Journal of Chromatography, 429 (1988) 155–176
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4155

REVIEW

CLINICAL ANALYSIS OF INDIVIDUAL STEROIDS BY COLUMN LIQUID CHROMATOGRAPHY

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(First received December 10th, 1987; revised manuscript received February 9th, 1988)

CONTENTS

LIST OF ABBREVIATIONS

GC	Gas chromatography
GC–MS	Gas chromatography–mass spectrometry
ID–MS	Isotope dilution–mass spectrometry
I.S.	Internal standard
LC	Liquid chromatography

0378-4347/88/$03.50

RIA	Radioimmunoassay
RP	Reversed phase
TLC	Thin-layer chromatography

1. INTRODUCTION

Until recently, very few chromatographic techniques were available for determination of steroids in blood for use in a routine clinical laboratory; therefore, measurement of urinary steroids was widely used. The urinary measurement of steroids and metabolites does usually indicate the amount of steroids secreted by the respective endocrine organs during the collection period. However, factors such as inconvenience and incompleteness of 24-h urinary collection, alteration in renal function, and secretion of same steroid hormone by more than one gland can complicate the interpretation of urinary steroid measurements. On the other hand, collection and handling of plasma samples is much more convenient and plasma assays are more appropriate for serial measurements or dynamic testing of steroid hormones. However, in certain situations, even steroid estimation in plasma may be of limited use because of rapid fluctuations in steroid levels. Thus, plasma concentrations of steroids may only represent the status of endocrine organ at the time of sampling. In certain situations, therefore, urinary determination of unconjugated steroids may reflect a better index of endocrine function, because it can represent the biologically active non-protein-bound free fraction of the hormone. For example, estimation of free cortisol in urine has found wide application in the assessment of adrenal cortical function.

A recent review article by Shackleton [1] has adequately described the status of steroid profiling by chromatographic techniques. Therefore, I will confine my discussion to the analysis of clinically important steroids. This review will also be limited to column liquid chromatographic (LC) techniques developed to measure these steroid hormones.

2. STEROIDS OF CLINICAL INTEREST

Steroids of clinical significance in normal and pathophysiological conditions fall into several distinct groups. This review will be limited to the routine analysis of endogenous and synthetic corticosteroids, endogenous estrogens and vitamin D isomers in plasma and urine matrices. The corticosteroids are physiologically probably the most important group of steroids. Most of these compounds possess a Δ^4-3-keto group, a dihydroxy acetone side-chain at C-17, and generally an oxygen function at C-11. The adrenal cortical steroids may be subdivided into glucocorticoids and mineralocorticoids. Cortisol and corticosterone are the most potent glucocorticoids. More than 90% of circulating cortisol is bound to proteins, of which about 85% is bound to corticosterone-binding globulin and 10% is bound to albumin. Less than 1% of total cortisol is excreted unchanged in urine; the rest is excreted as metabolites and conjugates. The most potent mineralocorticoid is aldosterone. A large number of analogues with glucocorticoid and mineralocorticoid activity have been synthesized for therapeutic use; some of them are more

potent than the naturally occurring hormones. The measurement of plasma cortisol levels is useful in evaluating suspected abnormality of glucocorticoid production in Addison's disease (glucocorticoid deficiency) or Cushing's syndrome (glucocorticoid excess). However, the measurement of urinary free cortisol appears to be a more sensitive and specific test for initial screening of Cushing's syndrome.

Generally, there are four different types of methods for the estimation of cortisol in plasma in a routine clinical laboratory. They are based on the Porter-Silber color reaction, the measurement of sulfuric acid–ethanol-induced fluorescence, the ligand assays, and chromatographic methods based on gas chromatography (GC), gas chromatography–mass spectrometry (GC–MS), and column LC [2]. The Porter-Silber reaction, a non-specific color reaction for cortisol determination, is therefore interfered by other steroids, non-steroid metabolites, and certain drugs [3]. Fluorometric anlaysis is still a widely used method in clinical laboratories; however, it is interfered with by corticosterone, deoxycorticosterone, and certain drugs [4]. Ligand assays generally lack specificity because cortisol antibodies may cross-react to a varying degree with other steroids such as cortisone, 11-deoxycortisol, 17α-hydroxyprogesterone, corticosterone, prednisone, and deoxycorticosterone [5]. Under normal conditions the concentrations of these steroids in serum are very small compared to that of cortisol, making these assays satisfactory. However, in a number of metabolic and endocrine disorders, the concentration of steroids that cross-react with cortisol antibodies are significant. Under these circumstances a more specific and sensitive assay for cortisol is highly desirable. GC–MS offers the desired specificity and sensitivity for cortisol analysis, but cost of equipment and the complicated preparation of samples make the routine clinical use of the GC–MS less attractive. On the other hand LC methods are less expensive, quite specific, and adequately sensitive for the assessment of both endogenous and exogenous glucocorticoids.

Estrogens are female sex hormones. They are responsible for the development and maintenance of female sex organs and also participate in the regulation of the menstrual cycle and in the maintenance of pregnancy. Many obstetricians believe that the measurement of estrogens is important in the assessment of fetal well-being. In this regard, the measurement of estriol is superior to estradiol. Estriol is most frequently measured to assess high-risk pregnancies. In either case, the monitoring is usually initiated in the third trimester, when estriol levels rise to measurable amounts. The serial measurements are more important than the absolute amount at a particular time. The urinary estriol is traditionally measured on a 24-h collection. However, it is unclear whether urinary estriol or plasma conjugated, unconjugated, or total estriol is the better parameter in evaluating the integrity of the feto-placental unit during the third trimester of pregnancy [2]. Several spectrophotometric, fluorometric, GC and LC methods have been reported for the measurement of urinary estriol [6]. Urinary estriol can be specifically measured by a variety of chromatographic techniques. The majority of them require a hydrolysis step prior to extraction and analysis. However, due to low concentration of unconjugated estriol in serum, many of these techniques are not applicable to the analysis of serum estriol. Therefore, during the last fifteen

years several radioimmunoassay (RIA) methods have been reported for the measurement of serum estriol. However, many of these methods suffered from nonspecificity and required chromatographic or solvent purification prior to RIA [7].

Vitamin D isomers include both vitamin D_2 and vitamin D_3. Vitamin D itself is biologically inert and is activated by two hydroxylation reactions in the liver and kidney. Vitamin D is first converted into 25-hydroxy-vitamin D in the liver, and then to 1,25-dihydroxy-vitamin D by the kidney. Therefore in addition to vitamin D, 25-hydroxy-vitamin D, and 1,25-dihydroxy-vitamin D are present in the blood. The characteristic *cis*-triene ultraviolet spectrum of vitamin D metabolites, with an absorption maximum at 265 nm, is a preferable wavelength to monitor the vitamin D metabolites. The nanogram per milliliter plasma concentrations of vitamin D and 25-hydroxy-vitamin D are easily measurable by LC using an ultraviolet detector at 254 nm [8]. The concentrations of potent 1,25-dihydroxy-vitamin D and 24,25-dihydroxy-vitamin D, however, are too low to be measured by UV detection. For these metabolites, competitive protein binding assays continue to be most widely used methods. Because of the lack of specificity of the binding protein, the dihydroxy metabolites are separated chromatographically before competitive protein binding assays. LC provides the capability to separate and measure 25-hydroxy metabolites of vitamin D_2 and vitamin D_3 [9]. Similarly, adsorption chromatography utilizing a silica column has been used to separate 1,25-dihydroxy metabolites of vitamin D prior to their estimation by competitive protein binding or RIA methods [10].

