 1-25 μL of pure aldosterone standard.

Three injections of each volume were made and each point on the curve represents the average of the three injections. (See Figures 18 and 19.)

The curve appears to be linear to 267 ng aldosterone. Using this standard curve, the amount of aldosterone found in urine specimens is determined.

B. Subjects' Urine Specimens and Chromatograms

A 24-h urine collection from subject A, a sixteen year old female, was prepared according to the method described in Chapter Four and a urine extract was obtained. A 5-μL injection of this urine extract was made. Figure 20 shows the chromatogram of this urine extract with aldosterone stock standard added (5 μg).

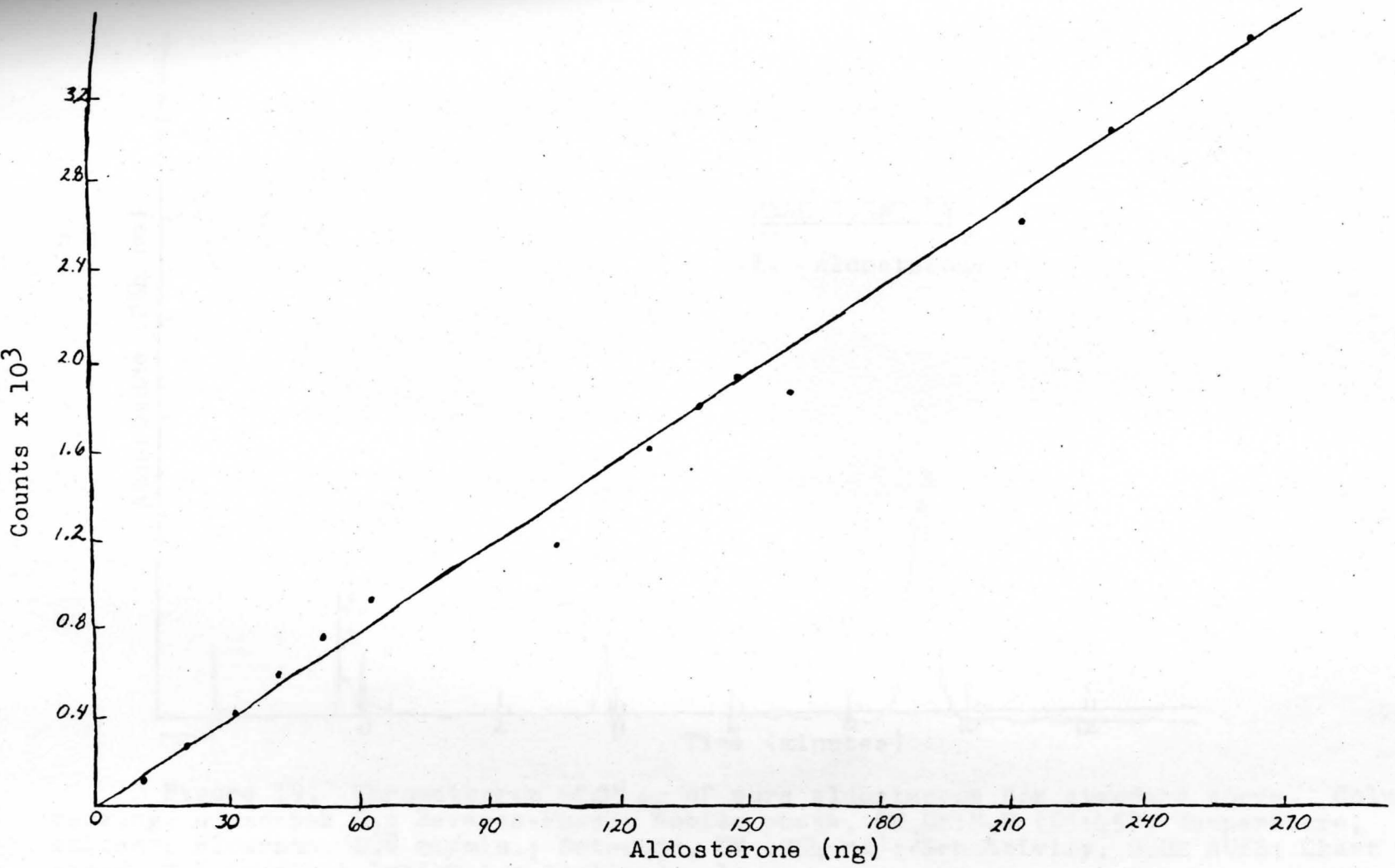


Figure 18. Standard curve of pure aldosterone. Injected volumes 1-25 μL of aldosterone stock standard (10.7 μg/mL). Column packing, μBondapak C_{18} Reverse-Phase; Mobile phase, CH_3OH:H_2O (55:45); Temperature, ambient; Flowrate, 0.8 mL/min.; Detector, UV (254 nm); Sensitivity, 0.02 AUFS; Chart speed, 0.5 in/min.;Integrator Amplitude, low.

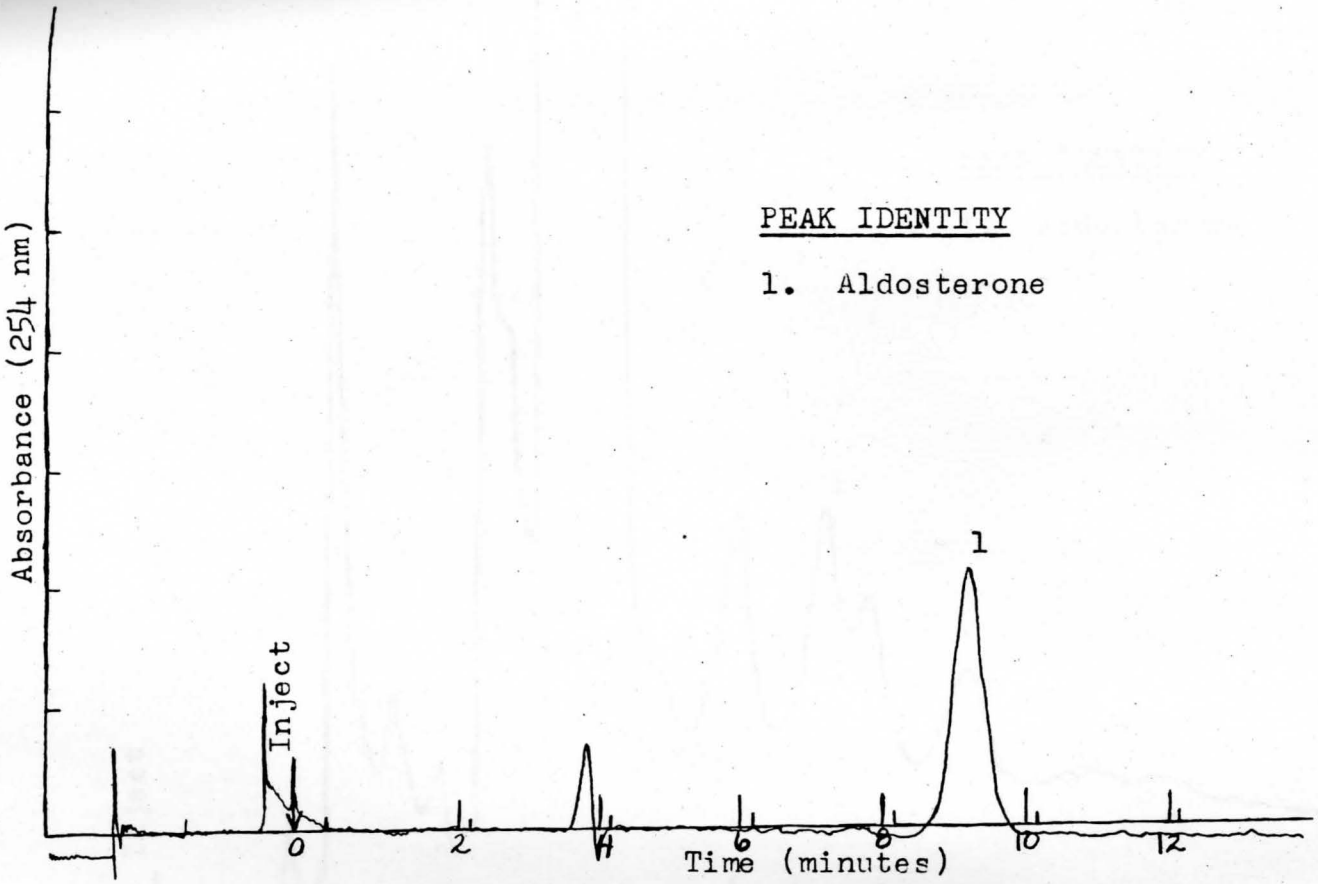


Figure 19. Chromatogram of .05 μg of pure aldosterone for standard curve. Column packing, μBondapak C_{18} Reverse-Phase; Mobile phase, $CH_3OH:H_2O$ (55:45); Temperature, ambient; Flowrate, 0.8 mL/min.; Detector, UV (254 nm); Sensitivity, 0.02 AUFS; Chart speed, 0.5 in/min.; Integrator Amplitude, low.

