THE ENZYMATIC SYNTHESIS OF ALDOSTERONE GLUCURONIDE AND ITS DETERMINATION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

by

Gracy Elias

Submitted in Partial Fulfillment of the Requirements

for the Degree of

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THESIS

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ABSTRACT

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Master of Science
Youngstown State University, 1986

This thesis describes the development of a possible procedure for the synthesis of aldosterone-18-glucuronide. Aldosterone is a steroid which is clinically important. Aldosterone-18-glucuronide is not available commercially. Without a pure standard, it is difficult to isolate the synthesized product using HPLC. The various procedures for glucuronide formation in the literature were studied and used in this research.

Aldosterone and UDPGA in the presence of the enzyme UDPGT from rabbit liver in Tris buffer at pH 7.4 incubated at $37^{0}C$ for one hour gave a positive result. To separate the products from the reactants, HPLC was used. HPLC separated the reactants from the newly found product. The newly found product was collected from the HPLC at its retention time and found that it was an acid-labile

conjugate of aldosterone because it released aldosterone when hydrolyzed at pH 1.0 at room temperature. The UVmax of this compound was found to be different from aldosterone and glucuronic acid. However, the test for glucuronic acid after hydrolysis of the synthesized aldosterone conjugate using HPLC gave reasons to think that the synthesized product might be a Tris complex of aldosterone-18-glucuronide. Suggestions for further work are discussed.

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I thank my husband and my son for their support, understanding and encouragement in the preparation of this thesis.

Finally, a word of thanks to Ms. Willa Mattern for her prompt typing of the manuscript.

DEDICATION

To my beloved husband, Thomas, my little son Joe, and my parents.

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LIST OF SYMBOLS

SYMBOL	DEFINITION
AG	Aldosterone glucuronide
AUFS	Absorbance units full scale
HPLC	High Performance (Pressure) Liquid Chromatography
IR	Infrared Absorption Spectroscopy
k'	Capacity factor
N	Theoretical plates
NEPD	Nonextractable polar derivatives
nm	Nanometers (10^{-9} m)
NMR	Nuclear Magnetic Resonance Spectroscopy
RIA	Radioimmunoassay
r.p.m.	Revolutions per minute
THAG	Tetrahydroaldosterone glucuronide
UDP	Uridine-5'-diphosphate
UDPGA	Uridine-5'-diphosphoglucuronic acid
UDPGT	Uridine diphosphoglucuronyl transferase

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CHAPTER I

INTRODUCTION

A. Definition and Structure of Aldosterone and Aldosterone-18-glucuronide.

The adrenal (suprarenal) glands, which are located superior to each kidney, produce numerous steroids. They have the common cyclopentanoperhydrophenanthrene ring (Fig. 1) as a part of their chemical structure (1).

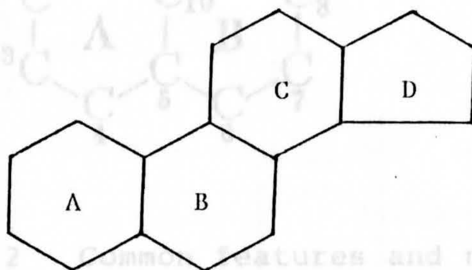

Fig. 1 Cyclopentanoperhydrophenanthrene ring (1)

These steroid hormones contain up to 21 carbon atoms (C_{21} steroids) numbered as shown in Fig. 2 (1).

Aldosterone is one of the steroids produced by the adrenal cortex. Aldosterone exists in three forms as shown in Fig. 3 (2).

Aldosterone is present in solution mainly in the tautomeric forms B and C, where the aldehyde group is protected by hemiacetal/ketal formation (2).

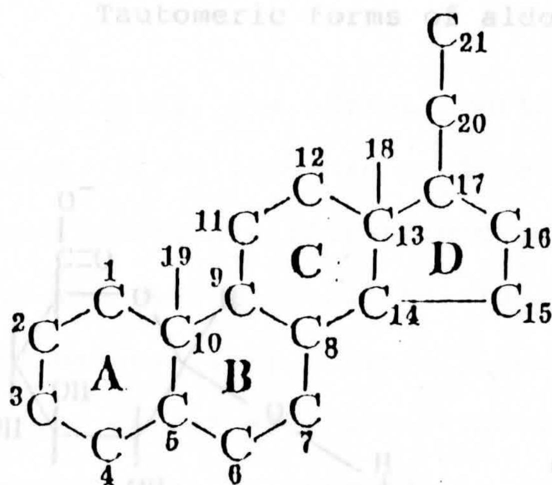

Fig. 2 Common features and numbering systems of steroids.

One of the two major urinary metabolites of aldosterone is aldosterone-18-glucuronide. The other metabolite is tetrahydroaldosterone glucuronide (3, 4). The structure of aldosterone-18-glucuronide is shown in Fig. 4.

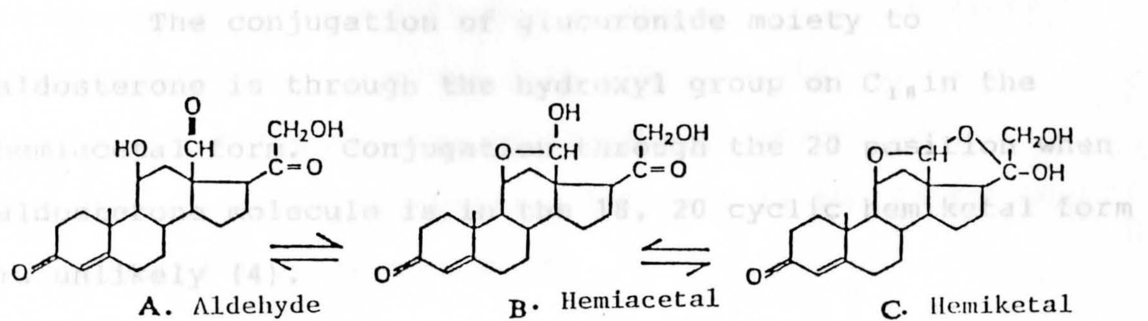

Fig. 3 Tautomeric forms of aldosterone (2).

Fig. 4 Structure of aldosterone-18-glucuronide (hemiacetal tautomer).

The conjugation of glucuronide moiety to aldosterone is through the hydroxyl group on C_{18} in the hemiacetal form. Conjugation through the 20 position when aldosterone molecule is in the 18, 20 cyclic hemiketal form is unlikely (4).

B. Tissue Origin of Aldosterone and Aldosterone glucuronide

Histologically, the adrenal cortex is subdivided into three zones. Each zone has a different cellular arrangement and secretes different groups of hormones. The outer zone, called zona glomerulosa, is arranged in arched loops or round balls. They primarily secrete a group of hormones that are called mineralcorticoids. The middle zone, zona fasciculata, is arranged in long, straight cords. It secretes mainly glucocorticoid hormones. The inner zone, the zona reticularis, contains cords of cells that branch freely. This zone synthesizes mostly sex hormones (gonadocorticoids), chiefly male hormones called androgens (5).

Aldosterone is the main mineralcorticoid responsible for 95% of the mineralcorticoid activity. Mineralcorticoids regulate salt and water metabolism. The other mineralcorticoid is deoxycorticosterone (5). Although

aldosterone is formed in the adrenals, it is immediately metabolized and converted into biologically inactive compounds. It undergoes metabolism in liver. But the liver as well as kidneys take part in the formation of aldosterone-18-glucuronide (6).

C. Physiological Function of Aldosterone

Aldosterone acts on the tubule cells in the kidneys and causes them to increase their reabsorption of sodium and water. As a result, sodium ions are removed from the urine and returned to the blood. On the other hand, aldosterone decreases reabsorption of potassium. So large amounts of potassium are lost in the urine. The sodium reabsorption leads to the elimination of H^+ ions, the retention of Na^+, Cl^-, and HCO_3^-, and the retention of water (5).

The control of aldosterone secretion is complex. Apparently, several mechanisms operate. One of these is the renin-angiotensin pathway shown in Fig. 5.

A decrease in blood volume from dehydration or Na deficiency brings about a drop in blood pressure. The low blood pressure stimulates certain kidney cells, called juxtaglomerular cells, to secrete into the blood an enzyme called renin. In this pathway, renin converts

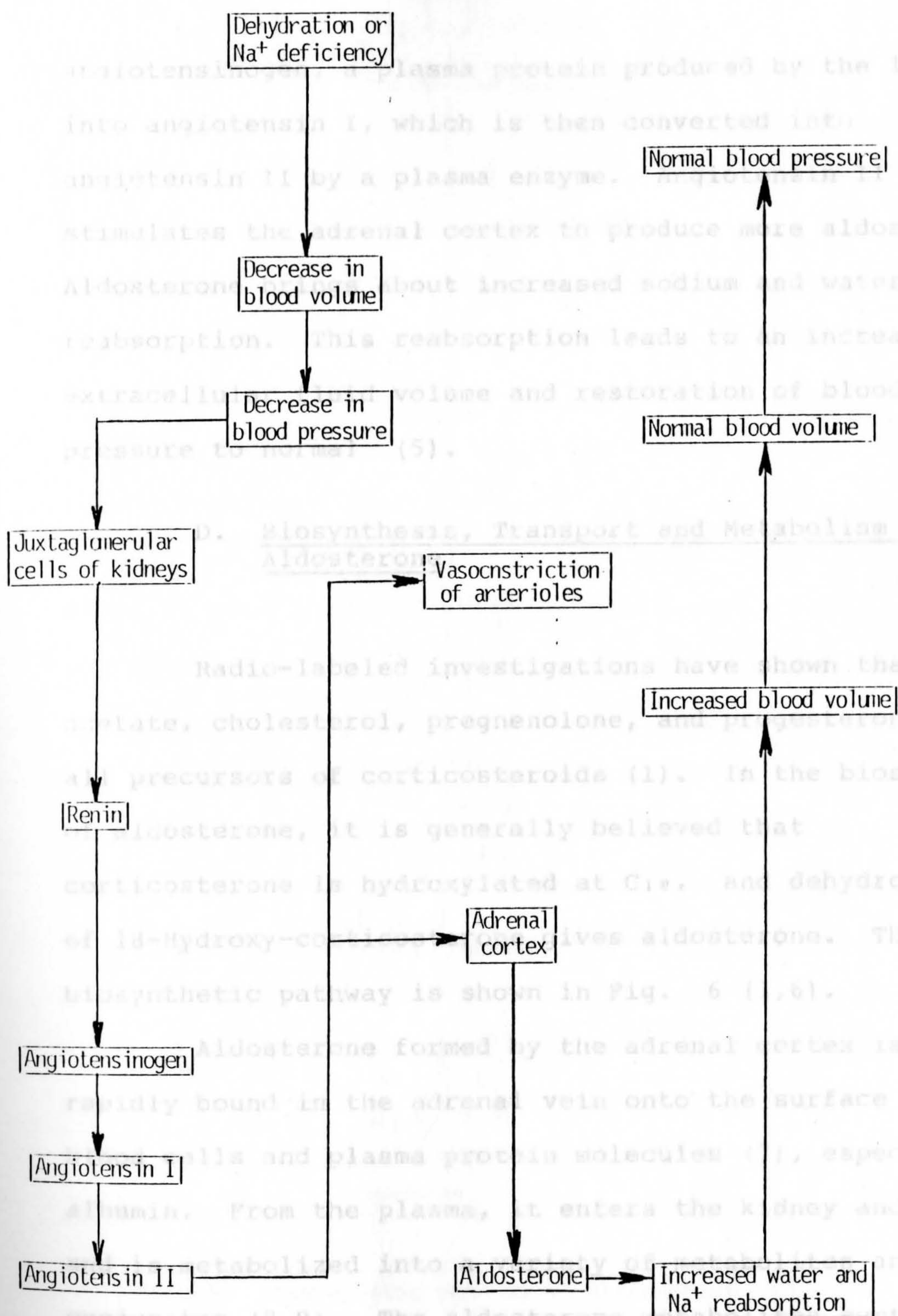

Fig. 5. Renin-angiotensin pathway (5).

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angiotensinogen, a plasma protein produced by the liver, into angiotensin I, which is then converted into angiotensin II by a plasma enzyme. Angiotensin II stimulates the adrenal cortex to produce more aldosterone. Aldosterone brings about increased sodium and water reabsorption. This reabsorption leads to an increase in extracellular fluid volume and restoration of blood pressure to normal (5).

D. Biosynthesis, Transport and Metabolism of Aldosterone

Radio-labeled investigations have shown that acetate, cholesterol, pregnenolone, and progesterone are all precursors of corticosteroids (1). In the biosynthesis of aldosterone, it is generally believed that corticosterone is hydroxylated at C_{18}, and dehydrogenation of 18-Hydroxy-corticosterone gives aldosterone. This biosynthetic pathway is shown in Fig. 6 (1,6).

Aldosterone formed by the adrenal cortex is very rapidly bound in the adrenal vein onto the surface of red blood cells and plasma protein molecules (7), especially albumin. From the plasma, it enters the kidney and liver and is metabolized into a variety of metabolites and conjugates (8,9). The aldosterone metabolites synthesized

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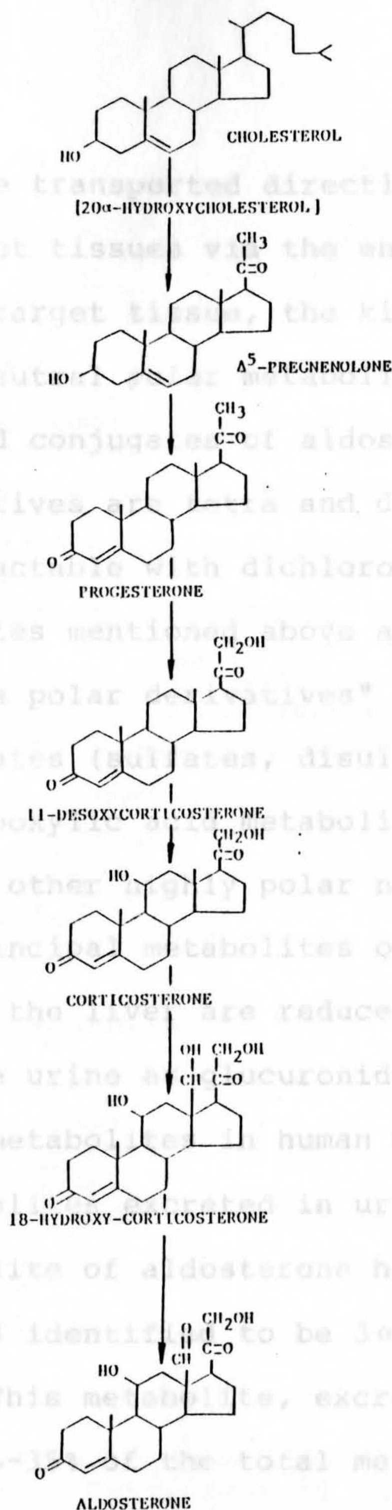
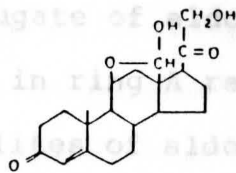


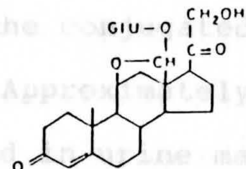
Fig. 6. Biosynthetic pathway of aldosterone

in liver may be transported directly to, or eventually reach the target tissues via the enterohepatic circulation (10,11). The target tissue, the kidney, contains reduced metabolites, neutral polar metabolites, carboxylic acid metabolites and conjugates of aldosterone (11). The reduced derivatives are tetra and dihydroaldosterone (5), which are extractable with dichloromethane, and all the other metabolites mentioned above are very water-soluble "nonextractable polar derivatives" (NEPD) (12). These NEPD include conjugates (sulfates, disulfates, and glucuronides) as well as carboxylic acid metabolites, hydroxylated compounds, and other highly polar neutral metabolites.

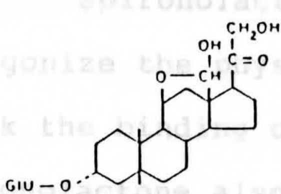
The principal metabolites of aldosterone synthesized in the liver are reduced in ring A and are excreted in the urine as glucuronides (11). Fig. 7 shows the principal metabolites in human urine and the percentage of total metabolites excreted in urine. The principal reduced metabolite of aldosterone has been isolated from human urine and identified to be $3\alpha, 5\beta$-tetrahydroaldosterone. This metabolite, excreted as a 3-glucuronide accounts for 25-35% of the total metabolites in human urine (11). The other major aldosterone metabolite found in human urine is an acid labile (pH 1) conjugate of aldosterone and has been identified as aldosterone-18-



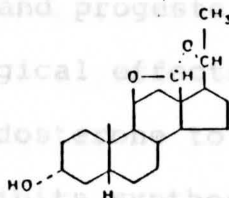
Free aldosterone (unconjugated) (0.1-0.5%)



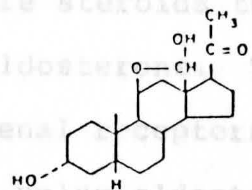
Aldosterone-18-glucuronide (7-12%)



Tetrahydroaldosterone 3-glucuronide (25-35%)

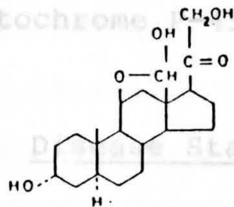


21-Deoxy-bicyclic acetal*

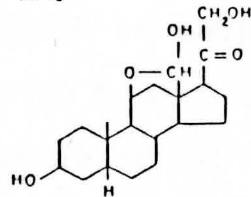


21-Deoxy-tetrahydroaldosterone

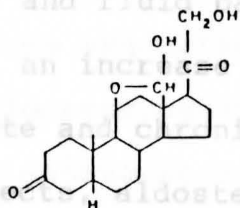
10%



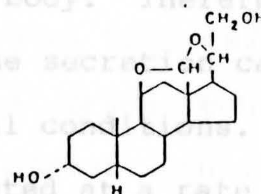
3α, 5α-Tetrahydroaldosterone



3β, 5β-Tetrahydroaldosterone



5β-Dihydroaldosterone



Bicyclic acetal*

Fig. 7. Principal Metabolites of Aldosterone in Human Urine. Percentage of total metabolites excreted in urine are shown in parentheses. *21-Deoxybicyclic acetal = 3α-Hydroxy-5β-pregnane-11β, 18S: 18S, 20α-Diepoxide. Bicyclic acetal = 3α, 21-Dihydroxy-5β-pregnane-11β, 18S:18S, 20α-Diepoxide.

glucuronide (11). It was earlier referred to as the 3-oxo-conjugate of aldosterone since the conjugated 4-en-3-one system in ring A remains intact. Approximately 10% of the metabolites of aldosterone excreted in urine may be accounted for as the 18-glucuronide.

