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A DETERMINATION OF SERUM CORTISOL BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

by

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THESIS

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TABLE OF CONTENTS

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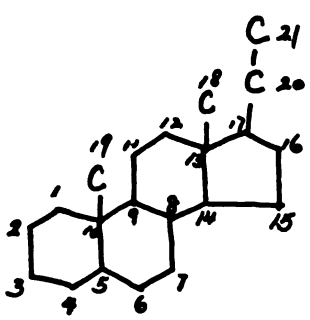
ABSTRACT

We describe a specific and precise method for measuring concentrations of cortisol in serum or plasma by liquid chromatography. Cortisol, together with an internal standard, equilenin, is extracted from 1 ml of serum or plasma and analyzed isocratically on a reversed phase column with a mobile phase consisting of acetonitrile/phosphate buffer, at a flow rate of 2.0 ml/min. The eluted cortisol is detected by its absorption at 254 nm; and quantitated by peak height measurements. Each analysis requires no longer than 15 min at the optimum column temperature of 50° C. The lower limit of detection for cortisol is about 2 ng/sample for a standard solution. A sensitivity of 5 µg/liter of serum is attained routinely. Analytical recoveries exceeded 95%, with good day-to-day precision (CV between 4% and 7%). Of more than 50 drugs and steroids tested for possible interference only the synthetic steroids prednisone and prednisolone may interfere with the analysis of cortisol.

GENERAL INTRODUCTION

A clinical syndrome resulting from destruction of the adrenal glands was first noticed by Thomas Addison (1). This observation attracted scientific attention to these organs for the first time. Seven years later Brown-Sequard (2) demonstrated that the removal of both adrenals from experimental animals resulted in the animal's instaneous death. Subsequent investigations demonstrated that these life-sustaining hormones were secreted by cells in the adrenal cortex. These hormones are broadly classified into: glucocorticoids, 17-ketosteroids, aldosterone, estrogens, androgens, and progestins. Some of these hormones are biologically very potent, whereas others lack such potent activity. Cortisol, a glucocorticoid hormone, is produced in the zona fasciculata. Dehydroepiandrosterone (DHEA), and androgen, is secreted by the zona reticularis. Aldosterone, a mineralocorticoid, is produced in the zona glomerulosa.

The steroid molecule contains a cyclopentanoperhydrophenanthrane nucleus as its basic structure. The following numbering system is widely used to identify substituents on the nucleus and its side chains.



Maintenance of the adrenal gland and its secretory activity is regulated by adrenocorticotropic hormone (ACTH) which is secreted by the anterior pituitary gland. ACTH secretion is in turn regulated by a corticotropin releasing factor (CRF) secreted by the hypothalamus. The hypothalamus, anterior

pituitary and adrenal cortex thus form a functional unit (3,4). Therefore, measurement of cortisol in blood can detect dysfunctions of the adrenal glands, the pituitary gland and the hypothalamus.

The purpose of this study was to develop a rapid accurate and precise method for the measurement of cortisol.

Biosynthesis:

Radio-labeled precursors have played very important roles in the elucidation of the biogenesis of steroid hormones. Labeled cholesterol has been widely used for *in vivo* and *in vitro* studies of steroidogenesis, and was found to be a precursor of steroid hormones. The major steps involved in the biosynthesis of adrenocorticosteroid are illustrated in Figure 1. Cholesterol, the main precursor, is converted into pregnenolone (5). Pregnenolone is an important intermediate and is utilized by two different pathways. The first pathway includes the following steps:

1) Dehydrogenation and isomerization of pregnenolone to progesterone (6), 2) 17-hydroxylation of progesterone to 17-hydroxyprogesterone (7), 3) 21-hydroxylation of 17-hydroxyprogesterone to 11-deoxycortisone (Compound "S"), and 4) 11-hydroxylation of deoxycortisone to cortisol (8). The second pathway includes the following steps: 1) 17-hydroxylation of pregnenolone to 17-hydroxypregnenolone, 2) oxidation of 17-hydroxypregnenolone to 17-hydroxyprogesterone, 3) the subsequent conversion steps to cortisol are similar to the first pathway (9), an alternative minor pathway involves 21-hydroxylation of progesterone to deoxycorticosterone, followed by 11-hydroxylation to corticosterone (compound "B"), and 18-hydroxylation of corticosterone to aldosterone (mineralocorticoid).

The rate limiting step of the above biosynthesis pathways is the conversion of cholesterol to pregnenolone.

Metabolism:

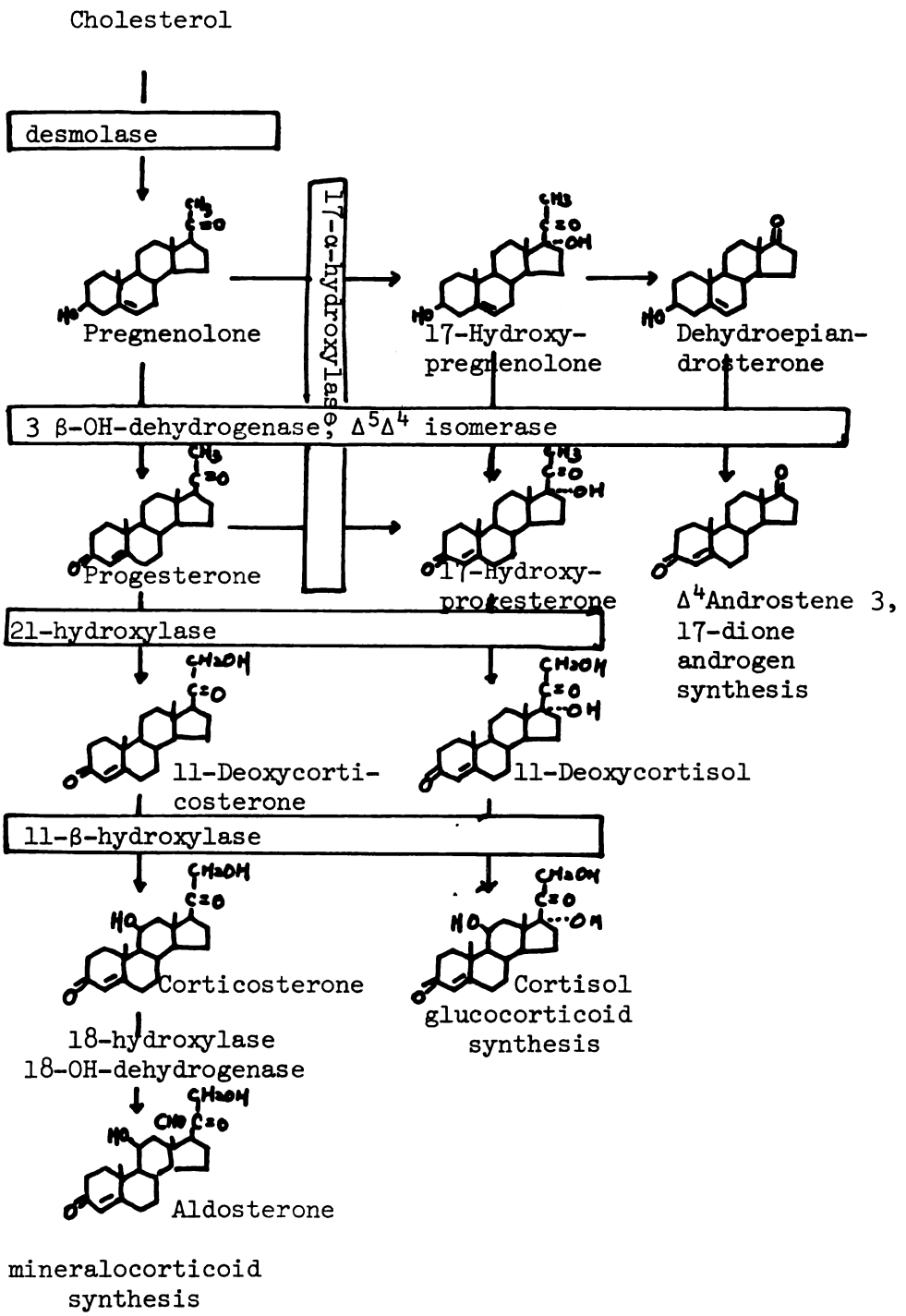


Figure 1: Pathways of Steroid Biosynthesis

The cyclopentanoperhydrophenanthrene nucleus remains intact in the human body. Steroid hormones are metabolized in the liver and transformed into inactive compounds. Fukushima (10) administered C-14 labeled cortisol to healthy human volunteers and found that 50 percent of the cortisol was reduced to tetrahydrocortisol and 25 percent of the cortisol was reduced to cortol or cortolone. Only minute amounts were excreted as free cortisol in the urine. Furthermore, 10-15 percent was excreted as 17-ketosteroids. The conjugation of steroid hormones was first demonstrated by Venning and Browne (11). Conjugation is very important for excretion into the urine. The equilibrium between cortisol and cortisone concentrations, maintained by the enzyme 11-dehydrogenase, is very important in regulating the level of circulating cortisol in the body.

The rate of metabolism in great measure determines the half life of cortisol, which in turn influences the secretion of this hormone. In cirrhosis and hypothyroidism the cortisol half life is unusually prolonged because of slowed metabolism. Although cortisol levels are lower in these patients, they still maintain normal physiological functions. On the other hand, in hyperthyroidism and simple obesity there is rapid excretion of cortisol resulting in a shorter half life. Although cortisol levels are higher in these patients, they still maintain normal physiological functions. The half life of cortisol in normal individuals is about 90 minutes (12).

Transport:

There are two independent corticosteroid binding systems in the body (13). A high affinity low capacity system, and a low affinity high capacity system. Albumin is the low affinity high capacity binding protein in human plasma. Transcortin (an α globulin) is the high affinity low capacity binding protein. Transcortin has high affinity for cortisol, progesterone, hydroxycorticosterone, corticosterone and some synthetic corticosteroid analogues.

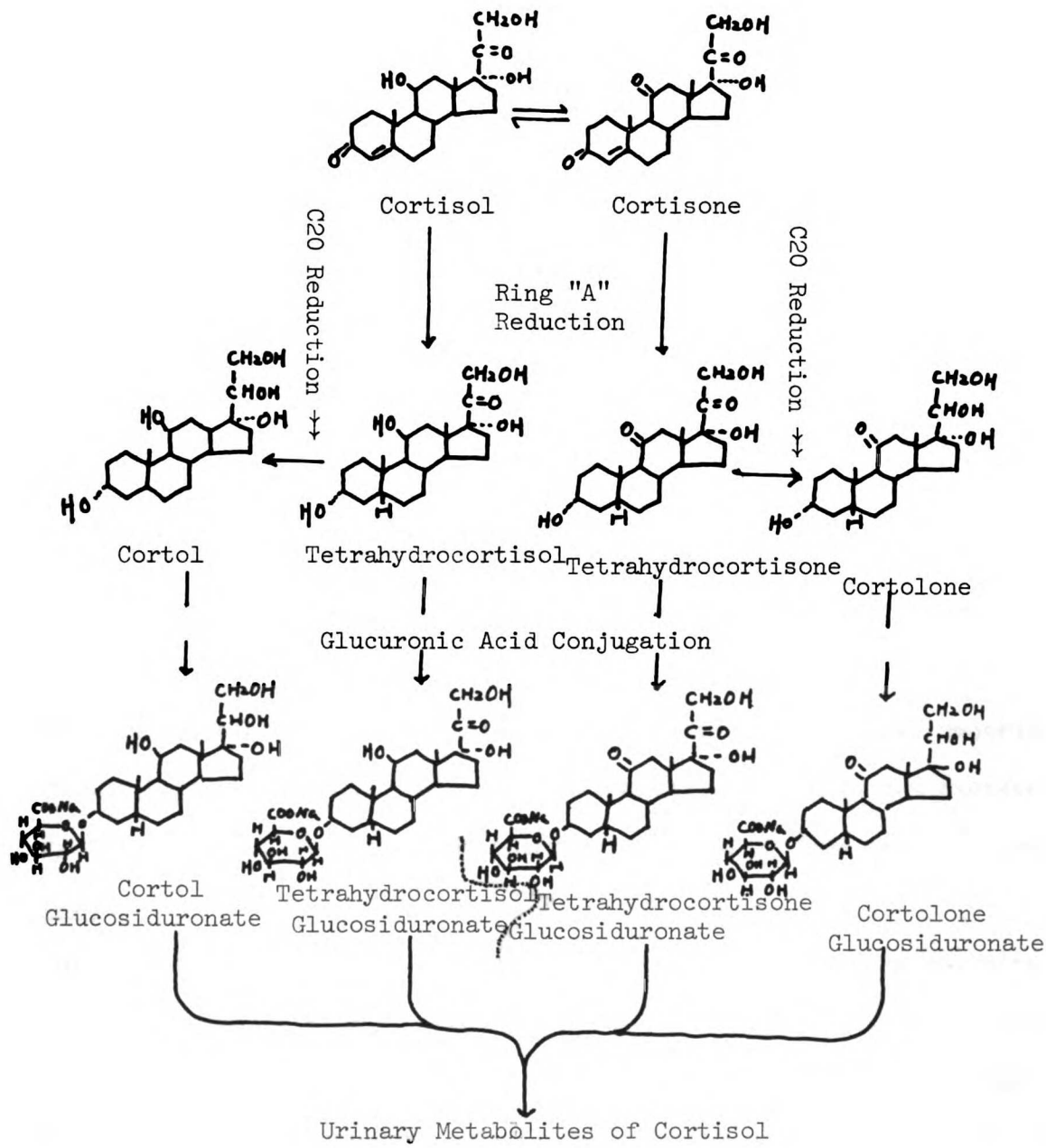


Figure 2: Metabolism of Cortisol in the Liver

Cortisol binding to protein serves as a buffer mechanism. Transcortin will release cortisol whenever free cortisol concentrations are low, and will bind cortisol whenever the concentrations of free cortisol are high. There are many advantages to this unique phenomenon, such as minimal loss of protein bound cortisol through glomerular filtration and prevention of target organs from possible toxic effects of free hormone.