3. ISOLATION PROCEDURES

Isolation of the steroid of interest from the physiological fluids is the rate-limiting step in the clinical analysis of these compounds. Because of the low concentration and complexicity of the matrix involved in the analysis, more complex isolation and sample preparation requirements must be met before chromatographic analysis. In a routine clinical analysis, the isolation procedure should meet the following criteria:

(1) Selectivity in isolating the steroids of interest or a class of steroids.

(2) Reproducibility and efficiency in terms of recovery.

(3) Speed of analysis.

(4) Simplicity with regard to actual manipulation steps involved in isolating the desired compounds.

(5) Cost-effectivity for routine analysis.

There are basically two types of isolation techniques involved in the analysis of clinically important steroids. The first one incorporating the principle of liquid–liquid extraction employing methylene chloride or diethyl ether is still widely used for the extraction of unconjugated steroids from plasma or urine. The second emerging technique employing the solid-phase extraction principle is becoming more popular for the extraction of both free and conjugated steroids from the physiological fluid. The advent of bonded-phase silica support expedited the growth of solid-phase extraction techniques in the routine clinical analysis. The silica gel offers many advantages over the other phases. Silica gel provides large

surface areas (approximately 500 m^2/g), which provides high capacity for the bonded-phase moieties. Silica gel is also mechanically stable, which allows it to withstand high or reduced pressure to expedite the sample preparation. The specificity and the capability of bonded-phase silica to isolate a wide range of compounds is attributed to the wide variety of chemical functionalities. The specific chemical interaction allows the analyst to achieve selective isolation of the steroid of interest.

3.1. Liquid–liquid extraction

Liquid–liquid extraction methods for the isolation and concentration of steroids from the specimen may vary from a simple one-step solvent extraction to complicated back-extraction in alkaline buffers. Generally, the type of extraction and amount of sample clean-up is determined by the efficiency and the selectivity of the chromatographic techniques used for the analysis. The more specific and efficient the chromatographic system, the less sample extraction and clean-up are necessary to obtain the desired results. Sometimes, the extraction steps are necessary to improve the sensitivity of the assay method by concentrating the desired steroid. In the various liquid–liquid extraction methods employed for the analysis of endogenous corticosteroids from plasma or urine, typically 0.1–2 ml of desired sample is extracted with non-polar organic solvents (such as methylene chloride, chloroform, diethyl ether) at a specified pH. After phase separation, the organic layer is evaporated and the residue reconstituted into a small amount of mobile phase or polar solvents. The liquid–liquid extraction methods eliminate most of the plasma proteins, and appropriate washes with sodium hydroxide also eliminate many of the interfering phenolic estrogens present in plasma or urine samples. Excellent recoveries were reported for several endogenous and synthetic corticosteroids using this extraction technique. Although the liquid–liquid extraction can provide a pure extract for the quantification of plasma cortisol, it is generally inadequate for urine samples. To overcome this difficulty, several modifications have been made for the quantitation of urinary free cortisol, often involving on-line extraction or column switching techniques to yield a clean extract.

LC assays for estrogens require an extraction and partial purification before analysis. The analysis of urinary estriol by LC usually involves hydrolysis of estriol conjugates prior to its extraction with either liquid–liquid partitioning or column extraction. The liquid–liquid extraction of urinary estriol or plasma unconjugated estriol usually involves extracting 1–2 ml of hydrolyzed urine or plasma sample with 10 volumes of organic solvents such as diethyl ether. The ether layer is washed with sodium hydroxide to remove some of the acidic interfering components. The ether layer is concentrated, reconstituted in a small amount of mobile phase, and injected onto the LC system for quantitative analysis. The liquid–liquid extraction procedures incorporating back-extractions are often plagued by poor recoveries, because of losses incurred during the neutralization steps.

The liquid–liquid extraction methods for vitamin D analysis are not very specific and therefore not widely used. Because of the lack of specificity of the binding protein and the existence of non-specific lipid interferences in serum,

measurement of 25-hydroxy-vitamin D in crude serum extracts gives higher values than those obtained after chromatographic purification.

3.2. Solid-phase extraction

In recent years, numerous sample preparation techniques using the solid-phase extraction approach have been introduced for the analysis of steroids. Examples of products employed for solid-phase extractions are XAD™ resins (Rohm and Haas, Philadelphia, PA, U.S.A), Sephadex, Carbopack, and cartridges of bonded silica gels such as Sep-Pak ™ (Waters Assoc., Milford, MA, U.S.A.) and Bond-Elut™ (Analytichem International, Harbor City, CA, U.S.A.) [11]. The utility of bonded-phase solid-phase extraction cartridges are due to their specific interactions with the compound of interest, a mechanism quite different from solubility, the basis of liquid–liquid extraction techniques. The solid phase also provides large surface area in which these specific interactions can take place. Thus, solid-phase extraction methods provide an isolation technique which is more selective and more efficient than liquid–liquid partitioning.

The use of bonded-phase cartridges for the extraction of corticosteroids from plasma and urine have been described by various investigators. Prasad et al. [12] used Bond-Elut C_{18} (500 mg) extraction cartridges to extract endogenous and synthetic corticosteroids from swine plasma. The Bond-Elut extraction cartridges were loaded with 2 ml of plasma, washed two times with 2 ml of water, followed by two washes of 2 ml each with 2% acetone in water. Finally the corticosteroids were eluted with 1 ml of acetonitrile, which was evaporated, the residue was redissolved in 150 μl of methylene chloride, and an aliquot of 100 μl injected onto the LC instrument. The corticosteroids (prednisolone acetate, prednisolone, prednisone, cortisone, and cortisol) were separated on a LiChrosorb Si 60, 5-μm silica column with a mobile phase of water-saturated methylene chloride–tetrahydrofuran–methanol–glacial acetic acid. The corticosteroids were detected at 254 nm. The use of Extrelut columns was advocated by Lawson [13] to isolate methyl prednisolone and its hemisuccinate from plasma and urine, while Schöneshöfer et al. [14] used these columns to isolate fifteen different free steroids from urine to assess their utility in the differential diagnosis of Cushing's syndrome. The corticosteroids were adsorbed on these columns and then eluted with either diethyl ether or ethyl acetate. Lawson [13] discovered that the recovery of methyl prednisolone and its hemisuccinate was only 52–65% in plasma, while the recovery from urine was between 83 and 97% . Schöneshöfer et al. [14] further purified the fifteen free steroids extracted by an Extrelut column by fractionating them on an automated LC system using a diol column. After fractionating the steroids, they were quantified by RIA techniques. The on-line sample pretreatment techniques were extensively exploited by Schöneshöfer et al. [15] to measure urinary free cortisol and other metabolites of cortisol. Various kinds of hydrophobic packing materials were utilized in isolating the cortisol from the urine matrix. The advantage of on-line sample pretreatment lies predominantly in the quantitative recovery of the steroid of interest and the ease of automation.