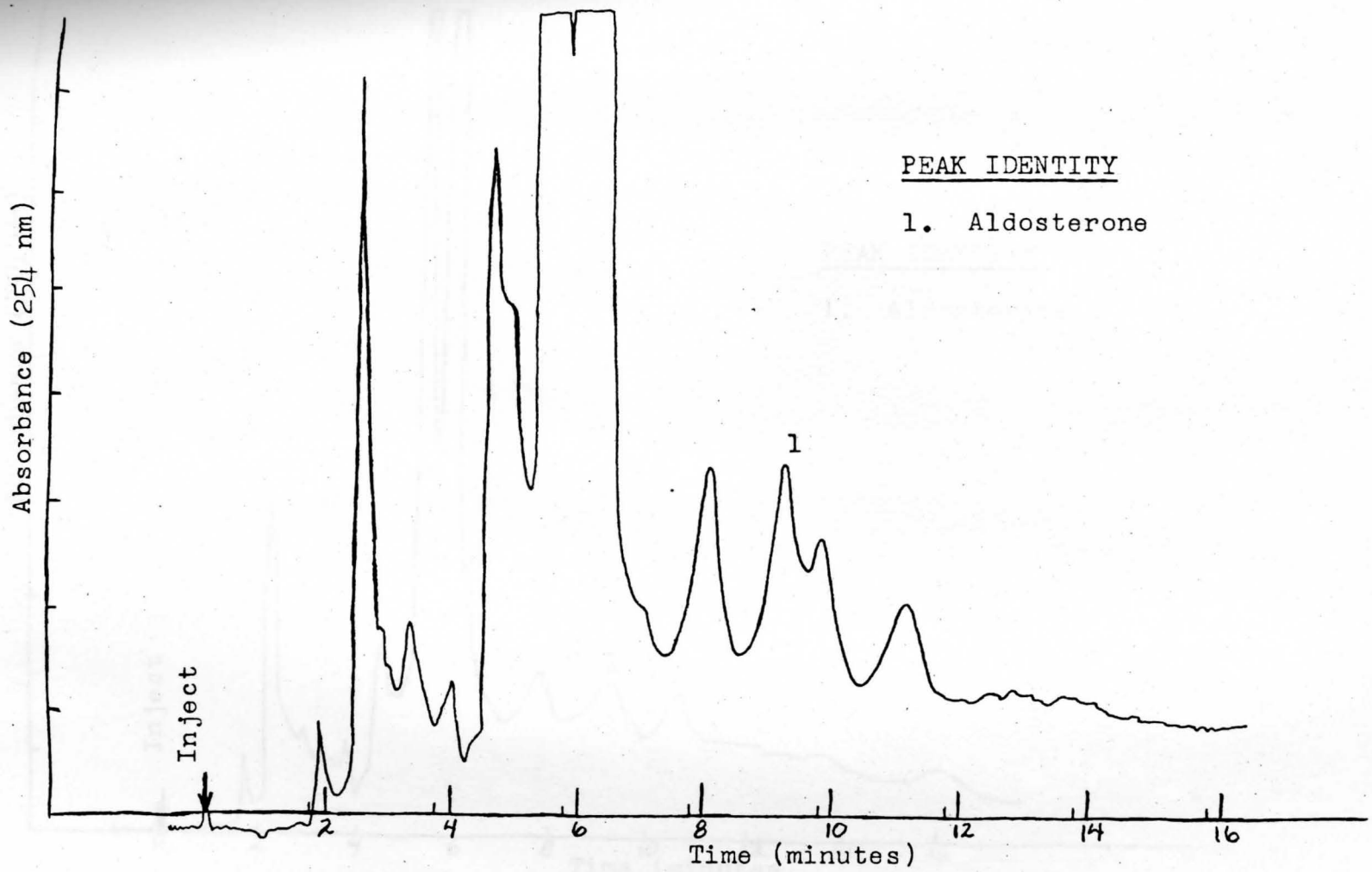


Figure 20. Chromatogram of subject A's (female) urine extract with aldosterone standard added. Column packing, μBondapak C_{18} Reverse-Phase; Mobile phase, $CH_3OH:H_2O$ (55:45); Temperature, ambient; Flowrate, 0.8 mL/min.; Detector, UV (254 nm); Sensitivity, 0.02 UAFS; Chart speed, 0.5 in/min.; Integrator Amplitude, low.