Mills et al. (14) reported a correlation between physiological actions and the concentration of free cortisol. This was verified by in vitro experiments (15,16). During pregnancy and estrogens treatment transcortin levels are increased (17). In these situations total cortisol levels are increased, but free cortisol levels are normal. The same is true for patients with nephrotic syndrome. Though transcortin and plasma cortisol levels are low, free cortisol levels are normal. These patients also retain normal adrenal function. Only when free cortisol levels are increased or decreased do patients show clinical symptoms of hyper or hypoadrenal disorders.

In blood cortisol is approximately 75 percent bound to transcortin, 15 percent bound to albumin and 10 percent free. Obviously the large majority of cortisol is protein bound. Since protein bound cortisol is not excreted, urinary free cortisol is a good indicator of circulating free cortisol levels.

Secretion:

Cortisol is the principle glucocorticoid secreted by the adrenal glands (18). The daily adult adrenal secretion of cortisol ranges between 15 and 30 mg. The volume of distribution for cortisol is approximately the same as that for the total extracellular fluid space. Plasma concentrations are dependent on the rate of secretion, the rate of metabolism and the rate of excretion of free cortisol. Cortisol has a pronounced diurnal variation, reaching its highest concentrations in the morning and then gradually declining to its lowest level between early morning and midnight (19). Loss

of diurnal rhythms indicates adrenal cortex abnormalities, such as Cushing's syndrome. The normal range of cortisol is 6.5 to 26.3 μg per 100 ml plasma (or 65 to 263 ng per 1 ml plasma) between 8 and 10 a.m., and 2 to 18 μg per 100 ml plasma (or 20 to 180 ng per 1 ml plasma) at 4 p.m. (20).

Functions:

Cortisol can diffuse freely through cell membranes into the cytoplasm. In the cytoplasm cortisol binds with cytoplasmic receptors and forms the cytoplasmic receptor complex (21). The steroid-receptor complex migrates into the nucleus where it attaches to specific areas of the chromatin, activating certain genes and allowing for the formation of new RNA. This RNA then directs the formation of new proteins, some of which are important in cell structure and cell replication. Some of these newly synthesized proteins are enzymes which regulate metabolic functions of the cell. Other proteins are transported out of the target cells as secretory products. This control-receptor mechanism is similar to that proposed earlier by Jensen and the others for the interaction of estrogens with uterine receptors (22). The binding process is specific and selective, the receptor recognizing very subtle differences in steroid structures. For this reason different tissue receptors show different affinities to various steroids.

Cortisol is the most potent glucocorticoid hormone in the body. The term "glucocorticoid" has been applied to steroids that have distinct effects on carbohydrate metabolism. Cortisol promotes gluconeogenesis (23,24), glycogen desposition in liver (25), and elevates blood glucose concentrations (26-29). However, some of these effects are still controversial (30,31).

In addition to the effects on carbohydrate metabolism, cortisol also affects other biological functions.

1. Protein-catabolism activity: Cortisol accelerates the catabolism of proteins such as albumin. If high cortisol levels are sustained for

a long period of time the secretion of growth hormone is suppressed and somatic growth is inhibited.

2. Anti-inflammatory activity: When present in high concentration cortisol inhibits inflammatory and allergic reactions. Cortisol stabilizes proteolytic enzymes released as a consequence of cellular injury (32).
3. Miscellaneous activities: Cortisol also affects the induction of enzyme production, stimulation of hematopoiesis, promotion of fat deposition, alteration of fat distribution (33,34), promotion of uric acid excretion, and maintenance of muscular activity.

Regulation:

Cortisol secretion is regulated by adrenocorticotropic hormone (ACTH). ACTH is a single chain polypeptide compound of 39 amino acids (35). Only the first 24 N-terminal amino acids are required for biological activity. In all species that have been studied so far these 24 amino acids are common, but minor species differences are observed in amino acid composition in the 25-39 portion of the molecule. There are at least three different mechanisms which control ACTH secretion.

1. Pituitary Adrenal Rhythms: The normal individual has higher blood ACTH levels in the morning than in the evening. This phenomenon explains the well known diurnal variation in cortisol secretion (19). The diurnal rhythm is affected by one's sleep-wake habits. The timing and duration of the pituitary-adrenal secretory cycle can be altered by consistant changing of the timing and duration of the sleep-wake schedule. Loss of this rhythm is one of the earliest features of Cushing's syndrome.
2. Feed-back mechanism: If cortisol levels are high ACTH secretion is suppressed and the adrenal cortex ceases its secretory activity until

cortisol levels return to normal. Conversely, if cortisol levels are low ACTH levels rise and the adrenal cortex secrets cortisol until blood levels are restored. This is a classical example of a negative feedback mechanism which is so important in maintaining homeostasis.

3. Stress: Superimposed upon all other regulators of ACTH secretion is the stimulatory influence of stress. It appears that this works through the central nervous system to stimulate ACTH (36). Regardless of the time of day and the level of plasma cortisol normal individuals respond to a major stress with large increases in ACTH secretion and a consequent increase in cortisol secretion.

Adrenal Cortical Disorders:

Hypercortisolism (Cushing's Syndrome)

The major feature of Cushing's syndrome is an excessive secretion of cortisol, whereas aldosterone production is almost always normal. There is sometimes an excessive secretion of adrenal androgens, but rarely, estrogens. The increased activity of the adrenal cortex in Cushing's syndrome may be due to four possible causes: excess pituitary ACTH syndrome, benign adenoma of the adrenal cortex, carcinoma of the adrenal cortex, and ectopic ACTH syndrome.

1. Excess pituitary ACTH syndrome: Adrenocortical hyperplasia secondary to excess ACTH secretion is the cause of 75 percent of the cases of Cushing's syndrome. In this common form of Cushing's syndrome an abnormality of the hypothalamic function affects the negative feedback control of cortisol. Hence ACTH secretion is influenced. Early in this syndrome plasma cortisol concentration may be normal, but the normal diurnal variation in cortisol secretion is lost. Later, the plasma cortisol concentration may be elevated throughout the day. Bilateral adrenal hyperplasia is the result of this disease.
2. Benign adrenocortical adenoma: Benign adrenal cortical adenoma is

found in 10 to 15 percent of patients with Cushing's syndrome. In these patients the opposite adrenal is often atrophied. The biochemical distinction between hyperplasia and adenoma is not always well defined, but low 17-oxosteroid excretion relative to high 17-oxogenic steroid excretion may sometimes give an indication of the presence of an adenoma. The response to metamethasone suppression of ACTH stimulation is variable; some adenomas behaving like adrenal hyperplasia, others behaving autonomously.

3. Adrenocortical carcinoma: The least common cause of Cushing's syndrome is a carcinoma of the adrenal cortex. In addition to excessive secretion of cortisol, the tumor often secretes very large amounts of androgens, leading to severe virilization. When urinary 17-oxosteroids are secreted in excess of several hundred mg. per day, an adrenal carcinoma is generally suspected.
4. Ectopic ACTH syndrome: A few non-endocrine malignant tumors produce an altered DNA which synthesizes a polypeptide substance immunologically and biologically similar to pituitary ACTH. This leads to bilateral adrenal hyperplasia and hypercortisolism. Carcinoma of the lung, thymus and kidney are the most frequently observed tumors to secrete these compounds. Though the clinical symptoms of Cushing's syndrome are not very distinct in these cases, plasma and urinary cortisol concentrations are very high, giving rise to hypokalemic alkalosis. The high cortisol level is unaffected by any of the suppression or stimulation tests (37).

Adrenal Cortical Hypofunction:

Cortisol secretion may be insufficient to maintain normal life because of 1) primary disease of dysfunction of the adrenal cortex (Addison's disease), or 2) deficient ACTH (Secondary adrenal insufficiency).

1. Primary dysfunction of the adrenal cortex: Over 90 percent of primary adrenal insufficiency is caused by a) bilateral tuberculosis of the adrenal glands, or b) bilateral atrophy of unknown etiology. The major manifestations are deficiencies of aldosterone and cortisol. The diagnosis of Addison's disease is confirmed by demonstrating a functional inability of the adrenal cortex to respond to ACTH.
2. Secondary adrenal insufficiency: In these patients, plasma cortisol concentrations and urinary 17-OHCS and 17-ketosteroids are characteristically low, but they rise slowly in response to exogenous ACTH.

Mixed Hypo- and Hyperadrenocorticism -- Congenital Adrenal Hyperplasia

The secretion of cortisol requires the integrity of biosynthetic pathways involving a long series of enzymatically regulated steps. If, through a genetic error, any one of these enzymes is defective, the corresponding biosynthetic step will be blocked, resulting in the accumulation of precursors immediately preceding the impeded step. Decreased cortisol secretion leads to increased secretion of ACTH with a resultant adrenal hyperplasia. These hyperplastic adrenals deviate towards the production of androgens. The characteristics of all congenital adrenal enzymatic defects are:

1. A varying degree of cortisol deficiency;
2. Adrenal hyperplasia;
3. Various clinical manifestations due to the excesses of intermediates.

There are five such defects known in the steroid biosynthetic pathway (see Figure 3).

1. Deficiency of desmolase: Since this is the first enzymatic reaction in steroid biosynthesis, deficiency of this enzyme is incompatible with life. At autopsy infants with this defect have been found to have large fat-laden adrenals.
2. Deficiency of 3-hydroxysteroid dehydrogenase: This is a rare form of

congenital adrenal hyperplasia (38). The defect in cortisol synthesis occurs at an early stage, the conversion of pregnenolone to progesterone. The mortality rate is very high with this enzyme defect.

3. Deficiency of 17 α-hydroxylase: The lack of 17 α-hydroxylase seems to involve both the adrenals and the ovaries, since both 17-oxosteroids and estrogens are almost totally absent from the urine. This defect leads to the failure of sexual development, and cortisol or other 17-hydroxylated compounds are usually absent.
4. Deficiency of C-21 hydroxylase: The commonest form of congenital adrenal hyperplasia is due to a block in hydroxylation at C-21. The intermediate compound, 17 α-hydroxyprogesterone, accumulates with a considerable increase in the excretion of its urinary metabolites, pregnane-3α, 17α, 20α, - triol, and 17-hydoxy-pregnanolone. The deficiency of C-21 hydroxylase is normally diagnosed by the excretion of high levels of 17-oxosteroids and pregnanetriol in the urine.
5. Deficiency of 11β- hydroxylase: Deficiency of 11β- hydroxylase leads to the accumulation of the intermediate precursors of cortisol and corticosterone (11-deoxycortisol and 11-deoxycortocosterone). 11-deoxycorticosterone is thought to be responsible for hypertension, a symptom often a clinical feature of this type of congenital enzyme defect.

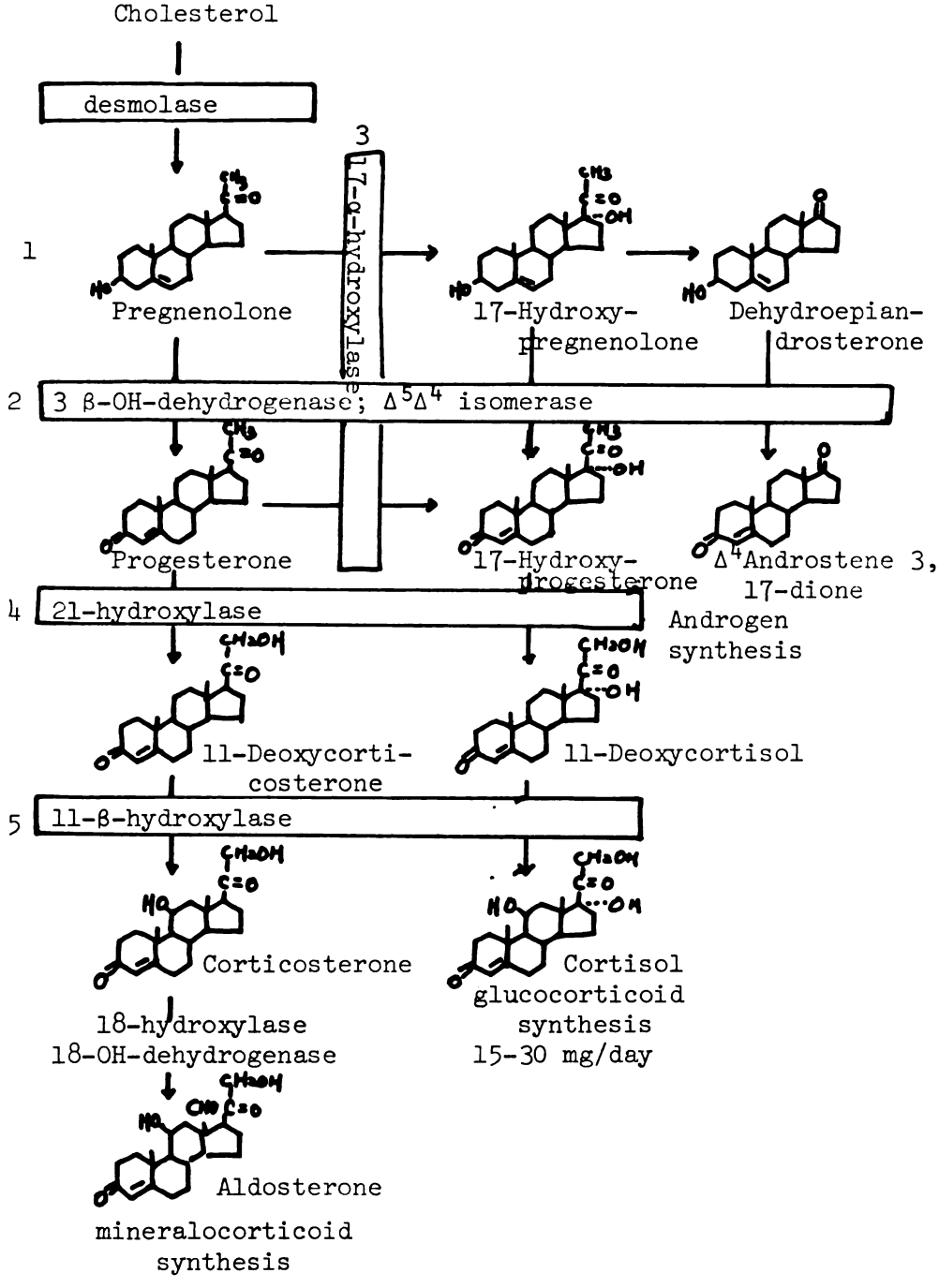
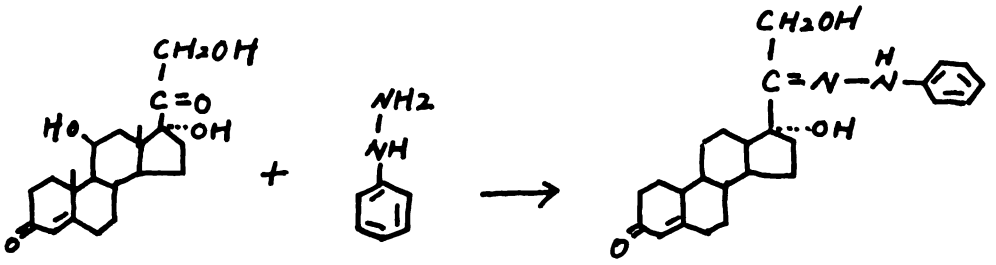


Figure 3: Five Adrenal Biosynthetic Disorders

REVIEW OF ANALYTICAL METHODS

Colorimetric Method: The Porter and Silber reaction is based on the fact that 17,21-dihydroxy-20-keto groups on steroid molecules will react with phenylhydrazine and sulfuric acid to form a yellow pigment (39).



