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Review

High-performance liquid chromatographic analysis of corticosteroids

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Abstract

This review presents recent developments in high-performance liquid chromatographic (HPLC) analysis of corticosteroids for the determination of clinically important steroids in biological specimens. Various sample preparation techniques are described.

Contents

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Abbreviations

Steroid abbreviations

Aldo	Aldosterone
B	Corticosterone
DOC	11-Deoxycorticosterone
21-DOE	21-Deoxycortisone
21-DOF	21-Deoxycortisol
DXM	Dexamethasone
E	Cortisone
F	Cortisol
18-OHB	18-Hydroxycorticosterone
18-OHDOC	18-Hydroxydeoxycorticosterone
18-OHF	18-Hydroxycortisol
17-OHP	17-Hydroxyprogesterone
P	Progesterone
S	11-Deoxycortisol
THE	Tetrahydrocortisone
THF	Tetrahydrocortisol

Other abbreviations

ACTH	Adrenocorticotropin
CAH	Congenital adrenal hyperplasia
CS	Cushing's syndrome
HPLC	High-performance liquid chromatography
LC	Liquid chromatography
MLC	Micellar liquid chromatography
11-OHD	11β-Hydroxylase deficiency
17-OHD	17α-Hydroxylase deficiency
21-OHD	21-Hydroxylase deficiency
SE	Solvent extraction
SPE	Solid-phase extraction

1. Introduction

Measurement of plasma and urine concentrations of corticosteroids are of clinical significance in adrenal and pituitary dysfunctions [1]. Saliva concentrations of corticosteroids have also been studied intensively but there are still problems in the interpretation of data to clearly define their potential value and limitations [2]. Corticosteroid concentrations are also affected by both naturally occurring and synthetic corticosteroids which are commonly used in medical practice [1].

A comprehensive evaluation of adrenal gland function requires the determination of as many steroids as possible from a single specimen. Previously used materials for column chromatography (e.g., Sephadex LH-20, Celite and Lipidex) for separating the steroids before quantification by radioimmunoassay (RIA), could not be operated with a high pressure [3]. So these columns were never fast to run and the separation of steroids on multiple columns was very time-consuming. Nowadays applications concerning the analysis of corticosteroids mainly use HPLC as a separation method with both normal-phase and reversed-phase chromatography and isocratic and gradient elution. Detection may be by UV absorbance [4–45], fluorescence [46–62], mass spectrometry [63–79] or RIA [80–92]. The predominant advantage of this technique arises in the feasibility of its automation, thus eliminating the laborious processing of the chromatographic step. As the quantities of most corticosteroid hormones in serum, urine and saliva are extremely low, RIA has been used for quantification in most studies. However, in recent methods the good detection limits for the steroids have resulted from the combined influences of the mobile-phase composition, columns and the high absolute extraction efficiencies of the analytes. Therefore also other types of detection have turned out to be sensitive enough for measurements of low steroid concentrations.

Recent applications in HPLC analysis of corticosteroids use on-line coupling of liquid chromatography with thermospray mass spectrometry (LC-TSP–MS) and atmospheric pressure ionization mass spectrometry (LC-API–MS) which enable structural information of the compounds for their identification. Direct analysis of steroids eluting from HPLC columns allows on-line detection of polar unconjugated molecules without derivatization and can be used for direct characterization of steroid glucuronides or sulfates without prior enzymatic hydrolysis. This provides distinct advantage for analysis of free and conjugated steroids from biological samples. By combining various detectors (e.g. diode-array and

MS), still more representative steroid profiles can be obtained from a single sample specimen.

2. Corticosteroids: chemistry and biosynthesis

The biosynthesis of steroids from cholesterol and acetate has been studied extensively. Their structure consists of a cyclopentaphenanthrene nucleus comprising three fused cyclohexane rings (A, B, C) in a non-linear arrangement and a terminal cyclopentane ring (D). The carbon atoms are numbered as shown in Fig. 1. Adrenocorticotropic hormone (ACTH) stimulates the human adrenal cortex to synthesize and secrete a large number of steroid hormones. Most of the reactions are catalyzed by mixed-function oxidases which contain cytochrome P_{450} and require NADPH and molecular oxygen. Many of the enzymes have been extensively studied and characterized [97–99]. The two major categories of compounds are the adrenal androgens (androstenedione, dehydroepiandrosterone and testosterone) and the corticosteroids. These C_{21} steroids of the pregnane series exert two main types of action, mineralocorticoid and glucocorticoid effects. All natural corticosteroids and most of the active synthetic analogs have a 21-hydroxy