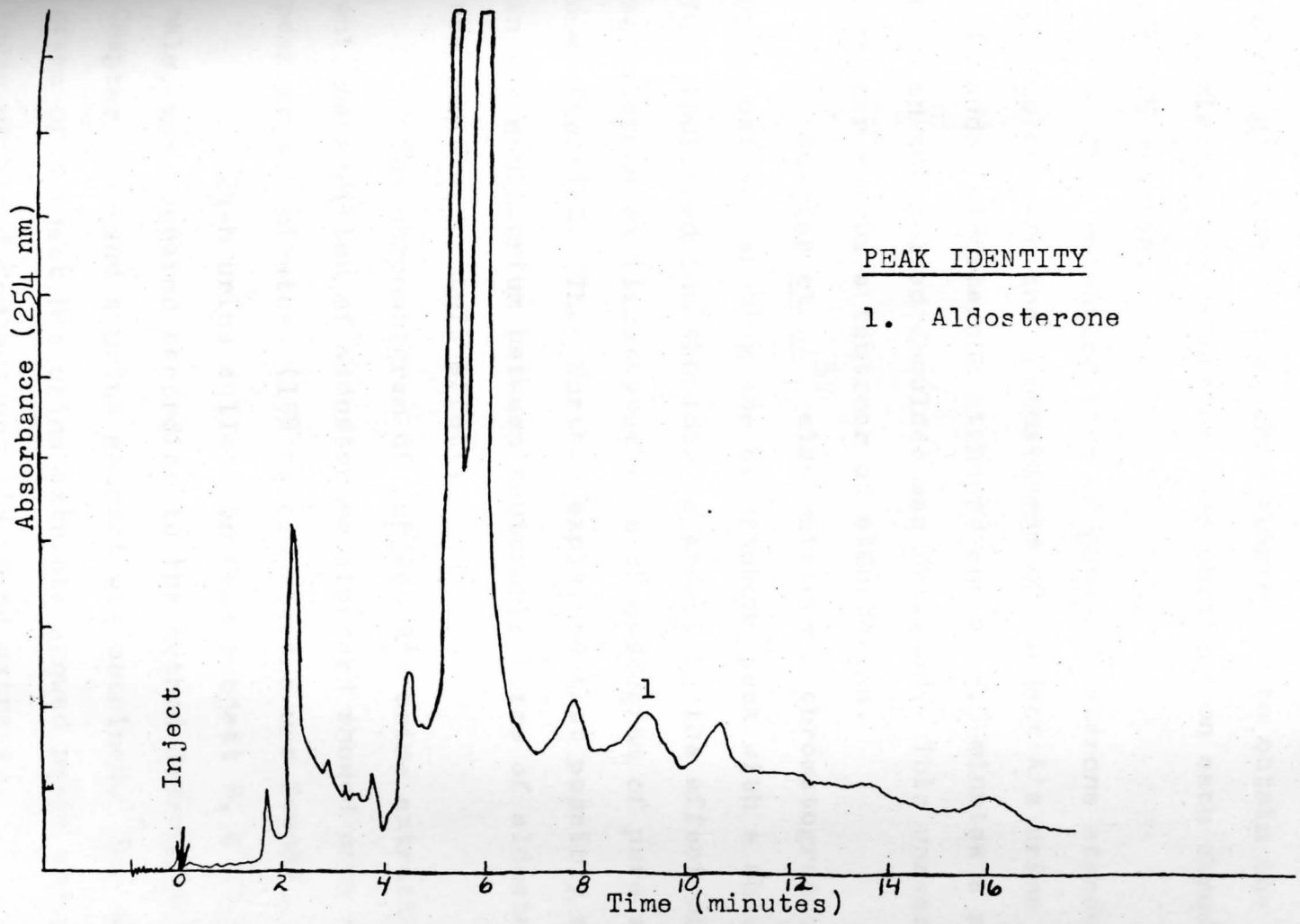


Figure 21. Chromatogram of subject A's (female) urine extract without aldosterone standard added. Column packing, μBondapak C_{18} Reverse-Phase; Mobile phase, $CH_3OH:H_2O$ (55:45); Sensitivity, 0.02 AUFS; Temperature, ambient; Flowrate 0.8 mL/min.; Chart speed, 0.5 in/min.; Integrator Amplitude, low.

Figure 21 shows the chromatogram of a 5- L injection of this urine extract without the addition of aldosterone standard.

Three 5- L injections of aldosterone stock standard (10.7 g/mL) were first chromatographed to obtain the retention times. The retention time obtained on each chromatogram was 9.5 minutes.

This retention time of pure aldosterone standard was used to compare the chromatogram of subject A's urine extract with added aldosterone standard and at 9.5 minutes a peak with an unresolved shoulder was obtained. This unresolved shoulder may be a tautomer of aldosterone.

DeVries et al[32] also obtained a chromatogram of a urine extract showing the aldosterone peak with a shoulder. They indicated the shoulder is caused by the effect of TLC on aldosterone as illustrated by a chromatogram of pure aldosterone after TLC. They further explained that possibly a shift in the equilibrium between tautomeric forms of aldosterone is responsible for this effect.

The chromatogram of subject A's urine extract without the addition of aldosterone standard showed only one broad peak at 9.5 minutes. (158 ng aldosterone was found).

A 24-h urine collection from subject B, a 17 year old male, was prepared according to the method described in Chapter Four and a urine extract was obtained. The chromatograms of subject B's urine extracts showed peaks similar to those obtained for subject A's urine extracts.

The researcher did not realize at the time the experiments were carried out that the unresolved shoulder obtained with subjects A and B's urine extracts could be a tautomer of aldosterone. See page 7. The aldosterone standard does not show a shoulder, only the aldosterone extracted from urine at pH 1. Perhaps the acidification of the urine to pH 1 promotes the tautomerism of the aldosterone, but, yet not observed in aldosterone in H_2O samples which were carried through the experimental protocol. Therefore, experimenting with the HPLC parameters to separate the aldosterone peak and shoulder followed.

The CH_3OH:H_2O concentration was changed manually using subject B's urine extracts with and without aldosterone standard to see if the peak plus shoulder could be resolved.

The best separation of the peak and shoulder was obtained with CH_3OH:H_2O (46:54) concentration.

Three chromatograms of pure aldosterone stock standard were always recorded before chromatograms of any urine extracts were obtained. The reason for this procedure was to determine the retention times of the pure aldosterone stock standard, then use this retention time to determine where aldosterone would be located on the urine extract chromatogram. The retention times for pure aldosterone would be located on the urine extract chromatogram. The retention time for pure aldosterone (16 minutes) increases as methanol concentration decreases. (See Figure 22.)

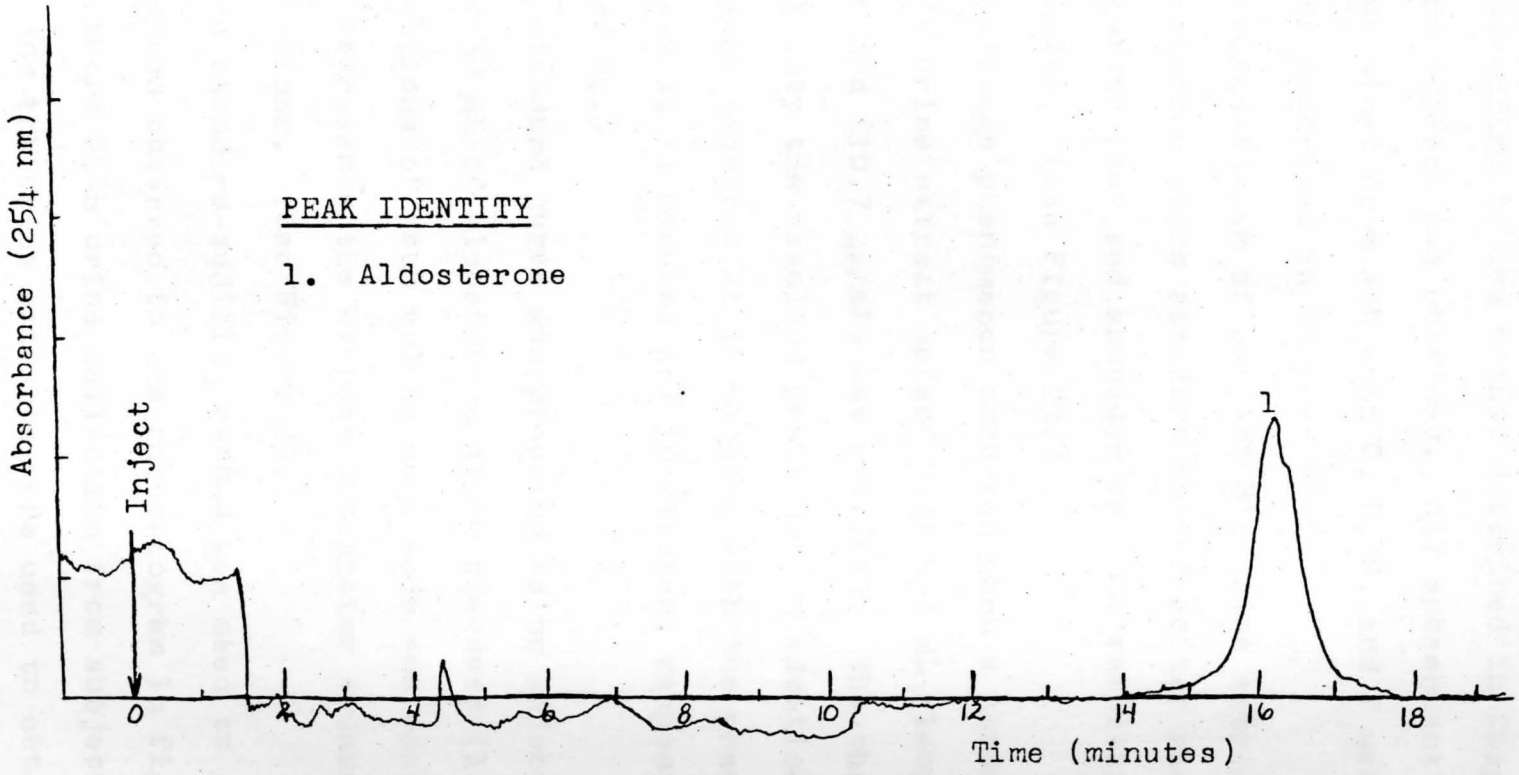


Figure 22. Chromatogram of pure aldosterone. Column packing, μBondapak C_{18} Reverse-Phase; Mobile phase, $CH_3OH:H_2O$ (46:54); Temperature, ambient; Flowrate 0.8 mL/min.; Detector, UV (254 nm); Sensitivity, 0.02 AUFS; Chart speed, 0.5 in/min; Integrator Amplitude, low.