The first successful assay of blood cortisol was based on this reaction. Initially column chromatography was utilized to isolate steroid molecules prior to analysis, but later, Silber and Porter (40) dispensed with the need for chromatography. Their original method was further improved by Peterson et al. (41).

A number of investigators have subsequently modified the original Porter and Silber method. 1) Gold et al. (42) evaporated the extraction solvent before addition of the color reagent. 2) Waximan (43) used column chromatography to separate cortisol and 11- desoxycortisol before measuring them by the colorimetric method. 3) Hadd (44) used p- hydrazinobenzenesulfonic acid/phosphoric acid reagents instead of the original Porter-Silber reagents. This modification increased sensitivity by 2-3 fold.

The Porter and Silber reaction is not specific for cortisol. It is interferred with by biologically inactive metabolites such as tetrahydrocortisone and tetrahydrocortisol, and also by synthetic corticosteroid such as prednisolone (42). However, 21-desoxycortisol, an interferring compound in the fluorimetric and competitive binding assays, is not measured by the

colorimetric method (42). Therefore, the Porter-Silber method will not overestimate cortisol in patients with a deficiency of 21-hydroxylase.

Fluorimetric Method:

The original fluorimetric method of De Moor et al. (45) was further modified by Mattingly (46). The procedure involves extraction of plasma by an organic solvent, followed by reaction with a fluorescence-inducing reagent. The method will measure both cortisol and corticosterone. Most corticosteroids such as tetrahydrocortisol, 11-deoxycortisol, and cortison do not fluoresce significantly. Drugs such as Spironolactone, Mepacrin, Fucidic acid and Carbamazepine will give falsely high results (47). Nonsteroidal impurities present in the organic solvent, NaOH solution, and fluorescene reagent may give a fluorescence background which may elevate cortisol levels to a significant extent (48,49).

The specificity of the original method was improved in several ways:

1. Incorporating paper or silica gel chromatography to separate cortisol and corticosterone (50-53).
2. Washing the methylene chloride extract with NaOH, or applying a phase-separation filter to eliminate impurities from the sample (52-54).
3. Forming an oxime derivative of cortisol and corticosterone to reduce the fluorescence background of plasma (55).
4. A selective cleavage by metaperiodate of 20, 21α-ketols to 17-carboxylic acids and formaldehyde to exclude some endogenous steroids (56,57).

A comparison between the commonly used fluorimetric and colorimetric techniques is shown in Table 1. The fluorimetric method is more sensitive, faster and can be used to estimate cortisol in the presence of other 17-hydroxycorticosteroids. On the other hand, the colorimetric method can be used

for the estimation of plasma 11-deoxycortisol in the metyrapone test.

Gas Chromatography:

Gas-liquid chromatography (GLC) is a widely used technique for steroid separation. Steroids are decomposed into 17-oxo steroids in the GC column (58). Preoxidizing the side chain of steroids before introduction into GC gives similar results (59). However, in order to obtain better resolution and greater thermal stability, steroids were derivatized before they were

TABLE 1

Comparison of the fluorimetric and colorimetric methods the estimation of plasma cortisol

	Fluorimetric (plasma 11-hydroxycorticosteroids)	Colorimetric (plasma 17-hydroxycorticosteroids)
Sample volume	1-2 ml	At least 5 ml
Working time	Six samples in 1-2 hours	Six samples in 3-4 hours and overnight color development.
Specificity	Less interference with drugs but variable amount of non-specific fluorescence in plasma.	Drugs interfere, especially paraldehyde. Probably less non-specific color from plasma only.
Steroids estimated	Cortisol and corticosterone. Not cortisone, 11-deoxycortisol or synthetic glucocorticoids.	17,21 dihydroxy-20-oxo steroids, e.g., cortisol, cortisone, 11-deoxycortisol. Not corticosterone.

chromatographed. Gardiner (60) converted steroidal ketones into O-methyloximes and then reacted them with hexamethyl disilazane to convert them into trimethylsilyl ethers. Both the 20-oxo and 21-hydroxyl groups were protected by forming these derivatives. Dimethylmethoxychlosilane and 1,1,3,3,-tetramethyldisilazane were used to form cyclic derivatives to protect the 17α,21-dihydroxy-20-oxo side chain of cortisol (61). Capillary columns were used to achieve higher resolution (62). Even then the steroids containing an α-ketol

side chains, such as cortisol, corticosterone, aldosterone, and their metabolites, decomposed at the higher temperatures necessary for optimum GC conditions.

Gas chromatography and mass spectroscopy was first utilized by Bjorkenem (63). He used radioisotope-labeled cortisol as an internal standard. Cortisol levels were determined by calculating the ratios at m/e 605 and m/e 607. This method is very accurate and specific, but the cost and complexity of the instrumentation limits its usefulness at this time.

Competitive Protein Binding Assay:

The principle of the competitive protein binding assay (CPB) has been widely applied for hormone assays since Yallow and Berson (64) first introduced radioimmunoassay to measure plasma insulin. A method for cortisol measurement using this technique was developed by Murphy (65-67). CPB method can be divided into three parts: 1) deproteinization, 2) competitive binding, 3) separation. Binding proteins in the sample are destroyed by deproteinization. Heat, alcohol, and methylene chloride can be used for this purpose. Then a fixed amount of tracer and cortisol binding globulin (CBG) are allowed to mix with the sample. A competitive binding occurs between tracer and sample steroid for the binding sites on CBG. Protein-bound and unbound steroids are separated and counted. A standard curve is obtained by plotting percentage bound fractions against known concentration of cortisol, and the amount in the unknown sample is then calculated from the curve. Several modifications have been introduced to increase the specificity (68,69) and sensitivity of the method (70). The original method was also simplified by several investigators (71,72).

The advantages of the competitive protein binding assay over fluorimetric methods are a) the smaller volume of plasma required, b) its greater sensitivity and c) relatively better specificity. However, there is still the problem of

cross-reactivity. CPB is interferred with by corticosterone, 11-deoxycortisol, cortisone, progesterone, androstenediol, prednisolone and prednisone. This method should not be used after metyrapone administration or for patients with congenital adrenal hyperplasia because under these conditions CPB will give erroneous results. It is evident that the method is not specific for cortisol and in cases where this is important, more specific methods should be used.

Radioimmunoassay:

The most essential feature of a useful cortisol radioimmunoassay is the production of a specific antibody. Cortisol by itself is not an immunogen because of its non-protein property and low molecular weight. A cortisol protein conjugate is required to stimulate immunological response in experimental animals. Pioneering work in the area of synthesizing steroid-protein conjugates was done by Erlanger et al. (73). They prepared steroid-protein conjugates by reacting steroids with bovine serum albumin. Cortisol-21-hemissuccinyl-bovine serum albumin has proven to be a favorable immunogen (74-77) for the production of antibodies in rabbits (78). However, these antibodies were found to cross-react with 11-deoxycortisol, corticosterone and 17α-hydroxyprogesterone. A chromatographic step was required to eliminate these interferences. Fahmy et al. (79) synthesized cortisol-3-(O-carboxymethyl) oximino-BSA which has the entire D ring intact with the C-17 side chain on the molecule to exert maximal influence as an antigenic determinant. Antibody produced against this antigen is much more specific than the one produced by cortisol-21-hemisuccinyl-bovine serum albumin. The chromatographic step can be eliminated without sacrificing the specificity of the assay.

A solvent extraction step is also required in most of the radioimmunoassays because corticosteroid binding globulin present in plasma will compete

with antibody for cortisol binding (77,80-85). However, Jiang et al. (86) reported similar results with and without an extraction step incorporated in the method, except in specimens with high corticosterone or 11-deoxycortisol concentrations.

Tritiated cortisol was used as a tracer in earlier radioimmunoassay methods. This was later substituted for by a gamma-emitting iodinated cortisol. ^{125}I-cortisol is easy to count, has high specific activity, and the assay can be automated quite readily. A 3-carboxymethylomine tyrosine methyl ester derivative labeled with iodine-125 has been used as a tracer (84), however, the antibody has different affinity for iodinated cortisol and unlabeled cortisol. Other modifications include double antibody techniques (87) and a coated tube method (88).

In conclusion, radioimmunoassay is a more sensitive, specific, and accurate method than the competitive protein binding or fluorimetric methods. It is also easier than gas chromatography. It suffers from a number of drawbacks such as the cross-reactivity of antibodies used, the relatively long time for analysis, the hazard of handling radioactive material and the expensive cost of the reagents used in the assay. Therefore, a new method that can overcome these problems will be a welcome addition to the existing methods of cortisol analysis.

High Performance Liquid Chromatography (HPLC):

Liquid chromatographic separation is based on the adsorption or partition of solute molecules between a stationary phase and a mobile phase. Thin-layer, paper, ion-exchange, and exclusion chromatography had been used for a long time, but these chromatographic techniques suffered from poor efficiency. However, with the introduction of efficient delivery systems for mobile phases, on-line detectors, and efficient columns, liquid chromatography is now experiencing a renaissance. High performance liquid

chromatography possessed efficiency, sensitivity, and specificity equivalent to that of gas liquid chromatography. Moreover, HPLC is ideally suited for a large number of biomolecules which are thermally unstable or non-volatile and therefore unsuitable for analysis by GLC. There are four basic types of liquid chromatographic separations:

1. Liquid-liquid or partition chromatography: Liquid-liquid chromatography can be either normal phase (polar stationary phase and non-polar mobile phase) or reverse phase (non-polar stationary phase and polar mobile phase). Normal phase is used to separate polar compounds, and reverse phase is used to separate non-polar compounds. Separation is based on the relative solubility of the compounds of interest in the two liquid phases according to their partition coefficient (k). The stationary phase may be either coated onto a support or chemically bonded to that support. The most difficult separations, involving closely related compounds, can be achieved utilizing this method due to its high selectivity and efficiency.
2. Liquid-solid or adsorption chromatography: This technique is based on interaction between the solute and fixed active sites on a finely divided solid adsorbent used as the stationary phase. The most widely used adsorbent, silica gel, has silanol (also called surface hydroxyl) groups which show varying degrees of acidity. Silica gel causes stronger retention of basic compounds, such as amines. Alumina is a basic adsorbent, owing to the presence of oxide ions. It preferentially retains acidic compounds, such as carboxylic acids and phenols. Liquid-solid chromatography is very widely used because of its broad applicability and low cost. It is similar to thin-layer chromatography (TLC) in many

respects. TLC separations can readily be adapted to HPLC.

3. Ion exchange chromatography: In this technique the stationary phase is an ion exchanger which is an ion-bearing resin material. These ions can be exchanged for an equivalent number of other ions of the same change provided by the eluting buffer. Ion exchange chromatography is thus particularly adapted to the analysis of ionized or ionizable compounds. Many biological components cannot be separated by gas chromatography because they are not volatile enough and/or they are thermolabile. Since compounds such as amino acids, nucleic acids and peptides are water-soluble and easily ionized by modifying pH, they are well suited to separation by ion exchange chromatography. Ion exchange resins bearing negatively charged groups are used for exchanging cationic species. Ion-exchange resins bearing positively charged groups are used for an ion exchange chromatography. The most commonly used functional groups for ion exchange chromatography are sulphonates (SO_3-) for cation exchange and quaternary amines for an ion exchange chromatography.

Sulphonate ion exchange resins are strong cationic exchangers. Similarly, quaternary ammonium is a strong anionic exchanger. When present in the OH- form their properties are similar to those of a strong base. These quarternary salts are completely dissociated and hence their exchange properties are not dependent on pH conditions. There are some exchange groups which possess weak acidic or basic properties, like a carboxylate ($-COO^-$) bearing resin which permits the exchange of cations only in acidic medium. Similarly tertiary amines($-NR_2$) bearing resins impart ion exchanging properties only in an acidic medium. The exchange capacity of

these weak acidic or basic exchangers depends upon pH.

4. Exclusion or gel filtration chromatography: Gel filtration chromatography separates solutes according to their molecular size and weight. Molecules which are smaller than the average pore size of the packing are retained while larger molecules are excluded from the pores. The major drawback of exclusion chromatography is its inability to separate compounds of the same molecular weight.