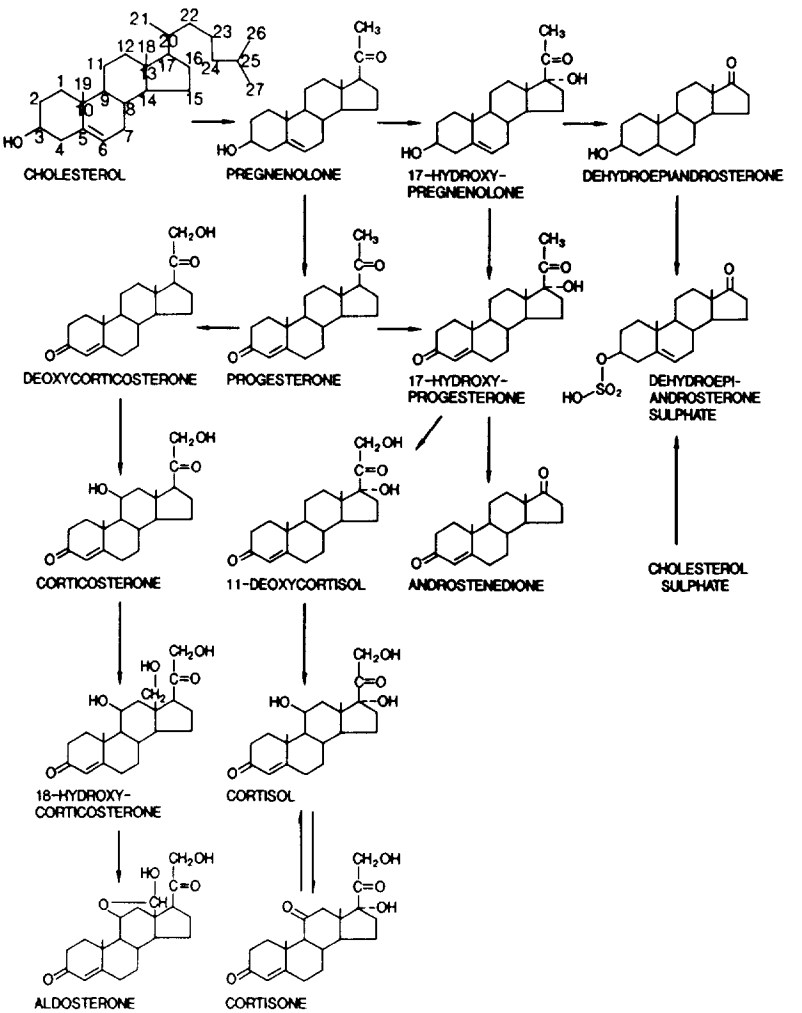

Fig. 1. Human adrenocortical steroidogenic pathways.

group. Orientation of the groups attached to the steroid ring system is important for the biological activity. The methyl groups at C18 and C19, the hydroxyl group at C11, and the two-carbon ketol side-chains at C17 project above the plane of the steroid and are designated β (full-line bonds). The hydroxy at C17 projects below the plane and is designated α (dotted bond).

It has been demonstrated that corticosteroids in plasma are largely bound to proteins, mainly corticosteroid-binding globulin (approximately 85%), and that these protein-bound corticosteroids are biologically inactive, whereas the non-bound fraction is active. Albumin has also binding capacity for corticosteroids but with relatively low affinity (10%). Less than 1% of total cortisol is excreted unchanged in urine, the rest is excreted as metabolites and conjugates. The level of urinary free corticosteroid has been said to reflect that of the non-bound fraction in plasma, because the free fraction in plasma is excreted by the kidney, whereas the protein-bound fraction is not [100]. The non-protein-bound or free fraction of hormones in blood is generally considered to be the biologically active moiety and a better indicator of physiological status than the total hormone concentration [101].

2.1. Endogenous steroids of interest

Cortisol, aldosterone and progesterone belong to the primary steroid hormones which have assigned biological functions (Table 1). These or their metabolites (tetrahydrocortisone, tetrahydrocortisol, 5α-tetrahydrocortisol, 20-dihydrocortisol, 6β-hydrocortisol, tetrahydroaldosterone) are frequently determined for evaluation of endocrine function. Measurement of other hormonal corticosteroids (17α-hydroxyprogesterone, 21-deoxycortisol, corticosterone, deoxycorticosterone, 18-hydroxydeoxycorticosterone, 18-hydroxycorticosterone, 18-hydroxycortisol) provides information on possible errors in corticosteroid biosynthesis [1].

Aldosterone is the major mineralocorticosteroid in man, but deoxycorticosterone (DOC) and corticosterone also have significant activity. Compounds such as 18-hydroxydeoxycorticosterone [82,88] and compounds modified at position 19 (19-hydroxy or 19-nor) may also contribute to the total mineralocorticoid action of the adrenocortical secretion [81]. The 18,19$(OH)_2$-corticosterone [89,117] and 18-OH-19-nor-corticosterone [116] compounds have been recently synthesized and also detected in human urine.

Cortisol is the major human glucocorticoid. Recently also its 20-dihydro isomers have been found in human urine [9] as well as 18-hydroxycortisol and 18-oxocortisol [84].