A second 24-h urine collection from subject B was prepared according to the method described in Chapter Four and a urine extract was obtained. All subsequent urine extracts obtained from subjects C, D, E, and F were all prepared as described in Chapter Four.

A chromatogram of subject B's urine extracts without added aldosterone stock standard showed better resolution of the aldosterone peak and shoulder and the retention time was 16 minutes. (See Figure 23.)

A strange phenomenon occurred when a chromatogram of subject B's urine extract spiked with a 1 mL aldosterone stock standard (10.7 μg/mL) was obtained. The chromatogram showed not only the resolved peaks but an additional unresolved peak appeared at 16 minutes with the previously resolved peaks at 14 minutes and 15 minutes, respectively. (See Figure 24.)

A standard curve was prepared using injection volumes of 1 μL to 30 μL of aldosterone stock standard (10.7 μg/mL). Three injections of each volume were made and each point on the curve represents the average integrator counts of the three injections. (See Figure 25.)

The standard-addition method was used to corroborate the phenomenon observed in the chromatogram in figure 24.

A third 24-h urine collection from subject B was prepared, and the urine extracts were used to obtain chromatograms of the standard additions.

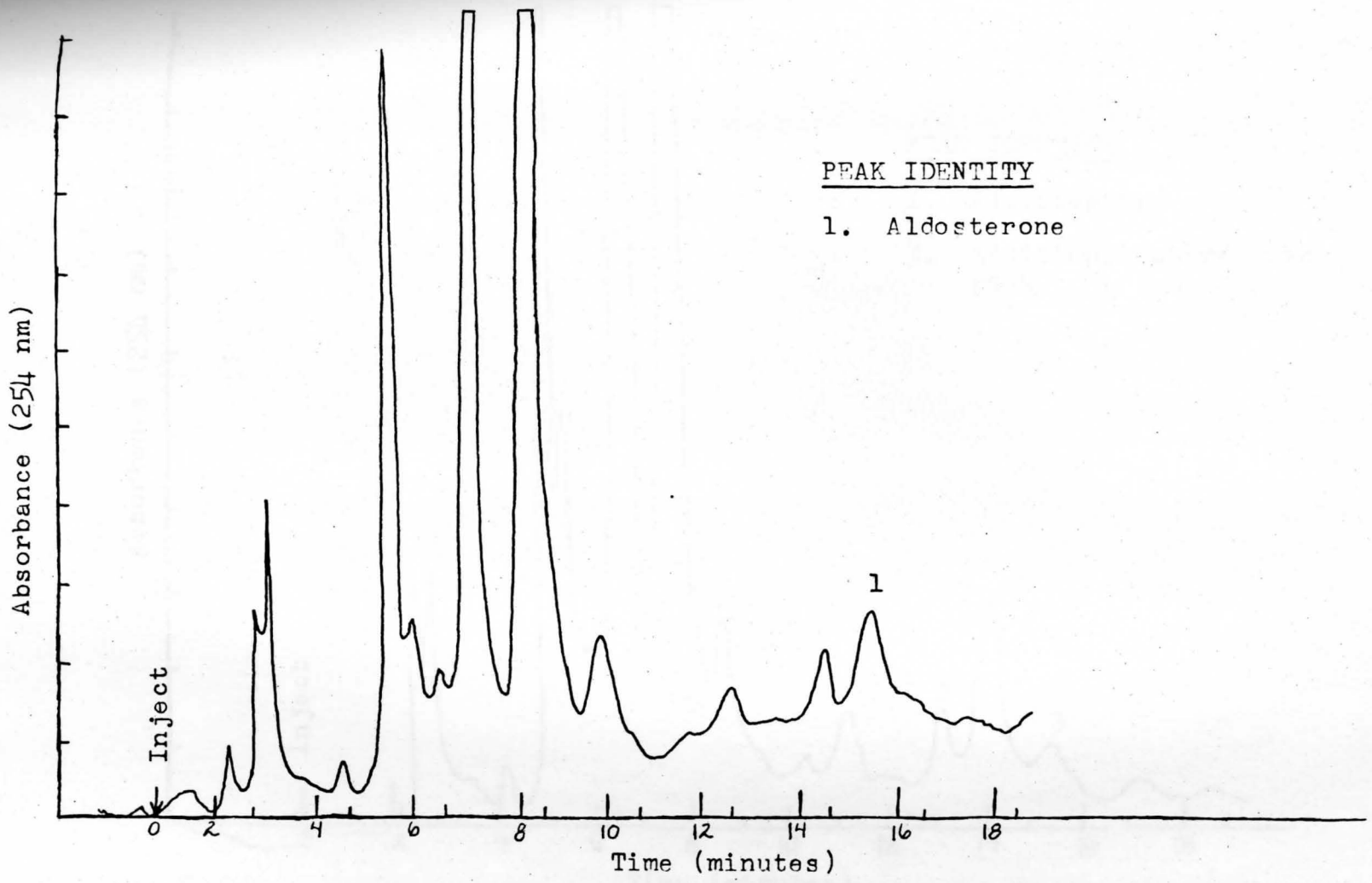


Figure 23. Chromatogram of subject B's (male) urine extract without aldosterone standard added. Column packing, Bondapak C_{18} Reverse-Phase; Mobile phase, $CH_3OH:H_2O$ (46:54); Temperature, ambient; Detector, UV (254 nm); Sensitivity, 0.02 AUFS; Flowrate, 0.8 mL/min.; Chart speed, 0.5 in/min.; Integrator Amplitude, low.