The basic components of a HPLC are as follows:

1. Pumping systems: There are two basic types of pumping systems available for HPLC, constant pressure pumps and volume pumps. Constant pressure pumps are not as accurate as constant volume pumps, but can be used where flow accuracy and reproducibility are less critical. The two types of constant volume pumps used in HPLC systems are continuous displacement (syringe type) pumps, and reciprocating pumps. The advantage of syringe type pumps is that it can provide a pulse-free flow. The major disadvantage is the finite solvent capacity. The advantage of reciprocating pump is the continuous solvent delivery. The major disadvantage is the generation of pulses during the solvent delivery. This disadvantage has been overcome in presentday pumps.

2. Injection systems: The three types of the injectors used for sample introduction are on-column syringe injectors, valve-loop injectors and syringe-loop injectors. On-column syringe injectors have the advantage of minimal band spreading with good efficiency, but they cannot be used at high pressures (over 2000 p.s.i.). Valve-loop injectors can be operated at pressure up to 5000 p.s.i. They can also be automated easily. The main disadvantage is the waste of

sample in loading. Syringe-loop injectors, which combine the flexibility of syringe injection with the use of a valve, are the most widely used injectors at present.

3. Columns: Column packings for HPLC fall into two categories - porous layer beads (PLB) are a solid core of glass bead surrounded by a thin porous outer shell of silica, alumina, or ion exchange groups. In the late 1960's and early 1970's PLB were the standard packings. However, with the production of the microparticles and the development of better packing techniques, microparticulate packings have now displaced the PLB in popularity. Compared to PLB, they offer better column efficiency, sample capacity, and speed of analysis.

Hydrocarbonaceous bonded phases are obtained by reacting silica gel with an alkyl-trichlorosilane. As a result of this reaction the hydrocarbonaceous moiety is covalently bound to the silica surface. The following equation shows a typical chlorosilane bonding reaction with a silanol group on the silica gel surface.

$$\equiv SiOH + Cl\,Si(CH_3)_2R \rightarrow \equiv SiO-Si(CH_3)_2R + HCl$$

A C_{18} alkyl chain is the most widely used packings for reverse phase columns. Similarly, other bonded phases with C_2, C_8, and phenyl hydrocarbonaceous moieties have been also introduced. A cyano (C_2H_4CN) phase can also be used as a nonpolar stationary phases under certain conditions. The longer the bonded alkyl chain, the greater the solute retention. In addition, they usually yield more stable columns. The alkyl chain length and the carbon content may also affect the selectivity of the stationary phase. In stationary phases containing short chains, e.g., C_2 or C_8-alkyl functions, the concentration of accessible silanol groups is probably greater than in C_{18}

phases having comparable surface coverage. Consequently, short chain phases have relatively high column efficiency. Phenyl phases can also be used in reverse phase liquid chromatography. They show lower selectivity for nonpolar compounds than do C_{18} phases. Nitrile bonded stationary phases can be used for reverse phase work if a more polar mobile phase is used. In the reverse phase mode nitrile phases show less retention than the more hydrophobic octadecylsilane functionality. Since they possess a certain degree of polarity, they can also be used in normal phase chromatography. The amino-alkyl moiety is particularly interesting in that, being basic it imparts a quite different chromatographic selectivity when compared to the acidic surface of the silica gel. In the reverse, phase mode polar compounds such as carbohydrates and peptides can be readily separated.

4. Detectors: There are two classes of detectors available in liquid chromatography: a) universal detectors which measure a property common to both the sample and the mobile phase, and b) specific detectors which measure a specific property of the solute. Examples of the first type of detectors are differential refractometers, conductivity detectors, and dielectric constant detectors. The second type of detectors include ultraviolet absorption, fluorescence, polarographic, radioactivity, and electrochemical detectors. UV detectors are, at present, the most widely used detectors in HPLC. They are, however, limited to solutes which absorb UV radiation. Since it is a specific detector, it is relatively insensitive to temperature and flow changes, and it is ideal for gradient elution. Some variable-wavelength detectors can be adapted for scanning a peak after stopping flow, resulting in a UV spectrum of the peak in question. Refractive index detectors are the most commonly used universal detectors. RI detectors

lack sensitivity, are not suitable for gradient elution, and require strict temperature control. Polarographic detectors record the current change between a polarizable electrode and a nonpolarizable electrode as a function of time. The solution must have electric conductivity and sometimes it is necessary to introduce a suitable electrolyte into the mobile phase for that purpose. Fluorescence detectors can measure several compounds such as amino acids, vitamins, steroids, and drugs (89). Fluorescent derivatives of several compounds can also be made (90,91). The fluorescence detector is fairly specific, nondestructive, quite sensitive (as high as 10^{-9} g/ml for strongly fluorescing compounds), and can be used with gradient elution. Radioactivity detectors are not widely used. Yet this detector has several advantages, such as a wide linear range, adaptability to gradient elution, and high sensitivity. The isotope contained in the effluent comes in direct contact with a scintillator which is packed in a tube. The light pulses generated by the interaction between the scintillator and the isotope can be detected by a photomultiplier tube. Conductivity detectors can detect sample in the mobile phase by changes in conductivity (92,93). Its sensitivity depends on the difference in conductivity between solvent and solute components. Therefore, this type of detector is useful mainly with aqueous elunts and ionic solutes, such as amino acids. The use of dielectric constant detectors is possible when there is a large difference in the polarity of sample and solvent. This type of detector is nondestructive, but not recommended with gradient elution, particularly if the gradient causes a change in solvent polarity.

Plasma cortisol was initially measured by Wortmann et al. (94) using HPLC. Cortisol, cortisone, and aldosterone were separated as a

group on a C_{18} reverse phase column. The column was eluted with methanol-water. However, Trefz et al. (95) showed that these compounds can also be separated by using normal phase chromatography. The extraction of cortisol was laborious, and the sensitivity of the method was poor. Van Den Berg (96) used normal phase chromatography with prednisolone as an internal standard to detect cortisol. Prednisolone is a widely prescribed synthetic steroid which will interfere with the assay and give falsely low cortisol concentrations. Recently, Reardon et al. (97) described a liquid chromatographic method for the analysis of cortisol and 11-deoxycortisol from serum samples. However, they did not report on a number of important analytical variables. Their method lacked internal standardization and a systemic interference study of drugs and synthetic steroids. No data on the sensitivity for the detection of cortisol in the proposed method was reported. They reported analytical recovery of cortisol at one concentration (100 µg/liter) only, ignoring the lower and higher concentrations widely prevalent in Addison's and Cushing's syndromes. The linearity of the method was evaluated on pure methanol cortisol standards rather than serum based standards. In addition, optimization of important chromatographic parameters (pH, and composition of the mobile phase) was not reported.

The method developed in our laboratory provides a practical application of HPLC for the measurement of plasma cortisol. It is simple and specific, and we paid special attention to the points outlined above. The method is sensitive enough to analyze 5 µg/liter of cortisol when 1 ml of serum is used, and it can be easily adapted for pediatric samples (300 µl serum).

MATERIALS AND METHODS

Chromatographic apparatus:

A model 601 (Perkin-Elmer Corp., Norwalk, Connecticut, 06856) high pressure liquid chromatograph equipped with a Model LC 15 detector (Perkin-Elmer) and a temperature controlled oven was used. The pre-packed column (μ-Bondapak C_{18}, 30 cm x 4 mm: Waters Associates, Inc., Milford, Massachusetts 07157) was mounted in the oven. A Honeywell Electric Model 194 (Honeywell, Inc., Fort Washington, Pennsylvania 19036) recorder was used. Samples were injected into a Rheodyne 7105 valve (Rheodyne, Berkeley, California 94710) mounted on the chromatograph. The column was eluted with acetonitrile/phosphate buffer (30/70 by volume) at a rate of 2.0 ml/min at 50° C, and the column effluent was monitored at 254 nm. The sensitivity of the detector was set at 0.016 absorbance units full scale.

Reagents and Standards:

Acetonitrile: Acetonitrile ("ultraviolet" grade), distilled in glass (Burwick and Jackson Laboratories, Inc., Muskegon, Michigan 49442).

Phosphate buffer (pH 3.2): was prepared by adding 300 μl of 1 mol/liter KH_2PO_4 to 1800 ml of distilled water, followed by titration with phosphoric acid to pH 3.2.

Mobile phase: This was a solution of 300 ml of acetonitrile in 700 ml of phosphate buffer.

Methylene chloride: Distilled in glass (Burdick and Jackson).

Radioimmunoassay kit: Cortisol Premix$_R$ RIA kit (^{125}I) was obtained from Diagnostic Products Corporation, Los Angeles, CA 90064.

Standards: All steroids used were purchased from Sigma Chemical Co., St. Louis, Missouri 63178. The chromatographic reference standard was prepared as follows: 4 mg of cortisol and 16 mg of equilenin were dissolved in 1 liter of methanol. This solution is stable at 4° C for at least 3 months.

The stock internal standard was prepared by dissolving 20 mg of equilenin in 1 liter of methanol. The working internal standard was prepared by diluting stock solution 10 fold with distilled water.

Procedure:

Extract 1 ml of serum or plasma containing 1 ml of working internal standard (2 µg of equilenin) with 9 ml of methylene chloride on a mechanical shaker for 15 min. Centrifuge at 2500 rpm (210 g) for 10 minutes, and transfer the organic phase into a labeled 13x100 mm glass tube. Evaporate under a stream of nitrogen gas or filtered air at 37° C. Dissolve the residue in 100 µl of methanol, and inject all of the solution into the chromatograph. Elute with the mobile phase at a flow rate of 2.0 ml/min. The column head pressure observed was about 10 MPa (1500 p.s.i.) (Figure 4 illustrates chromatogram obtained by this procedure at three different concentrations).

Pediatric samples were processed as above, except 300 µl of serum and an equal volume of working internal standard were extracted with 8 ml of methylene chloride. The detector was set at 0.004 absorbance units full scale.

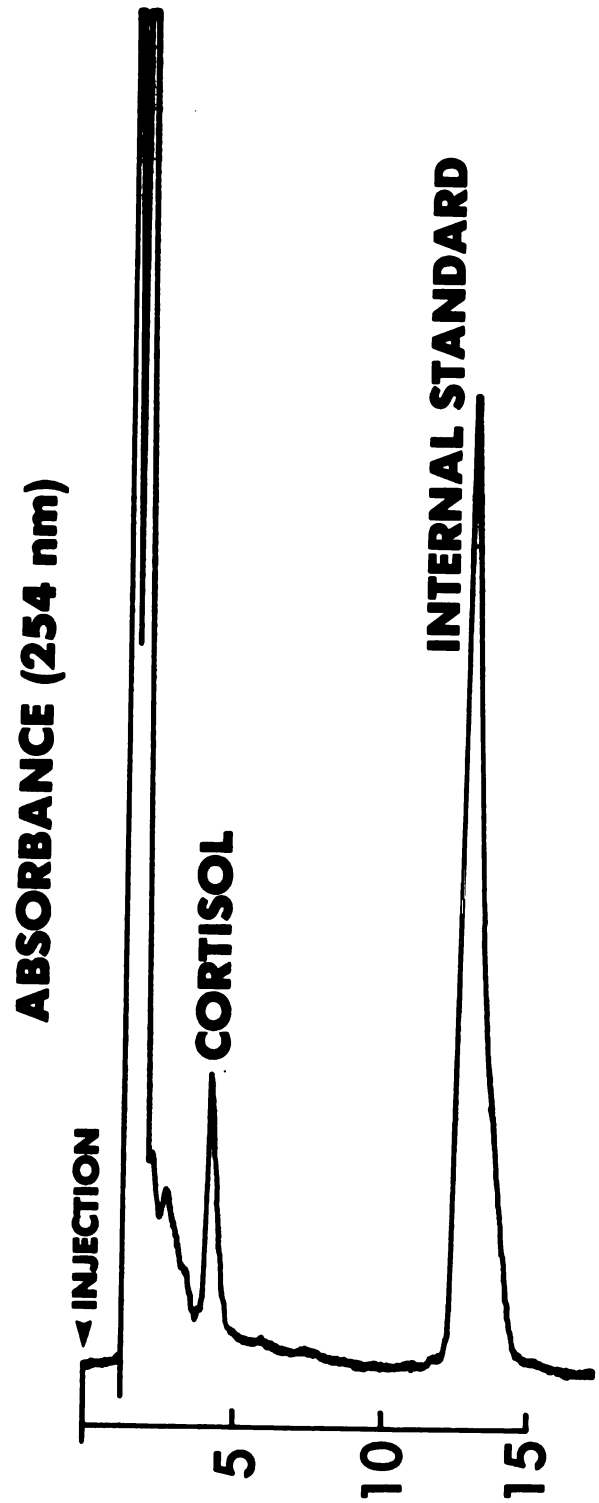


Figure 4-a: Chromatogram obtained from a patient's serum containing 78 µg/liter of cortisol.