2.2. Corticosteroid drugs

Most of the corticosteroids possess a Δ^4-3-keto group, a dihydroxyacetone side-chain at C17, and generally an oxygen function at C11. A large number of analogues with glucocorticoid and

Table 1
Principal adrenocorticosteroids

Aldosterone	11β,21-Dihydroxy-3,20-dioxopregn-4-en-18-al
Cortisol (hydrocortisone)	11β,17α,21-Trihydroxypregn-4-ene-3,20-dione
11-Deoxycortisol	17α,21-Dihydroxypregn-4-ene-3,20-dione
Cortisone	17α,21-Dihydroxy-4-pregnene-3,11,20-trione
Corticosterone	11β,21-Dihydroxy-4-pregnene-3,20-dione
Deoxycorticosterone	21-Hydroxy-4-pregnene-3,20-dione
18-Hydroxycorticosterone	11β,18,21-Trihydroxy-4-pregnene-3,20-dione
Pregnenolone	3β-Hydroxypregn-5-en-20-one
Progesterone	4-Pregnene-3,20-dione
17-Hydroxyprogesterone	4-Pregnen-17α-ol-3,20-dione

Table 2
Some synthetic analogues of adrenocorticosteroids

Betamethasone	9α-Fluoro-16β-methyl-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione
Dexamethasone	9α-Fluoro-16α-methyl-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione
Fludrocortisone acetate	9α-Fluoro-11β,17α,21-trihydroxypregn-4-ene-3,20-dione 21-acetate
Fluocinolone acetonide	6,9-Difluoro-11,21-dihydroxy-16,17-[(1-methylethylidene)bis(oxy)]-pregna-1,4-diene-3,20-dione
Fluoromethalone	(6α,11β)-9-Fluoro-11,17-dihydroxy-6-methylpregna-1,4-diene-3,20-dione
Fluprednisolone	6-Fluoro-11,17,21-trihydroxypregna-1,4-diene-3,20-dione
Halcinonide	21-Chloro-9α-fluoro-11β-hydroxy-16α,17α-isopropylidenedioxypregn-4-ene-3,20-dione
Halomethasone	2-Chloro-6α,9-difluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione
Halopredone acetate	2-Bromo-6β,9α-difluoro-11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione 17,21-diacetate
Meprednisone	17,21-Dihydroxy-16β-methylpregna-1,4-diene-3,11,20-trione
Methylprednisolone	11,17,21-Trihydroxy-6-methyl-1,4-pregnadiene-3,20-dione
Paramethasone	6α-Fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione
Prednisone	17α,21-Dihydroxy-1,4-pregnadiene-3,11,20-trione
Prednisolone	1,4-Pregnadiene-11β,17α,21-triol-3,20-dione

mineralocorticoid activity have been synthesized for therapeutic use, some of them are more potent than naturally occurring hormones (Table 2). Minor molecular modification of e.g. hydrocortisone gives prednisolone and prednisone.

3. Biomedical applications of measurement of corticosteroids

HPLC offers many advantages over older types of chromatography such as higher resolution, a higher degree of reproducibility and, as has been proposed in recent years, the possibility of full automation for the separation of a number of corticosteroids. The big advantage of an HPLC method is that one can simultaneously measure many endogenous and exogenous steroids. A steroid profile, in which the individual corticosteroids are measured, is a valuable screening test in cases in which dysfunction of steroid-forming organs (adrenals and gonads) is suspected. Valid ratios between precursors and products in the key steps of steroidogenesis can be easily obtained with an HPLC method. Stress is also known to induce enhanced activity of the pituitary–adrenal axis, resulting in increased secretion of corticosteroids from the adrenal cortex [102]. The control of adrenocortical function has been the subject of several comprehensive reviews [103,104].

3.1. Free and total concentrations

Free and total steroid concentrations have been determined by analysis of samples before and after hydrolysis. Steroids are generally present as water-soluble conjugates of glucuronic and sulphuric acid. Hence, when total steroid concentrations are required, the hydrolysis of ether and ester linkages is an obligatory step. Removal of proteins and other large macromolecules without precipitation can be achieved using ultrafiltration with cone-shaped membranes. This can be used to measure the concentrations of free or non-protein bound analyte in plasma or serum. Dialysis can separate an analyte from the matrix by diffusion through a semi-permeable membrane rather than centrifugal force such as with ultrafiltration. Dialysis can only be applied to compounds that are relatively weakly protein-bound. Strongly protein-bound solutes require the binding to be disrupted.

Two general procedures exist for the hydrolysis of conjugates. The acidic hydrolysis is rapid and has been used with excellent recovery for the measurement of serum cortisol [105]. 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 [106]. One of

the major problems with the enzymatic hydrolysis is the slowness of the enzymatic reaction. However, enzymatic hydrolysis has been used widely for the corticosteroids which are labile in strong mineral acid. It is also useful for avoiding interfering substances produced by acid hydrolysis.

3.2. Clinical diagnosis

Adrenal diseases such as congenital adrenal hyperplasia (CAH), Cushing's and Conn's syndromes and adrenal carcinoma can be diagnosed by measuring steroid excretions in pathological concentrations. According to recent publications, a comprehensive evaluation of adrenal gland function requires the determination of as many steroids as possible from a single sample. Examples of possible errors in biosynthesis are: 17α-hydroxylase deficiency (17-OHD), 21-hydroxylase deficiency (21-OHD), 11β-hydroxylase deficiency (11-OHD), 17,20-lyase deficiency, cortisol oxidase deficiency, cortisone reductase deficiency [1].