Spironolactone and progesterone are steroids that antagonize the physiological effects of aldosterone. They block the binding of aldosterone to its renal receptors. Spironolactone also inhibits synthesis of polar aldosterone metabolites by liver microsomes, a process that is dependent on cytochrome P-450 (12).

E. Disease States

Aldosterone plays an important part in monitoring electrolyte and fluid balance in the body. Therefore, a decrease or an increase in aldosterone secretion can produce acute and chronic pathological conditions. In normal subjects, aldosterone is secreted at a rate of 10.5 μg per day (13). Increased quantities of aldosterone and its metabolites are present in the urine of patients with diseases such as primary and secondary hypertension, congestive heart failure, cirrhosis of the liver and nephrosis, as well as in the urine of pregnant women (12).

Elevation of the aldosterone level in the blood

plasma due to increased formation of aldosterone by the adrenals causes 'Primary aldosteronism' or Conn's syndrome. This may be an adrenal disease due to single or multiple adenomas, adrenal carcinoma, or bilateral adrenal hyperplasia. Extra-adrenal diseases, such as congestive cardiac failure, the nephrotic syndrome, or cirrhosis with ascites, may also cause overproduction of aldosterone. This is secondary aldosteronism (1). Cushing's syndrome and congenital adrenal hyperplasia are "mixed" forms of aldosteronism (6). In all these cases, the continuous excessive secretion of aldosterone results in sodium retention, potassium loss, and eventually hypertension (1).

Selective hypoaldosteronism occurs if (i) adrenal aldosterone secretion decreases because of deficient hormone synthesis as a consequence of impaired stimulation of aldosterone production or primarily as the result of deficient aldosterone synthesis by the adrenal cortex; (ii) the rate of metabolic destruction of aldosterone is increased; (iii) the effector organs are refractory to aldosterone (6).

F. Statement of the problem

Various methods have been developed for the determination of aldosterone (6). Among them

Neher-Wettstein (14) methods using paper and column chromatography were popular until the nineteen-sixties. The double derivative method of Kilman and Peterson (15), and isotope methods were used in the sixties. The gas chromatographic method was also developed and improved in the late sixties.

All these methods are tedious and long. A method which is less tedious and less time-consuming for the determination of aldosterone is the goal of many researchers. The development of bonded-phase silica derivatives made the extraction of aldosterone and its conjugates from urine easier. There are some improved methods for the determination of aldosterone using RIA (16) and also using HPLC (17,18). Aldosterone-18-glucuronide (AG) and tetrahydroaldosterone glucuronide (THAG) are the two major metabolities that are excreted in urine. Urine also contains small amounts of free aldosterone. For diagnosis of hyperaldosteronism the sum of aldosterone-18-glucuronide and free aldosterone is usually assayed by hydrolyzing the urine at pH 1 for 18-24 hours, followed by extraction. Successful determination of aldosterone and aldosterone-18-glucuronide was done with HPLC using a radiolabeled steroid (17,18).

Without radiolabeled isotopes, the determination of

aldosterone by HPLC as the glucuronide might be possible using aldosterone-18-glucuronide as a standard. But a standard is not available commercially to the best of this researcher's knowledge. If aldosterone-18-glucuronide were available as a standard, then urine could be analyzed for aldosterone by HPLC without the required hydrolysis step and subsequent time-consuming cleanup steps prior to HPLC (14, 19).

The purpose of this research is to develop a procedure for the enzymatic synthesis of aldosterone-18-glucuronide (AG) based on previous work involving the synthesis of α-naphthol glucuronides by To and Wells (20), and the synthesis of steroid glucuronides by Dutton et al (21). The separation and identification of aldosterone-18-glucuronide will then be attempted using reverse-phase HPLC with ultra-violet detection at 240 nm with fraction collection of the suspected peaks. If the retention time of aldosterone-18-glucuronide is known, then when a urine extract is injected onto the reversed-phase column, a peak with the same retention time will indicate the presence of aldosterone-18-glucuronide. Quantitation of the peak will then permit the determination of the amount of AG in the patient's urine. This can be compared to a normal range of AG and the total value of aldosterone as found by RIA or

hydrolytic methods.

CHAPTER II

REVIEW OF LITERATURE

Aldosterone, which is excreted in urine mainly as its metabolites, has been a topic of research for scientists in different fields. One of its main metabolites is the glucuronide conjugate,aldosterone-18-glucuronide (6). Analysis of steroids of a defined conjugate class is more informative than analysis after initial hydrolysis of the total mixture (22). The following paragraphs discuss the work done in the area of steroid glucuronides in general and aldosterone-18-glucuronide in particular.

A. Extraction Methods

The determination of various xenobiotic substances in biological fluids usually involves protein precipitation, filtration, and solvent extraction in order to prepare the sample for analysis by either spectrometric or chromatographic techniques. These cleanup procedures are required to remove many of interfering compounds. The removal of these interfering substances is essential in order to obtain a pure sample for subsequent procedures or, if quantitative studies are required, a signal-to-noise

ratio of sufficient intensity to ensure accurate and reproducible measurements (23).

Underwood et al (24) describe the solvent extraction procedure for aldosterone after the hydrolysis of the urine sample. They compared various solvents and found that methylene chloride and chloroform are the most suitable solvents for extraction of aldosterone.

Solid-phase extractions have many technical advantages over liquid-liquid extractions. Development of solid-phase extraction methods has accelerated in the last few years and has involved three main types of adsorbents: polystyrene resins, substituted crosslinked dextrans, and substituted porous silica.

The description by Bradlow (25) of a method for extraction of steroids by the neutral polystyrene resin, Amberlite XAD-2, represented a major advance in steroid analysis. Neutral Amberlite resins (XAD-2, XAD-4, XAD-7) have now been widely used for many years for the extraction of steroids from aqueous solutions. Aldosterone-18-glucuronide was separated from urine using Amberlite XAD-2 columns (18, 26). Urine was passed through several Amberlite XAD-2 columns, the columns were washed with water and the steroids were eluted with methanol. The methanol eluate was evaporated and the residue dissolved in phosphate buffer. This solution was then washed with methylene chloride and the aqueous portion was then incubated with

β-glucuronidase to bring about the hydrolysis of other steroid glucuronides. This incubation mixture was again washed with methylene chloride and the aqueous solution was applied to an Amberlite XAD-2 column and the column then washed with distilled water. The aldosterone-18-glucuronide on the column was then eluted with methanol.

Sephadex gels substituted with alkyl chains form good stationary phases in alcohol-water mixtures (27). Gels with an appropriate degree of substitution to be just wetted by water, e.g., Lipidex 1000 (containing 10%, w/w, of C_{12}-C_{14} alkyl chains), function as nonpolar adsorbents. If water containing lipid-soluble compounds is passed through a small bed of Lipidex 1000, these compounds are retained and can then be eluted with methanol or chloroform-methanol. Conjugated steroids, because of their water solubility, are poorly adsorbed by Lipidex 1000. But the addition of decyltrimethyl ammonium bromide to the aqueous solution results in formation of ion pairs that can be adsorbed by Lipidex 1000 (28).

Substituted porous silica, the so-called bonded phases, can be used for extraction of substances from aqueous solutions. Shackleton and Whitney (29) described a method for the rapid and quantitative extraction of free and conjugated steroids from urine using Sep-pak C_{18} cartridges. These cartridges are small columns containing octadecyl silane bonded phase packing retained between

filters. The lengthy extraction procedure with Amberlite XAD-2 resin is replaced by these bonded phase cartridges. Risk and Holland (17) used different bonded-phase silica derivatives (Sepralytes) for the preparation of aldosterone glucuronide (AG) and tetrahydroaldosterone glucuronide (THAG). They processed the urine sample through a C_2 (ethyl) column. A SCX (strong cation exchanger) column was positioned below the C_2 column, and the steroid glucuronates were eluted with distilled water. This eluent at pH 7.5 was then passed through a SAX (strong anion exchanger) followed by a CF (chromatofocusing) column and a C_2 column using a step gradient.

Free, sulfated and glucuro-conjugated steroids are extracted from serum, ovarian follicles, lutein cyst, and peritoneal fluids using Sep-Pak C_{18} cartridges (30). These three classes are then further separated from each other by successive extractions with ethyl acetate. Free steroids are recovered first; after hydrolysis of the water layer, sulfated steroids are recovered and, finally, after β-glucuronidase enzymatic hydrolysis, steroid glucuronides and mixed conjugates are obtained (30).

The method used by Axelson et al (31) to extract unconjugated and conjugated steroids from urine samples is shown in a flow scheme in Fig. 8.

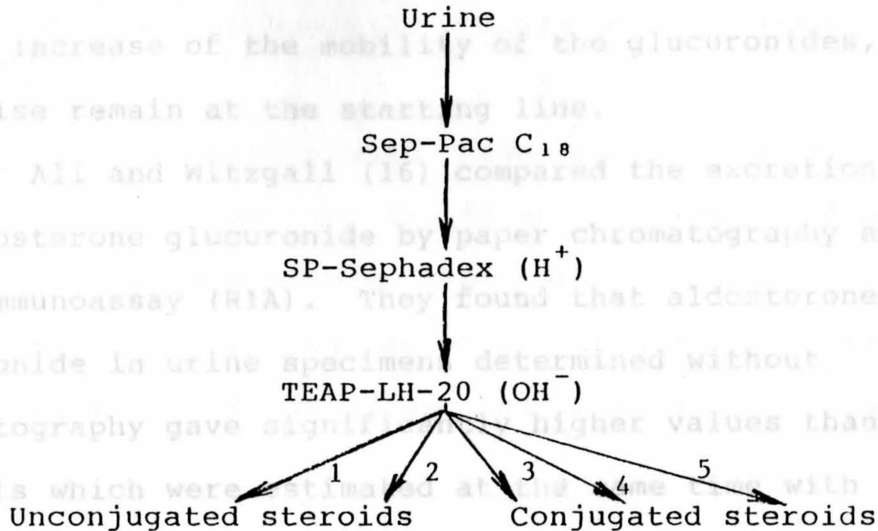


Fig. 8 General flow scheme for analysis of steroids in urine.

SP - Sephadex (H^+) was prepared as follows:
SP - Sephadex C-25 was converted into the sodium form and washed with 20% and 50% aqueous ethanol at 70°C and then stored in ethanol at 4°C. Prior to use it was converted into the H^+ form with 0.5 M hydrochloriacid.
Sodium hydroxide added to Triethylaminohydroxypropyl Sephadex LH-20 (TEAP-LH-20) in suitable solvents and at the proper temperature made TEAP-LH-20 (OH^-).
The numbered steroids in this figure are: (1) Neutral (2) Phenolic (3) Glucuronides (4) Monosulfates (5) Disulfates.

B. Paper and Open Column Chromatographic methods

Mattox et al (32) described the use of "liquid ion exchangers" as components of solvent systems for separation of steroid glucosiduronic acids. Addition of tetraheptyl ammonium chloride to the mobile phase and KCl to the

stationary phase of Bush-type systems (33) results in a marked increase of the mobility of the glucuronides, which otherwise remain at the starting line.

Ali and Witzgall (16) compared the excretion rates of aldosterone glucuronide by paper chromatography and radioimmunoassay (RIA). They found that aldosterone glucuronide in urine specimens determined without chromatography gave significantly higher values than aliquots which were estimated at the same time with chromatography. Grose et al (34) used DEAE-Sephadex column for the separation of aldosterone-18-glucuronide from aldosterone sulfate. They separated these two compounds also by paper chromatography.

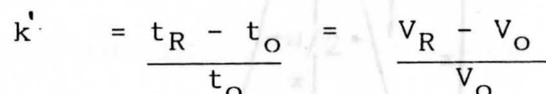
Morris and Tsai (18) used diethylaminohydroxypropyl-Sephadex-LH-20 (Sephadex DEAP-LH-20) resin column chromatography in separating aldosterone and its metabolites. They determined the percentage of aldosterone glucuronide in the liver, kidney, plasma, bile and urine of rats.

C. High Performance Liquid Chromatographic Methods

Liquid chromatography is the chromatographic process in which the moving phase is a liquid which percolates over a stationary bed. High pressure liquid chromatography is a liquid chromatographic technique in which the solution is pumped through the column at high

pressures. In reversed-phase partition chromagraphy, the stationary phase is nonpolar and the mobile phase is highly polar (35).

For effective liquid chromatographic separations, a column must have the capacity to retain samples, the ability to separate sample components, and efficiency of operation. The capacity factor of a column is a measure of sample retention by the column and is defined by the expression:



$$k' = \frac{t_R - t_o}{t_o} = \frac{V_R - V_o}{V_o}$$

where t_o is the hold up time and v_o is the void volume. The hold up time or void volume is the volume of the column not occupied by the packing and is equal to the total volume of solvent eluting from the column between the time of injection and the appearance of the unadsorbed species. t_R is the retention time, that is the time from injection to that at which the maximum of the solute peak appears on the recorder chart paper. V_R is the retention volume. If k' values are too low, the components elute too quickly. Therefore, the solvent is too strong. Conversely, if the k' values are too great, the elution times are too long. The long retention times can usually be decreased by increasing the solvent strength (35).

A separation factor α, which is a measure of a column's ability to separate two components, is expressed as a ratio of the capacity factors for the two components 1 and 2.

$$\alpha = \frac{k'_1}{k'_2}$$

If α is 1, the peaks coincide and there is no separation (35).

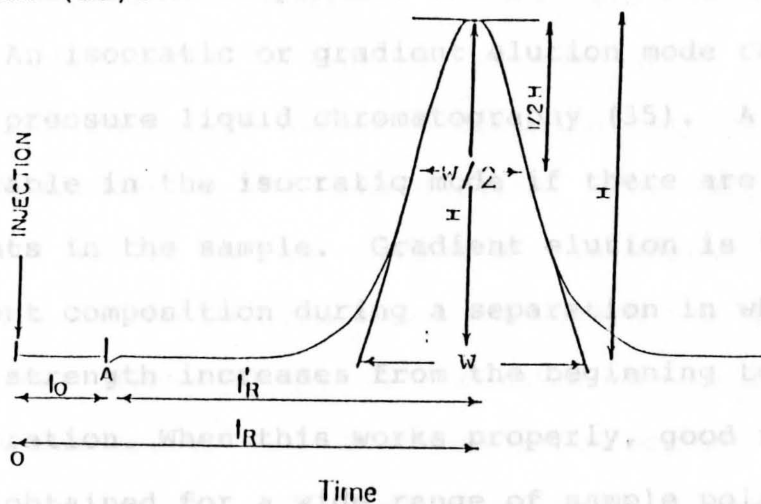


Fig. 9 Illustration of chromatographic terms

Column efficiency is quantitatively described by the number of theoretical plates N (35).

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

where t and W are defined in Fig. 10. For good resolution, narrow baseline widths (minimum band spreading) are desirable. Another column efficiency measure is the height equivalent to a theoretical plate, HETP, which is obtained by dividing the length of the column, L, by the number of

theoretical plates:

$$HETP = L/N \ (35).$$

Reversed-phase systems are more applicable for compounds that have hydrocarbon character and are sparingly soluble in water. Steroids have been satisfactorily analyzed on normal phase as well as reversed phase systems. However, a reversed-phase system is preferred if the major differences of the components are in the side chains (36).

An isocratic or gradient elution mode can be used in high pressure liquid chromatography (35). A weak eluent is desirable in the isocratic mode if there are different components in the sample. Gradient elution is the change of solvent composition during a separation in which the solvent strength increases from the beginning to the end of the separation. When this works properly, good resolution will be obtained for a wide range of sample polarities.

The selection of the optimum solvent system is important for good chromatographic separations (37). Viscosities of solvent should be kept below 0.4 - 0.5 centipoise. Solvent strength is also important in partition chromatography. If the separation of two bands is to be optimized from the standpoint of maximum resolution per unit time, the k' values of the two bands must fall close to an optimum value, which is usually between 2 and 5.

A grouping of solvents in order of chromato-

graphic strength is called an eluotropic series (37).

The HPLC of steroids has been reviewed by Heftmann and Hunter (38). Some literature that describes the isolation and analysis of conjugated steroids using HPLC is discussed below:

Van der Wal and Huber (40) have made extensive studies of the separation of estrogen conjugates and have obtained impressive results. Very rapid separations of estrogen conjugates (2 minutes) were achieved on octadecyl silica with phosphate buffer containing cetyltrimethylammonium bromide as the mobile phase. The latter was assumed to adsorb to the surface of the substituted silica and act as an ion-pairing agent to retard the steriod conjugates. Musey et al. (41) studied the separation of estrogen conjugates with a strong anion exchanger bonded on silica. Hermansson (42) studied reversed-phase systems in which a stationary phase of pentanol was coated on octadecyl silica. Phosphate buffer containing a quaternary ammonium ion was used as a mobile phase. Reversed-phase adsorption systems in which a low percentage of pentanol was added to the mobile phase in the absence of the hydrophobic counterion were also studied (43).

Shaw and Elliott (44) separated bile acid conjugates by HPLC on silica columns in basic systems that limited the life span of the columns, and also in acidic systems that showed tailing with the taurine conjugates

because of their strong interaction with silanol groups on the silica surface. The order of elution in the separation of the glucuronides and sulfates of (conjugated) bile acids on non-polar adsorbents with a low concentration of organic modifier in the mobile phase was recently confirmed experimentally (45).

Gradient elution with dioxane-water solvent system was selected for resolving polar adrenal steroids including mineralcorticoids such as aldosterone (46, 47). D'Agostino et al. (48) separated aldosterone and 18-Hydroxycorticosterone from a cell suspension of human adrenocortical tumour cells. They used a ternary mobile phase which was computer optimized.

Morris and Tsai (18) separated metabolites of (^{14}C) aldosterone synthesized in vitro by rat liver microsomal preparations, dog liver microsomal preparations, and human liver microsomal preparations using a C_{18}-μBondapak column in HPLC. They also separated polar neutral radiometabolites of (^{3}H) aldosterone.

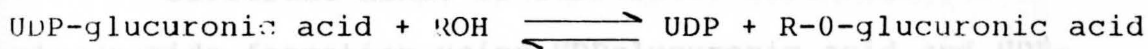
D. Miscellaneous methods

Axelson et al (31) analyzed steroids in different conjugate fractions from the urine of a healthy woman during follicular phase. They used gas liquid chromatography (GLC) and gas chromatography-mass spectrometry (GC-MS). Radioimmunoassay (RIA) was performed with a

highly specific antibody against aldosterone (16). RIA is the preferred method today.