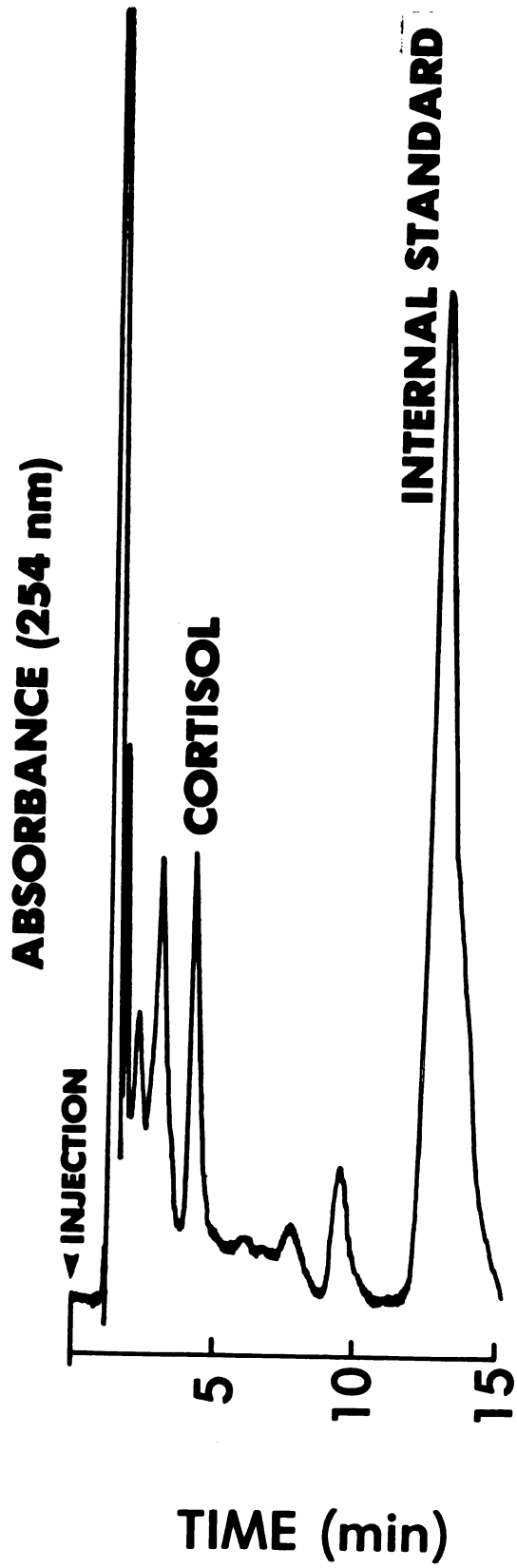


Figure 4-b: Chromatogram obtained from a patient's serum containing 112 µg/liter of cortisol. Peak eluting at 9.5 min corresponds to 11-deoxycortisol.

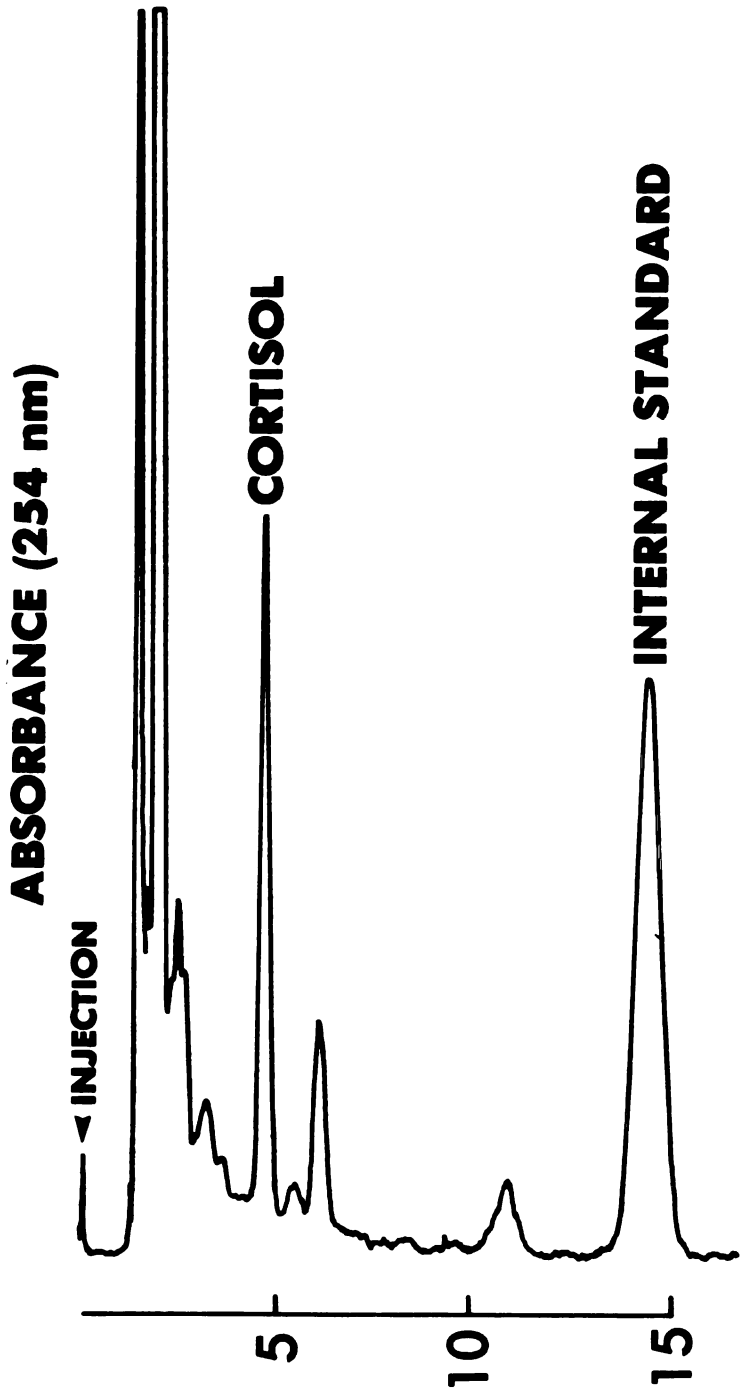


Figure 4-c: Chromatogram obtained from a patient's serum containing 360 µg/liter of cortisol.

RESULTS

Calculations and Quantitation:

200 ng of cortisol and 800 ng of internal standard (I.S.) were injected as reference standard to ascertain the adequacy of the chromatographic conditions. Since the peaks were sharp and symmetrical, the peak height measurement was sufficient for quantitation. This chromatogram (Figure 5) was used to calculate the relative retention time (RRT) and response factor (RF) as follows:

$$RRT = \frac{\text{Retention time of cortisol from the injection point}}{\text{Rention time of the I.S. from the injection point}}$$

$$RF = \frac{\text{Peak height of I.S.}}{\text{Peak height of cortisol}}$$

The RRT was used to identify cortisol in the plasma samples, and RF was used to quantitate the drugs as follows:

$$\text{ng/ml of cortisol in plasma(serum)} = \frac{\text{Peak height of cortisol} \times RF \times \text{concentration of I.S.}}{\text{Peak height of I.S.}}$$

Precision:

Precision of the assay was determined by repeated analysis of plasma samples containing different concentrations of cortisol. Analyses were performed either on the same day to determine within-day variation, or on consecutive days to assess day-to-day variation. The standard deviation (SD) was calculated by the following equation:

$$SD = \left[\frac{\Sigma X^2}{N} - \left(\frac{\Sigma X}{N}\right)^2\right]^{\frac{1}{2}}$$

X= values in ng/ml

N= # of values

The coefficient of variation (CV) was calculated as follows:

$$CV\% = \frac{SD}{\bar{X}}$$

$\bar{X}$= mean

Table 2 illustrates the results obtained in this study. Within-day precision at three different cortisol concentrations was <4%. Day-to-day precision was

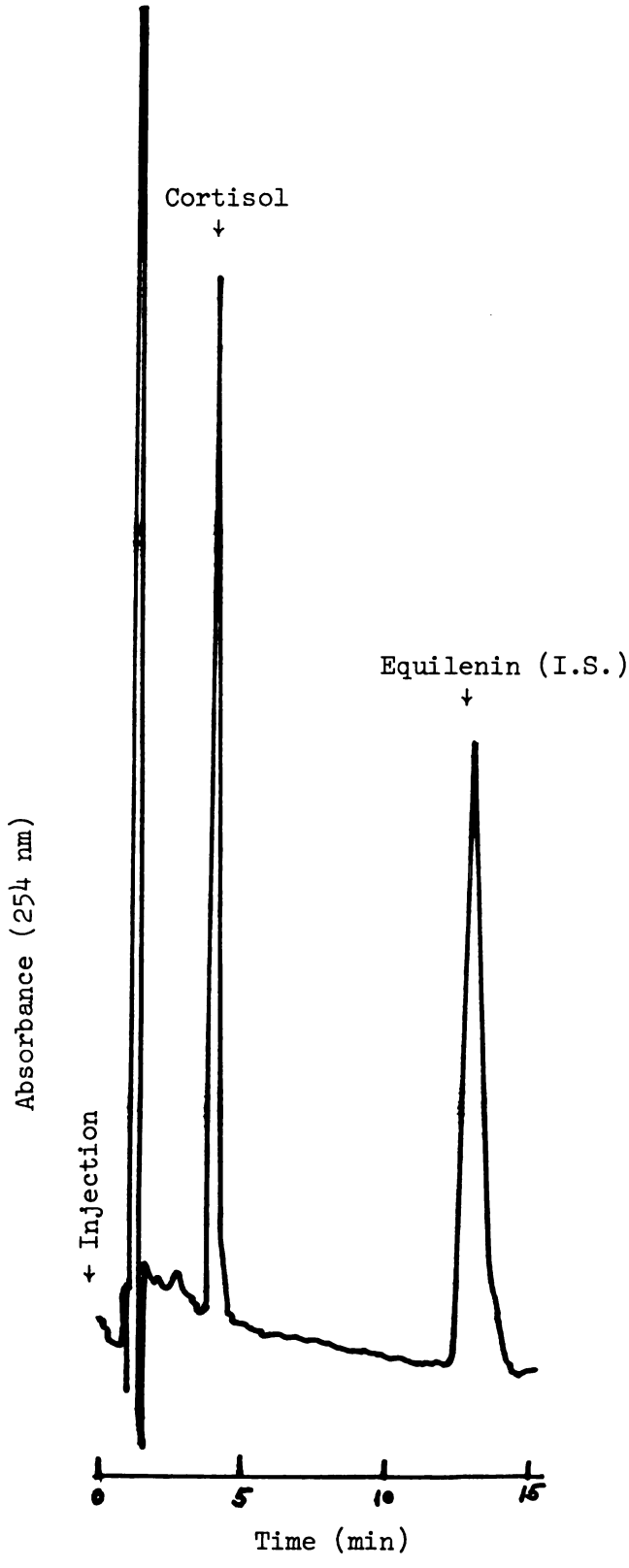


Figure 5: Chromatogram obtained from a standard reference mixture of 0.32 µg cortisol and 1.280 µg Equilenin.

≤7%. The higher day-to-day CV was expected since more systematic and random errors were introduced over a period of several days.

Analytical Recovery:

Known amounts of cortisol in methanol were added to a normal serum pool to achieve the concentrations shown in Table 3. A constant amount of internal standard was added to each sample and processed as described. Analytical recoveries are tabulated in Table 3. Analytical recoveries exceeded 95% over the entire range.

The absolute recovery of cortisol from serum was evaluated in the following manner. Cortisol was added to serum to achieve concentrations of 60, 80, 200, and 300 µg/liter. These samples were then analyzed by our procedure without adding any internal standard. Carefully measured aliquots of the extract were then chromatographed and peak height determined. Absolute recovery was calculated by comparing these peak heights with peak height obtained by the direct injection of pure cortisol standard. Absolute recovery of cortisol ranged from 70-76% and absolute recovery of internal standard equilenin ranged from 72-75%.

Linearity:

Cortisol was added to a serum pool in amounts equivalent to 80 µg to 720 µg/liter. A constant amount of internal standard was added to each sample, which was then processed as described. Concentrations and peak-height ratios were linearly related over this entire range (Figure 6).

Sensitivity:

Cortisol is detected and can be measured at a minimum concentration of 5 µg/liter when 1 ml of plasma or serum sample is extracted. 2 ng of cortisol standard could be detected at a signal to noise ratio of 5 by monitoring at 0.002 absorbance full scale.

TABLE 2

Precision of Assays for Cortisol in Serum[a]

Within days		Day-to-day	
Range + SD µg/liter	CV, %	Range + SD µg/liter	CV,%
76.1 + 2.5	3.3	81.0 + 5.7	7.0
178.8 + 6.7	3.7	178.4 + 7.2	4.0
369.2 + 9.2	2.5	354.2 + 22.9	6.5

[a] n = 10 in each case for within-day, and day-to-day.