The major steroids involved in mineralocorticoid disorders are 11-deoxycorticosterone (DOC), 11-deoxycortisol (S), corticosterone (B), aldosterone (Aldo), 18-hydroxycorticosterone (18-OHB), progesterone (P) and cortisol (F). Schöneshöfer et al. [80] and Lejeune-Lenain et al. [83] have developed automatic procedures capable of estimating these steroids simultaneously. Fig. 2 illustrates the separation achieved for thirteen reference steroids and the gradient profile applied. An endogenous steroid profile consisting of F, cortisone (E), B, S, DOC, 17-hydroxyprogesterone (17-OHP) and P has been found quite useful in a detailed study of different types of adrenal hyperplasia by Ji-ging et al. [14]. The analysis of E, F and the precursors 17-OHP and S may be helpful for the prenatal diagnosis of adrenal enzyme deficiencies. Such a diagnosis has relied on steroid determination in amniotic fluid in previous studies [85].

Patients with CAH have a large variety of steroids in their serum, many of which are quite similar, such as cortisol and 21-deoxycortisol [160]. 17-OHD is a rare form of CAH. Deficiency of 17α-hydroxylase leads to reduced production of glucocorticoids and sex steroids, resulting in an increased secretion of ACTH. The elevated steroids in 17-OHD are DOC, 18-OHDOC, B, and 18-OHB, a major by-product of adrenal aldosterone biosynthesis [43,88]. Patients with a complete deficiency have also almost undetectable excretions of cortisol and its metabolites. The major urinary steroids are metabolites of B [107]. In cases of 11-OHD, the enzymatic system is missing in the mineralocorticoid and in the glucocorticoid pathways of the adrenal cortex, leading to impaired production of F and B. Carpené et al. [88] used F and B or F and S as a diagnostic tool in cases of 17-OHD and 11-OHD. In 21-OHD or 11-OHD CAH, the high levels of 17-OHP and S (Fig. 3) can be rapidly measured by HPLC for the precise diagnosis [8,11]. DOC was identified as the cause of mineralocorticoid hypertension in 11-OHD and 17-OHD [108]. It has been shown that there is an increased relative excretion of unconjugated metabolites of cortisol by patients with juvenile hypertension due to apparent 11β-hydroxysteroid dehydrogenase defect. The major unconjugated steroids excreted in this disorder are F, 6β-OHF and 20α-DHF. Cortisone metabolites (with 11-carbonyl group) are excreted in reduced amounts in this disorder [109,110]. In recent years, mass-screening for CAH, especially for 21-OHD (Fig. 4), has been performed to facilitate early diagnosis and treatment [26].

Disorders of the pituitary–adrenal axis can result in either overproduction (Cushing's syndrome) or underproduction (e.g. Addison's disease) of cortisol [1]. The measurement of urinary free cortisol appears to be a most sensitive and specific test for initial screening of Cushing's syndrome (CS). Schöneshöfer et al. [82] found the simultaneous estimation of urinary free cortisol and dehydroepiandrosterone (DHEA) to be useful in distinguishing patients with adrenal adenoma from those with Cushing's disease and ectopic corticotropin syndrome. Elevated plasma and urinary cortisol levels have also been postulated to play a role in the hypertension of CS. Similarly, 19-OH-, 19-nor- and related derivatives of steroid hormones have been