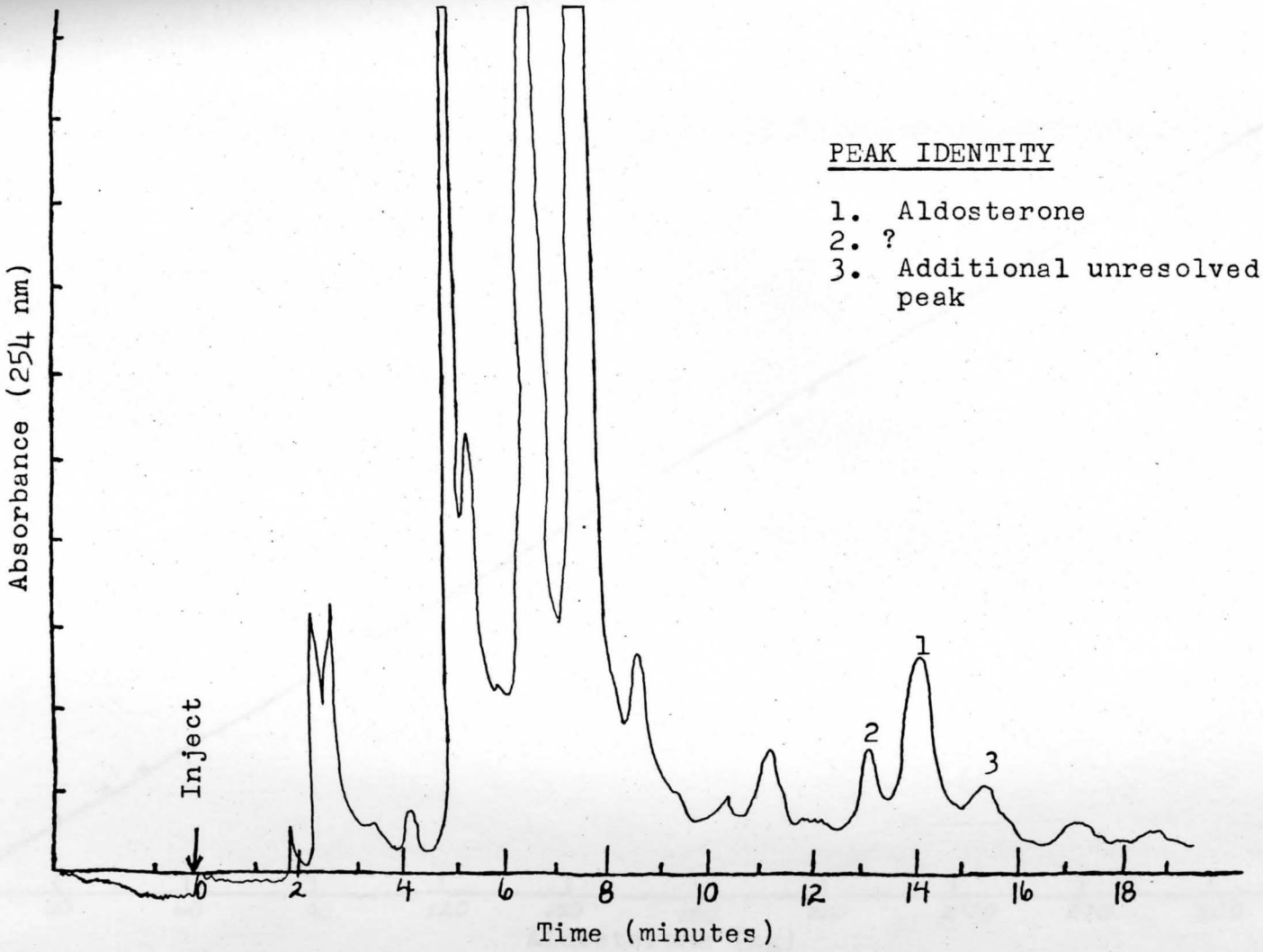


Figure 24. Chromatogram of subject B's urine extract spiked with 1 mL aldosterone stock standard (10.7 μg/mL). Column packing, μBondapak C_{18} Reverse-Phase; Mobile phase, $CH_3OH:H_2$) (46:54); Temperature, ambient; Flowrate, 0.8 mL/min.; Detector, UV (254 nm); Sensitivity, 0.02 AUFS; Chart speed, 0.5 in/min.; Integrator Amplitude, low

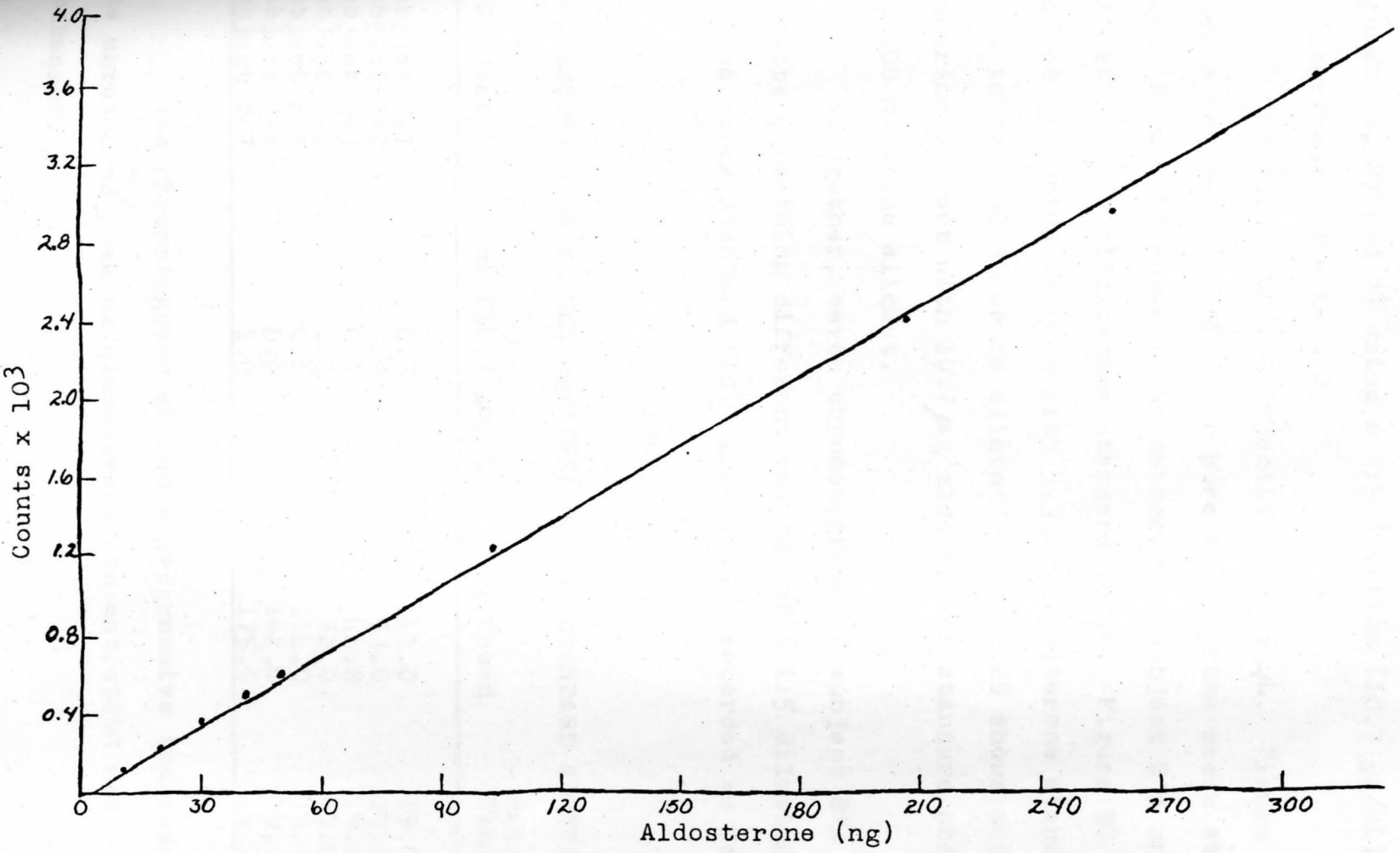


Figure 25. Standard curve of pure aldosterone. Injected volumes 1-30 μL of aldosterone stock standard (10.7 μg/mL). Column packing, Bondapak C_{18} Reverse-Phase; Mobile phase, $CH_3OH:H_2O$ (46:54); Temperature, ambient; Flowrate, 0.8 mL/min.; Detector, UV (254 nm); Sensitivity, 0.02 AUFS; Chart speed, 0.5 in/min.; Integrator Amplitude, low.

Three representative chromatograms are shown in figures 26, 27 and 28 using a 1:5 dilution (10.7 μg/mL) aldosterone stock standard.

The volume of all injections was 5 μL. Figure 26 shows a chromatogram of 53 ng pure aldosterone stock standard. Figure 27 shows a chromatogram of subject B's urine extract without aldosterone standard added. Figure 28 shows subject B's urine extract with 5.3 μg aldosterone standard added to the 100 mL urine aliquot. Figure 29 shows subject B's urine extract with 10.7 μg aldosterone standard added to the 100 mL urine aliquot.

Altogether, seven chromatograms of subject B's urine extracts containing different volumes of a 1:5 dilution aldosterone stock standard (10.7 μg/mL) were recorded as follows:

TABLE II

CHROMATOGRAMS OF VALUES OBTAINED OF THE STANDARD ADDITIONS

Subject	mL (10.7 μg/mL)	ng found	Retention Time (t_R)
Subject B-1	0.5	141.0	19.0 min.
Subject B-2	0.0	53.0	19.0 min.
Subject B-3	0.1	46.0	19.0 min.
Subject B-4	0.3	52.0	19.0 min.
Subject B-5	0.6	114.0	19.0 min.
Subject B-6	0.8	162.0	19.0 min.
Subject B-7	1.0	175.0	19.0 min.

The chromatograms showed a progressive increase in the unresolved peak as aldosterone concentration was increased.