In our laboratories, Bond-Elut extraction cartridges (C_{18}, 100 mg) were used

to isolate urinary free cortisol prior to the chromatographic separation and fluorescence detection. We used the extraction cartridges in series to isolate first the cortisol from urine, followed by the isolation of the fluorescent derivative. This isolation and purification scheme yielded a very clean extract for the routine analysis of urinary free cortisol. The Bond-Elut extraction columns (100 mg, C_{18}) were used by Hofreiter et al. [16] to isolate cortisol from plasma or serum sample. The cortisol is extracted from the extraction cartridge by eluting it with 300 μl of methanol. The recovery is adversely affected if the plasma sample is directly passed through the cartridge (recovery as low as 10%); however, if plasma is first treated with methanol, then the recovery improves dramatically (96–103%).

Nakamura and Yakata [17] used two-cycle LC to quantitate cortisol in urine. In the first chromatographic step, they separated the fractions containing cortisol and internal standard on a normal-phase silica column. These fractions were then concentrated and further analyzed on a reversed-phase cyano column. This kind of approach imparts a high degree of specificity to the analysis. Oka et al. [18] used a rapid-flow fractionation technique for isolating cortisol and corticosterone from plasma. They used a column packed with diatomaceous earth granules which was eluted with diethyl ether. The recovery was almost quantitative.

The use of bonded-phase cartridges for the extraction of estrogens and estrogen conjugates has been adequately treated in the review article by Shackleton [1]. Heikkinen et al. [19] used a Sep-Pak C_{18} cartridge for the extraction of estrogens from urine and plasma. The estrogens were eluted from the extraction cartridge with 3.0 ml of methanol. The recovery of estrogen ranged form 96 to 99%. Andreolini et al. [20] utilized Carbopack B (graphitized carbon black) columns to adsorb the estriol and its conjugates from urine before they were quantified by LC with fluorescence detection. A pretreatment column packed with copolymer beads was used by Dohji et al. [21] to isolate estradiol and estriol from maternal serum. The estriol and estradiol were adsorbed on the porous beads, whereas other serum components such as proteins and carbohydrates were washed off with pretreatment buffer. After 6 min, the eluent was allowed to flow through the pretreatment column to transfer the estrogens onto the analytical column.

Because of the extremely low concentrations of physiologically active vitamin D isomers, they are usually not measured by chromatographic techniques. However, the isolation and purification of both 1,25-dihydroxy-vitamin D and 24,25-dihydroxy-vitamin D isomers is accomplished with either normal or reversed-phase chromatography. Most of the published procedures have utilized normal-phase LC techniques to purify 1,25-dihydroxy-vitamin D isomers prior to the receptor binding assay. Recently, a number of methods have been developed to assay small amounts of vitamin D isomers from serum after extraction with Sep-Pak cartridges, thus eliminating the need to use LC [22]. Hollis and Frank [23] and Kao and Heser [24] used chromatographic mode sequencing techniques in which the vitamin D metabolites are first extracted from a serum sample using the C_{18} sorbent. The vitamin D metabolites are then resolved on a silica or amino sorbent cartridge. The extraction from a second cartridge purifies and also separates 25-hydroxy-vitamin D and 1,25-dihydroxy metabolites with good analytical recovery.

3.3. Hydrolysis of conjugates

Shackleton [1] in his review has already discussed the use of various β-glucuronidases available for use in the hydrolysis of steroid glucuronides. Enzymatic hydrolysis often does not yield quantitative recovery of hydrolyzed steroids, because some of the conjugated steroids occur as sulfate conjugates. Often a mixture of β-glucuronidase with sulfatase is used to hydrolyze the mixture of sulfate and glucuronide conjugates. One of the major problems with the enzymatic hydrolysis is the slowness of the enzymatic reaction, which is a major concern in the routine clinical analysis. However, in certain situations, the hydrolysis process can be substantially speeded up by utilizing higher temperature. In the routine clinical analysis, estriol conjugates are usually hydrolyzed prior to the chromatographic analysis of urinary estriol. Both acidic and enzymatic hydrolysis have been widely used for this purpose. Even though the acidic hydrolysis is rapid and efficient, it yields poor recovery and higher background for the measurement of estriol by chromatographic procedures. In our laboratories, we used β-glucuronidase powder derived from *Escherichia coli* to hydrolyze estriol conjugates in urine at 60°C for 30 min. The hydrolytic conditions were optimized by varying the concentration of enzyme, duration of incubation, and temperature of incubation. The hydrolysis was complete when 1800 U of β-glucuronidase were added to 5 ml of urine and incubated at 60°C for 30 min [25].

4. ANALYSIS OF PLASMA STEROIDS

4.1. Endogenous corticosteroids

Cortisol is the predominant corticosteroid present in the plasma. It represents about 80% of the circulating 17-hydroxycorticosteroids. Most of the circulating cortisol is bound to plasma proteins, primarily to a corticosteroid-binding globulin and to a smaller extent to albumin. Determination of serum or plasma cortisol concentration is useful in assessing adrenocortical function. Many analytical methods are currently available for the determination of plasma cortisol. The advantages and deficiencies of these analytical techniques have already been discussed in this review article. LC offers the desired sensitivity and specificity for the analysis of cortisol. During the last few years numerous LC procedures employing either liquid–liquid or solid-phase extraction techniques have been reported.

Reardon et al. [26] described an LC procedure for the simultaneous analysis of cortisol and 11-deoxycortisol from serum. After addition of 10 ml of methylene chloride to 1 ml of serum, the organic layer was washed with 0.1 *M* sodium hydroxide and water, and then dried under a stream of nitrogen at 40°C. The extract was dissolved in mobile phase, and cortisol and 11-deoxycortisol were separated on a reversed-phase C_{18} column with the detection wavelength set at 254 nm. No provision was made for the use of an internal standard in this analysis. A similar kind of reversed-phase LC method employing a liquid–liquid extraction technique was developed in our laboratories for the determination of serum cortisol

[27]. The method had a sensitivity of 5 ng/ml and use of internal standard gave day-to-day precision with a coefficient of variation of $<7\%$ (Fig. 1). The chromatographic time of 20 min and requirement of at least 300 μl of serum prompted us to develop a more sensitive and rapid method for the analysis of serum cortisol [28]. In this improved method, we formed a fluorescent derivative of cortisol by reacting it with sulfuric acid and ethanol, then measuring the resultant derivative on a fluorescence detector. The method only required 50 μl of serum and the chromatography was complete in 7 min (Fig. 2). A normal-phase chromatographic method was developed by Matsuzawa et al. [29] for the analysis of serum cortisol. After addition of prednisolone as an internal standard, 0.1 ml of serum was extracted with methylene chloride in the presence of 10 μl of 1.25 *M* sodium hydroxide. The extracted cortisol was separated on a μPorasil column with a mobile phase of methylene dichloride–ethanol–methanol–water (232:5:10:3, v/v), and detected at 254 nm. The analytical column was reactivated after every thirty samples.