E. Biosynthetic method of glucuronide formation

Glucuronide formation is an important reaction in metabolism of endogenous and xenobiotic compounds. Hepatic microsomal uridine diphosphate glucuronyl transferase (UDPGT) catalyzes the transfer of glucuronic acid from uridine diphosphoglucuronic acid (UDPGA) to various substrates (49). The major biosynthetic route for simple glucuronides is shown in the following equation:



UDP-glucuronic acid + ROH ⇌ UDP + R-O-glucuronic acid

UDPglucuronyl transferase's important physiological and pharmacological roles in vertebrate tissues have been comprehensively reviewed (49). Liver is the most active tissue for glucuronidation (50). Adult mammalian liver probably contains the highest UDPglucuronyltransferase activity per milligram portion of all tissues, whether as homogenate or washed microsomes (49). The transferase can be activated by its known endogenous activator, UDP-N-acetylglucosamine (UDPGlcNAc) (21). Transferase activity is increased approximately 30% by 2 to 20 mM magnesium ions (50) also. Boutin et al. (51) studied a

correlation between conjugation velocity and geometry. The most rapidly metabolized molecules are planar, and the bulkier the molecule, the more slowly it is conjugated.

The dependency of UDP-glucuronyl transferase on phospholipids is discussed (52) by Singh and coworkers. Substantial differences exist in rat and rabbit liver microsomal preparations with respect to the rate of 3-OH and 17-OH steroid glucuronidation (53). The form of UDPGT present in rabbit liver microsomes catalyzes the 3-OH glucuronidation of estrone and β-estradiol. However, two forms of UDPGT activity are present in rat liver microsomes which catalyze both the 17- and 3-OH positions of steroids (53).

Different kinds of substrates were used for glucuronide formation using UDPglucuronic acid and UDP-glucuronyl transferase as catalyst. The direct measurement of the conjugate formation was possible only with one compound (49). The conjugate formation was measured in the other compounds by measuring the diminution in free substrate concentration which occurred on the addition of UDPglucuronic acid (49).

The suggestions of Dutton (49) for the synthesis of glucuronides are useful. These suggestions are:

(i) UDPglucuronic acid should be of the highest purity.

(ii) The most suitable buffer is trishydroxy-

methylamino-methane (tris), at pH 7.4 - 7.8 . Magnesium ions can stimulate the reaction under certain conditions.

(iii) Nitrogen is the gas phase, to minimize enzymic oxidation of UDP-glucose in the homogenate, or oxidative side reactions of the substrate. The temperature should be $37^{0}C$ or $30^{0}C$.

Tris/maleate/$MgCl_2$ buffer, UDP-glucuronic acid, UDPGT from rat liver, and labelled substrates were the components in the incubation mixture for making the glucuronides of 1-naphthol and testosterone (21).

Tris-HCl containing magnesium ions, UDP-glucuronic acid, UDPGT from rabbit liver, and labelled substrates were the components in the incubation mixture for the glucuronide synthesis of estrone and 4-nitrophenol (50).

The solvents used to terminate the reactions were different for each compound. The synthesized glucuronides were determined quantitatively by liquid-scintillation counting techniques (21, 50).

Matsui et al (54) synthesized the glucuronides of 4-nitrophenol, phenolphthalein, and testosterone and developed a HPLC method for their quantitative determination. The incubation medium contained microsomal fractions from rat liver, Tris-HCl buffer, EDTA, $MgCl_2$, and UDPGA. The incubation was carried out at $37^{0}C$ and terminated by heating in a boiling water bath for 1-5 minutes. The amounts of the enzymatically formed

glucuronides were calculated by subtracting the unreacted substrates from the incubated substrates.

Paracetamol (acetaminophen) glucuronide was also synthesized enzymatically and determined by HPLC (56).

A simple method for the biosynthesis of α-naphthol glucuronide using UDPGA and UDP-glucuronyl transferase is described by To and Wells (20). Here the α-naphthol glucuronide was identified by HPLC, using a reversed-phase (RP) C_{18} column. The incubation mixture contained α-naphthol (the substrate), Tris buffer (pH 7.4) with magnesium chloride, DMSO, and UDP-glucuronyl transferase from male CD-1 mice. The reaction was stopped by the addition of ice cold methanol containing the internal standard, β-naphthol. The reaction was quantitative. The effect of substrate concentration and incubation time on enzyme activity was studied.

CHAPTER III

MATERIALS AND APPARATUS

A. Solvents and Reagents

All chemicals and solvents used were of the highest purity available.

Doubly deionized ultrafiltered water was used for the preparation of the mobile phase. Deionized water distilled from basic permanganate was collected and redistilled in glass apparatus (triply distilled) and was used for the preparation of all other reagents.

Solvents and reagents were prepared or used as described below.

Acetonitrile and methylene chloride (Burdick and Jackson Laboratories, Inc., Muskegan, MI, `distilled in glass', chromatoquality) were filtered through nylon filters of 0.45 μm pore size.

HPLC grade methanol was further filtered through the nylon filters. 50 mM Tris buffer was made from Tris (THAM). Certified A.C.S. Dimethyl Sulfoxide (DMSO) was also used. These reagents were purchased from Fisher-Scientific Company, Fairlawn, NJ.

Magnesium chloride (Allied Chemical, General Chemical Division, Morristown, NJ) was used in the

incubation mixture.

Hydrochloric acid (Mallinckrodt Inc., Paris, KY) was used to prepare 6N hydrochloric acid and 0.1 M HCl.

Sodium carbonate was used to prepare a 0.05 N Na CO solution. Sodium carbonate and α-Naphthol were from J.T. Baker chemical Co., Phillipsburgh, NJ.

Uridine 5′-Diphosphate, Uridine 5′-Diphospho-glucuronic acid (Na salt), Uridine 5′-Diphospho glucuronyl transferase from rabbit liver and bovine liver (Type II, lyophilized crude microsomal preparation containing approximately 30% buffer as potassium chloride and reduced glutathione), glucuronic acid, and α-naphthol glucuronide were purchased from Sigma Chemical Co., St. Louis, MO. β-naphthol was from Sargent-Welch Scientific Co., Cleveland, OH.

Nitrogen (The BOC Group, Inc., Murray Hill, NJ) was used to evaporate solvents from the samples. Helium (The BOC Group, Inc.; Montvale, NJ) was used over the solvent system.

B. <u>Steroids</u>

The following steroids were used in the experiment: D-Aldosterone, Aldosterone monoacetate, Aldosterone diacetate, Prednisolone (Sigma Chemical Co., St. Louis, MO) Prednisone (Alltech Associates, Inc., Deerfield, IL).

C. Apparatus

The High Pressure Liquid Chromatographic instrument, IBM model LC/9533 and the variable UV detector, model LC/9533, were purchased from IBM Instruments, Inc., Danbury, CT. A block diagram of the instrument is shown in Fig. 10.

The column used was the Econosphere Reversed-Phase, C_{18} (RP, C_{18}) with the dimensions (25 cm x 4.6 mm) and the particle size was 5 microns. It was protected by a guard column (5.0 cm x 4.6 mm) also containing octadecylsilica. Both came from Alltech Associates, Inc., Deerfield, IL. The recorder connected to the instrument was Sargent-Welch model XKR (Sargent Welch Scientific Co., Cleveland, OH).

The Millipore filtration apparatus (1000 mL size) (Millipore Corporation, Bedford, MA) was used for the filtration of all samples. Nylon 66 filters of 0.45 μm size and 47 mm diameter were used for solvent filtration, and nylon filters of 0.2 μm size and 25 mm diameter were used for sample filtration prior to injection (Alltech Associates, Inc., Deerfield, IL).

Micropipettes of sizes ranging from 10 μL to 1000 μL (Centaur Chemical Co., Stanford, CT) were used for sample preparations.

Microliter syringes (Rainin Instrument Co., Inc., Woburn, MA) in 50 μL and 100 μL sizes were used to inject samples into the HPLC.

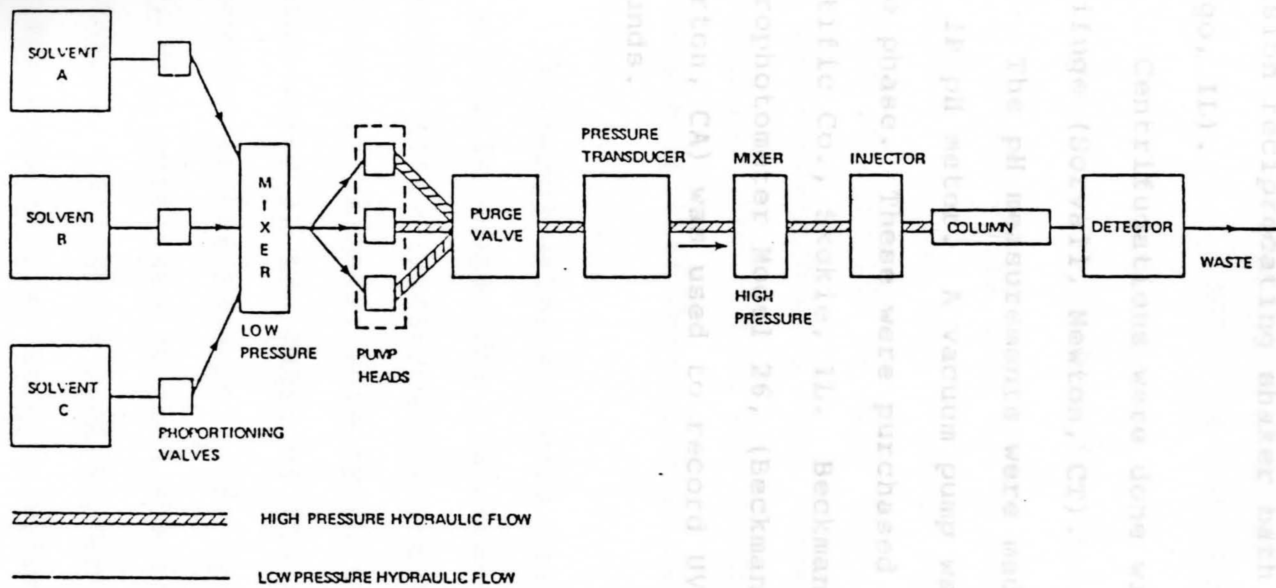


Fig. 10. Block Diagram of LC/9533

5 cc plastipak syringes (Becton-Dickinson Co., Rutherford, NJ) were used for sample filtration.

The incubation mixture was kept at 37°C in a Precision reciprocating shaker bath (GCA Corporation, Chicago, IL).

Centrifugations were done with a Sorvall GLC-1 Centrifuge (Sorvall, Newton, CT).

The pH measurements were made on a Sargent-Welch model IP pH meter. A vacuum pump was used to degas the mobile phase. These were purchased from Sargent-Welch Scientific Co., Skokie, IL. Beckman UV-Visible recording spectrophotometer Model 26, (Beckman Instruments, Fullerton, CA) was used to record UV spectra of several compounds.

CHAPTER IV

EXPERIMENTAL METHODS

A. Preparation of Mobile Phase

To and Wells used 0.1 M acetic acid-methanol (55:45, v/v) as the mobile phase for the identification of α-naphthol glucuronide by HPLC (20). This mobile phase was tried for aldosterone by this investigator and was found not suitable. The column was clogged several times because of some precipitate formation of aldosterone with this solvent.

Methanol-water mixtures and acetonitrile-water were used as mobile phases for aldosterone by a previous researcher (56). Different proportions of methanol-water and acetonitrile-water were tried in this research. Methanol-water in the ratio of (55:45,v/v), and acetronitrile-water in the ratio of (65:35, 50:50, 40:60, 35:65, 30:70, v/v) were used to find the retention time of aldosterone. All these mobile phases gave good symmetrical peaks for aldosterone, and the retention time increased as the water content in the mobile phase increased. Acetonitrile-water (25:75, v/v) was also used, but the peak was not symmetrical. The mobile phase which gave a reasonable

retention time with a good symmetrical peak was selected. That was acetonitrile-water (30:70, v/v), which was used as the mobile phase throughout the research.

Glass-distilled acetonitrile and deionized water were filtered as described in Chapter 3. The filtered solvents were thoroughly mixed in the proper ratio. The mobile phase was then transferred to the 1-liter bottle which was provided in the solvent tray of the LC/9533 and was degassed using a vacuum pump. Then a layer of helium was placed over the solvent.

B. Preparation of Solutions

The experimental work was started with 0.1 M acetic acid-methanol (55:45, v/v) as the mobile phase. α-naphthol, α-naphthol glucuronide and β-naphthol were the main components that appeared in the chromatogram when α-naphthol glucuronide was synthesized by To and Wells (20). α-naphthol glucuronide (6 mg) β-naphthol (6 mg), and α-naphthol (6 mg) were prepared in separate 20 mL aliquots of 0.1M acetic acid-methanol (55:45, v/v). Chromatograms were obtained with the above components.

Tris buffer (50 mM) was made by dissolving 0.6145 g Tris (THAM) in 100 mL triply distilled water, and the pH was adjusted to 7.4 with 6 M hydrochloric acid. $MgCl_2$ (0.2146 g) in 100 mL of triply distilled water made a 10 mM $MgCl_2$ solution. The UDPGA solution used was 4 mM. UDPGA,

Na salt (0.029 g/mL), UDP (0.15 mg/mL), D-Glucuronic acid, Na salt (0.15 mg/mL) were prepared in triply distilled water. D-Glucuronic acid, Na salt (0.19 mg/mL) in 50 mM Tris buffer was also prepared. Aldosterone (7.1 mg) in 50 mL methanol kept in the freezer was used. Rabbit liver enzyme (4 mg/mL) and 4 mg/mL bovine liver enzyme were made in Tris buffer.

C. Preparation of Incubation Mixture

Preparation of incubation mixture was based on the works done by To and Wells (20) and Dutton *et al* (21). Table 1 gives the volumes and concentrations of the components used in the incubation mixtures. First 25 μL (3.55 ug) or 9.86 nmoles of aldosterone stock solution were placed in a 15 mL glass centrifuging tube and dried under nitrogen at room temperature. Then 20 μL of Tris buffer (50 mM) and 20 μL of $MgCl_2$ (10 mM) were added. Since aldosterone is a slightly polar compound, 3% (v/v) DMSO (1.2 μL) was added for adequate solubilization in the aqueous medium. 25 μL of 4 mM UDP glucuronic acid (100 nmoles) was added and mixed well and the solutions pre-incubated in the centrifuging tube at 37^0 C for 5 minutes in a shaker water bath. 50 μL of UDP-glucuronyl transferase from rabbit liver (4 mg/mL) was then added and the mixture incubated for one hour. This was incubation mixture (I). The reaction was then stopped by adding 1 mL

TABLE 1

Volumes of the components in the incubation mixture

	aldosterone (7.1 mg/50 mL) dried under N_2	50 mM Tris buffer	10 mM $MgCl_2$	4 mM UDPGA	DMSO	UDP-glucuronyl transferase (4 mg/mL)	Total
Incubation mixture (I)	25 μL	20 μL	20 μL	25 μL	1.2 μL	50 μL	116.2 μL
Blank mixture		66.2 μL	——	——	——	50 μL	116.2 μL
Incubation mixture (II)	100 μL	80 μL	80 μL	100 μL	4.8 μL	200 μL	464.8 μL

of ice-cold methanol. The enzyme was precipitated and the supernatant was collected after centrifuging for 15 minutes at 1000 RPM in the Sorvall GLC-1 centrifuge.

The same amount of enzyme solution was also incubated under the same conditions with buffer solution without any other components at the same time and the supernatant was collected after adding 1 mL methanol. The volume of the incubation mixture (I) was increased four times to make incubation mixture (II).

D. Preparation of Samples for HPLC

The supernatants from the incubation mixtures were dried under nitrogen at room temperature and dissolved in 300 μ L of mobile phase. They were then filtered through 25 mm Nylon 66 filter of 0.2 μ size. A volume of 50 μ L of this filtered sample was injected onto the HPLC column.

E. HPLC Method

The experiment used the IBM Model LC/9533 (Fig. 10). Alltech C_{18} Reversed phase column (length: 25 cm; diameter: 4.6 mm; particle size: 5 microns) preceded by a guard column was used. The column was maintained at room temperature. The mobile phase consisted of acetonitrile-water (30:70, v/v). The column inlet pressure and the

guard column pressure were 159-179 bars. The flow rate was 1.0 mL/minute and the chart speed was 1.0 cm/minute. The IBM model LC/9523 variable U.V. detector was used. The sensitivity of the UV detector, which was set at a wavelength of 240 nm, was 0.05 Absorbance Units full scale deflection. A 50 μ L full volume injection loop was used, and a constant volume (50 μ L) of samples was injected.

CHAPTER V

RESULTS AND DISCUSSION

Success with high performance liquid chromatography depends on the efficiency of the column and the mobile phase used. The RP-C_{18} column purchased from Alltech Associates Inc., was used in this research. Along with the column, Alltech provided a sample, RP-Mix-D to make a quality assurance chromatogram. The sample contained six components in different concentrations. Sharp symmetrical peaks were obtained with good resolution and in good agreement with Alltech (see Fig. 11).

All samples were analyzed using a 25 cm x 4.6 mm I.D. reversed-phase (RP) C_{18} column with a particle size of 5 microns. The procedure followed by To and Wells (20) to synthesize α-naphthol glucuronide was the initial gûideline for this research. They used (55:45, v/v) 0.1 M acetic acid-methanol as the mobile phase and β-naphthol as the internal standard. This solvent system was used by this investigator to check the adaptability of their system to this research on aldosterone glucuronide synthesis. The chromatograms of the HPLC resolutions of α-naphthol glucuronide and β-naphthol are shown in Fig. 12 and 13.