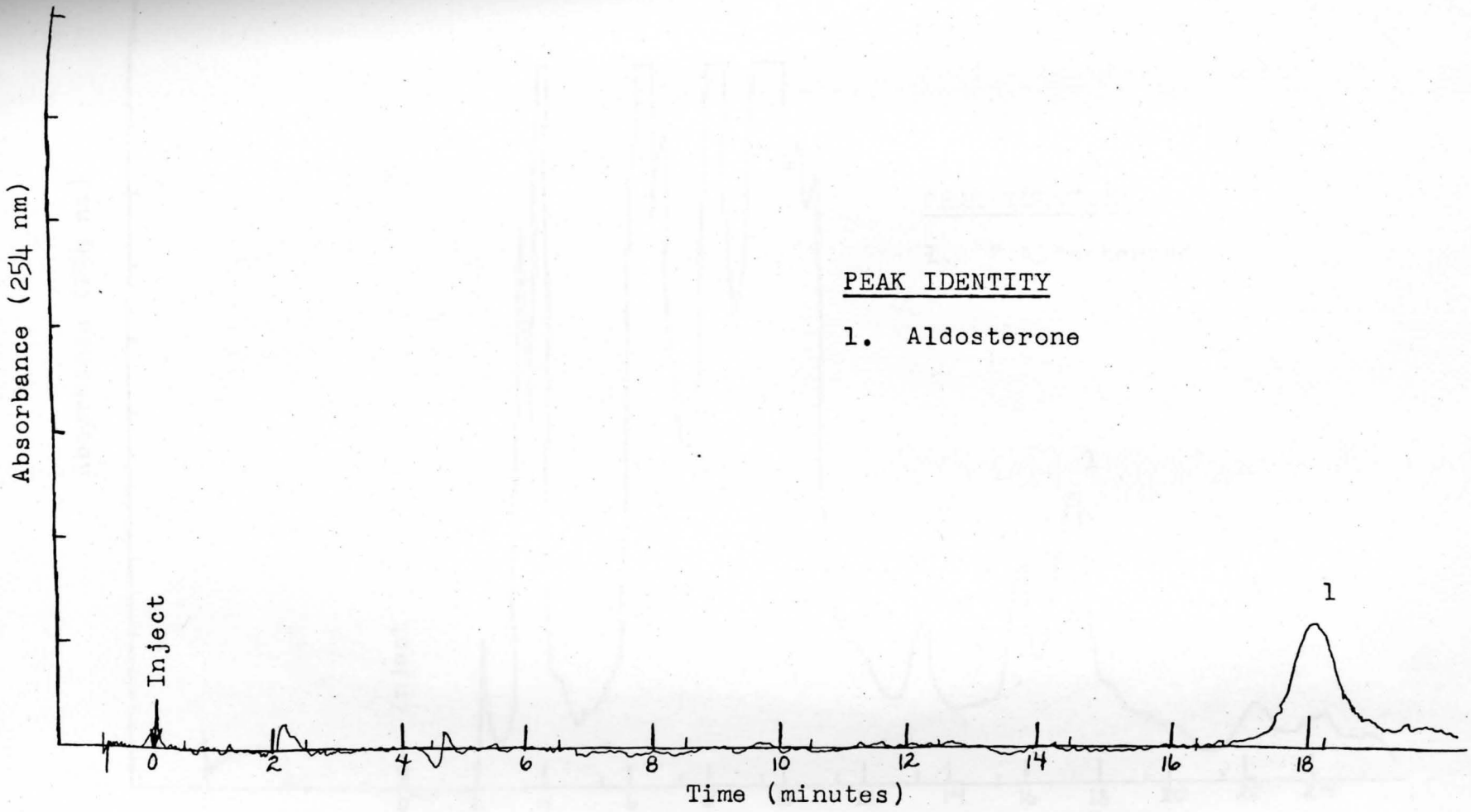


Figure 26. Chromatogram of 5 μL aldosterone stock standard (10.7 μg/mL). Column packing, μBondapak C_{18} Reverse-Phase; Mobile phase, $CH_3OH:H_2O$ (46:54); Temperature, ambient; Flowrate, 0.8 mL/min.; Detector, UV (254 nm); Sensitivity, 0.02 AUFS; Chart speed, 0.5 in/min.; Integrator Amplitude, low.

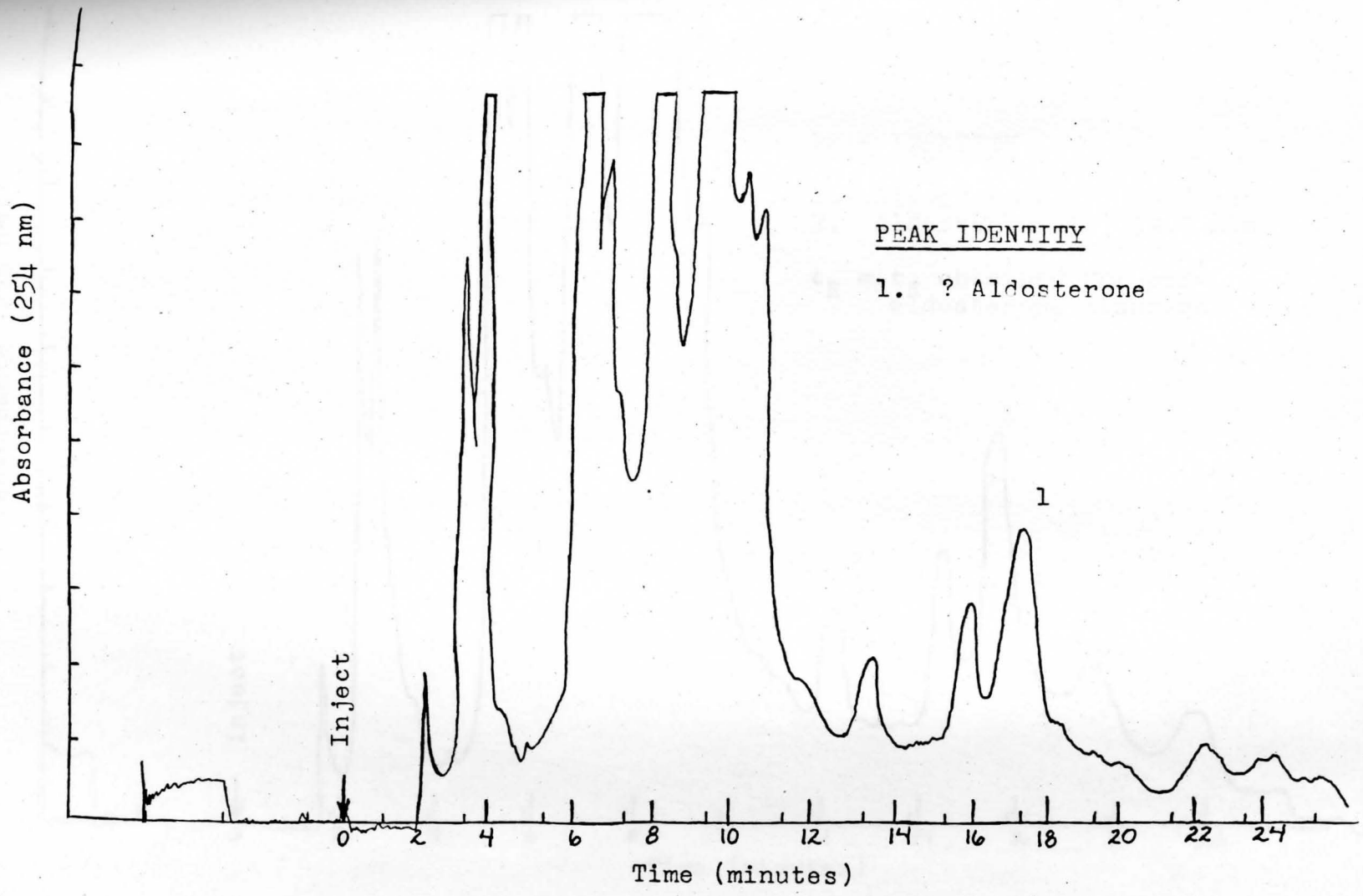


Figure 27. Chromatogram of subject B's urine extract without aldosterone standard added. Column packing, μBondapak C_{18} Reverse-Phase; Mobile phase, $CH_3OH:H_2O$ (46:54); Temperature, ambient; Flowrate, 0.8 mL/min.; Detector, UV (254 nm); Sensitivity, 0.02 AUFS; Chart speed, 0.5 in/min.; Integrator Amplitude, low.