A reversed-phase LC method incorporating two different internal standards

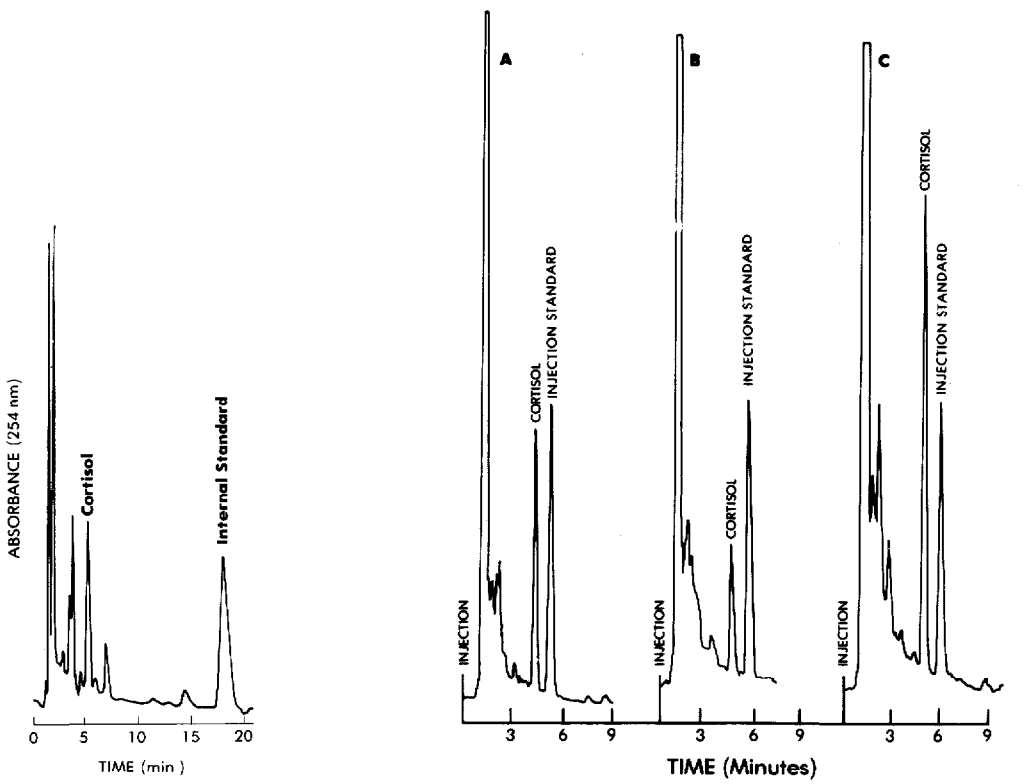

Fig. 1. LC profile of plasma cortisol. Chromatogram of plasma containing 300 μg/l cortisol with equilenin as internal standard; ultraviolet detection at 254 nm. (From ref. 27 with permission.)

Fig. 2. Chromatograms for (A) the reference standard and (B and C) serum samples containing, respectively, 80 and 300 μg/l cortisol; fluorescence detection. (From ref. 28 with permission.)

(19-nortestosterone and 6α-methyl prednisolone) was reported by Lambert et al. [30]. This accurate and specific LC method was compared with RIA, fluorometry, and isotope dilution–mass spectrometry (ID–MS). There was excellent correlation between LC and ID/MS methods. Van den Berg et al. [31] developed an LC method for serum cortisol using a selective chemically bonded stationary phase. After extracting cortisol from plasma by methylene chloride, the cortisol was resolved on a modified silica or Nucleosil NO_2 phase with a detection limit of 10 μg/l. The specificity of the method was assessed with field desorption mass spectrometry. A simultaneous normal-phase LC method for the determination of endogenous corticosteroids (cortisol and cortisone) and exogenous glucocorticoids (dexamethasone, prednisone, prednisolone, and methyl prednisolone) was reported by Frey et al. [32]. The steroids were extracted from 1 ml of plasma with methylene chloride–diethyl ether, washed with acid and base, and separated isocratically on a silica column.

Solid-phase extraction and LC analysis of cortisol and synthetic corticosteroids were reported by Prasad et al. [12]. The use of solid-phase extraction columns results in a faster, more efficient, and convenient method than traditional liquid–liquid extraction techniques. Oka et al. [18] utilized rapid-flow fractionation using diatomaceous earth granules as stationary support material and diethyl ether as mobile phase to extract cortisol and corticosterone from plasma prior to chromatographic analysis on silica column. The analytical recovery approached 100%.

Aldosterone is a potent mineralocorticoid which regulates salt and water metabolism. The interaction of renin, angiotensin, and aldosterone is important in the regulation of extracellular fluid volume, blood pressure, and sodium and potassium homeostatis. The exact role of aldosterone in the pathogenesis of essential hypertension is not completely understood. Additionally, the lack of a reliable method for the estimation of aldosterone compounded this problem further. In most of the RIA procedures routinely used for the estimation of aldosterone, the antibodies used in the assay exhibit significant and variable degree of cross-reactivity with other aldosterone and glucocorticoid metabolites. Various thin-layer chromatographic (TLC) purification steps were incorporated prior to the RIA determination to improve the specificity of the antibodies. De Vries et al. [33] developed an LC procedure for the assay of aldosterone in urine. After the hydrolysis of aldosterone conjugates with sulfuric acid at room temperature for 24 h, the steroid was extracted three times with methylene chloride, the organic layer was then washed with sodium hydroxide and saturated sodium chloride solution. The organic layer was separated, evaporated, the residue redissolved in 70% methanol, washed three times with toluene, and evaporated to dryness. The residue was then purified by TLC using three different solvent systems. Prednisolone was added as an internal standard and analyzed on an LC instrument using a silica column and mobile phase of chloroform–methanol (98.5:1.5, v/v). The reported detection limit of about 1.5 ng/ml of urine would make the assay suitable for clinical purpose, but the extraction and purification protocol is too cumbersome for routine use. Similarly, LC was also used in the RIA estimation of urinary tetrahydroaldosterone, which is the major urinary metabolite of aldosterone. The

RIA determination of tetrahydroaldosterone is difficult since large amounts of other tetrahydro metabolites of cortisol and cortisone are also released by the glucuronidase hydrolysis. These potentially cross-reacting steroids must be separated chromatographically before the RIA analysis. Therefore, the tetrahydroaldosterone was purified either on a diol or a reversed-phase column. The fraction containing tetrahydroaldosterone was evaporated and assayed by RIA [34].