TABLE 3

Analytical Recovery of Cortisol from Serum

Added µg/liter	Recovered	Recovery,%
25	27.5	110
50	51.6	103
100	99.6	100
200	193.1	97
350	363.1	104
500	488.4	98

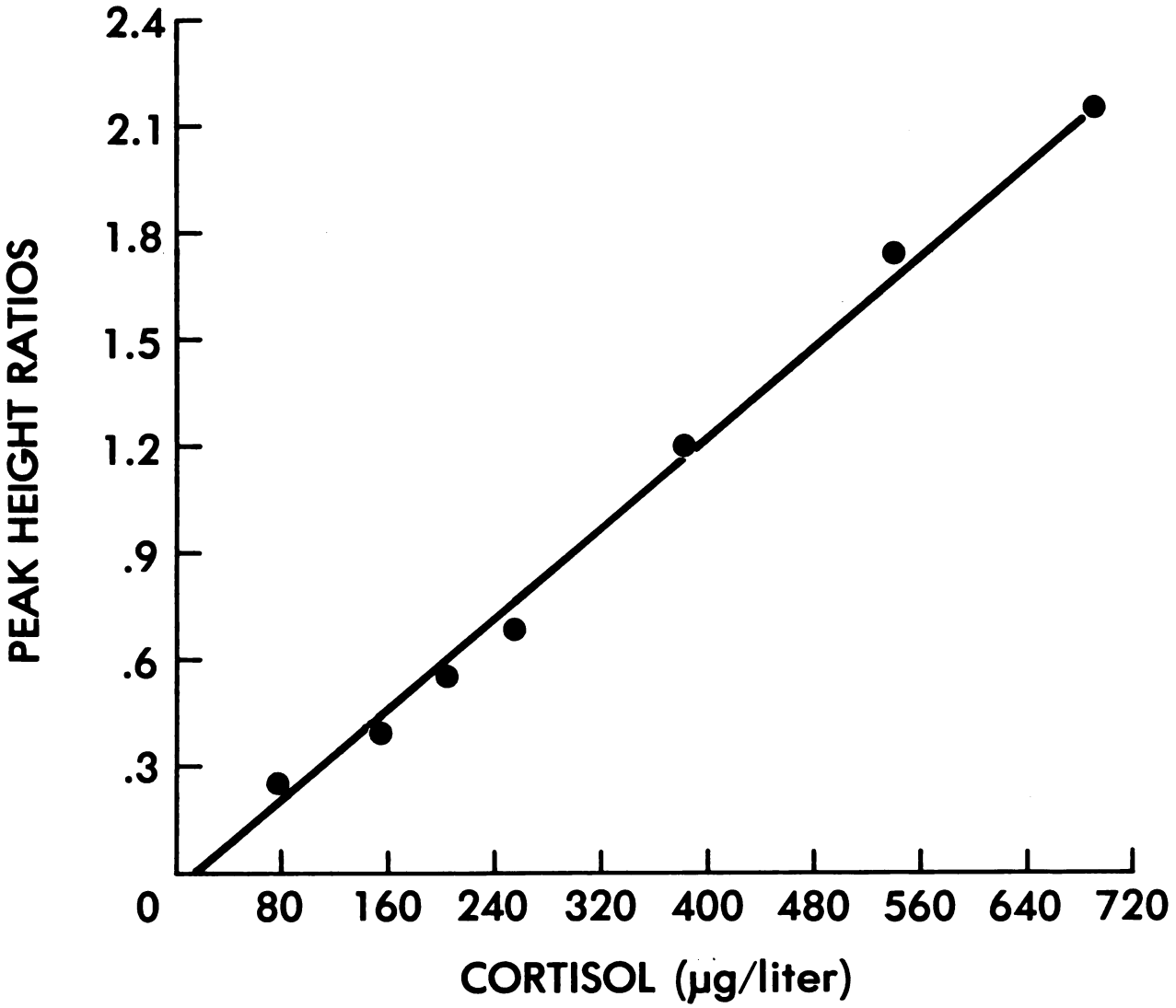


Figure 6: Peak-height ratios (cortisol/internal standard) plotted vs. concentration of cortisol.

Analysis of clinical samples:

Twenty eight serum samples were analyzed by the present method and by RIA. RIA analysis was performed in our laboratory using a cortisol Premix RIA kit (^{125}I) purchased from Diagnostic Products Corporation. A correlation coefficient of 0.966 was obtained between the proposed HPLC and RIA method, with a slope of 0.904 and Y-intercept of 2.51 µg/liter using the samples obtained from our laboratory (Figure 7). Samples obtained from Bio-Science Laboratory were analyzed by the HPLC method and compared with the results obtained from Bio-Science Laboratory (RIA method). A correlation coefficient of 0.912 was obtained between the two methods. This poorer correlation was possibly due to several factors. That samples were collected over a period of several weeks, and that some of them appeared to have been contaminated with mold and/or bacteria.

Interference:

Potential interference by other drugs and steroids was investigated by chromatographing each drug and steroid individually. Any drug or steroid that eluted sufficiently close to cortisol or the internal standard was further studied by adding known amounts of the interfering steroid or drug to a plasma cortisol standard and evaluating the quantitative effect on cortisol. Table 4 and 5 list retention times for the various steroids and drugs studied. Of the 51 steroids and drugs chromatographed so far, only synthetic derivatives such as prednisone and prednisolone eluted close to cortisol. These two synthetic steroids were not completely resolved from cortisol and hence could give false high results if present in the sample. However, these interfering steroids could be readily recognized from the chromatogram. The pH's of the mobile phase was varied to eliminate the interference by prednisone and prednisolone. Figure 8 illustrates chromatograms obtained at pH 2.8 and pH 3.2, Prednisone and prednisolone still had similar resolution at both pHs. These

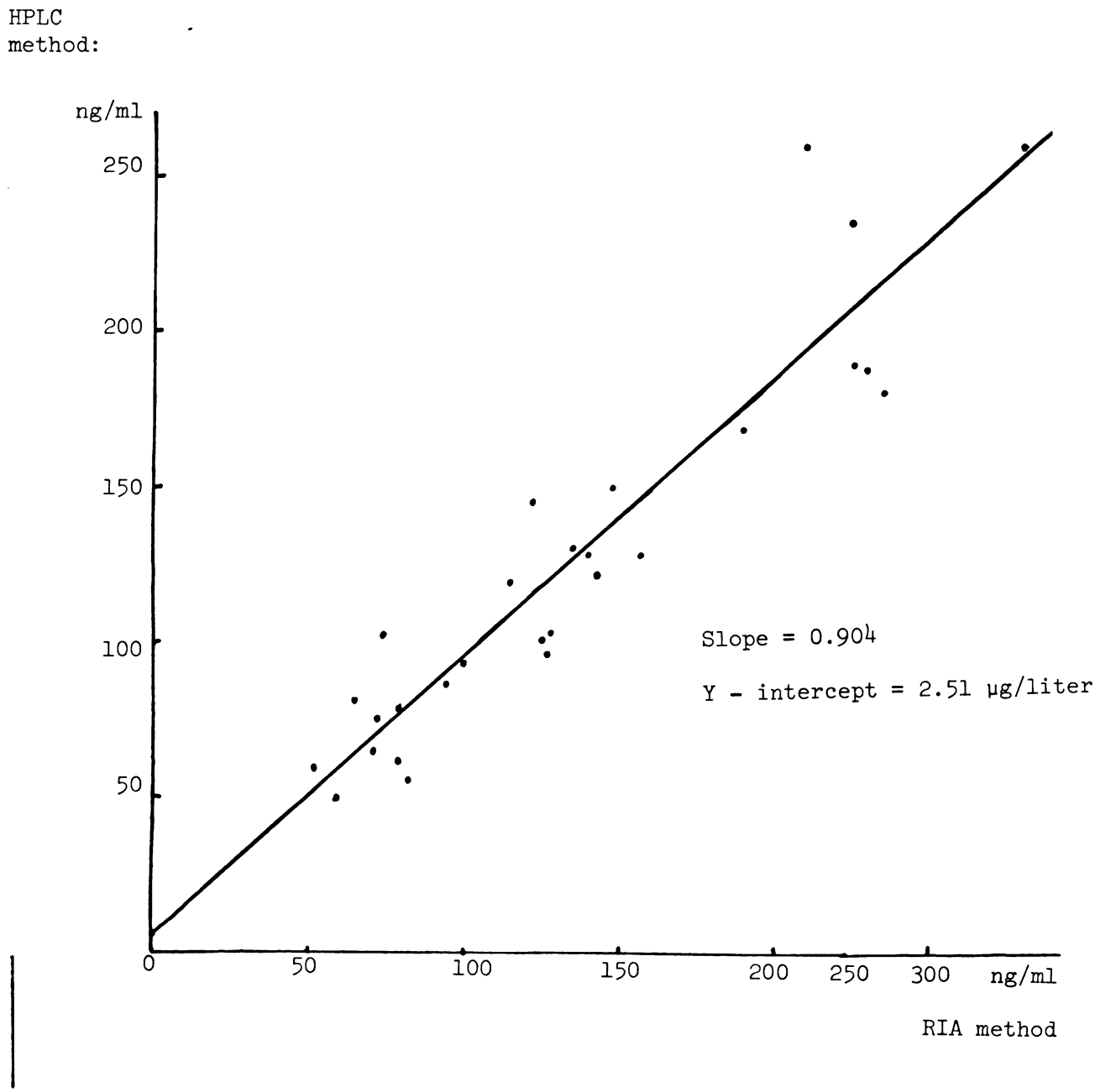


Figure 7: Comparison between RIA and HPLC method (n = 28).

TABLE 4

Retention Times for some Steroids

Steroids	Retention Time Min.
Cortisol	4.5
Equilenin	14.4
Corticosterone	8.4
Prednisone	4.3
Prednisolone	4.3
Deoxycorticosterone	18.5
11-Dehydrocorticosterone	7.5
Testesterone	18.6
Androsterone	9.1
17 α-Hydroxyprogesterone	22.8
19-Nortestosterone	12.7
11-Deoxycortisol	9.2
Δ^4-Androsten-3-17-dione	19.1
Aldosterone	3.6
11-Deoxycorticosterone	6.4
Tetrahydrocortisone	N.D.[a]
α-Cortolone	N.D.
β-Cortol	N.D.
11β -Hydroxyandrostrone	N.D.
5β -Pregnan-3 α. 17α 21-triol-20-one	N.D.
Estrone	N.D.
Estriol	N.D.
Estradiol	N.D.
Progesterone	N.D.

[a]N.D., not detectable

TABLE 5

Retention Times for some Drugs

(Retention time)

Drugs	Min.
Primidone	1.3
Salicylic Acid	2.6
Acetaminophen	1.7
Theophylline	1.6
Caffeine	1.8
Codeine	2.0
Phenobarbital	2.9
Phenytoin	5.4
Carbamazepine	5.7
Quinidine	3.0
Pentobartital	5.4
Amobarbital	5.4
Meperidine	2.7
Methaqualone	8.4
Diazepam	17.0
N-desmethyldiazepam	10.3
Glutethimide	6.0
Chlordiazepoxide	5.3
Amitriptyline	11.5
Nortriptyline	8.4
Imipramine	11.0
Desipramine	11.0
Haloperidol	8.4
Amphetamine	1.9
Propoxyphene	20
Methadone	20

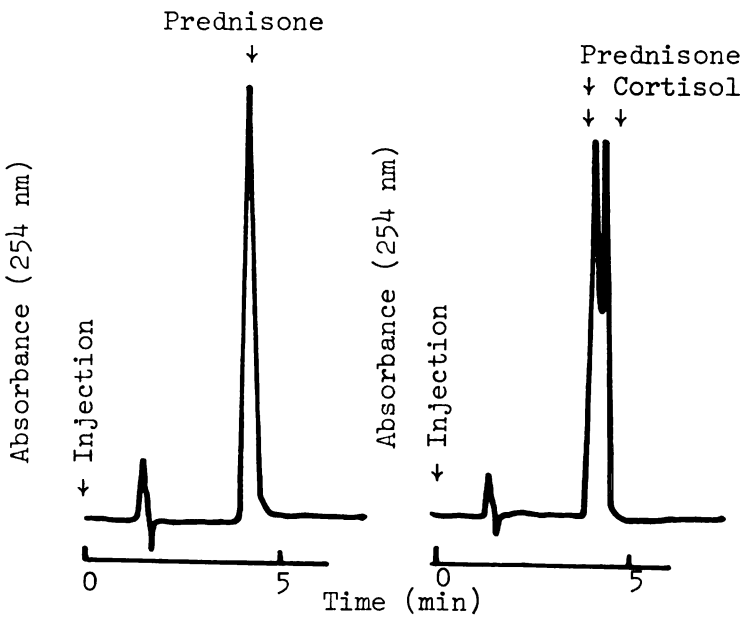


Figure 8a: Chromatogram showing prednisone interference with cortisol at pH 3.2.

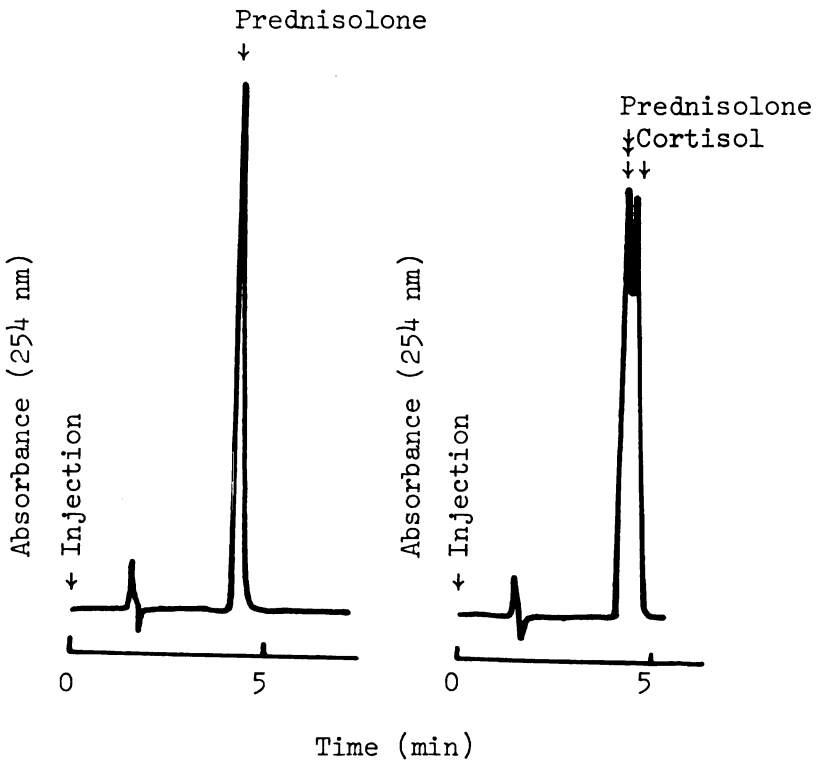


Figure 8b: Chromatogram showing prednisolone interference with cortisol at pH 3.2.

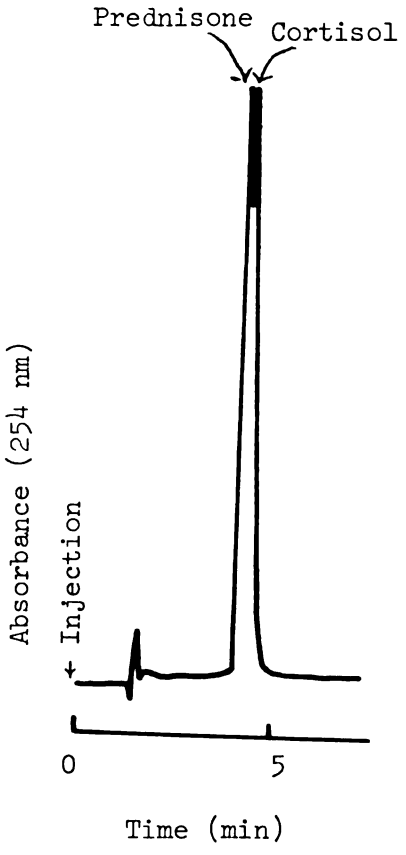


Figure 8c: Chromatogram showing prednisone interference with cortisol at pH 2.8.