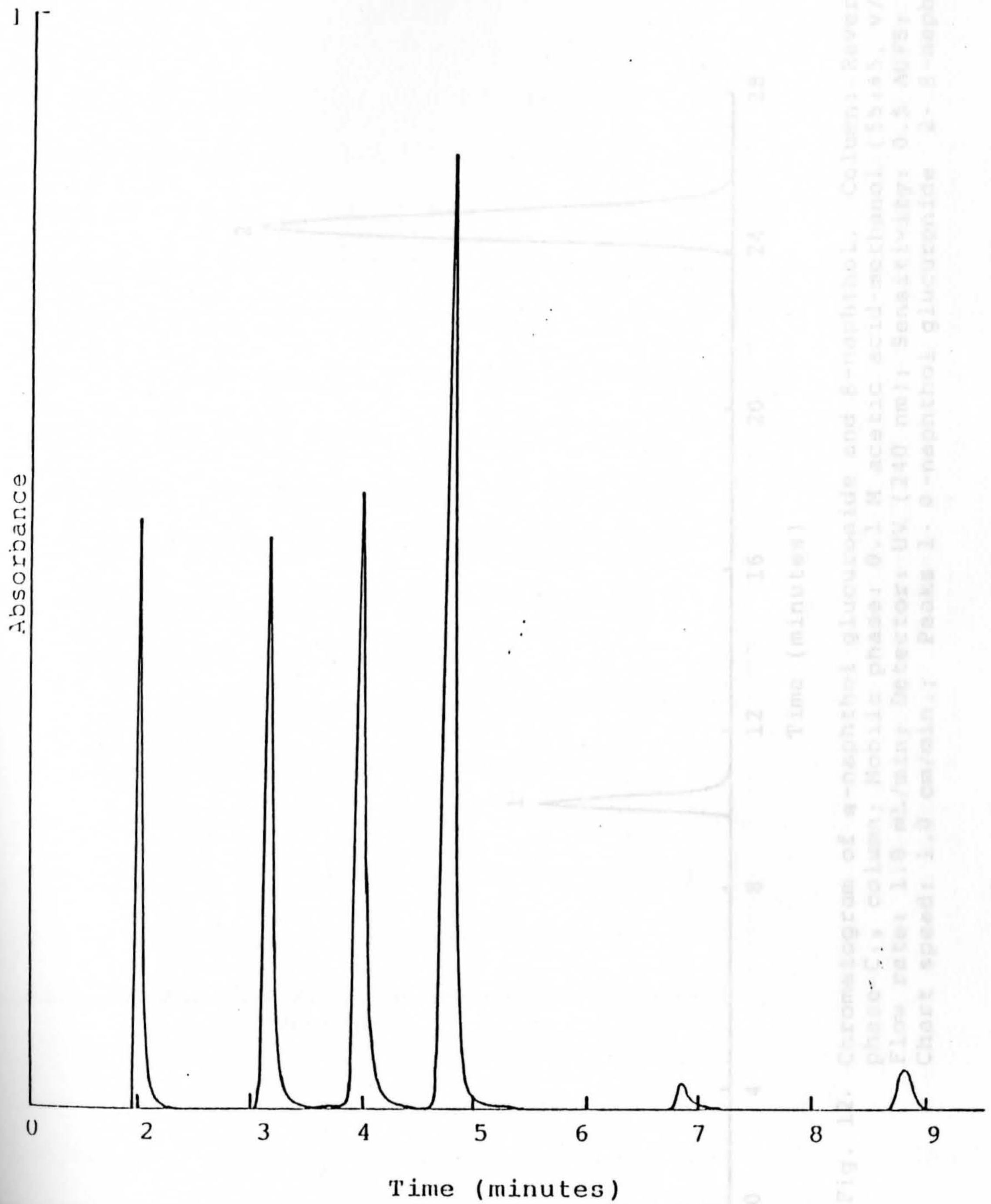


Fig. 11. Chromatogram of RP-mix D. Column: Reversed-phase C_{18} column; Mobile phase: Acetonitrile-water (60:40, v/v); Flow rate: 1·2 mL/minute; Detector: UV (254 nm); Sensitivity: 1.0 AUFS; Chart speed: 2.0 cm/minute.

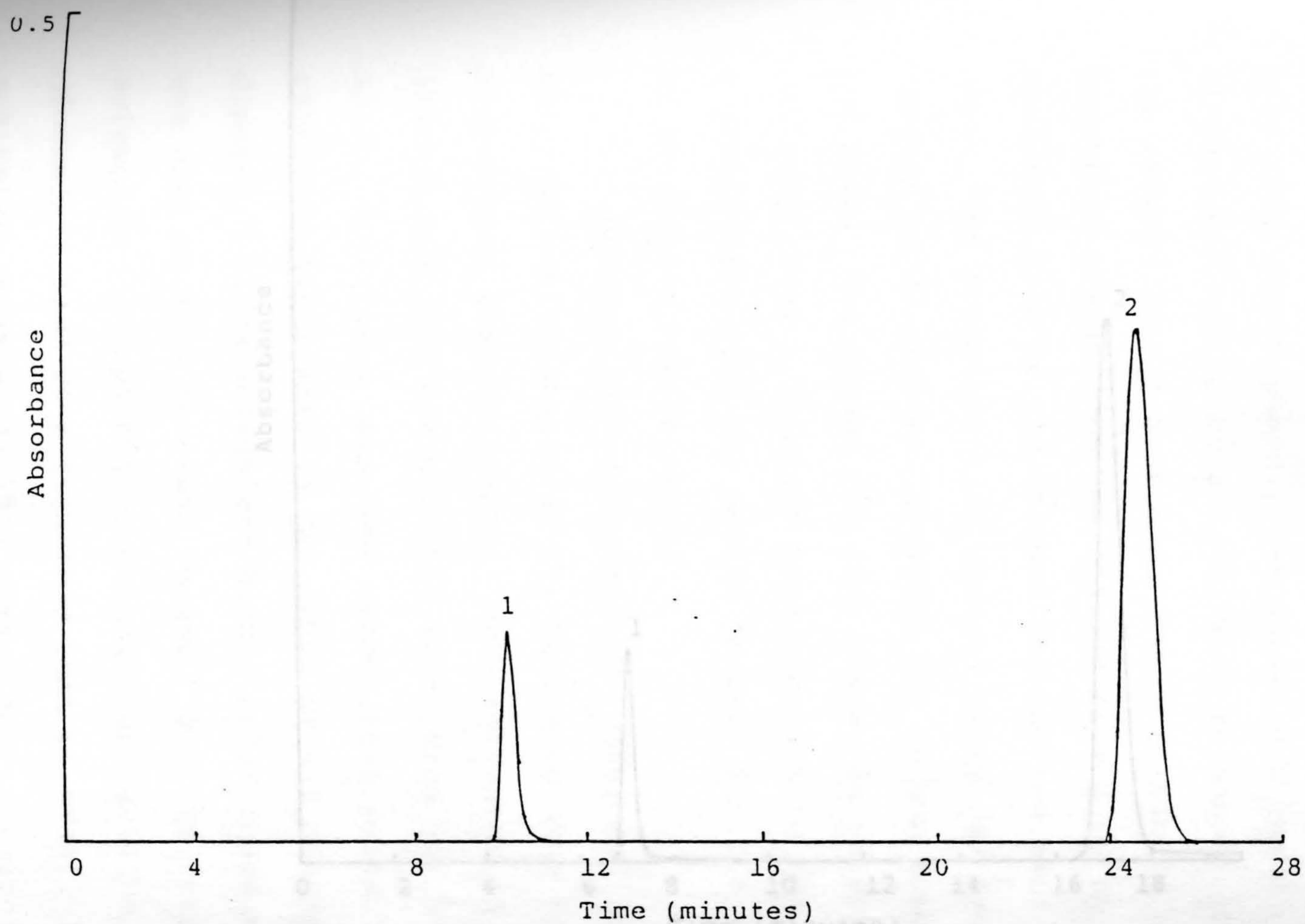


Fig. 12. Chromatogram of α-naphthol glucuronide and β-naphthol. Column: Reversed-phase-C_{18} column; Mobile phase: 0.1 M acetic acid-methanol (55:45, v/v); Flow rate: 1.0 mL/min; Detector: UV (240 nm); Sensitivity: 0.5 AUFS; Chart speed: 1.0 cm/min.; Peaks 1. α-naphthol glucuronide 2. β-naphthol.

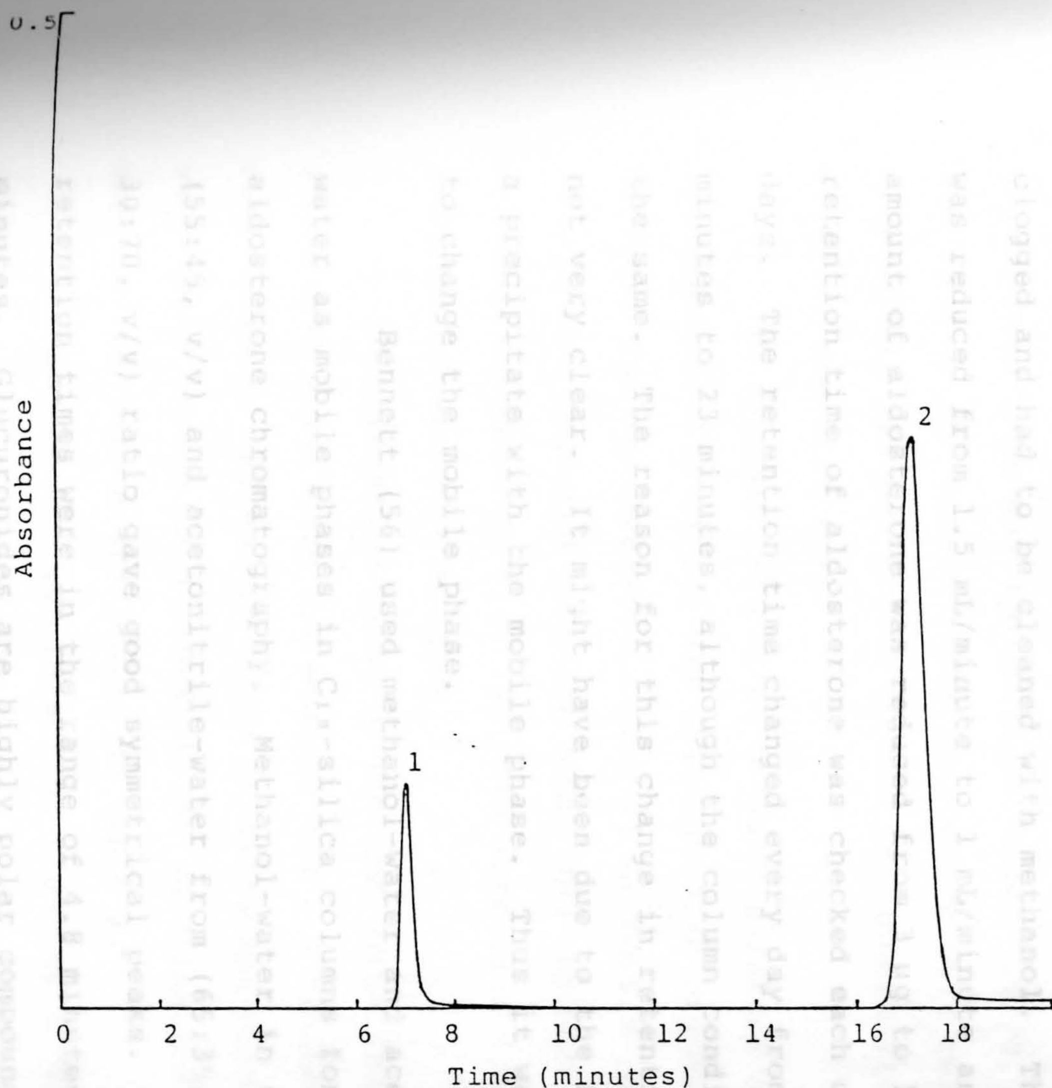


Fig. 13. Chromatogram of α-naphthol glucuronide and β-naphthol. Column: Reversed-phase C_{18} column; Mobile phase: 0.1 M acetic acid-methanol (55:45, v/v); Flow rate: 1.5 mL/min.; Detector: UV (240nm); Sensitivity: 0.5 AUFS; Chart speed: 1.0 cm/min.; Peaks: 1· α-naphthol glucuronide 2· β-naphthol

The only difference between the two chromatograms is the flow rate. 3 μg of aldosterone in 20 μL was injected onto the column. The pressure rose to a maximum of 400 bars with this mobile phase. The column and guard column became clogged and had to be cleaned with methanol. The flow rate was reduced from 1.5 mL/minute to 1 mL/minute and the amount of aldosterone was reduced from 3 μg to 150 ng. The retention time of aldosterone was checked each day for six days. The retention time changed every day from 6.9 minutes to 23 minutes, although the column conditions were the same. The reason for this change in retention time was not very clear. It might have been due to the formation of a precipitate with the mobile phase. Thus it was necessary to change the mobile phase.

Bennett (56) used methanol-water and acetonitrile-water as mobile phases in C_{18}-silica columns for aldosterone chromatography. Methanol-water in the ratio of (55:45, v/v) and acetonitrile-water from (65:35, v/v to 30:70, v/v) ratio gave good symmetrical peaks. The retention times were in the range of 4.8 minutes to 10.6 minutes. Glucuronides are highly polar compounds. Uracil was used to find the void volume. But the retention volumes of α-naphthol glucuronide and Uridine diphosphoglucuronic acid were less than that of Uracil in acetonitrile-water (50:50, v/v). The retention time of Uridine-5′-diphosphate (UDP) was less than that of UDP-

glucuronic acid. Since AG is a steroid glucuronide, it should be separable from UDP and UDPGA because it is less polar than either one. The mobile phase which gave an optimal retention time for aldosterone with a good symmetrical peak was acetonitrile-water (30:70, v/v). This was used as the mobile phase throughout this research. The wave-length was set at 240 nm and the sensitivity of the UV detector was 0.05 AUFS.

An aliquot (75 μL) of a stock solution of aldosterone (142 ng/μL) was evaporated to dryness under nitrogen and redissolved in 1010 μL of mobile phase, and 50 μL were injected onto the HPLC. The chromatogram obtained is shown in Fig. 14. The retention time of aldosterone was 10.6 minutes.

The retention times of UDP and UDPGA (UDP-glucuronic acid) were 2.1 minutes and 2.3 minutes respectively. Retention times of different compounds which were tried during this research for checking chromatographic resolution are listed in Table 2.

Once the desired retention times were determined, a standard curve for the aldosterone was constructed. The area of the peak was determined using the formula 1/2 BH for a triangle. The amounts used were 70, 159, 352, 527 and 703 ng. Table 3 shows the peak areas for each amount. The standard curve for aldosterone is presented in Fig. 15. The standard curve is linear up to 703 ng, which differs

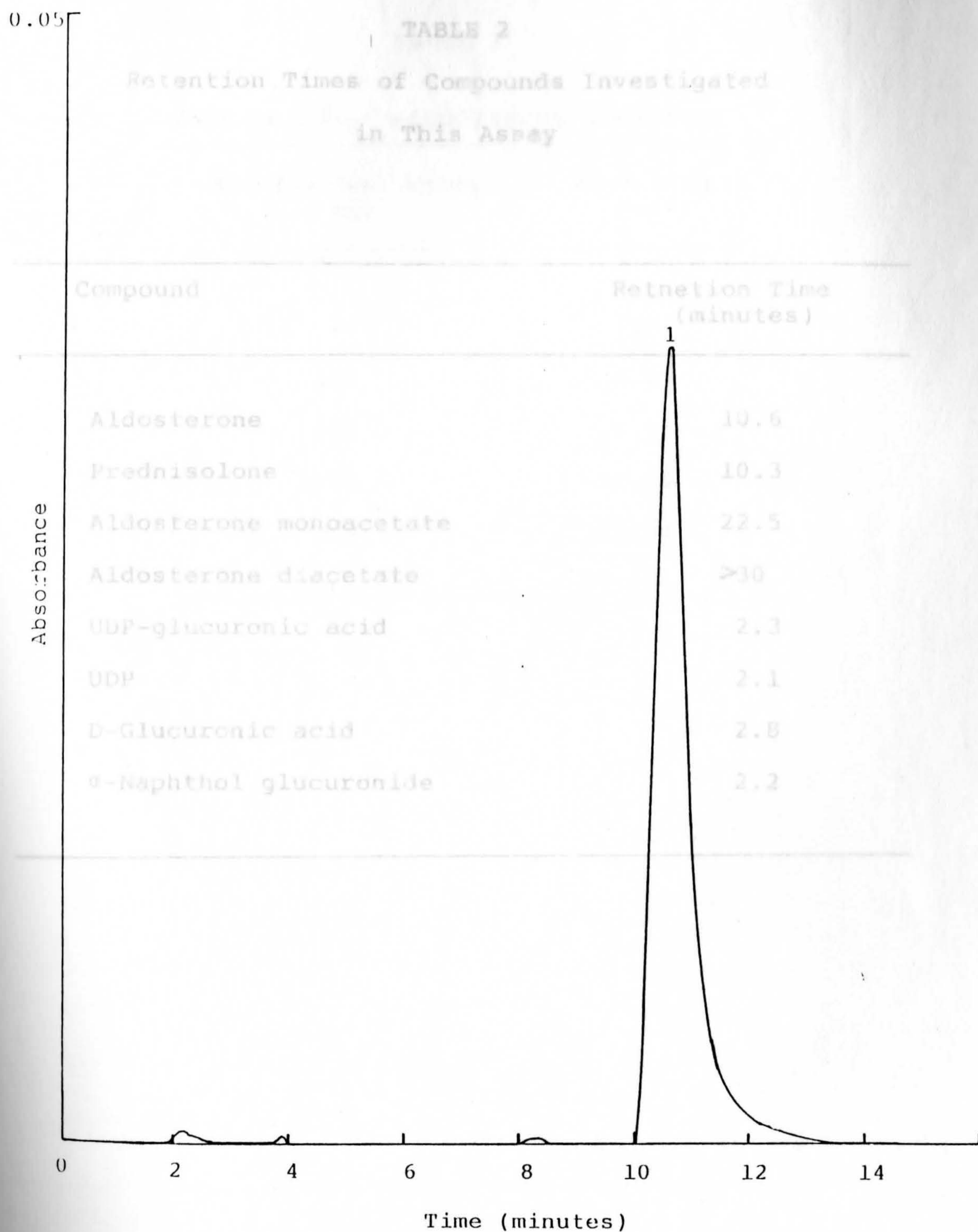


Fig. 14. Chromatogram of pure aldosterone working standard (527 ng). Column: C_{18} Reversed-phase; Mobile phase: Acetonitrile-water (30:70, v/v); Temperature: Ambient; Flow rate: 1.0 mL/min.; Chart speed: 1.0 cm/min.; Detector: UV (240 nm); Sensitivity: 0.05 AUFS.
Peak 1. Aldosterone