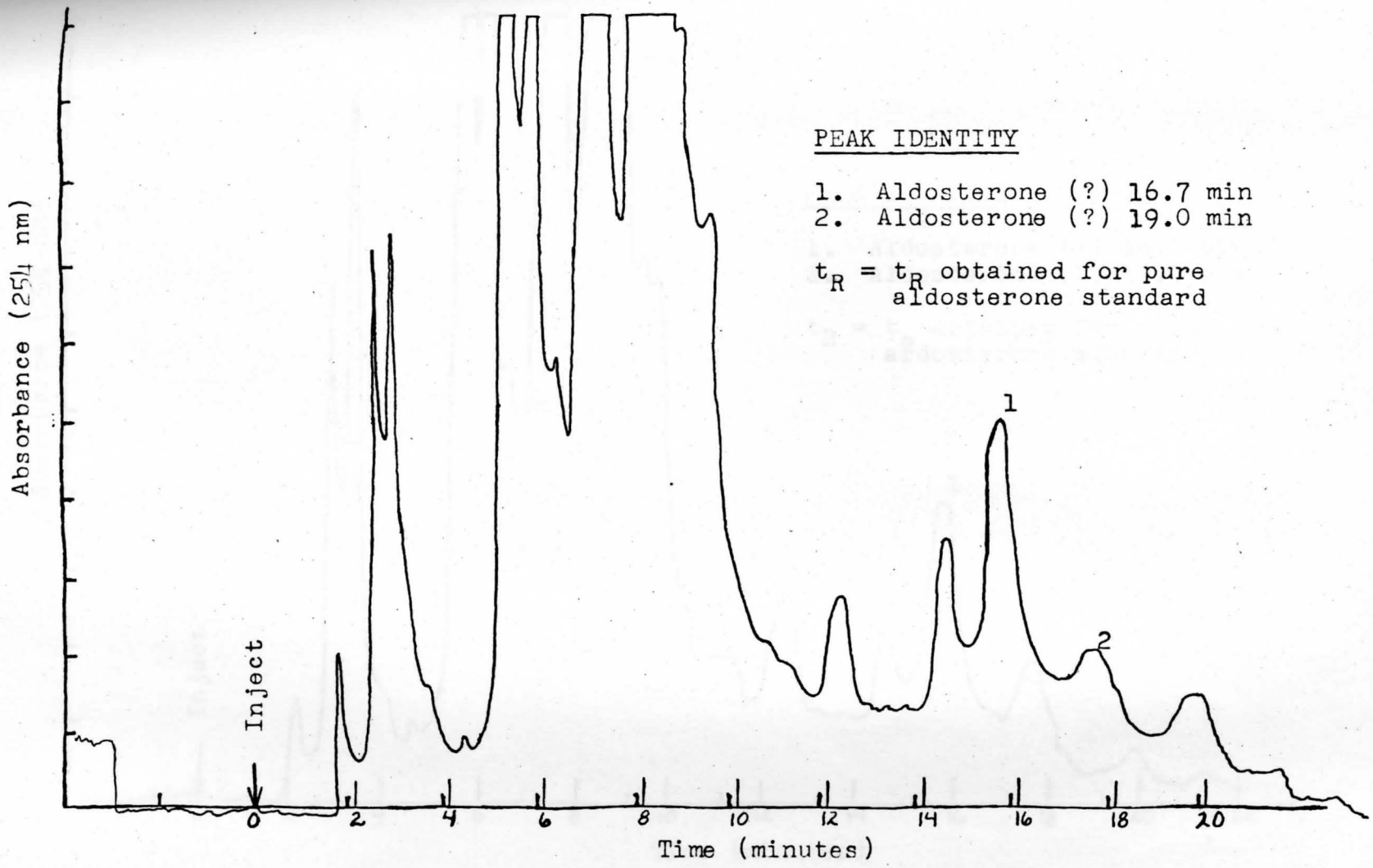


Figure 28. Chromatogram of subject B's urine extract with 5.3 μg aldosterone standard added to the 100 mL urine aliquot. Column packing, μBondapak C_{18} Reverse-Phase; Mobile phase, $CH_3OH:H_2O$ (46:54); Temperature, ambient; Flowrate, 0.8 mL/min.; Detector, UV (254 nm); Sensitivity, 0.02 AUFS; Chart speed, 0.5 in/min.; Integrator Amplitude, low.

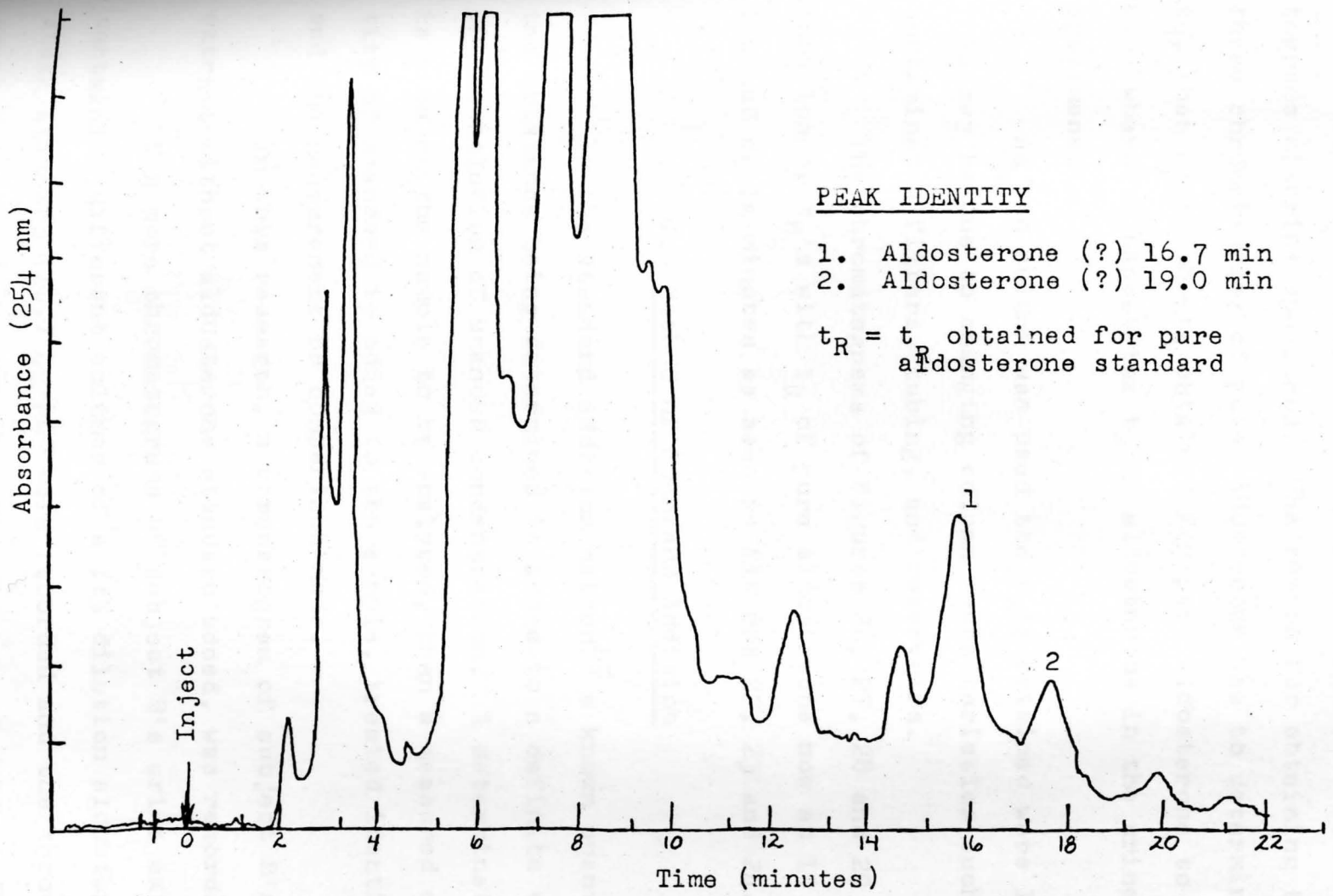


Figure 29. Chromatogram of subject B's urine extract with 10.7 μg aldosterone standard added to the 100 mL urine aliquot. Column packing μBondapak C_{18} Reverse-Phase; Mobile phase, CH_3OH:H_2O (46:54); Temperature, ambient; Flowrate 0.8 mL/min.; Detector, UV (254 nm); Sensitivity, 0.02 AUFS; Chart speed 0.5 in/min.; Integrator Amplitude, low.