Several other LC procedures have been described for the determination of corticosterone, 17-hydroxyprogesterone, and 11-deoxycortisol. Elevated levels of 11-deoxycortisol and 17-hydroxyprogesterone are diagnostic for congenital adrenal hyperplasia due to 21-hydroxylase or 11β-hydroxylase deficiency [18,35,36]. Corticosterone is a major glucocorticoid in laboratory rodents. Most of these LC procedures incorporate a liquid–liquid extraction procedure followed by some kind of reversed-phase separation of the desired steroid. These steroids are not routinely monitored in the clinical laboratories, but are mostly assayed in the specialized endocrinology laboratories.

4.2. Synthetic corticosteroids

Synthetic glucocorticoids have been used both in low and high dose for the treatment of several diseases. These synthetic corticosteroids include dexamethasone, prednisone, prednisolone, methyl prednisolone, and their water-soluble esters. To study the pharmacokinetics of these synthetic corticosteroids, various sensitive assays have been devised. LC has been used very widely to monitor the low concentrations of these steroids. Both normal-phase and reversed-phase LC with either liquid–liquid or solid-phase extraction procedures have been reported for the analysis of these steroids. Many of the reported procedures are also capable of measuring simultaneously both endogenous and synthetic corticosteroids. Frey et al. [32] reported a normal-phase simultaneous assay for cortisol, cortisone, dexamethasone, prednisone, prednisolone, and methyl prednisolone. They reported a lower detection limit of 10 ng/ml from 1 ml of plasma sample. The dexamethasone suppression test is frequently used in the diagnosis of hypercortisolism (Cushing's syndrome). Sometimes the interpretation of this test is questionable, therefore some investigators emphasize the need to monitor cortisol and dexamethasone simultaneously. Due to the low dose of dexamethasone, the resulting serum levels are extremely low, thus requiring a very sensitive method. A highly sensitive (<1 ng/ml) reversed-phase LC method has been reported by Lamiable et al. [37]. They extracted 2 ml of plasma with 9 ml of methylene chloride containing 25 ng of equilenin as an internal standard. The reversed-phase separation and detection at 246 nm provided the reported sensitivity. Loo and Jordan [38] reported a normal-phase LC method for the simultaneous determination of prednisone, prednisolone, and dexamethasone. The lowest level of detection reported for these synthetic corticosteroids was 25, 25, and 100 ng/ml for dexamethasone, prednisone, and prednisolone, respectively. The lack of sensitivity for dexamethasone detection limits the utility of this method in the dexamethasone suppression test. Recently, a number of LC methods have been reported for the analysis of methyl prednisolone and it soluble prodrug esters in

plasma and other body fluids. The various methods utilize liquid–liquid or solid-phase extraction procedures followed by normal- or reversed-phase chromatography. Many of these methods have been used to study the pharmacokinetics of these synthetic corticosteroids [39–41].

4.3. Endogenous estrogens

Measurement of total urinary estrogens has been used in assessing the integrity of the feto-placental unit during the third trimester of pregnancy. Problems with collecting accurate 24-h urine have prompted the development of assays for serum estriol. Unconjugated estriol in serum is derived almost exclusively from its synthesis in the fetus and by the placenta. The short half-life of about 20 min makes estriol a sensitive marker of current feto-placental function [6]. Most of the earlier methods developed for the measurement of urinary estrogens were not suitable for the measurement of estriol or estradiol in serum. During the last ten to fifteen years several RIA methods have been reported for the measurement of serum estriol [42,43]. However, many of these methods suffered from non-specificity and required either chromatographic or solvent extraction prior to radioimmunoassay [44].

Several LC methods using either fluorescence or electrochemical detection have been reported for the analysis of unconjugated estriol. In our laboratories, we developed an LC procedure using fluorescence detection for the analysis of unconjugated estriol in serum [45]. In this procedure, 1 ml of serum or plasma was extracted by using a Clin-Elut column. The Clin-Elut column was eluted twice with 4 ml of methylene choride–propanol-2 (95:5, v/v). The two extracts were combined and evaporated under a stream of nitrogen at 45°C to dryness. The residue was reconstituted in 50 μl of mobile phase and then 40 μl were exactly injected onto the LC instrument. The Ultrasphere octyl column was eluted with acetonitrile–phosphate buffer (23:77, v/v) at a flow-rate of 3.0 ml/min at 50°C and detected at the excitation and emission wavelengths of 280 and 308 nm, respectively. The recovery of estriol from spiked plasma samples averaged between 86 and 96% with a sensitivity of <1 ng/ml. When compared with RIA, the LC results were consistently lower. The total chromatographic time of <8.0 min and good reproducibility of this assay (Fig. 3) provided us with a specific and simple method. With the Clin-Elut column extraction, we were able to complete the analysis of ten serum samples in less than 2 h, which is comparable to RIA methods. Kaplan and Hohnadel [46] reported an LC assay for unconjugated serum estriol using electrochemical detection. They extracted 2 ml of serum with diethyl ether, separated the ether phase, evaporated the ether layer, and reconstituted the residue with 250 μl of a 500 μg/l methanolic solution of propiophenone (internal standard). An aliquot containing 10–50 μl was injected onto the LC system. The estriol was resolved on a reversed-phase C_{18} or C_8 Radial-Pak cartridge and detected by the glassy carbon electrode of the electrochemical detector set at 0.75 V. They reported a sensitivity of 1 ng/ml with almost quantitative recovery of unconjugated estriol from serum samples.

A polymer based pretreatment column was used by Dohji et al. [21] to adsorb

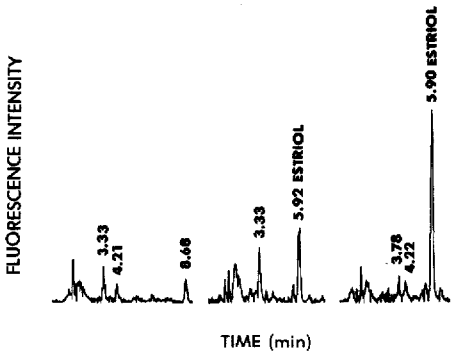

Fig. 3. Chromatograms of (left) an estriol-free male serum, (middle) the serum of a pregnant woman with 5.3 μg/l unconjugated estriol, and (right) the serum of a pregnant woman with 13.3 μg/l unconjugated estriol; fluorescence detection. (From ref. 45 with permission.)

estradiol and estriol from serum, while hydrophilic components such as proteins and carbohydrates were excluded from the pretreatment column. This automated pretreatment mode coupled with fluorescence detection required only 150 μl of serum with a reported sensitivity of 1 ng/ml. This procedure eliminated the cumbersome and time-consuming pretreatment steps used in other chromatographic methods. Use of Carbopack B (graphitized carbon black) was reported by Andreolini et al. [20] to extract estriol and its conjugates from physiological fluids prior to LC determination. In this method, 1 ml of serum is deproteinized with cold methanol, then the supernate passed through the Carbopack column. The estriol is then eluted with chloroform–methanol containing tetrapropylammonium bromide. The estriol and its conjugates are separated by gradient elution on a reversed-phase column and detected by measuring their fluorescence at excitation and emission wavelengths of 275 and 302 nm, respectively.