TABLE 2

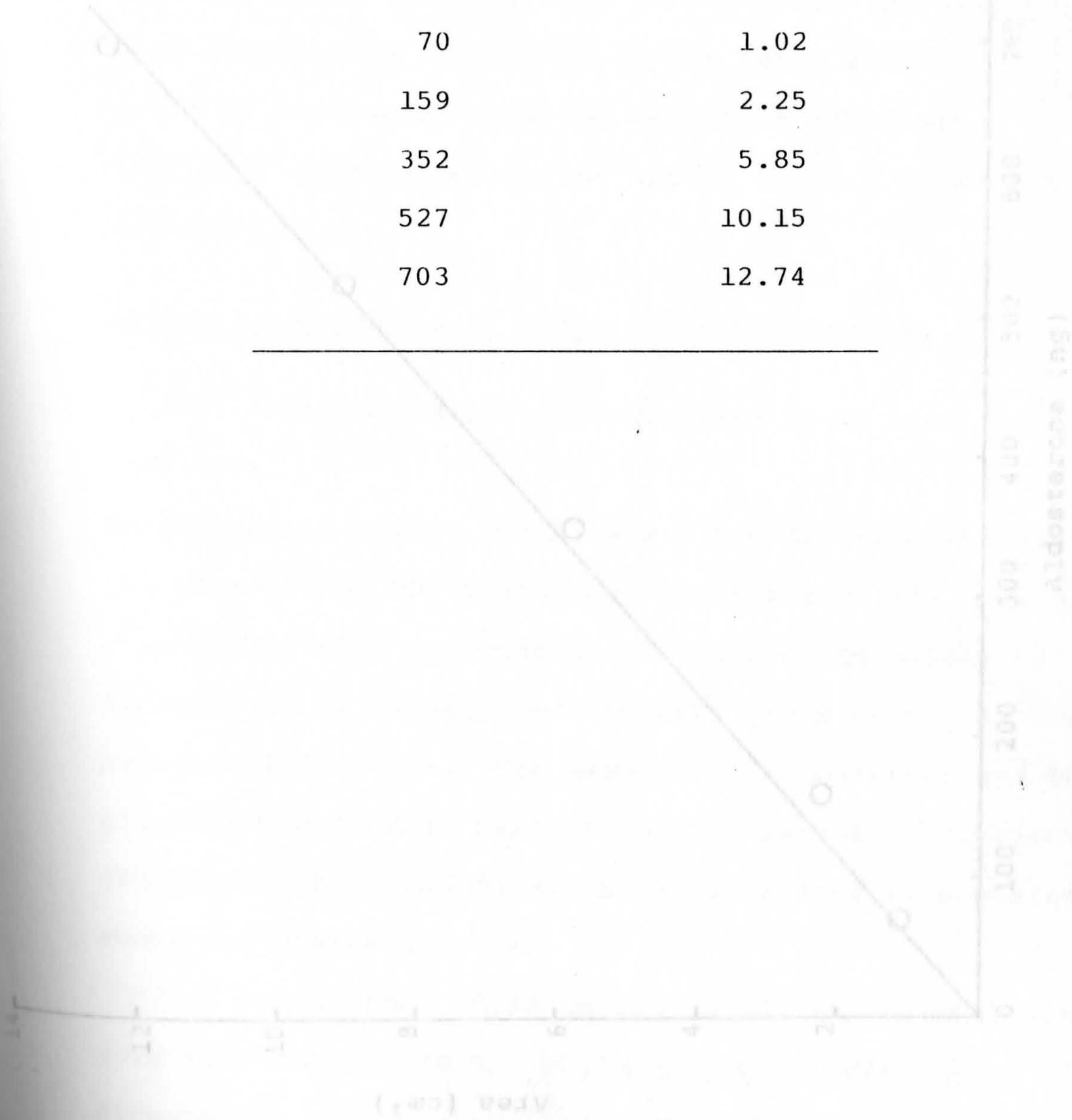
Retention Times of Compounds Investigated in This Assay

Compound	Retnetion Time (minutes)
Aldosterone	10.6
Prednisolone	10.3
Aldosterone monoacetate	22.5
Aldosterone diacetate	>30
UDP-glucuronic acid	2.3
UDP	2.1
D-Glucuronic acid	2.8
α-Naphthol glucuronide	2.2

TABLE 3

PEAK AREAS OF ALDOSTERONE STANDARDS

Aldosterone Amount (ng)	Peak Area (cm^2)
70	1.02
159	2.25
352	5.85
527	10.15
703	12.74



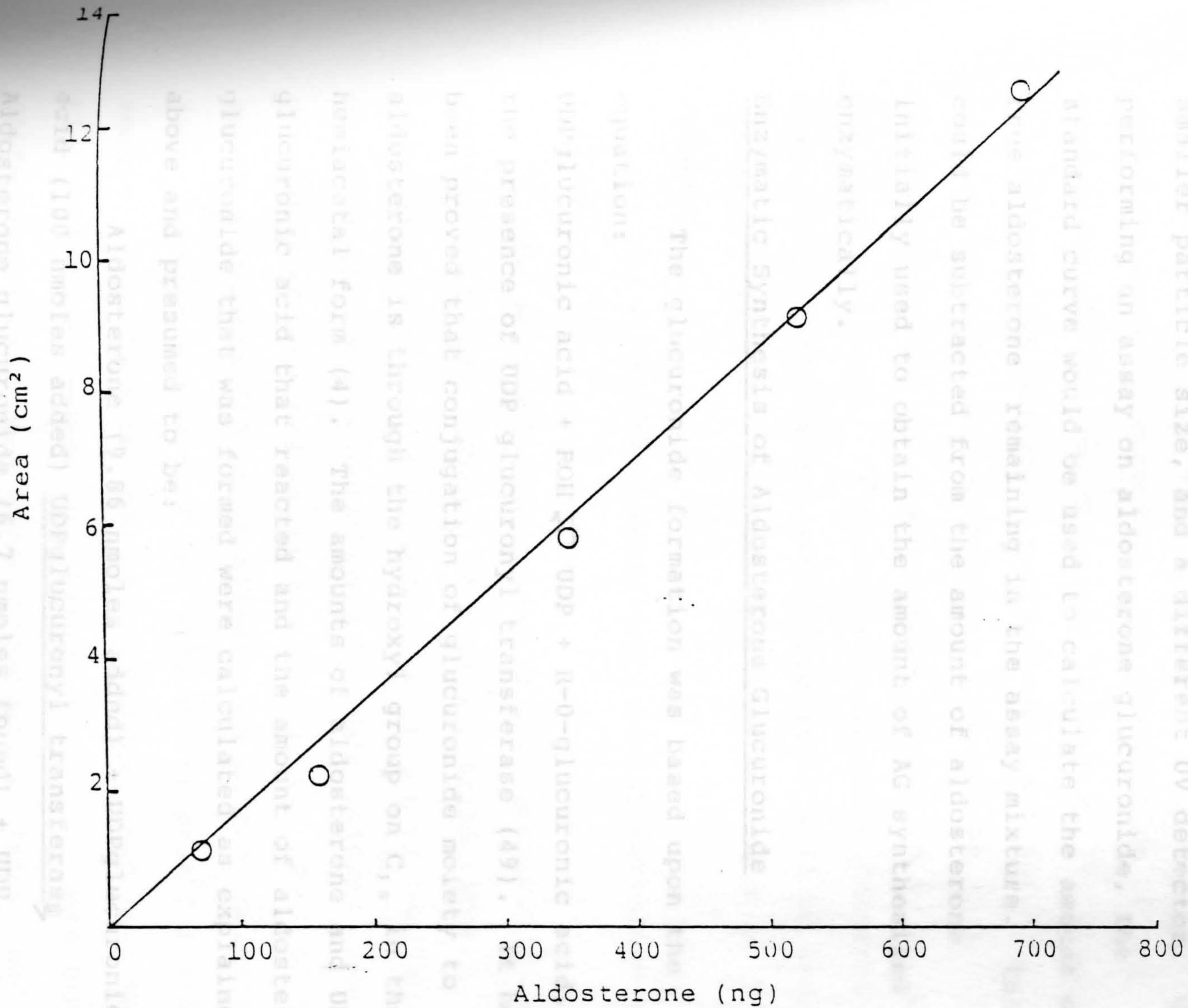


Fig. 15. Aldosterone Standard Curve
Mobile phase: Acetonitrile-water (30:70, v/v); Flow rate:1.0 mL/min.; Detector: UV (240 nm); Sensitivity: 0.05 AUFS; Chart speed: 1.0 cm/min.

from Bennett's (56) results, which were linear only to 150 ng aldosterone. The linearity to a higher concentration could be due to the different HPLC used, a column with smaller particle size, and a different UV detector. When performing an assay on aldosterone glucuronide, the standard curve would be used to calculate the amount of free aldosterone remaining in the assay mixture. This could be subtracted from the amount of aldosterone initially used to obtain the amount of AG synthesized enzymatically.

Enzymatic Synthesis of Aldosterone Glucuronide

The glucuronide formation was based upon the equation:

UDPglucuronic acid + ROH ⇌ UDP + R-O-glucuronic acid, in the presence of UDP glucuronyl transferase (49). It has been proved that conjugation of glucuronide moiety to aldosterone is through the hydroxyl group on C_{18} in the hemiacetal form (4). The amounts of aldosterone and UDP-glucuronic acid that reacted and the amount of aldosterone glucuronide that was formed were calculated as explained above and presumed to be:

Aldosterone (9.86 nmoles added) + UDPglucuronic acid (100 nmoles added) $\xrightarrow{\text{UDPglucuronyl transferase}}$ Aldosterone glucuronide (6.7 nmoles found) + UDP.

During the incubation period aldosterone was not

completely converted to aldosterone-18-glucuronide (approximately 68% conversion in one hour). Thus after incubation, the incubation mixture contained unreacted aldosterone, excess uridine diphosphoglucuronic acid, UDP, and aldosterone glucuronide. The preparation of the incubation mixture was discussed in Chapter IV. Twenty minutes was the maximum time for incubation for α-naphthol glucuronide (20) and testosterone glucuronide (54). The incubation period was changed from 20 minutes to 30 minutes and then to 1 hour. If there is glucuronide formation, the peak area of aldosterone after incubation will be less than before incubation. This was true only at one hour incubation.

The glucuronide assay was initally performed as follows:

The methanol supernatant of the incubation mixture (I) was collected. 300 μL of this was dried under N_2 at room temperature and redissolved in 300 μL of mobile phase. 50 μL of this was injected onto the HPLC column. The chromatogram obtained is shown in Fig. 16. 50 μL of this sample contained 159 ng of aldosterone before incubation. Fig. 17 shows the size of the aldosterone peak for this amount. Fig. 18 shows the UPD-glucuronic acid that was in Fig. 16 before incubation. Matsui <u>et al</u> (54) used the difference in the peak sizes of substrates before and after incubation to calculate the amount of glucuronides formed.

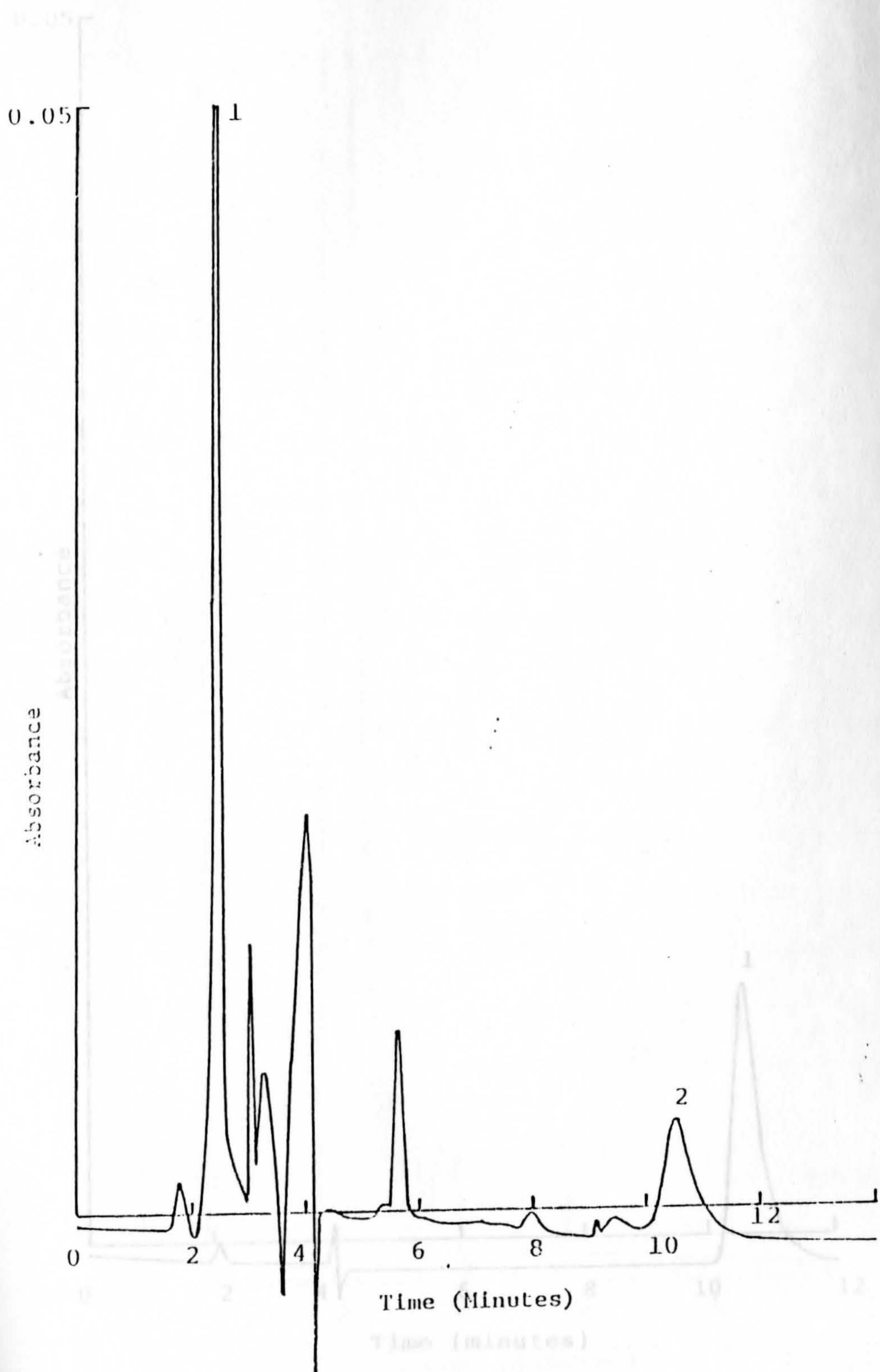


Fig. 16. Chromatogram of residue from the supernatant of the incubation mixture (I). Mobile phase: Acetonitrile-water (30:70, v/v): Flow rate: 1.0 mL/minute; Detector: UV (240 nm); Sensitivity: 0.05 AUFS; Chart speed: 1.0 cm/min.

Peaks 1. UDP and UDP glucuronic acid together 2. Aldosterone

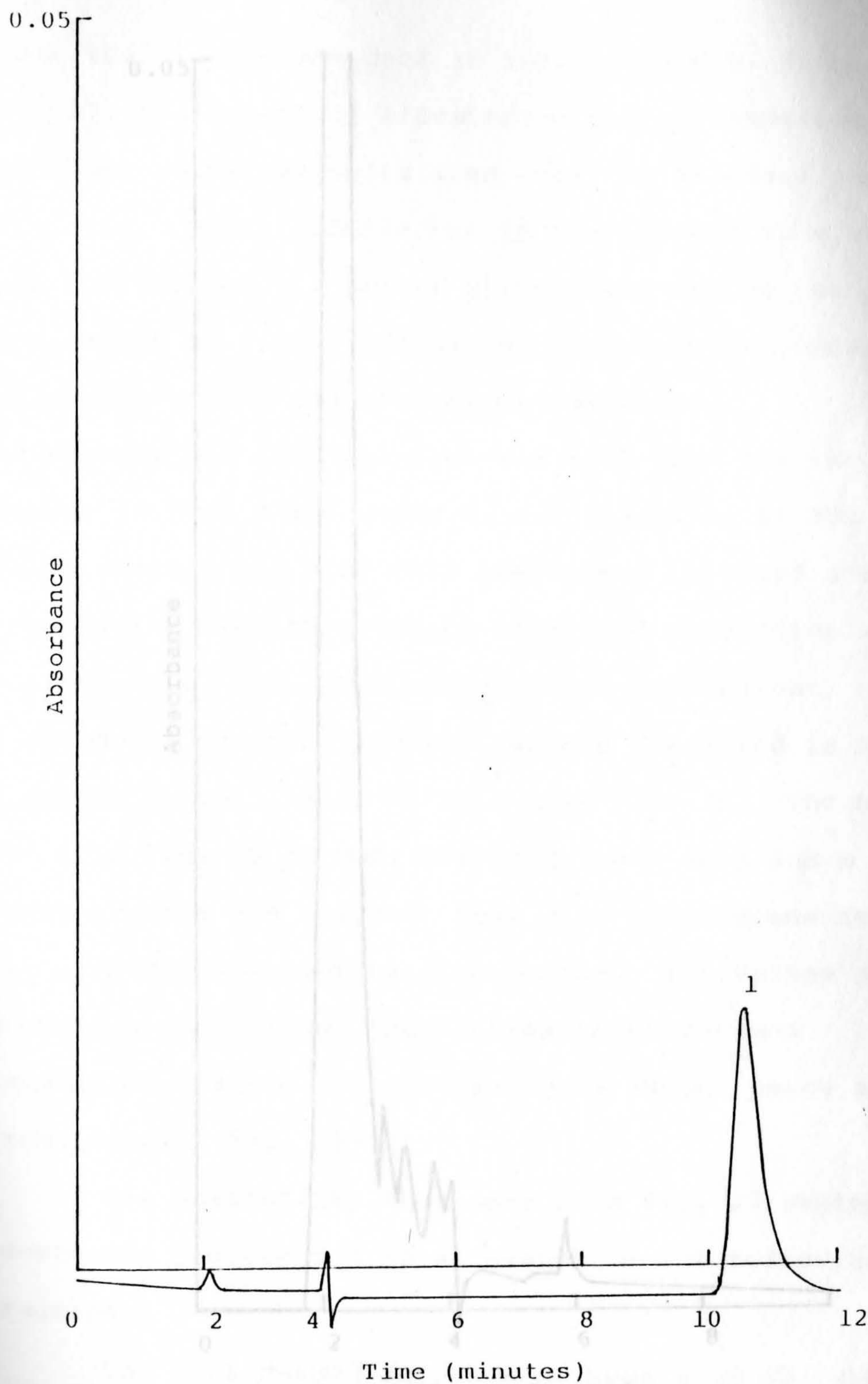


Fig. 17. Chromatogram of aldosterone (159.6 ng) used in the incubation mixture (I).
Mobile phase: Acetonitrile-water (30:70, v/v); Flow rate: 1.0 mL/min.; Detector: UV (240 nm); Sensitivity: 0.05 AUFS: Chart speed: 1.0 cm/min.
Peak 1. Aldosterone