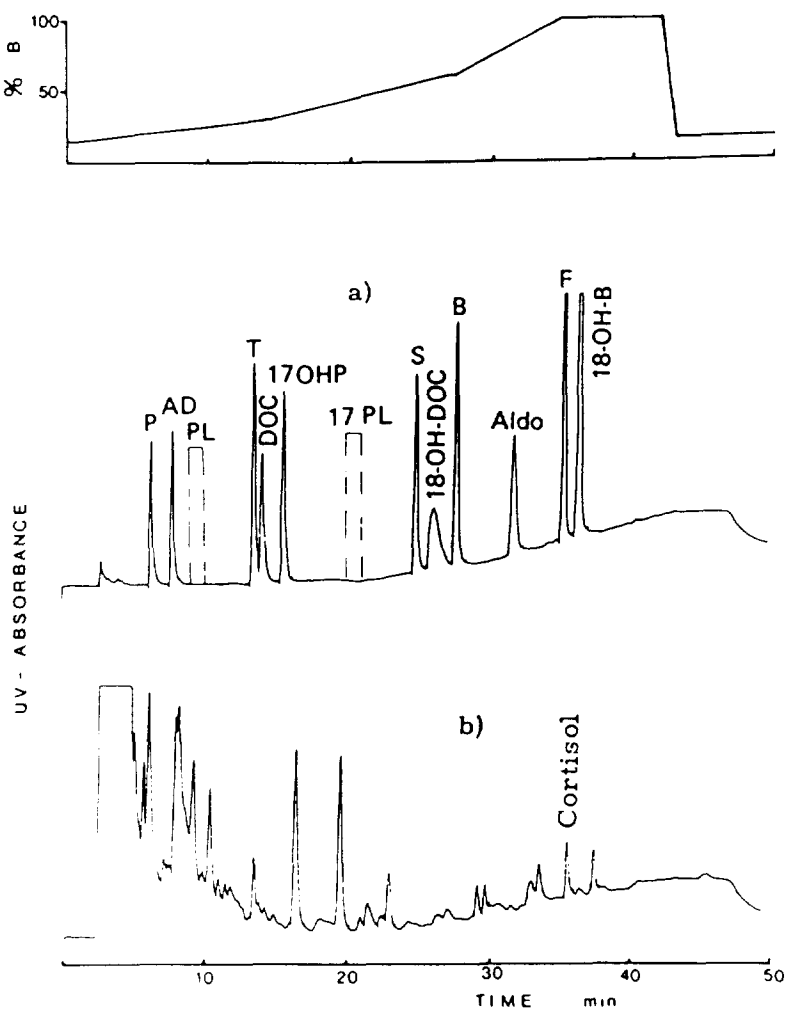

Fig. 2. Chromatograms of a mixture of steroid standards (a) and of the ether extract of a normal serum sample (b). Amount of the individual steroids injected (a) was 500 ng. Steroids not detectable by UV absorption were located by [^{3}H]-radioactivity measurement of fractions eluted (dotted lines). Upper part represents the gradient profile applied. DIOL column and *n*-hexane–isopropanol were used for HPLC. (From Ref. [80] with permission.)

suggested to play a role in hypertension. Dale et al. [111] isolated and quantitated 19-nor-deoxycorticosterone (19-nor-DOC) in a neutral fraction of human urine and it was found elevated in patients with low-renin essential hypertension and primary aldosteronism [112]. Griffing et al. [81] reported that their results clearly demonstrated a defect in 17α-hydroxylation associated with markedly elevated urinary 19-nor-DOC, which was under ACTH regulation. According to Gomez-Sanchez et al. [84], the excretion of 18-hydroxycortisol (18-OHF) correlated well with the excretion of cortisol, 18-oxocortisol and especially 19-nor-DOC. Chu and Ulick [113] isolated 18-hydroxycortisol (18-OHF) as a major steroid in the urine of patients with primary aldosteronism due to an aldosterone-producing adenoma. Since then, it has been shown to be an important adrenal steroid: in patients with Conn's adenoma and those with hypermineralocorticoid hypertension correctable by glucocorticoid suppression [157]. The latter patients had high urinary excre-