*4.4. Vitamin D isomers**

Measurement of vitamin D isomers is important in assessing the vitamin D status. Since the concentration of dihydroxy-vitamin D isomers are usually very low in plasma, they cannot be measured by chromatographic methods. However, as described in a previous section, isolation and purification of these isomers are usually accomplished by solid-phase extraction or LC techniques. Once the desired dihydroxy isomers are selectively isolated by these techniques, their concentration can be measured in plasma by receptor binding or RIA methods. On the other hand, concentration of 25-hydroxycalciferol can be measured by LC with ultraviolet detection. Kao and Heser [24] reported an assay for simultaneous determination of 25-hydroxy- and 1,25-dihydroxy-vitamin D from a single sample by dual-cartridge extraction. The extract obtained from an amino cartridge was chromatographed on a silica column, and the concentration of 25-

*Additional information regarding vitamin D estimation can be found in the review of De Leenheer et al. [47].

hydroxy-vitamin D was measured by measuring its absorption at 254 nm. The recovery losses were compensated for by using tritium-labelled 25-hydroxy-vitamin D. The dual cartridge extraction separated the 25-hydroxy isomer from 1,25-dihydroxy-vitamin D, and hence the LC analysis was appropriate for the quantitation of 25-hydroxy-vitamin D. The measurement of 10–20 ng/ml 25-hydroxy-vitamin D in plasma is easily achievable by LC methods.

5. ANALYSIS OF URINARY STEROIDS

5.1. Urinary free cortisol

Clinically, measurement of urinary free cortisol has been shown to be the best discriminating test for the diagnosis of Cushing's disease. Analytically, the measurement of this steroid hormone is difficult, because of its low concentration and the presence of large amounts of similar metabolites in urine. At present, RIA is frequently used to measure urinary free cortisol. However, because of the non-specific interaction of cortisol antibody with closely related metabolites, an extensive extraction and purification scheme is necessary for reliable measurement of urinary free cortisol. A method combining chromatographic isolation of cortisol followed by RIA is more specific than RIA alone; however, this requires two steps, limiting its application in routine clinical analysis.

Canalis et al. [48] have modified their serum cortisol LC method so that urinary free cortisol can be measured. In this method, they extracted a 2-ml aliquot of a 24-h urine collection with 20 ml of methylene chloride, washed it with 2 ml of 0.1 *M* sodium hydroxide and 2 ml of water. The methylene chloride phase was evaporated to dryness, reconstituted in mobile phase, and cortisol was separated on a reversed-phase C_{18} column with a mobile phase of tetrahydrofuran–methanol–water (24.5:2.5:73, v/v). The smallest amount of cortisol detectable at 254 nm was reported to be 5 ng. The correlation between LC and RIA was poor ($r=0.59$), with RIA values being 1.4–4.3 fold greater than LC. An LC assay using fluorescence detection was developed by Shihabi et al. [49], in which urinary free cortisol was extracted with methylene chloride; the fluorescent derivative of cortisol obtained by treating it with sulfuric acid was then separated on a reversed-phase C_{18} column and monitored at an excitation wavelength of 390 nm and emission wavelength of 475 nm. The advantage of fluorescence detection over the UV detection, of course, was the greater sensitivity and specificity. A fully automated LC method for the measurement of urinary free cortisol was developed by Schöneshöfer et al. [50] in which 1 ml of urine is concentrated and prepurified on a reversed-phase precolumn with alkaline, acid, and organic washes. After selective elution, the cortisol-containing fraction is focused on the top of the second reversed-phase precolumn. From this precolumn, the cortisol is backflushed by the mobile phase, separated from the closely related compounds on a reversed-phase column, and detected by ultraviolet absorbance at 254 nm. This on-line sample pretreatment eliminates time-consuming manual extraction techniques utilized in other LC procedures. Additionally, the intensive prepurification almost completely eliminated the non-specific background from the urine matrix.

Lantto [51] compared the results obtained by LC and RIA for urinary free cortisol with ID–MS and reported that LC method gave the most comparable results ($r=0.86$) with a highly specific reference method based on ID–MS. The urine sample containing prednisolone as internal standard was extracted with methylene chloride and the organic phase separated and evaporated. The residue was then subjected to TLC. The zone containing cortisol and internal standard was scraped off and extracted with 5 ml of ethyl acetate. After evaporation, the residue was dissolved in 100 μl of methylene chloride–ethanol and injected onto an LC instrument equipped with a Nucleosil-NO_2 column. The use of tritiated prednisolone compensated for recovery losses in liquid–liquid and TLC extraction steps. A two-cycle LC determination of urinary cortisol was described by Nakamura and Yakata [52], in which 1 ml of urine containing 200 ng of dexamethasone as internal standard was extracted with 8 ml of methylene chloride. The organic layer was washed with water and evaporated under a stream of nitrogen at 37°C. The residue was dissolved in 100 μl of the mobile phase and chromatographed on a silica column. The specific fraction containing cortisol and internal standard was collected, evaporated, the residue redissolved in methanol–water (40:60), and chromatographed on a cyano column. The "two-cycle" chromatograms thus obtained were sufficiently specific for the quantitation of urinary cortisol. The minimum concentration of cortisol detectable by this method was 5 μg/l.

In our laboratories, we have developed a fluorescence LC method for the determination of urinary free cortisol. In this method, 1 ml of urine is passed through

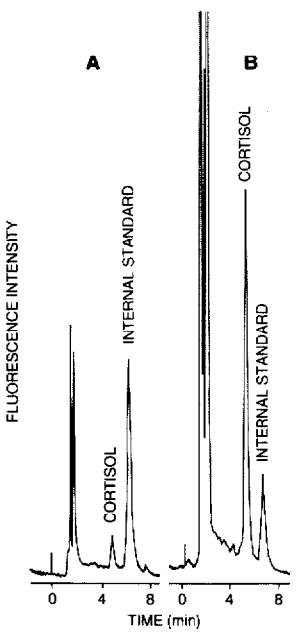

Fig. 4. LC profile of urinary free cortisol. (A) Standard containing 10 μg/l cortisol; (B) urine sample containing 260 μg/l cortisol; fluorescence detection.