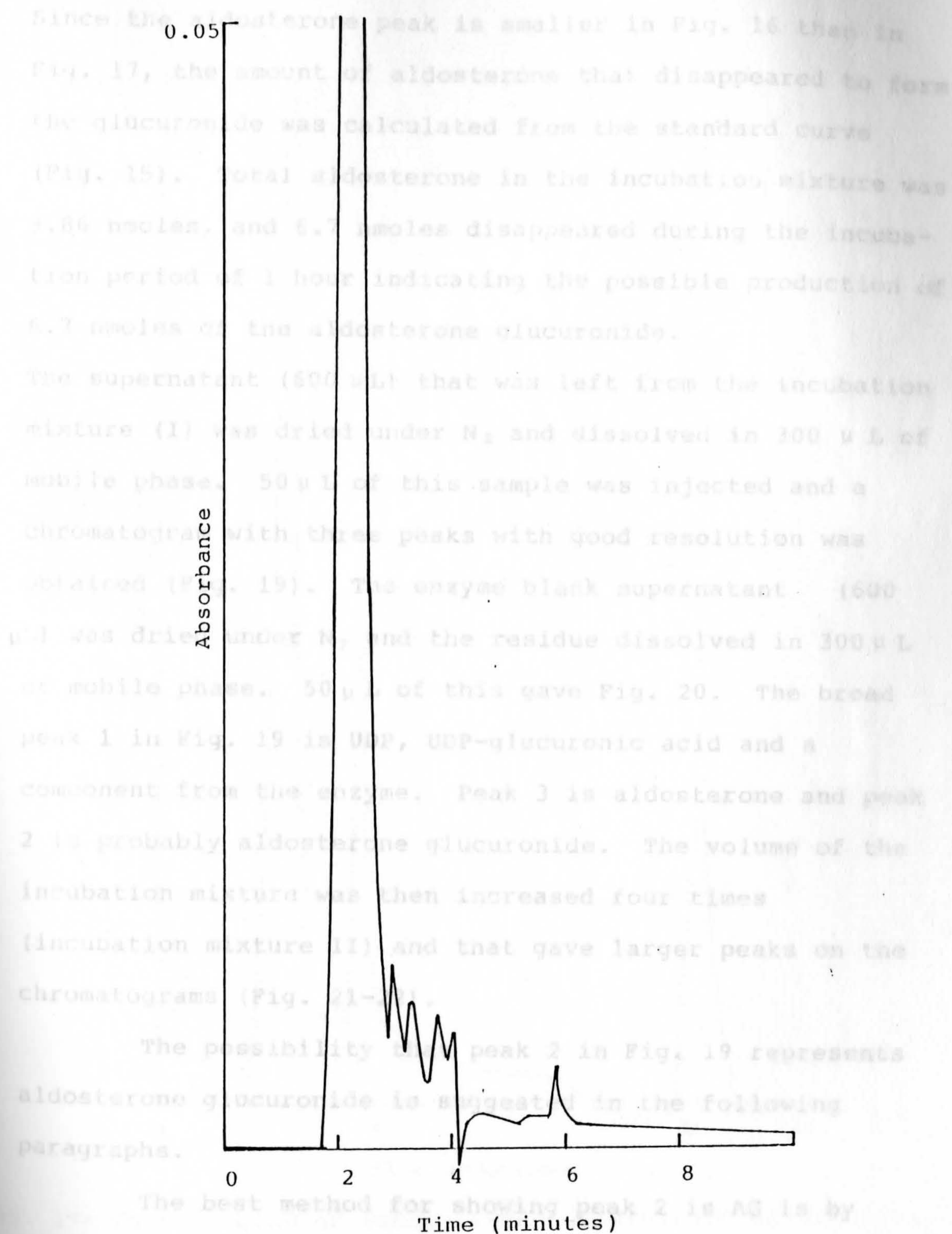


Fig. 18. Chromatogram of UDP-glucuronic acid used in the incubation mixture (I).
Mobile phase: Acetonitrile-water (30:70, v/v); Flow rate: 1.0 mL/min.; Detector: UV (240 nm); Sensitivity: 0.05 AUFS; Chart speed: 1.0 cm/min.

Since the aldosterone peak is smaller in Fig. 16 than in Fig. 17, the amount of aldosterone that disappeared to form the glucuronide was calculated from the standard curve (Fig. 15). Total aldosterone in the incubation mixture was 9.86 nmoles, and 6.7 nmoles disappeared during the incubation period of 1 hour indicating the possible production of 6.7 nmoles of the aldosterone glucuronide.

The supernatant (600 μL) that was left from the incubation mixture (I) was dried under N_2 and dissolved in 300 μL of mobile phase. 50 μL of this sample was injected and a chromatogram with three peaks with good resolution was obtained (Fig. 19). The enzyme blank supernatant (600 μL) was dried under N_2 and the residue dissolved in 300 μL of mobile phase. 50 μL of this gave Fig. 20. The broad peak 1 in Fig. 19 is UDP, UDP-glucuronic acid and a component from the enzyme. Peak 3 is aldosterone and peak 2 is probably aldosterone glucuronide. The volume of the incubation mixture was then increased four times (incubation mixture II) and that gave larger peaks on the chromatograms (Fig. 21-22).

The possibility that peak 2 in Fig. 19 represents aldosterone glucuronide is suggested in the following paragraphs.

The best method for showing peak 2 is AG is by comparing the chromatogram to one produced by a pure standard. But there is no commercially available standard

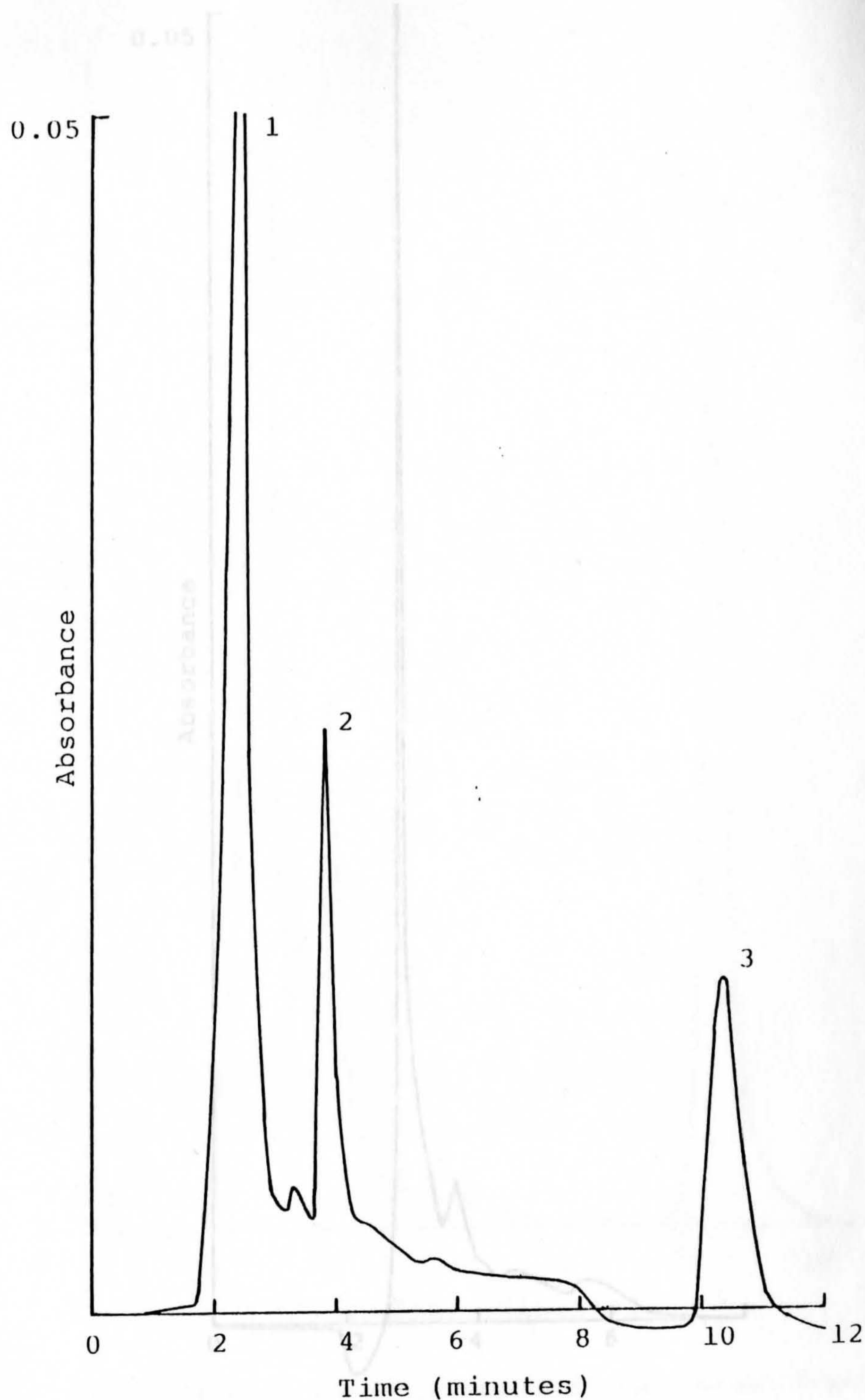


Fig. 19. Chromatogram of concentrated supernatant of incubation mixture (I).
Mobile phase: Acetonitrile-water (30:70 v/v); Flow rate: 1.0 mL/minute; Detector: UV (240 nm); Sensitivity: 0.05 AUFS; Chart speed: 1.0 cm/min.
Peaks 1. UDP, UDP-glucuronic acid and a component from enzyme, 2. putative aldosterone glucuronide, 3. aldosterone.

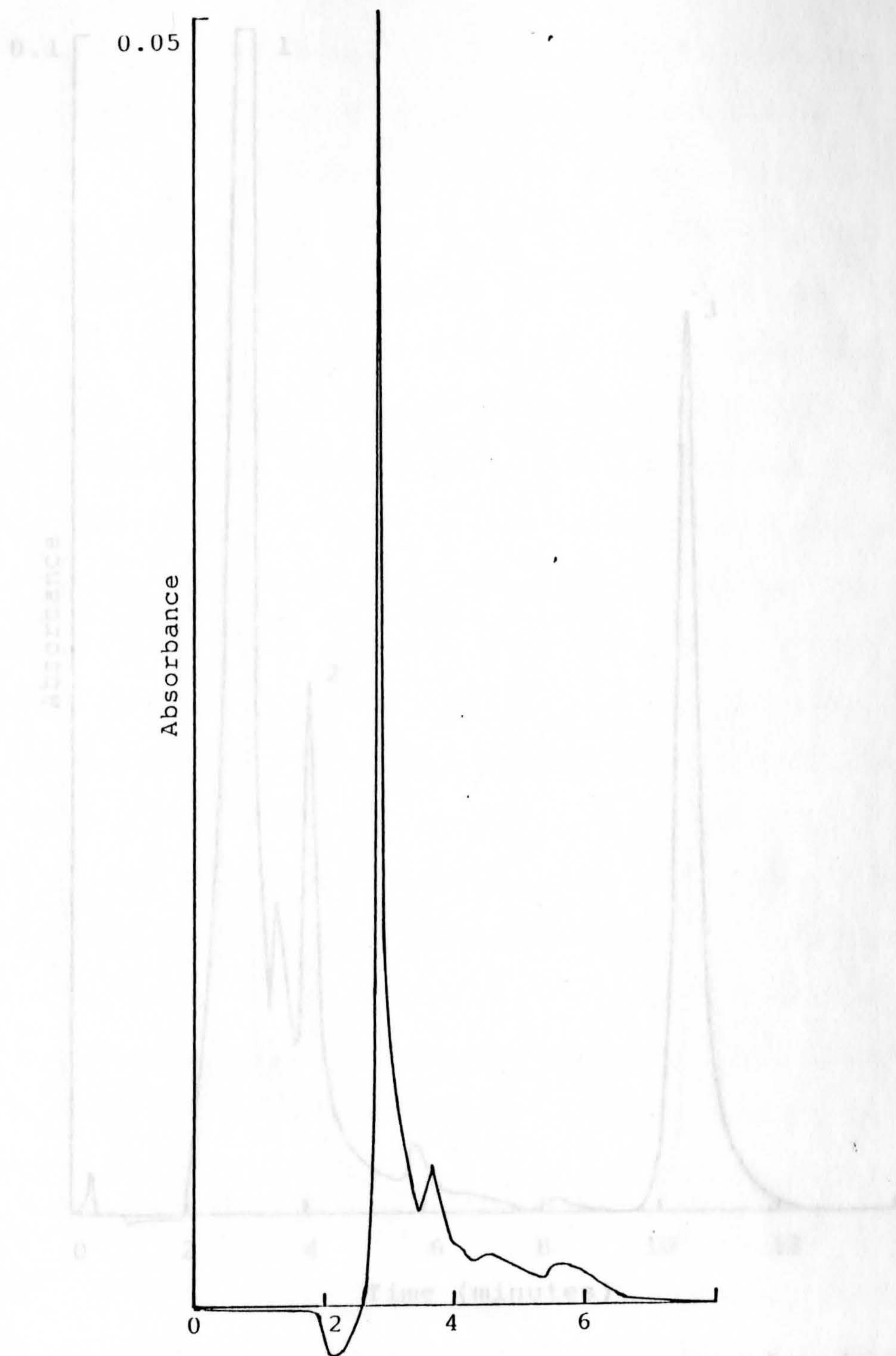


Fig. 20. Chromatogram of concentrated enyme blank supernatant. Mobile phase: Acetonitrile-water (30:70, v/v); Flow rate: 1.0 mL/min.; Detector: UV (240 nm); Sensitivity: 0.05 AUFS; Chart speed: 1.0 cm/min.

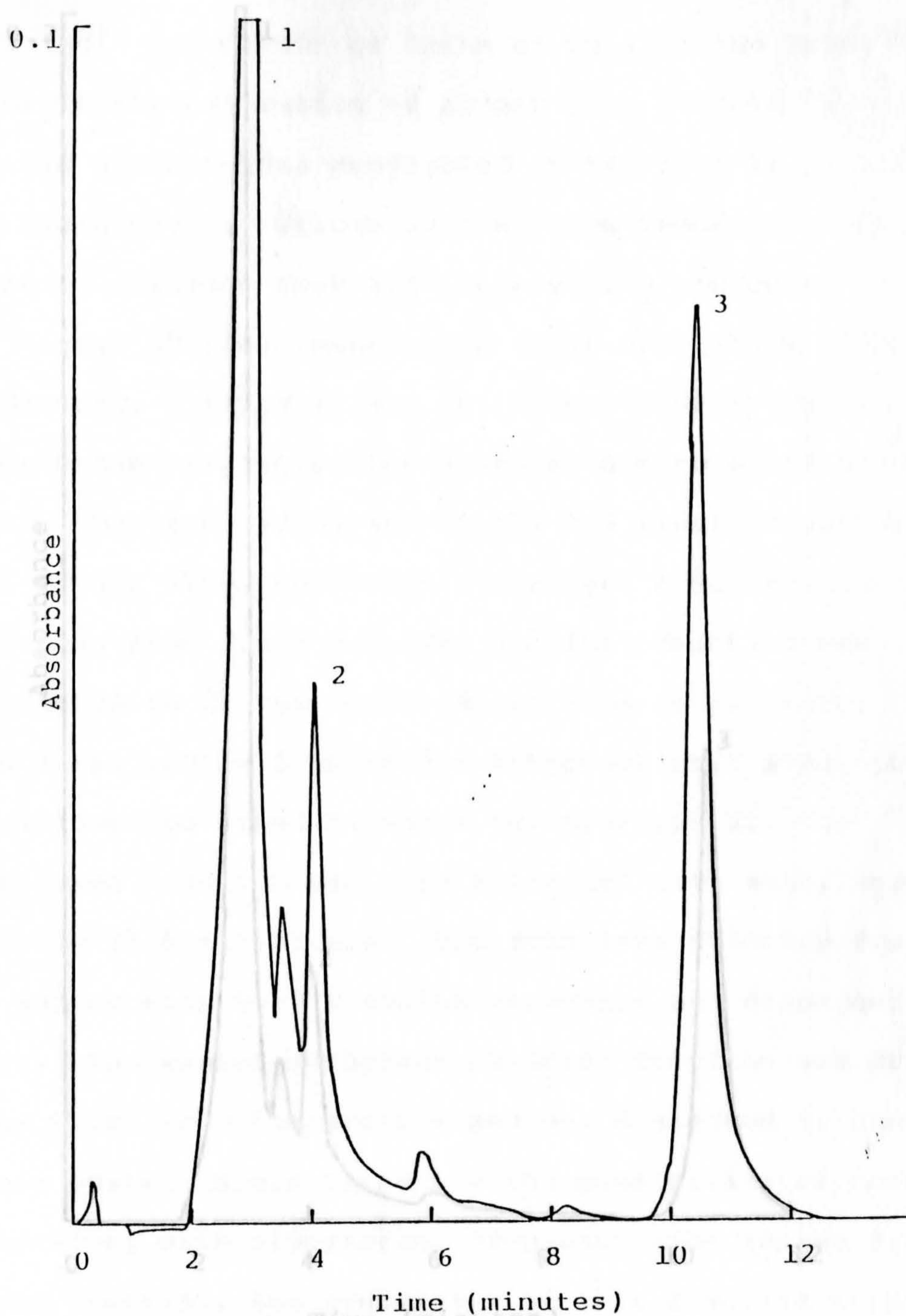


Fig. 21. Chromatogram of a more concentrated supernatant from incubation mixture (11).
Mobile phase: Acetonitrile-water (30:70, v/v); Flow rate:1.0 mL/min.; Detector: UV (240nm); Sensitivity: 0.05 AUFS; Chart speed:1 cm/min.
Peaks 1. UDP, UDP-glucuronic acid and a component from enzyme, 2. Putative aldosterone glucuronide, 3. Aldosterone.