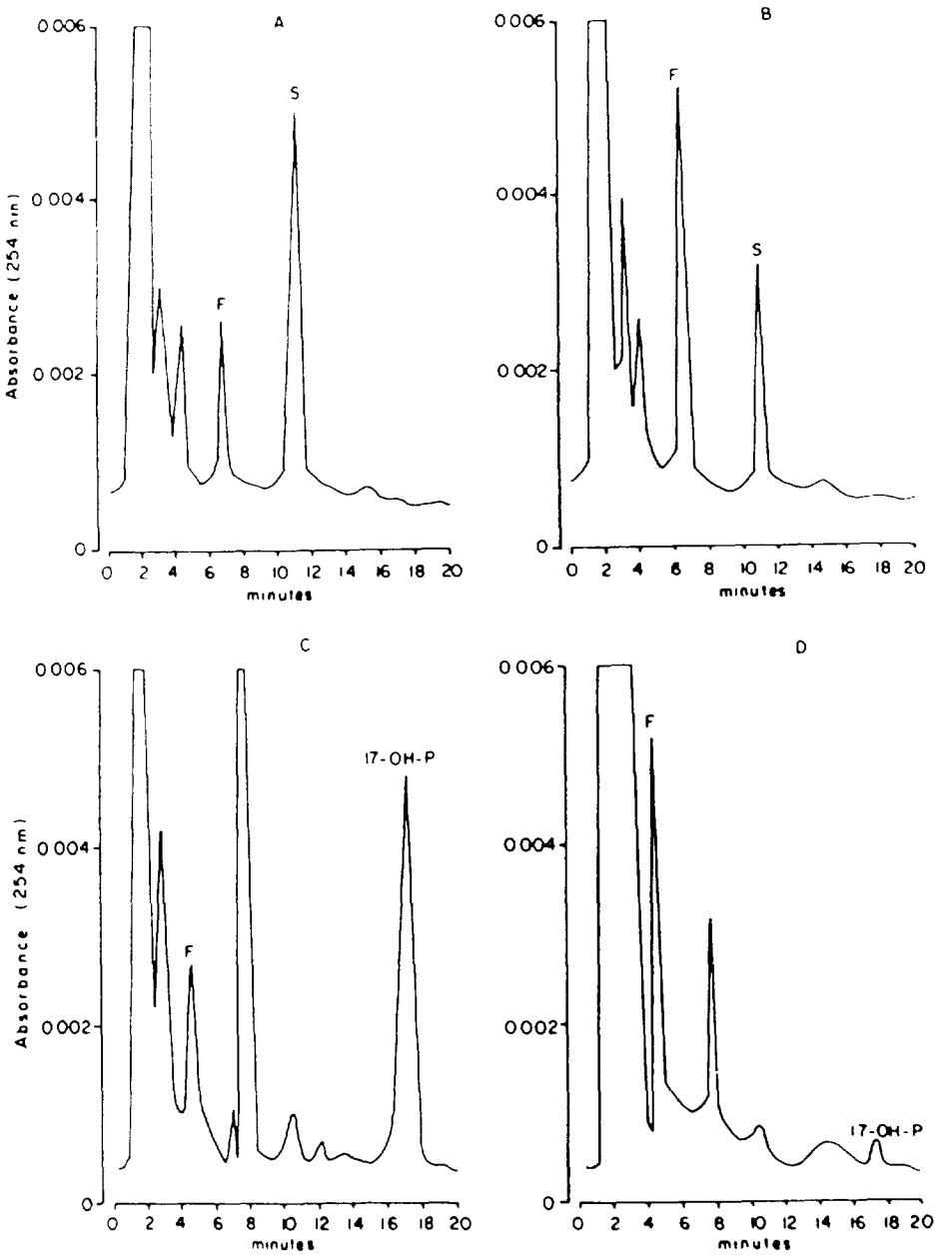

Fig. 3. Characteristic chromatograms of serum extracts obtained from patients with 11-β-hydroxylase deficiency or with 21-hydroxylase deficiency, before treatment (A and C) and following treatment with hydrocortisone (B and D). Mobile phase was methanol–water (60:40, v/v) in panels A and B and acetonitrile–water (43:57, v/v) in panels C and D. C_{18}-μBondapak column was used for determination. (From Ref. [8] with permission.)

tion of 18-OHF and low excretions of F and 20α-DHF. Liberato et al. [72] showed that the patients with juvenile hypertension had high excretions of cortisol and 20α-dihydrocortisol, but 18-OHF was undetectable. Small amounts of 6β-hydroxycortisol have been found in the urine of normal subjects, and it has been one of the major urinary excretory products in patients with