TABLE 1

REVIEW OF LC PROCEDURES FOR ENDOGENOUS CORTISOL ANALYSIS

Sample preparation	Column	Detection	Remarks	Ref.
1.0 ml serum extracted with 10 ml CH_2Cl_2	C_{18} RP	254 nm	Measures cortisol and deoxycortisol, no I.S.	26
0.1 ml serum + 10 μl 1.25 *M* NaOH + 1 ml CH_2Cl_2	μPorasil	254 nm	Prednisolone as I.S.	29
0.2 ml serum + 100 μl 0.1 *M* NaOH + 4 ml CH_2Cl_2	C_{18} RP	254 nm	Two I.S., 6-methyl prednisone, 19-nortestosterone	30
1.0 ml serum + 0.1 ml 0.25 *M* NaOH + 7 ml CH_2Cl_2	Nucleosil NO_2	240 nm	Prednisolone as I.S.	31
1.0 ml serum + 8 ml CH_2Cl_2	C_{18} RP	254 nm	Equilenin as I.S.	27
50 μl serum + 1 ml CH_2Cl_2	C_{18}RP	Fluorescence: ex. = 366 nm, em. = 488 nm	Fluorescent derivative of equilenin as I.S.	28
1.0 ml serum + 9 ml CH_2Cl_2–diethyl ether (2:1, v/v)	Silica	254 nm	Dexamethasone as I.S.	32
2.0 ml serum + Bond-Elut C_{18} extraction cartridge + elution with 1.0 ml acetonitrile	Silica	254 nm	Dexamethasone as I.S.	12
0.5 ml serum + diatomaceous earth material + elution with diethyl ether	Silica	245 nm	Rapid-flow fractionation	18
2 ml urine + 20 ml CH_2Cl_2 + wash with NaOH and water	C_{18} RP	254 nm	No I.S.	48
0.5 ml urine + 500 μl CH_2Cl_2 + evaporate + 50 μl of conc. H_2SO_4	C_{18} RP	Fluorescence	No I.S.	49
1 ml urine concentrated on an RP phase PRP-1 resin + 2nd preclumn packed with ODS silica	C_{18} RP	254 nm	No I.S.	50
2 ml urine + 100 ng prednisolone as I.S. + 8 ml CH_2Cl_2	Nucleosil NO_2	254 nm	Residue subjected to TLC prior to LC analysis	51
1 ml urine + 200 ng dexamethasone as I.S. + 8 ml CH_2Cl_2	Cyano	254 nm	Two-cycle LC; residue first chromatographed on silica, then collected fraction analyzed on cyano column	52

a Bond-Elut C_{18} column, from which interfering substances are washed off, and the cortisol is eluted with methylene chloride containing a fluorescent derivative of equilenin as the internal standard. Cortisol and the internal standard are back-extracted into sulfuric acid, in which they form fluorescent derivatives. The sulfuric acid-induced fluorescent derivatives are then diluted with water and once again passed through the Bond-Elut C_{18} column, which adsorbs the fluorescent derivatives. The column is eluted with methanol, an aliquot is injected onto the LC system, and fluorescent derivatives are separated on a reversed-phase C_{18} column and detected at an excitation wavelength of 366 nm and emission wavelength of 488 nm. The solid-phase extraction of cortisol and cortisol fluorescent derivatives yield a very clean extract suitable for the analysis of the majority of urine samples (Fig. 4). A general survey of various LC methods for the analysis of cortisol is presented in Table 1.

5.2. Urinary estriol

During pregnancy, the placenta is the major source of estrogen synthesis, particularly estriol is produced in milligram quantities. However, it needs adrenal C-19 steroid precursors derived from either maternal or fetal biosynthetic pathways. Because the pathways for estriol formation during pregnancy involve both fetus and placenta, the estriol measurements can be a sensitive clinical indicator of fetoplacental status. The normal range of urinary estriol during pregnancy is very wide. Therefore, an isolated determination of estriol has only limited clinical value. Serial determinations of estriol, therefore, are more meaningful to evaluate the trend of estriol production in high-risk pregnancy. A decline of 30% or more from a preceding determination or a trend of continuous fall for three or more consecutive days generally reflects fetal distress.

Measurement of both plasma and urinary estriol has proved to be equally useful in predicting fetal problems. Therefore, the selection of the method to be used for routine clinical analysis should be based on the availability of a reliable method, same-day turn-around time, and clinical experience. Most of the estriol present in urine is excreted as sulfate or glucuronate conjugates (80–95%). Most of the chromatographic methods, therefore, require the hydrolysis of estriol conjugates prior to analysis. Many LC procedures have been reported for the analysis of unconjugated as well as estriol conjugates. Gotelli et al. [25] reported a reversed-phase LC method for the analysis of urinary estriol, in which a 5-ml aliquot of 24-h urine collection was hydrolyzed with β-glucuronidase derived from *Escherichia coli* at 60°C for 30 min. After hydrolysis, 1 ml of 25 μg/ml carbamazepine was added as internal standard and the hydrolysate was extracted with 20 ml of diethyl ether–petroleum ether (60:40, v/v). The organic phase was separated and evaporated at 60°C, the residue was reconstituted in 200 μl of methanol, from which 20 μl were injected onto the LC system. The reversed-phase C_{18} column was eluted with a mobile phase consisting of acetonitrile–phosphate buffer, pH 4.4 (22:78, v/v), at a flow-rate of 3 ml/min. The column was maintained at 50°C, and the column effluent was monitored at 280 nm. The chromatography was

complete in less than 15 min, and precision of the assay was excellent, with a coefficient of variation $<4\%$ (Fig. 5).

Dolphin and Pergande [53] used both normal- and reversed-phase chromatography for the analysis of estrogenic steroids in pregnancy urine. The urine was hydrolyzed with concentrated hydrochloric acid, and the estrogens were extracted into diethyl ether. The extract was concentrated and analyzed by LC on either a silica or reversed-phase C_{18} column. The column effluent was monitored at 280 nm The method was plagued with poor extraction efficiency (27%) and relatively large standard deviation.

Bowers and Johnson [54] accomplished on-line hydrolysis of urinary estriol conjugates with immobilized β-glucuronidase before reversed-phase LC analysis of estriol. The β-glucuronidase was covalently bound to controlled pore glass. A 20-μl aliquot of urine mixed with phosphate buffer (pH 6.8) was injected onto the LC system. During the first 5 min, the immobilized enzyme reactor is on-line at a flow-rate of 1.0 ml/min, after which the flow-rate is increased to 2 ml/min to elute any remaining material from the reactor. At the end of 15 min, a linear gradient elution is started to separate estriol on the reversed-phase column. This on-line reactor allows complete hydrolysis of urinary estriol glucuronides before its analysis by LC. The advantage of on-line hydrolysis is that it eliminates manual sample manipulation and on-line hydrolysis gives samples which are free of background from unwanted reaction. Estriol and its conjugates also were determined in pregnancy urine by extraction with Carbopack B and analysed by LC and fluorescence detection [20]. A 70-μl urine sample is diluted, and estriol and its conjugates are adsorbed onto graphitized carbon black (Carbopack B). After