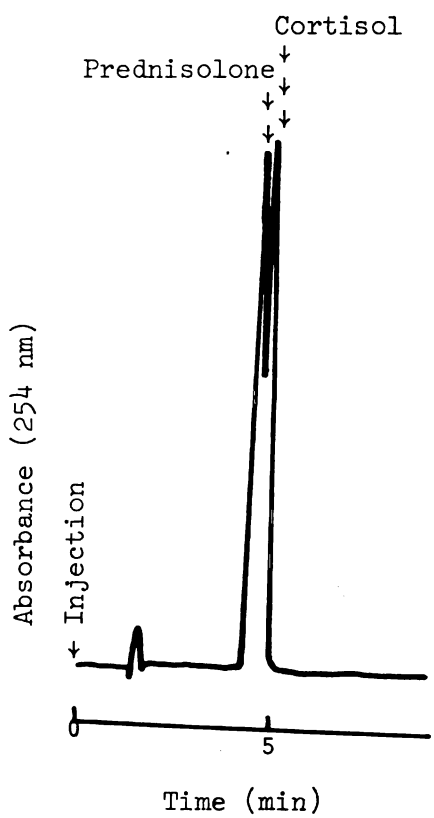


Figure 8d: Chromatogram showing prednisolone interference with cortisol at pH 2.8.

DISCUSSION

Various chromatographic conditions were evaluated by injecting 200 ng of cortisol in 50 µl of methanol. The composition of the mobile phase, and the pH of the mobile phase were varied to ascertain optimum chromatographic conditions (Figure 4).

Mobile phase variation included various ratio of acetonitrile/phosphate buffer, namely 50/50, 45/55, 40/60, and 35/65 parts by volume. Elution order of cortisol and internal standard was unaffected, but cortisol eluted too close to the solvent front when concentrations of acetonitrile exceeded 40 parts by volume, and it could not be adequately separated from normal serum constitutions. A mixture of acetonitrile/phosphate buffer (30/70 by volume) gave the desired separation in 15 minutes. Although adequate separation was achieved with lower acetonitrile concentrations, these concentrations resulted in long chromatographic times. Therefore, a composition of 30/70 volume was selected as a compromise which provided adequate resolution and a reasonable chromatographic time.

The effect of pH was most marked for some of the plasma or serum constituents. These plasma constituents coeluted and interfered with cortisol between pH 4 and 5 (Figure 9). However, when pH was lowered to 3.2 the interfering peaks eluted close to the solvent front and did not interfere with cortisol.

254 nm was selected as the wavelength for detection, a wavelength readily accessible with fixed wavelength detector. When cortisol was scanned in the mobile phase, a maximum absorbance from 210-265 nm was obtained.

No significant difference in cortisol concentration was observed when using either heparinized plasma or serum. However, oxalated plasma gave significantly lower cortisol levels. Figure 10 shows cortisol concentrations of heparinized plasma, oxalated plasma, and serum from the same patient. The results were 80 ng/ml for heparinized plasma, 88 ng/ml for serum and 40 ng/ml

for oxalated plasma.

Equilenin, a synthetic steroid, was selected as the internal standard because it was extracted in dichloromethane, and was eluted in less than 15 min. Moreover, no interference was observed at the place where equilenin eluted. Prednisolone and prednisone (synthetic steroids) were used as internal standard by several investigators, but these are routinely prescribed drugs and may interfere with the analysis.

Various extraction procedures have been tried, isooctane and NaOH washing steps were incorporated in several reported methods. These steps were necessary to eliminate several interfering compounds. But, as illustrated in Figure 11, isooctane and NaOH washes were not necessary for the present method. On the other hand, equilenin (internal standard) was soluble in NaOH, and was not extracted by methylene chloride. Therefore, no washing steps were incorporated in the present method.

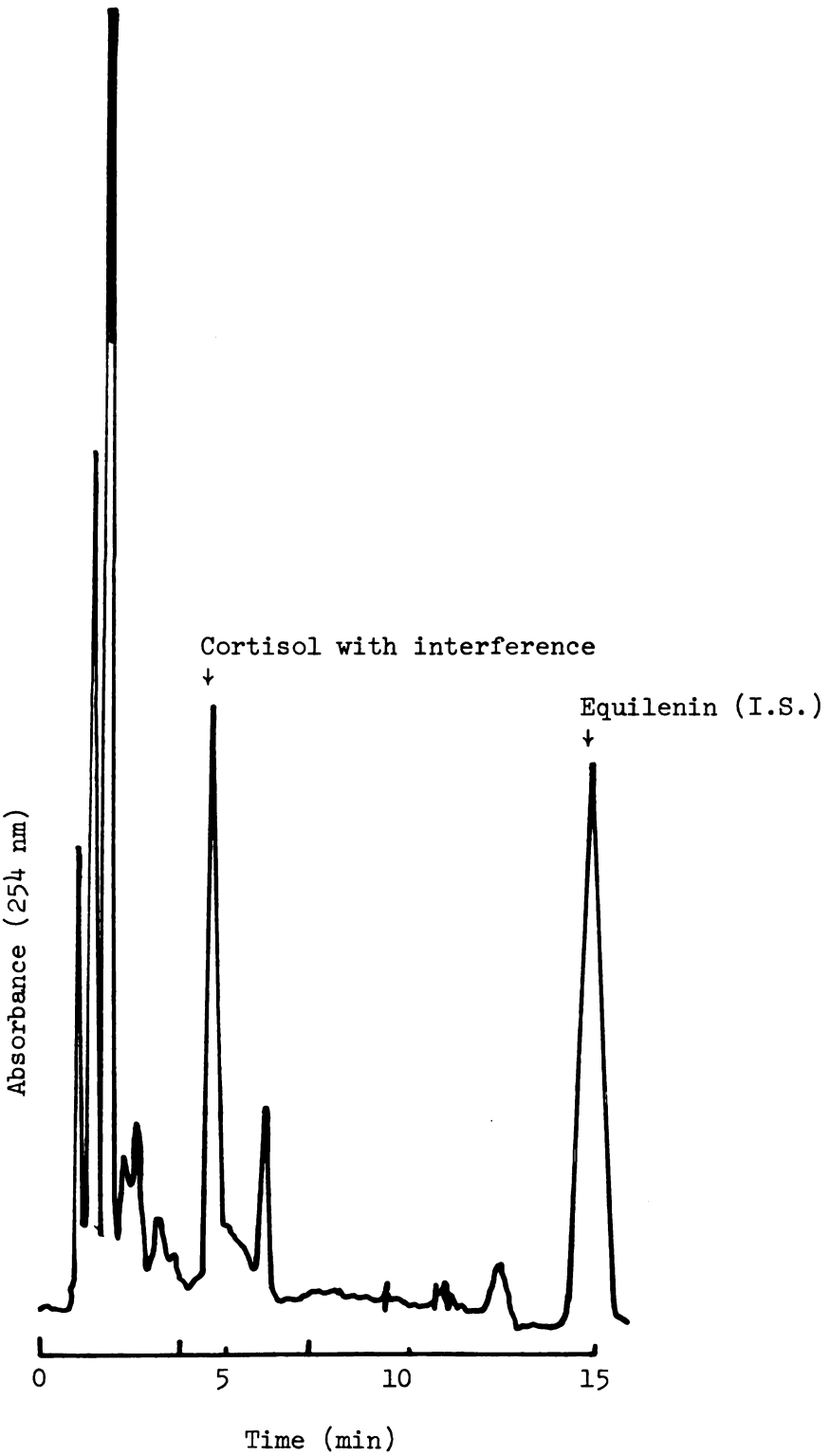


Figure 9: Chromatogram obtained from a patient's serum containing 78 µg/liter of cortisol at pH 4.4 showing interference.

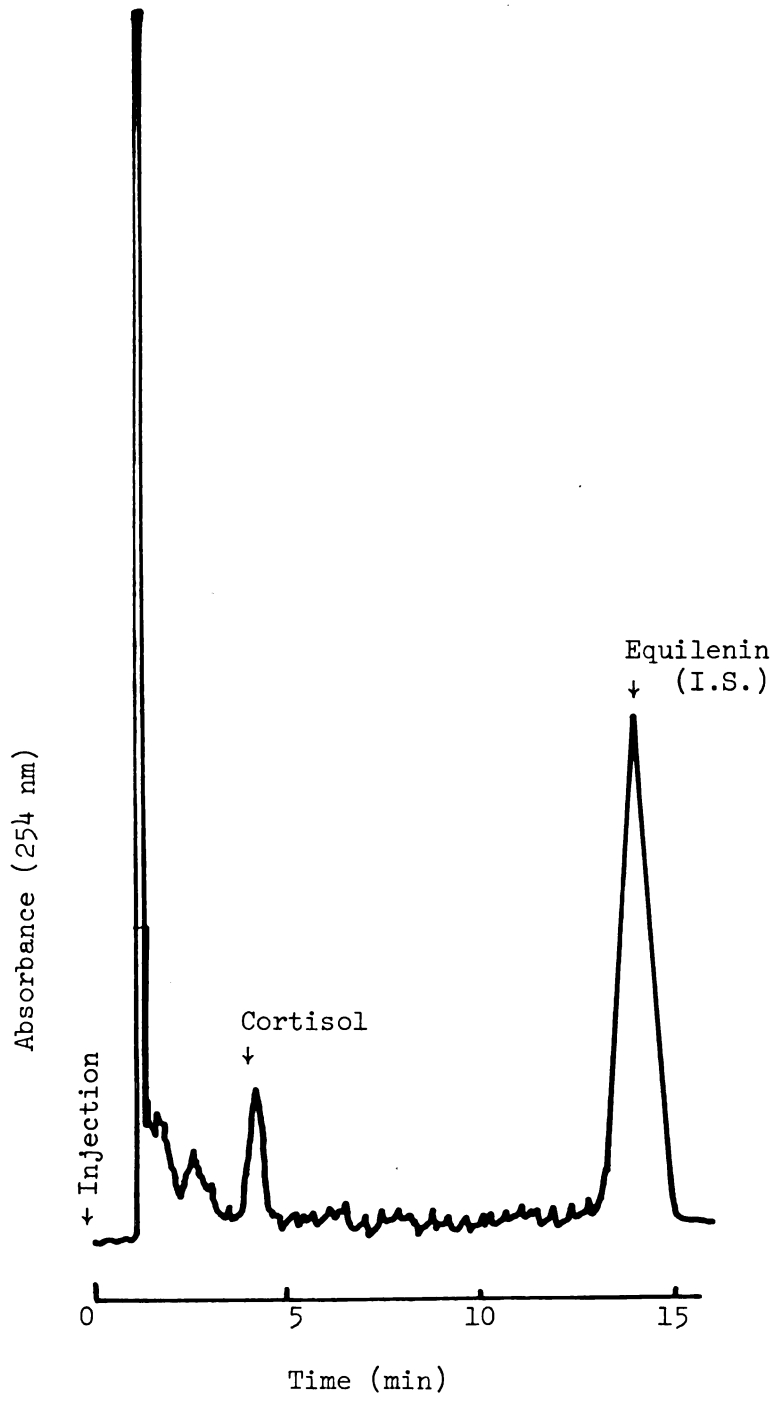


Figure 10a: Chromatogram obtained from a patient's serum containing 88 ng/ml of cortisol.

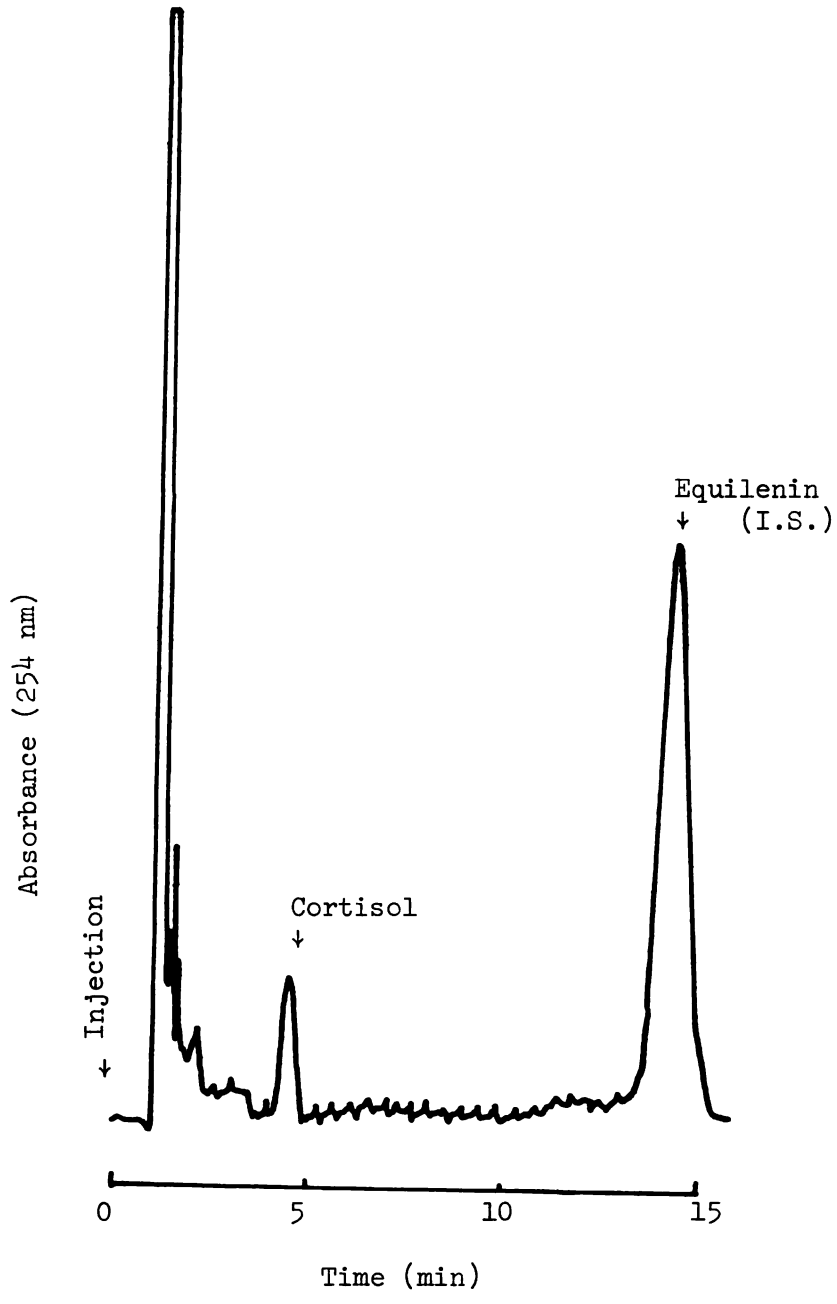


Figure 10b: Chromatogram obtained from the same patient plasma (heparinized) containing 80 ng/ml of cortisol.