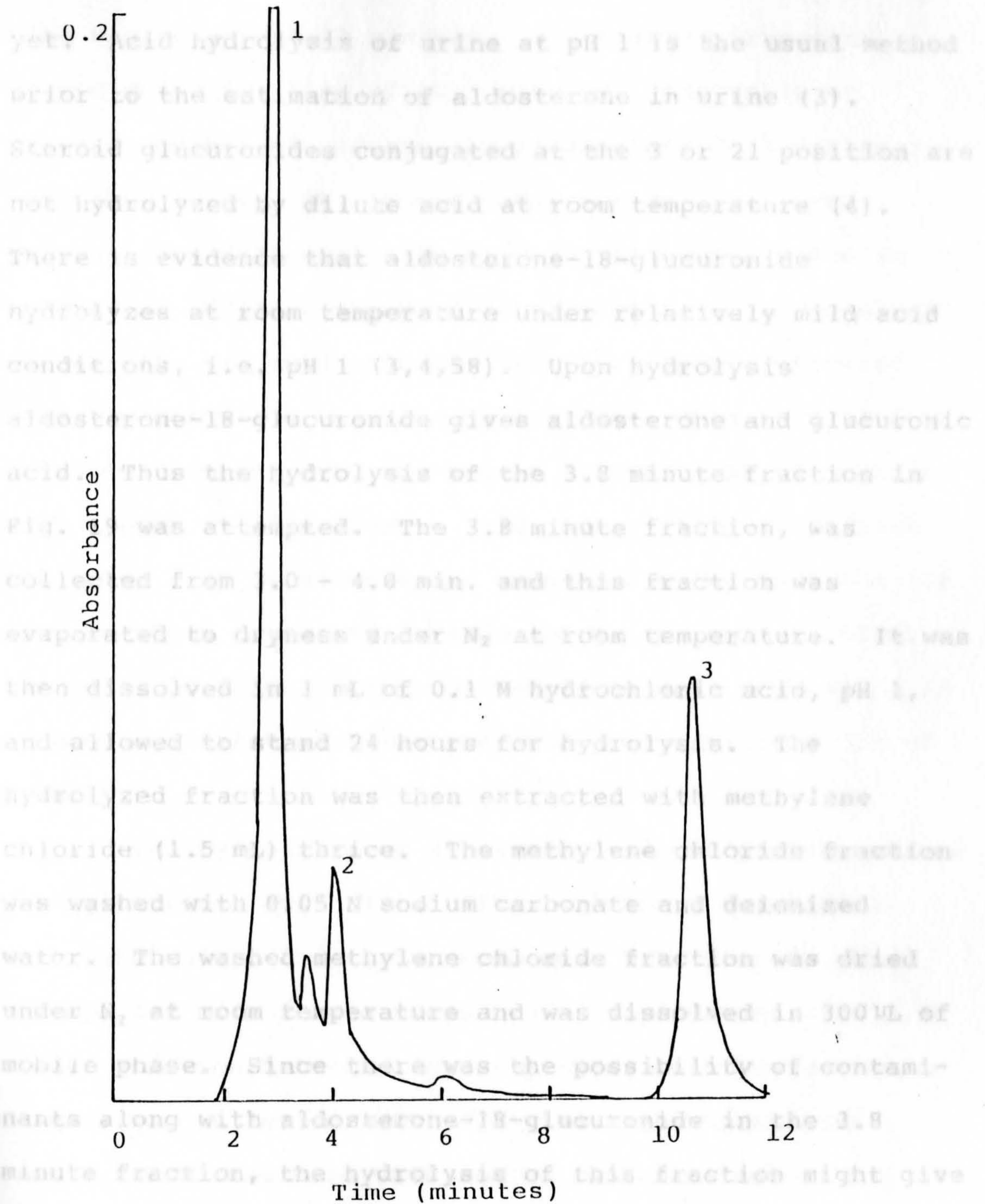


Fig. 22. Chromatogram of more concentrated supernatant from incubation mixture (II).
Mobile phase: Acetonitrile-water (30:70, v/v); Flow rate:1.0 mL/min.; Detector: UV(240nm); Sensitivity: 0.05 AUFS; Chart speed:1 cm/min.
Peaks 1. UDP, UDP-glucuronic acid and a component from enzyme, 2. Putative aldosterone glucuronide, 3. Aldosterone.

yet. Acid hydrolysis of urine at pH 1 is the usual method prior to the estimation of aldosterone in urine (3). Steroid glucuronides conjugated at the 3 or 21 position are not hydrolyzed by dilute acid at room temperature (4). There is evidence that aldosterone-18-glucuronide hydrolyzes at room temperature under relatively mild acid conditions, i.e. pH 1 (3,4,58). Upon hydrolysis aldosterone-18-glucuronide gives aldosterone and glucuronic acid. Thus the hydrolysis of the 3.8 minute fraction in Fig. 19 was attempted. The 3.8 minute fraction, was collected from 3.0 - 4.0 min. and this fraction was evaporated to dryness under N_2 at room temperature. It was then dissolved in 1 mL of 0.1 M hydrochloric acid, pH 1, and allowed to stand 24 hours for hydrolysis. The hydrolyzed fraction was then extracted with methylene chloride (1.5 mL) thrice. The methylene chloride fraction was washed with 0.05 N sodium carbonate and deionized water. The washed methylene chloride fraction was dried under N_2 at room temperature and was dissolved in 300 μL of mobile phase. Since there was the possibility of contaminants along with aldosterone-18-glucuronide in the 3.8 minute fraction, the hydrolysis of this fraction might give peaks other than aldosterone. Thus an internal standard had to be used. Prednisolone was found to have a retention time of 10.3 minutes which interfered with the aldosterone peak. Aldosterone diacetate was tried, and its retention

time was more than 30 minutes. Aldosterone monoacetate gave a retention time of 22.5 minutes. The ratio of retention times of aldosterone monoacetate to aldosterone was found to be 2.16. Since there was no other good internal standard available at this time which could be used as an internal standard for aldosterone, aldosterone monoacetate was used with the 300 μL of sample prepared from the methylene chloride extraction. From this 50μL was injected onto the HPLC column. A chromatogram was obtained with a peak at 10.6 minutes (Fig. 23). This is the same retention time as aldosterone which indicates that the 3.8 min. fraction contained an aldosterone conjugate. Also, the ratio of aldosterone monoacetate to this peak was 2.18 in good agreement with the previously determined value of 2.16.

The hydrolysis of aldosterone-18-glucuronide should give glucuronic acid also. After the above experiments, the guard column had to be replaced. With the new guard column, and with a more concentrated supernatant, the 3.8 minute fraction (changed to 3.6 minutes with the new guard column) was collected and hydrolyzed. The hydrolyzed sample was neutralized with sodium carbonate solution. 50 μL of this aqueous solution was injected onto the HPLC column. The chromatogram obtained is shown in Fig. 24. The retention time of the peak obtained was 3 minutes. It has to be proved to be sodium glucuronate. The retention

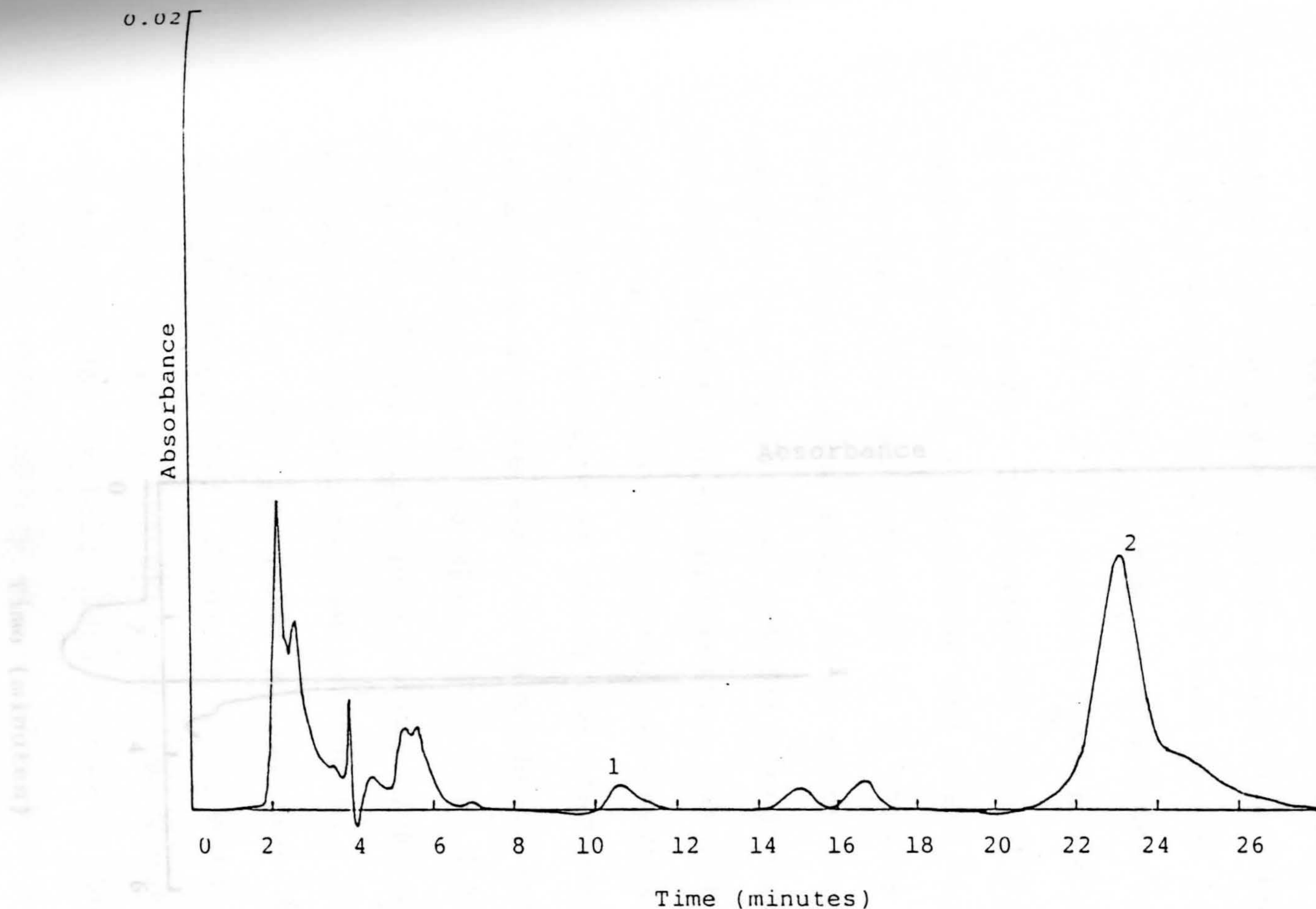


Fig. 23 Chromatogram of extracted methylene chloride fraction from the putative aldosterone glucuronide fraction.
Mobile phase: Acetonitrile-water (30:70, v/v); Flow rate: 1.0 mL/min.; Detector: UV (240 nm); Sensitivity: 0.02 AUFS; Chart speed: 1.0 cm/min.

Peaks 1. aldosterone, 2. aldosterone monoacetate

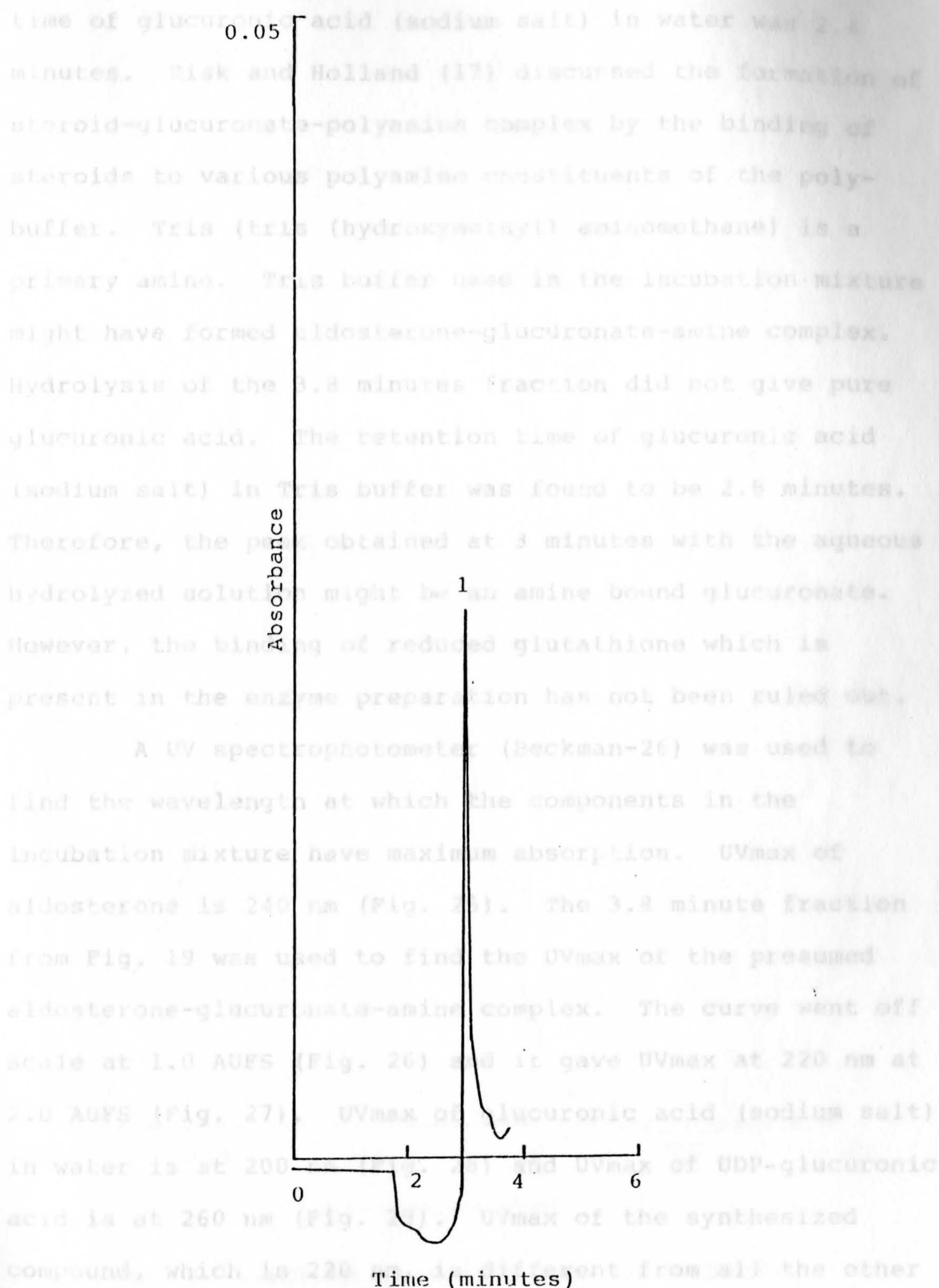


Fig. 24. Chromatogram of the aqueous solution of the hydrolyzed 3.8 min. fraction.
Mobile phase: Acetonitrile-water (30:70, v/v); Flow rate: 1.0 mL/min.; Detector: UV (240 nm); Sensitivity: 0.05 AUFS; Chart speed: 1.0 cm/ min.
Peak 1. Possible glucuronide complex.

time of glucuronic acid (sodium salt) in water was 2.4 minutes. Risk and Holland (17) discussed the formation of steroid-glucuronate-polyamine complex by the binding of steroids to various polyamine constituents of the poly-buffer. Tris (tris (hydroxymethyl) aminomethane) is a primary amine. Tris buffer used in the incubation mixture might have formed aldosterone-glucuronate-amine complex. Hydrolysis of the 3.8 minutes fraction did not give pure glucuronic acid. The retention time of glucuronic acid (sodium salt) in Tris buffer was found to be 2.8 minutes. Therefore, the peak obtained at 3 minutes with the aqueous hydrolyzed solution might be an amine bound glucuronate. However, the binding of reduced glutathione which is present in the enzyme preparation has not been ruled out.

A UV spectrophotometer (Beckman-26) was used to find the wavelength at which the components in the incubation mixture have maximum absorption. UVmax of aldosterone is 240 nm (Fig. 25). The 3.8 minute fraction from Fig. 19 was used to find the UVmax of the presumed aldosterone-glucuronate-amine complex. The curve went off scale at 1.0 AUFS (Fig. 26) and it gave UVmax at 220 nm at 2.0 AUFS (Fig. 27). UVmax of glucuronic acid (sodium salt) in water is at 200 nm (Fig. 28) and UVmax of UDP-glucuronic acid is at 260 nm (Fig. 29). UVmax of the synthesized compound, which is 220 nm, is different from all the other compounds in the incubation mixture. However, To and Wells