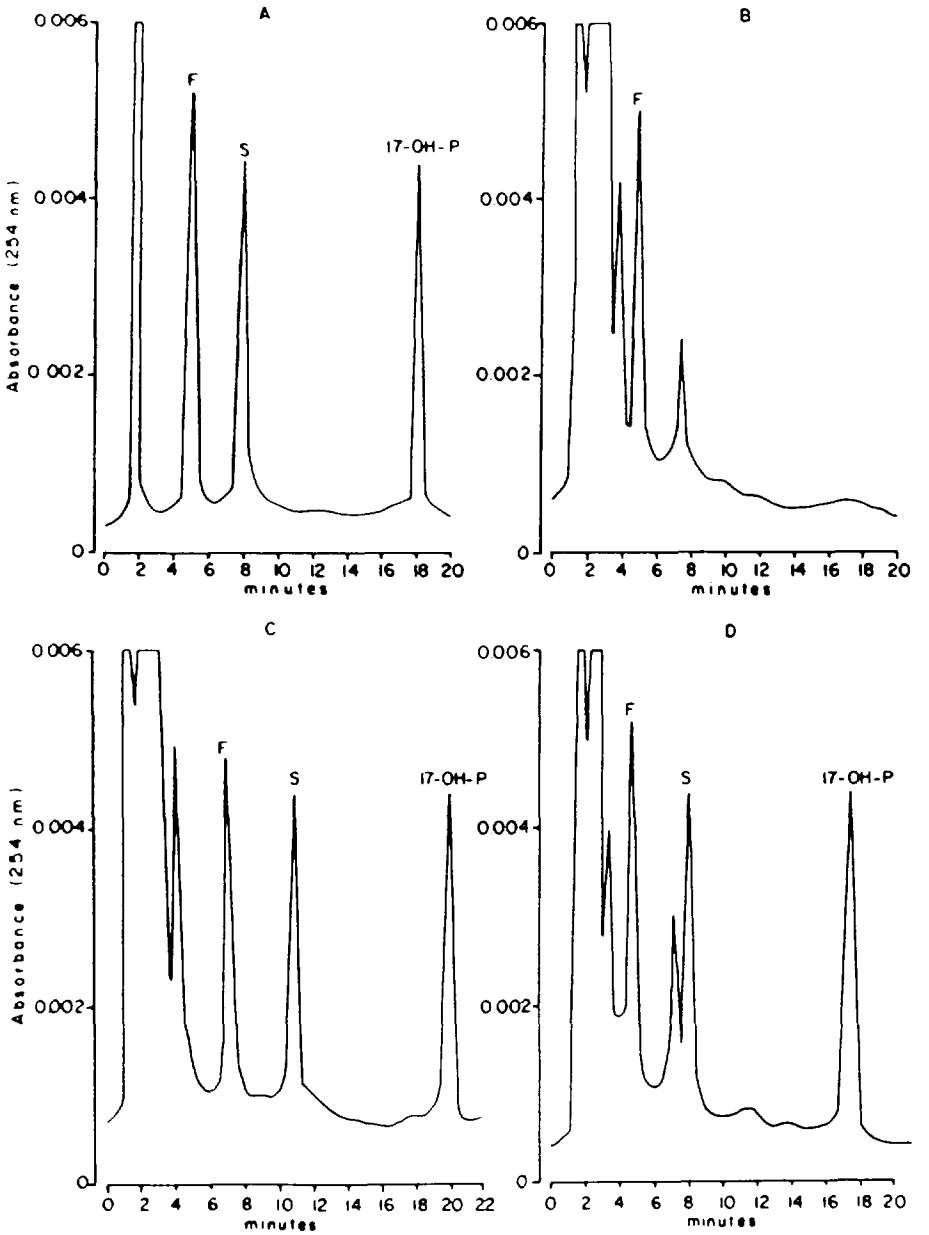

Fig. 4. Characteristic HPLC elution profiles. (A) A mixture of cortisol (F), 11-deoxycortisol (S), and 17-hydroxyprogesterone (17-OHP) utilizing acetonitrile–water (43:57, v/v) as mobile phase. (B) A serum extract obtained from a normal subject (acetonitrile–water). (C) The same serum extract with the addition of 40 ng of 11-deoxycortisol and 17-hydroxyprogesterone eluted with methanol–water (60:40, v/v) or (D) acetonitrile–water. C_{18}-μBondapak column was used for determination. (From Ref. [8] with permission.)

adrenal hyperfunction [114]. Schöneshöfer et al. [9] suggested the simultaneous monitoring of urinary cortisol (and cortisone) and its 20-dihydro isomers as a valuable tool for the definitive biochemical diagnosis of chronic hypercortisolism.

Aldosterone, which is one of the most potent mineralocorticoids, is occasionally elevated in patients with adrenal tumors producing CS, but it is rarely elevated in pituitary CS. DOC and 18-OH-DOC, on the other hand, may be increased in patients with both pituitary and adrenal CS [106]. The 19-noraldosterone, shown recently to be synthesized and produced in the human adrenal gland, possesses potent mineralocorticoid activity and has been detected with high urinary values in patients with Conn's syndrome [115,116]. In addition, 18,19-dihydroxycorticosterone (18,19(OH)$_2$B), a possible precursor for 19-noraldosterone, has been identified in human urine [117]. Urinary excretions of 18.19(OH)$_2$B and 18-OH-19-nor-B were elevated in the patients with primary and secondary aldosteronism compared with that seen in normal subjects and in subjects with essential hypertension and liver cirrhosis without aldosteronism [116]. Marked elevation of 18-OHB has also been found in primary aldosteronism due to an adrenal adenoma and with 17-OHD, but not in patients with idiopathic aldosteronism due to adrenal hyperplasia and with normal and low renin essential hypertension. Thus, measurement of plasma or urinary 18-OHB together with Aldo has proved very useful in the differential diagnosis of these hypertensive disorders [107].

Stewart et al. [161] have shown a markedly abnormal ratio of the urinary metabolites of cortisol and cortisone, a relationship controlled by 11β-hydroxysteroid dehydrogenase. The oxidation and reduction reactions are said to be controlled by separate enzymes. Most patients with cortisol oxidase deficiency are children and diagnosis is readily achieved by noting the low excretion of THE (relative to the THFs) and elevated excretion of unconjugated cortisol metabolites (e.g., cortisol, 6β-hydroxycortisol and 20α-dihydrocortisol). Patients with cortisone reductase deficiency convert all their cortisol into cortisone and its reduced metabolites [9,45].

Inactivity of the enzymes involved in conversion of corticosterone into aldosterone is a cause of hypoaldosteronism. Patients with this kind of deficiency have elevated excretions of the metabolites of B and 18-OHB. The measurement of urinary free Aldo, Aldo-18-glucuronide, THAldo, 18-OHB and 18-OHDOC was studied in distinguishing primary aldosteronism from essential hypertension in patients with typical and atypical primary aldosteronism and in patients with essential hypertension. The discriminating function of the THAldo determination was the best [118]. The clinical and laboratory findings in DOC-secreting adenoma, carcinoma and hyperplasia are similar to primary hyperaldosteronism. The difference is only in the mineralocorticoid produced [108]. Levels of various adrenal steroids in adrenal hyperfunction, adrenal enzymatic defects and adrenal carcinoma are shown in Ref. [119].

3.3. Pharmacotherapy

Corticosteroids are commonly used in medical practice. They are used in the form of steroid hormones as well as their synthetic analogues both for systemic treatment and for topical application.