As previously indicated, three chromatograms of pure aldosterone were always obtained prior to obtaining chromatograms of urine specimens. The reason for obtaining the three chromatograms of pure aldosterone was to determine the t_R, then using the t_R obtained for pure aldosterone to determine where to expect the t_R of aldosterone in the urine specimens.

As the column was used the t_R's obtained were longer. This may be due to changing column characteristics such as contaminated filters, tubing, and reservoirs.

The chromatograms of figures 26, 27, 28 and 29 all show longer t_R's with t_R of pure aldosterone now at 19 minutes instead of 16 minutes as seen in figures 22, 23 and 24.

C. Method of Standard Addition

In the standard addition method[65] a known quantity of the substance being determined is added to a definite volume of the solution of unknown concentration. A determination is made on the sample to be analyzed, then a measured quantity of standard is added to the sample, treated identically, and the measurement or comparison made.

In this research, a chromatogram of subject B's urine extract without aldosterone standard added, was recorded.

Six more chromatograms of subject B's urine extracts containing different volumes of a 1:5 dilution aldosterone stock standard (10.7 μg/mL) were recorded and the chromatograms were compared with the chromatogram of subject B's

urine extract without aldosterone standard added.

The new standard curve was used to determine the quantity (in ng) aldosterone found. (See Figure 25.)

Further examination of the chromatograms obtained revealed the retention times of aldosterone standards and urine extracts increased as more chromatograms were recorded.

Baselines became so erratic at one point, this researcher contacted the Waters Associates[66] to help determine the cause.

It was suggested, 2.0 mL dimethylsulfoxide (DMSO) be injected once a week to strip the column of any large polar molecules, for example, proteins.

As suggested by the Waters Associates personnel, DMSO (2.0 mL) was injected onto the column, but the problem was not resolved. Therefore, all filters, tubing and the reference and sample cuvettes were cleaned with a 6N HNO_3 (Nitric acid) solution. The distillation apparatus used to prepare the glass-distilled H_2O used in this procedure, and all reservoirs were also cleaned. This resolved the problem of erratic baselines.

Before each urine extract injection, 2 mL of H_2O followed by 2 mL of methanol were injected onto the column, to clean the column.

All this trouble-shooting resulted in reducing the retention times for aldosterone standards and urine extracts to 15 minutes.

Unfortunately, time did not permit to rerecord the aldosterone standards and urine extracts.

Twenty-four hour urine collections were obtained from:

TABLE III

Subject	Gender	Age (years)
Subject-A	Female	16 y
Subject-B	Male	17 y
Subject-C	Female	51 y
Subject-D	Male	26 y
Subject-E	Male	46 y
Subject-F	Male	23 y

All urine collections were prepared according to the method described in Chapter Four and urine extracts obtained.

The chromatograms of the urine extracts showed essentially the same results as obtained for subjects A and B.

The loss of aldosterone was determined in triplicate by adding no aldosterone, 5 μg, 26.7 μg, and 53.4 μg of pure aldosterone stock standard respectively, to 100 mL aliquots of 2X's glass-distilled water. Each 100 mL aliquot of water was then treated as a urine specimen following all the required steps. Twelve determinations were carried out as outlined in Chapter Four. The percent recovery was determined as follows:

$$\% \text{ recovery} = \frac{\text{mass of aldosterone found}}{\text{mass of aldosterone introduced into the sample}} \times 100$$

No peak plus shoulder was observed. The percent recovery was 64-71%.

CHAPTER VI

CONCLUSIONS AND SUGGESTIONS FOR FURTHER RESEARCH

A. Detection of Aldosterone Using HPLC

The chromatogram obtained of a 5 μL injection of pure aldosterone stock standard is shown in figure 15.

The shape of the aldosterone peak is symmetrical and the retention time is 3.8 minutes. The operational conditions were described previously.

The separation of a 1:2 mixture of pure aldosterone and cortisol, injected directly onto the column is shown in figure 16, the shape of both peaks is symmetrical.

Chromatograms of urine extracts were obtained, which showed a peak with an unresolved shoulder, at the same t_R as was obtained with pure aldosterone.

Figures 15 and 16 and all subsequent chromatograms recorded, are evidence that urinary aldosterone can be detected using HPLC. However, in order to suppress interferences from matrix elements of a urine extract completely, further experimentation would be required.

B. Problems Encountered

No difficulty was encountered when pure aldosterone, aldosterone and cortisol mixture, and other pure steroids were first injected onto the HPLC column.

Although μBondapak C_{18} has a monomolecular layer of octadecyltrichlorosilane chemically bonded via Si-C bonds to Waters Associates' new small diameter (10 microns) packing material (μPorasil), and their claim that the stability of these chemical bonds prevents bleeding and pooling, evidence of column deterioration was observed.

The retention times were not reproducible after only eight to twelve runs.

Erratic baselines were observed on the chromatograms.

It was suggested by the Waters Associates, that the column be cleaned weekly by the injection of 2 mL of dimethylsulfoxide (DMSO) to strip the column of protein build-up, also, to clean the UV cell.

Cleaning the column with DMSO helped, however, the column had to be regenerated by injection of 2 mL H_2O followed by 2 mL CH_3OH after each urine extract was run in order to obtain reproducible retention times for a day's run.

In order to prolong the life of the column, a precolumn or guard column may be desirable to be included between the injection port and the analytical column, especially for biological fluids.

Cleaning the UV Detector reference and sample cells, as well as the tubing of all the modules with 6N HNO_3 resolved the problems.

High system back pressure occurred frequently by the column becoming plugged by particulate matter building up in the inlet end-fitting filter.

Even though a U6K septumless injector was in the system, the column end-fitting filter had to be replaced frequently.

The reservoir had to be cleaned frequently and the solvent changed.

The reservoir filters and the inlet filter had to be cleaned frequently.

C. Suggestions For Future Research

At the time these experiments were carried out, disposable cartridges containing XAD-2 resin for purification of the urine samples were not available to this researcher.

Further experimentation, for example, using the disposable XAD-2 column cartridges to extract and concentrate the urinary aldosterone from the individual specimens, followed by the methods and procedures described in Chapter Four and further experimentation by varying HPLC parameters should produce more satisfactory results.

DeVries et al,[32] in their assay of aldosterone in urine by HPLC performed thin-layer chromatography (TLC) prior to HPLC to purify the urine extracts.

This researcher did not use TLC because TLC would add another time-consuming step to the procedure.

Whitney et al,[68] developed a single-step procedure for extraction and concentration of hydrophobic metabolites from plasma, serum, bile and amniotic fluids using disposable Sep-Pak C_{18} cartridges.

Further experimentation using Sep-Pak C_{18} extraction would be an extremely prudent course of action to take. The savings in time and solvents, combined with the simplicity, high capacity, excellent efficiency and broad range of Sep-Pak extractions, would provide an exceptional tool for use in determining human urinary aldosterone by HPLC.

Dr. Spiegel's conversation with Joanne Whitney disclosed that 98% of radioactive aldosterone conjugates adsorb onto Sep-Pak C_{18}.

The development of the Sep-Pak C_{18} cartridges (Waters Associates, Inc.) gives new hope for a simple and rapid determination of human urinary aldosterone by High-Pressure Liquid Chromatography.

Some modification of the procedure developed plus further experimentation by varying HPLC parameters should produce a simple, rapid determination of urinary aldosterone by HPLC.

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