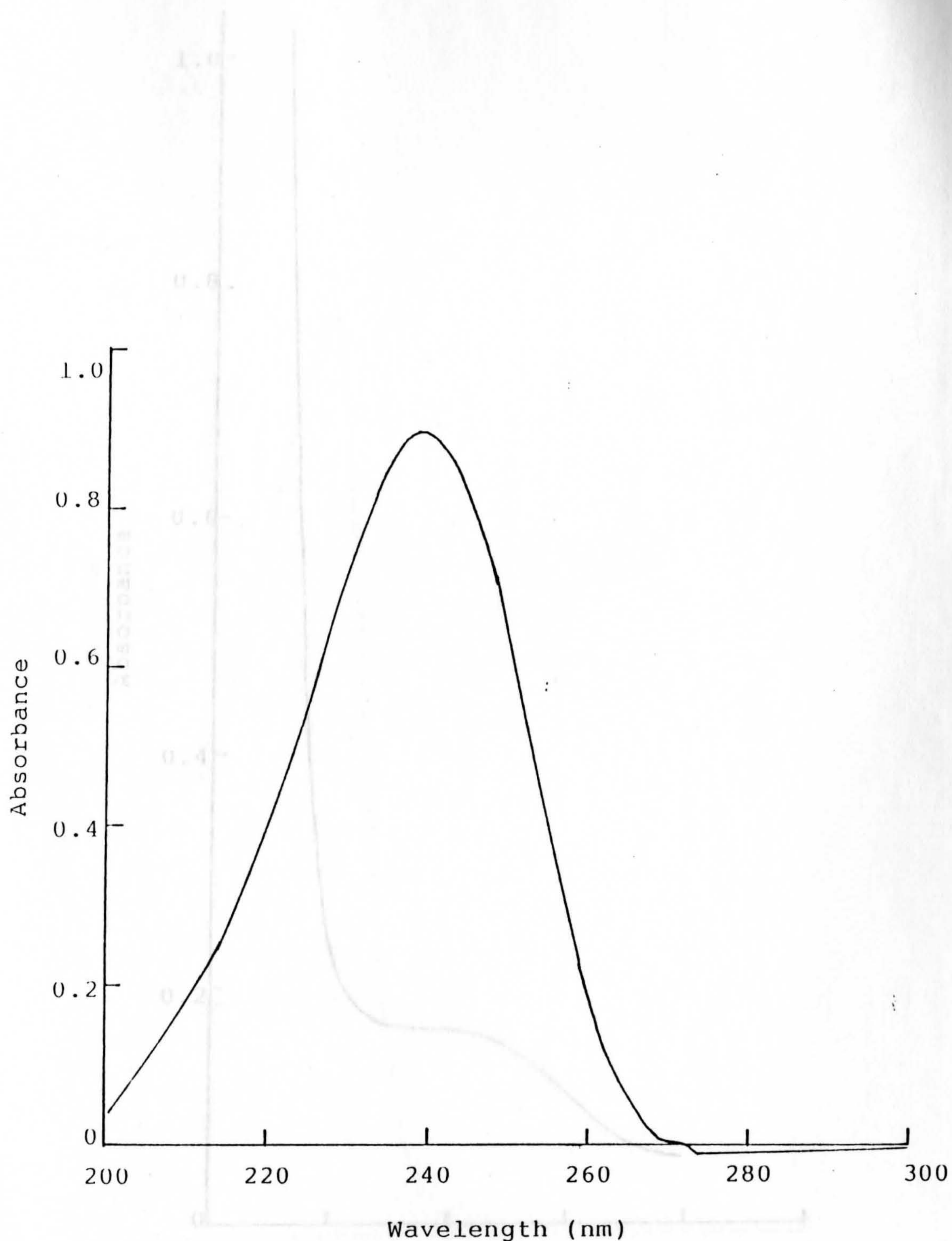


Fig. 25. UV spectrum of aldosterone in methanol

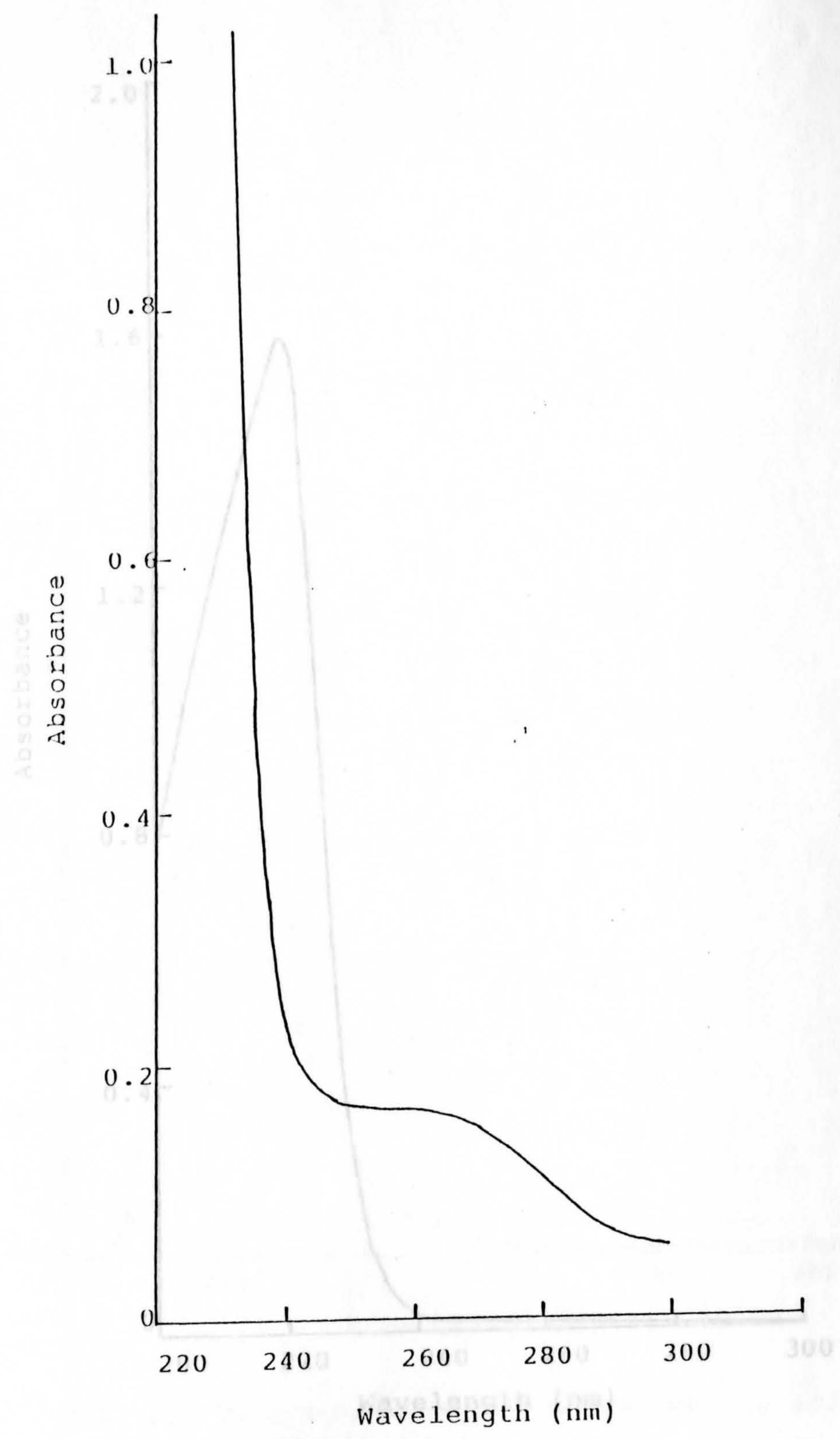


Fig. 26. UV spectrum of 3-4 min. fraction from HPLC of incubation mixture in 30% Acetonitrile in water.

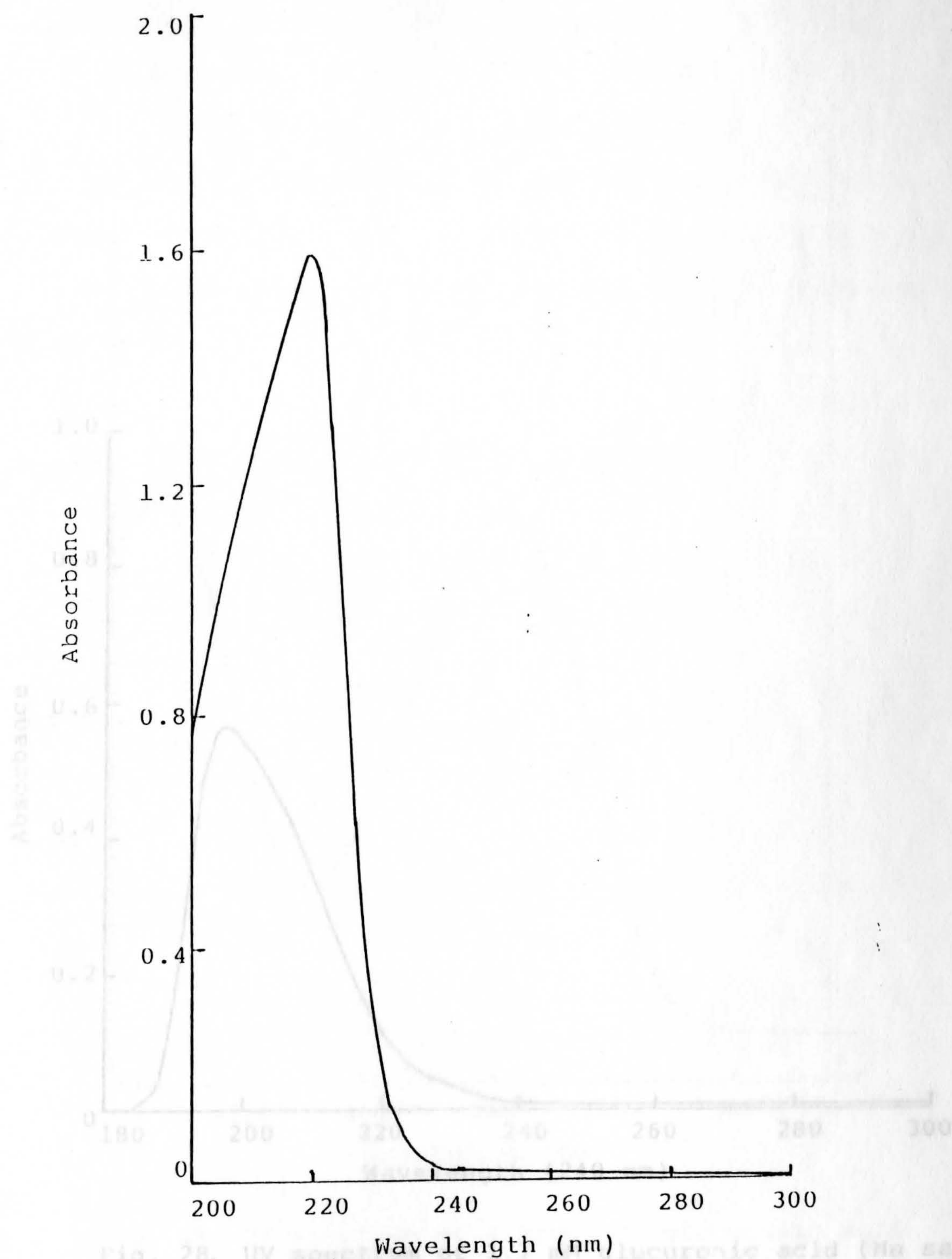


Fig. 27. UV spectrum of 3-4 min. fraction from HPLC of incubation mixture in 30% Acetonitrile in water.

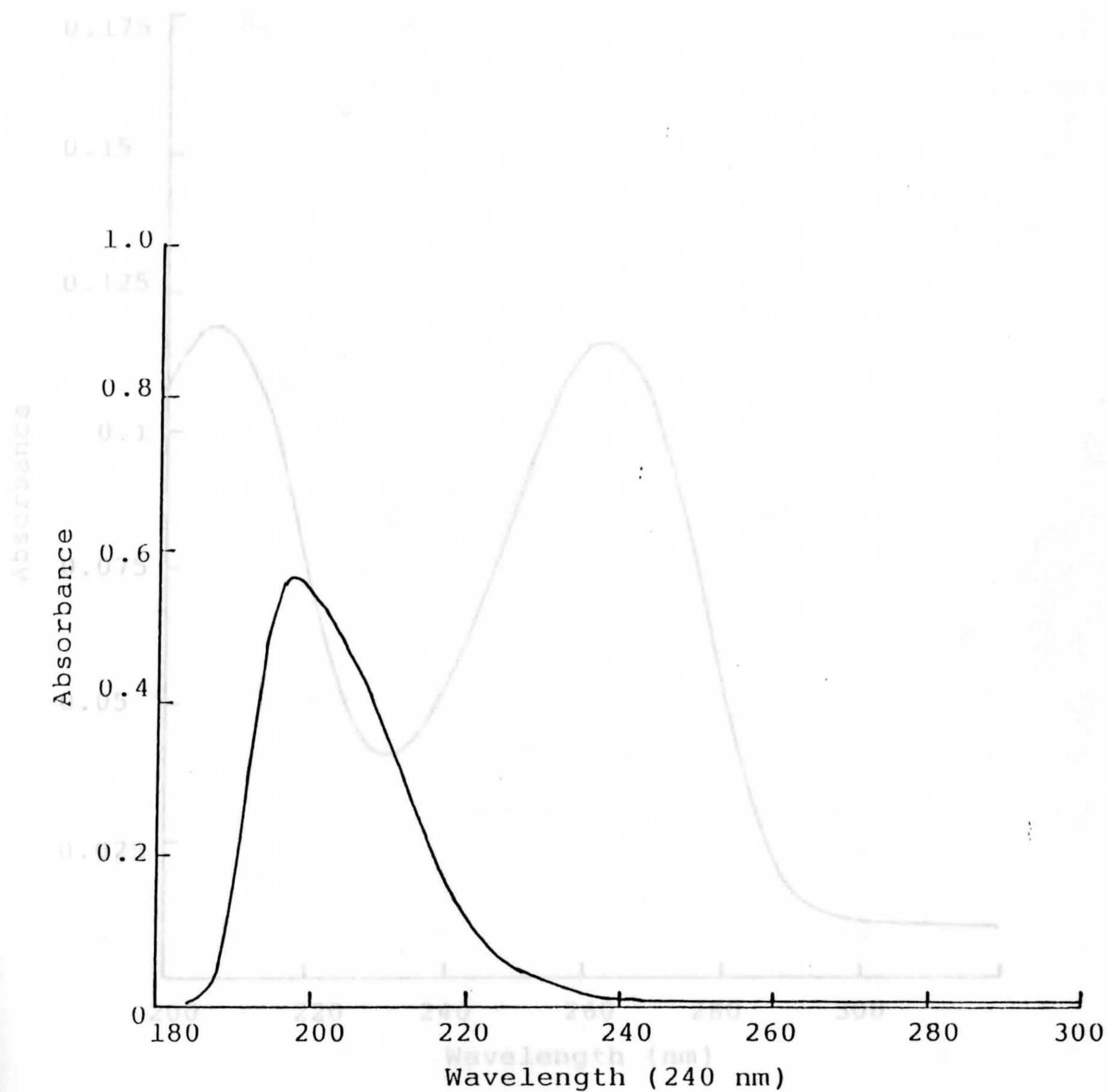


Fig. 28. UV spectrum of 1.7 mM glucuronic acid (Na salt) in water.

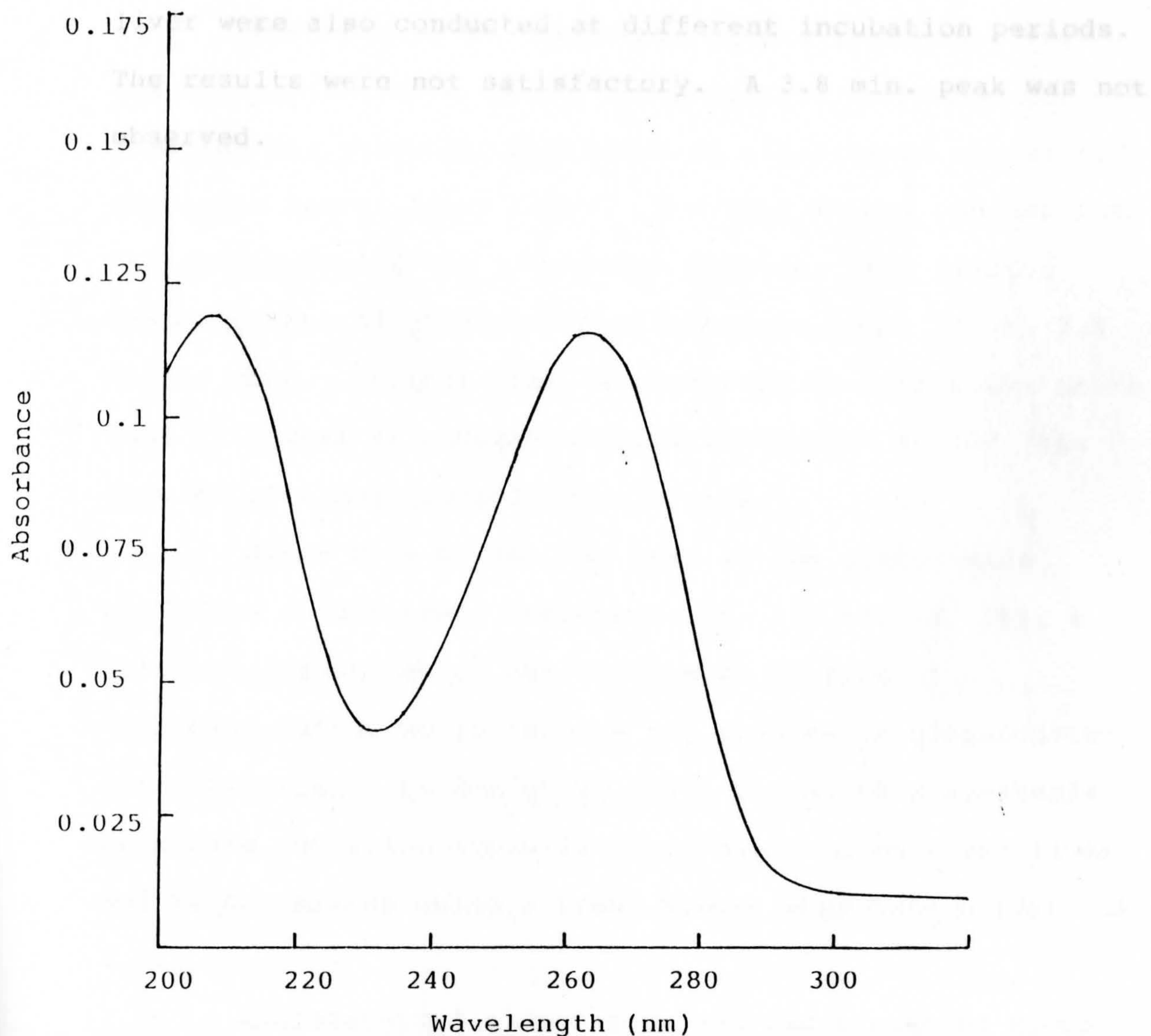


Fig. 29. UV spectrum of 0.01mM UDP-glucuronic acid in water.

(20) found that α-naphthol glucuronide gave a maximum absorbance of 225 nm which is certainly different from the UV spectrum of either α-naphthol or glucuronic acid.

The source of UDP glucuronyl transferase in these experiments was rabbit liver. Experiments with bovine liver were also conducted at different incubation periods. The results were not satisfactory. A 3.8 min. peak was not observed.

CHAPTER VI

CONCLUSION

The results of this research has shown that HPLC can be utilized as a simple and sensitive technique for studying the enzymatic synthesis of aldosterone glucuronide using the rabbit liver UDPGT. The HPLC method can separate the components of the incubation mixture. The results indicate that aldosterone-18-glucuronide might be the 3.8 minute peak. Further work is necessary to completely prove that this peak is aldosterone-18-glucuronide or the Tris complex of aldosterone-18-glucuronide.

Since Tris buffer was used in the glucuronide synthesis of different compounds (20, 21, 50, 54, 55), a thorough investigation has to be made to find if a different buffer would resolve the problem of glucuronate identification. Sandor et al. (57) discussed a synthesis procedure for aldosterone-18-glucuronide using human liver and kidney slices using a Krebs Ringer phosphate buffer, pH 7.4.

Radiolabelled aldosterone and radiolabelled UDPGA could be used in this synthesis. This would help in the isolation and identification of the synthesized product. Pasqualini et al. (58) isolated aldosterone-18-glucuronide

from human urine. A positive Tollens reaction, a positive blue tetrazolium reaction, detection of glucuronic acid by paper chromatography, acid hydrolysis of the conjugate at pH 1, and quantitative analysis indicating one molecule of glucuronic acid to one molecule of aldosterone gave proof that this isolated conjugate was aldosterone-18-glucuronide. The synthesized products can be checked by following their procedure after purification. Van der Wal and Huber (59) observed that many steroid conjugates absorbed UV light below 210 nm. The synthesized product in this research absorbed UV light at 220 nm. The aldosterone-18-glucuronide isolated by Pasqualini et al. (58) was shown to absorb UV light at 254 nm. A future researcher can synthesize enough material to find the structure of this compound by NMR, IR and mass spectroscopy.

After purifying the synthesized aldosterone-18-glucuronide, it can be used as a standard to detect aldosterone-18-glucuronide in urine samples. It is possible to extract this conjugate from urine using solid phase extraction. There are several pigments and metabolites of steroids which will coelute with aldosterone-18-glucuronide from urine samples. An important step is to separate this from other polar metabolites by a suitable extraction procedure.

Instead of isocratic elution, which was used in

this work, a gradient elution probably will give a good resolution of peaks from the urine samples. A reduction in flow rate from 1 mL/min to 0.5 mL/min may also improve the resolution. To separate the reactants and products better, one could change the % of acetonitrile in the mobile phase from 30% to 25% to 20% and/or change the aqueous phase from pure water to a suitable buffer.

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