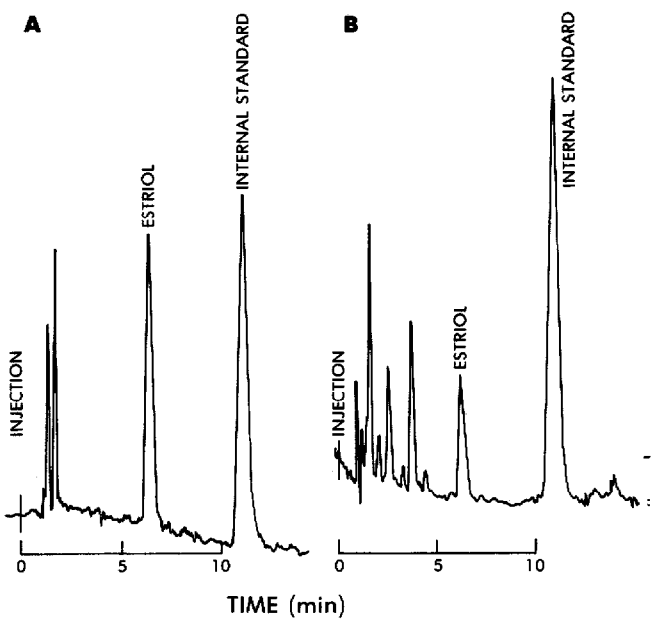

Fig. 5. LC profile of urinary estriol. (A) The working estriol standard; (B) a pregnancy urine containing 9.2 mg/l estriol; UV detection at 280 nm. (From ref. 25 with permission.)

TABLE 2

REVIEW OF LC PROCEDURES FOR ENDOGENOUS ESTRIOL

Sample preparation	Column	Detection	Remarks	Ref.
Plasma or serum				
1 ml plasma + Clin-Elut column + elution 2 × 4 ml CH_2Cl_2 and propanol	RP C_8	Fluorescence: ex. = 280 nm, em. = 308 nm	No I.S.	45
2 ml plasma + 125 ng propiophenone (I.S.) + diethyl ether extraction	RP C_{18} RP C_8	Electrochemical: 0.75 V		46
0.15 ml serum + pretreatment copolymer column	RP polymer column	Fluorescence: ex. = 220 nm, em. = 320 nm	Copolymer beads synthesized	21
1 ml serum + deproteinization with methanol + supernate passed through Carbopack column + elution with $CHCl_3$ + methanol	RP C_{18}	Fluorescence: ex. = 275 nm, em. = 302 nm	No I.S.	20
Urine				
5 ml urine hydrolyzed with β-glucuronidase + extraction with 20 ml diethyl ether–petroleum ether	RP C_{18}	280 nm	Carbamazepine as I.S.	25
Urine hydrolyzed with conc. HCl + extraction with diethyl ether	Silica RP C_{18}	280 nm	No I.S., estradiol and estrone can be measured	53
20 μl urine hydrolyzed on-line with β-glucuronidase	RP C_{18}	Fluorescence	No I.S.	54
70 μl urine diluted, estriol adsorbed on Carbopack, eluted with chloroform–methanol containing tetrapropylammonium bromide as ion-pair reagent	RP C_{18}	Fluorescence: ex. = 275 nm, Em. = 302 nm	No I.S.	20
20 ml urine hydrolyzed with conc. HCl + extraction with diethyl ether	Silica	Electrochemical: 0.4 V	Tetraalkylammonium salts added to mobile phase for conductivity	55
2 ml urine hydrolyzed with β-glucuronidase at 50 °C + extraction with 35 ml ether	RP C_{18}	Fluorescence: ex. = 220 nm, em. = 304 nm	No I.S.	56

a couple of washings, the analytes are desorbed with chloroform–methanol (60:40, v/v) containing tetrapropylammonium bromide. After evaporation, the conjugates are separated on a reversed-phase column with a mobile phase containing tetrapropylammonium bromide as an ion-pair reagent. The column effluent is monitored by measuring the fluorescence intensity of estriol conjugates. This method is capable of measuring estriol and its glucuronide and sulfate conjugates.

Electrochemical detection has also been used in the analysis of urinary estriol. Gunasingham et al. [55] measured urinary estriol after acid hydrolysis by normal-phase LC with electrochemical detection. To overcome the difficulty of low dielectric constant of the mobile phase in normal-phase LC, they added tetraalkylammonium salts as the supporting electrolyte to the eluent. They have claimed an order of magnitude better sensitivity with electrochemical detection compared with fluorescence detection. An LC method with fluorescence detection was reported by Taylor [56]. In this method, a 2-ml aliquot of urine is hydrolyzed at 55°C for 30 min, after the hydrolysis estriol is extracted with 20 ml of diethyl ether in the presence of 1.0 ml of 10 *M* sodium hydroxide. The ether layer is discarded, the remaining aqueous layer is acidified with 1.5 ml of concentrated hydrochloric acid, which is then extracted with 35 ml of diethyl ether. The ether layer is back-extracted with 3 ml of carbonate buffer, the ether layer is separated, evaporated, and the residue reconstituted in 1.0 ml methanol. A 10-μl aliquot is injected onto the LC system, and estriol separated on a reversed-phase column. The effluent is monitored by a fluorescence detector set at an excitation wavelength of 220 nm and emission wavelength of 304 nm. Ager and Oliver [57] have surveyed different LC methods for the separation of estrogen conjugates in urine and synthetic mixtures. The readers are urged to refer to this review article as well as the paper by Van der Wal and Huber [58] for the separation of estrogen conjugates. A general survey of various LC methods for the analysis of estrogens is illustrated in Table 2.

6. GENERAL DISCUSSION

It is evident from the discussion in this review, that there is not a single LC technique or procedure available for measuring the common steroids of interest in the clinical laboratories. Most of the clinical laboratories use some kind of RIA, homogenous immunoassay, or competitive protein-binding methods for the analysis of endogenous corticosteroids, endogenous estrogens, and vitamin D isomers. These techniques can be readily adapted for batch analysis and are usually less demanding of the expertise available in the clinical laboratories. However, they usually suffer from non-specific interferences from closely related compounds or from compounds of unknown origin. Therefore, in many analyses a chromatographic or solvent purification is often necessary to accurately measure small amounts of many of these naturally occurring steroid hormones. LC can carry out the function of isolating and quantitating the small amount of these compounds without much difficulty. However, this technique has the disadvantage that batch processing is not possible. However, with automated sample processing equipment interfaced with LC instrumentation, the analysis can be run on

24-h basis. The LC procedures are usually much more specific and can be used for the analysis of plasma or urine cortisol, plasma or urinary estriol, and isolation and purification of vitamin D metabolites prior to their measurement by receptor binding or RIA method.

7. SUMMARY

At present, there are various LC methods available for the determination of estriol, aldosterone, and cortisol in urine, and for cortisol, cortisone, 11-deoxycortisol, corticosterone, 17-hydroxyprogesterone, estriol, vitamin D isomers, and various exogenous glucocorticoids in serum. The LC methods are more specific than the currently available RIAs or homogenous immunoassays. However, whether the data obtained by more specific LC methods are better clinically than the commonly used immunoassays for these steroids must still be proven. In this review, I have critically evaluated various LC methods currently available for the routine determination of clinically important steroids in the clinical laboratories. A complete evaluation and advantages and disadvantages of alternative techniques are beyond the scope of this review. However, the readers are urged to refer to the review articles and chapters listed in the reference section.

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