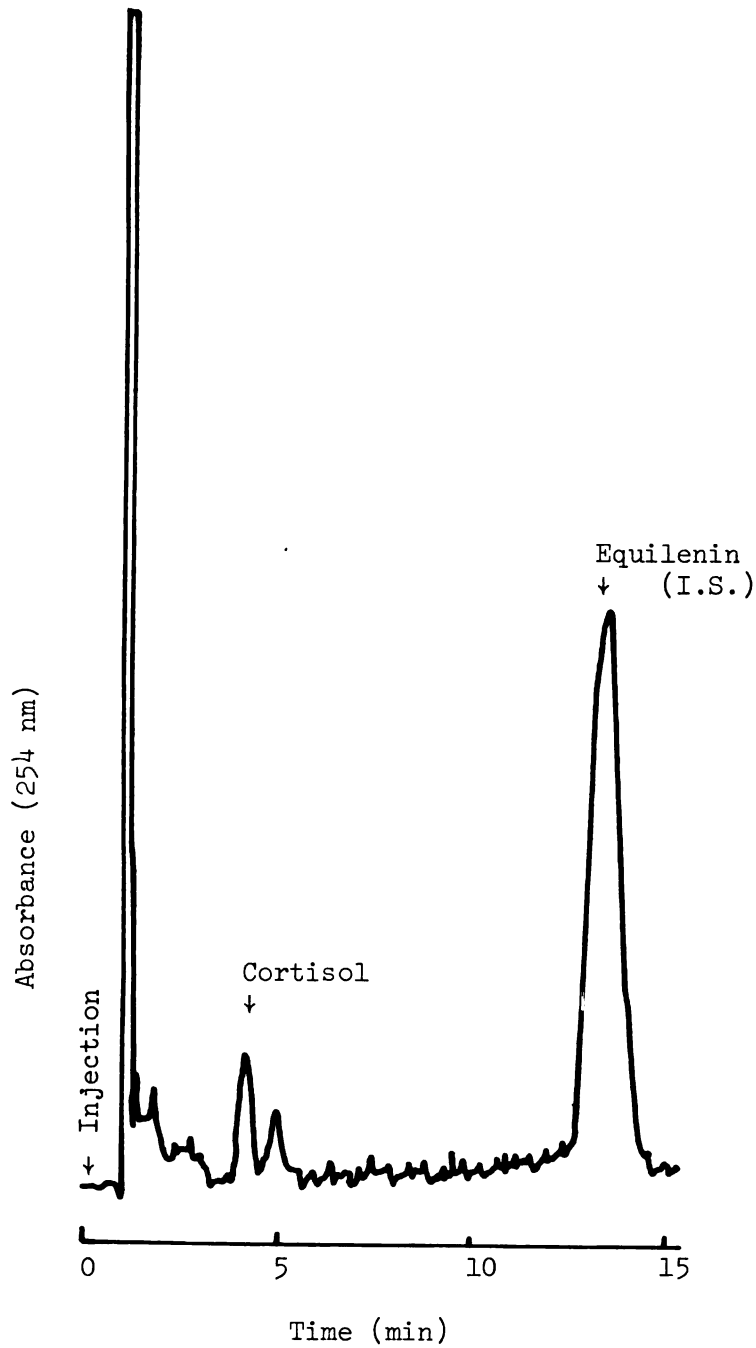


Figure 10c: Chromatogram obtained from the same patient's plasma (oxalated) with cortisol concentration calculated at 40 ng/ml.

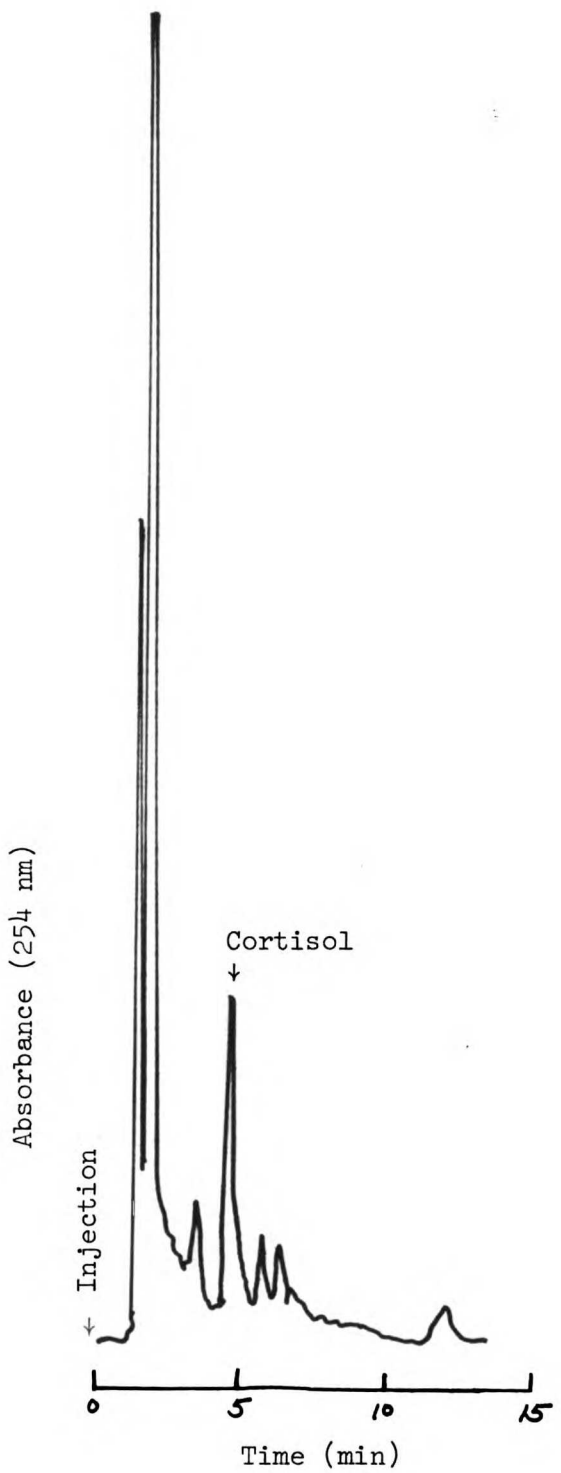


Figure 11a: Chromatogram obtained from a patient's serum after isooctane and NaOH wash.

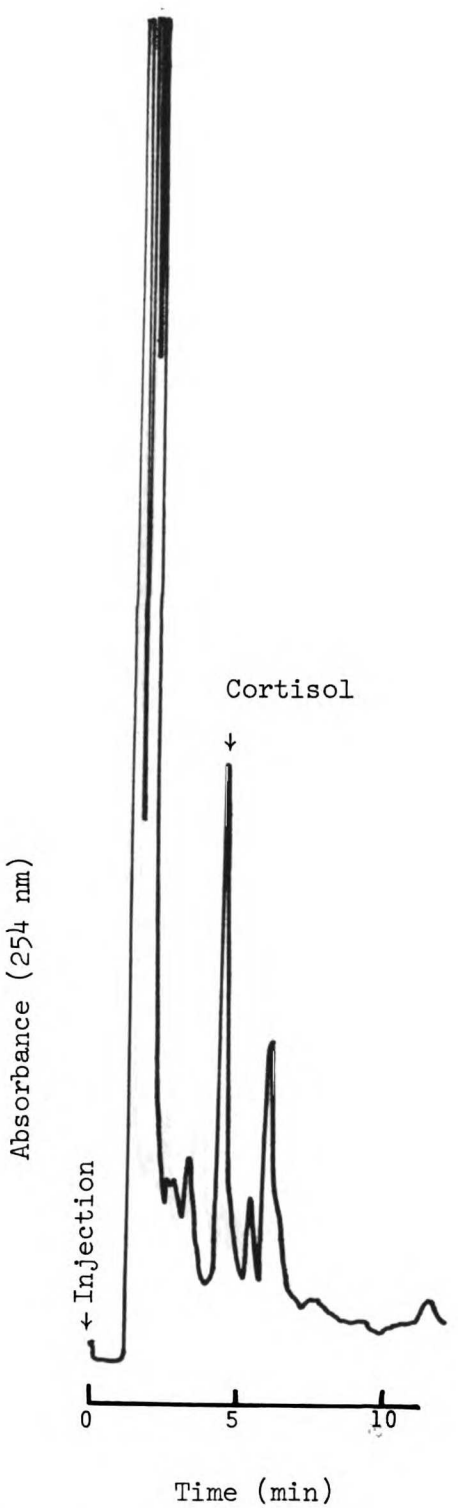


Figure 11b: Chromatogram obtained from a patient's serum after NaOH wash.

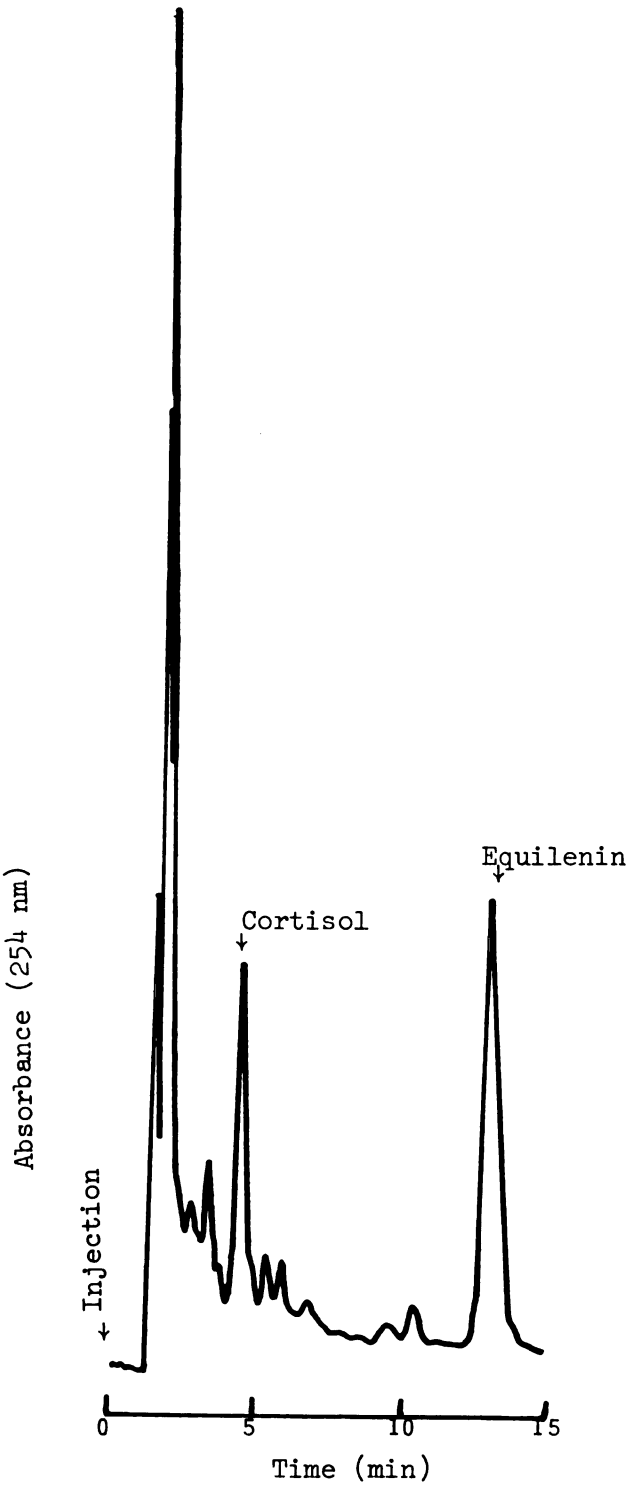


Figure 11c: Chromatogram obtained from a patient's serum after isooctane wash.

CONCLUSION

Most clinical laboratories use either fluorimetric or radioimmunoassay technique for the measurement of plasma cortisol. None of these methods are totally specific for cortisol and they are usually interfered with by other steroids and metabolites. These problems have been eliminated to a large extent by the proposed HPLC method. The method is sensitive enough to measure 5 μg/liter of cortisol when 1 ml of serum is used. It can also be easily adapted for pediatric samples (0.3 ml serum). The procedure described above offers the advantage of simple sample preparation and relatively short analysis time. These time-saving factors are important for laboratory cost reduction. Recovery, interference, and precision studies support the usefulness of this method in a routine clinical laboratory. In conclusion, it is apparent that this method is a suitable and practical method for routine blood cortisol measurement.

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