Synthetic corticosteroids, e.g. prednisone, prednisolone, methylprednisolone and their water-soluble esters, have been used both in low and high doses [17,40,41]. Prednisone is both a prodrug and a metabolite of the active drug prednisolone [23]. It is the most widely used synthetic glucocorticoid in immunosuppressive therapy [29]. Synthetic corticosteroids such as 6-methylprednisolone (MP) have long been used in the treatment of a wide variety of disorders [1]. Methylprednisolone acetate (MPA), the sodium hemisuccinate salt (MPHS) and 21[8-[methyl-(2-sulfoethyl)amino]-8-oxooctanoate] sodium salt (MPSO) have been used extensively for treatment of endocrine, rheumatic and hematological disorders, collagen diseases, as well as other indications. Betamethasone (BTM), dexamethasone (DXM) and their prodrugs are highly potent fluorinated glucocorticoids and are widely used for the treatment of inflammation, allergies and adrenal cortex insufficiency [1]. Prolonged therapy with corticosteroids may result in suppression of pituitary–adrenal function that can be slow in returning to normal [1]. The incidence

of undesirable side effects seem to be related to dosage and duration of therapy [93].

The administration of the prodrug results in the hydrolysis of the ester to the pharmacologically active compound. Therefore, the ability to measure simultaneously the ester prodrug and the parent alcohol to examine their disposition would be valuable [15]. Various HPLC methods are widely used for pharmacokinetic studies of these drugs [17] as well as to provide valuable information about the presence of undeclared corticosteroids in commercial preparations [33]. For drug release testing of topical forms extremely sensitive methods are required. Methods have been developed based on both reversed-phase [20–22,24,29–31,33,38] and normal-phase elution [10,32,38,39] and UV detection. Hill and Langner [94] studied HPLC diode-array UV detection for toxicological drug analysis. Pre-column concentration has been used for increased sensitivity [95]. Valvo et al. [96] tested thirty corticosteroids which can be found in pharmaceutical preparations either as active principles and/or related impurities. Combined HPLC–RIA methods have been described recently for DXM and its prodrug [91,92].

4. Sampling

4.1. Sample collection and storage

The most frequently encountered specimens are urine, serum, plasma and, more recently, saliva. Corticosteroids are sufficiently stable so that no special precautions are necessary during collection of biological samples. However, 24-h urine specimens are collected in the presence of a preservative such as boric acid and ascorbic acid to prevent bacterial degradation of steroids and hydrolysis of steroid conjugates, or with no preservatives at 4°C. Erythrocytes should be separated rapidly by centrifugation in order to minimize steroid metabolism following sampling of blood. Although inconvenient to collect, analysis of a 24-h urine specimen represents the average steroid excretion over 24 h, thus avoiding the problem of circadian variation of steroid concentrations. Salivary sampling regimens, on the other hand, have the advantage of frequent, easy collection by non-invasive techniques which obviate difficulties of ensuring completeness of a 24-h urine collection [37,158]. Samples may be stored overnight at 4°C. For longer periods, storage at −20°C in airtight containers is adequate [11,80]. Repeated freezing and thawing can cause hydrolysis of conjugates and yield false high values for unconjugated steroids [103]. Homogenized tissue of adrenal glands and adrenal tumors have been analyzed after ether extraction [4].

4.2. Techniques for sample preparation

When there is a sufficiently high concentration of an analyte and there is a specific detection system, then dilution can be a very simple and effective means of sample preparation. Otherwise, there are basically two types of isolation techniques involved in the analysis of corticosteroids. The first one incorporating the principle of solvent extraction (SE) eliminates most of the plasma proteins, and appropriate washes with sodium hydroxide also eliminate many of the interfering phenolic estrogens and non-steroidal lipids present in plasma or urine samples. The major disadvantage of SE is emulsion formation which causes loss of compounds, leading to lower and variable recoveries. Because of the diverse range of polarities of the steroids, careful selection of extracting solvent provides limited selectivity and, thus, a crude fractionation. In the various SE 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 and ethyl acetate at a specific pH. In general for extraction of biological samples, the less polar the solvent the more selective it is, therefore the solvent of choice is usually the least polar one in which the analyte is still soluble. For example, hexane extraction of plasma recovers 85% of progesterone but only 1% of cortisol and corticosterone. Recoveries of ca. 100% have been obtained for F, E, B, 18-OHB, S and ca. 90% for

Pred, 18-OHDOC, DOC, Aldo and DXM with diethyl ether–dichloromethane (6:4, v/v). The distribution ratio between the two phases will be influenced by the choice of the extracting solvent, pH value of the aqueous phase and the ratio of the volumes of the organic to aqueous phases. If there is a low recovery of the analyte this can be enhanced by successive extractions of the sample to produce acceptable recoveries, but in practice it is often the case that a large excess of extracting solvent can be used in order to save time and achieve the same result. The recovery of polar steroids and steroid conjugates is improved by addition of inorganic salts (e.g. sodium chloride, ammonium sulphate and ammonium carbonate) to samples. Extraction of steroids from tissues requires generally a mixture of polar solvents such as ethanol and acetone or chloroform and methanol, although cleaner extracts are generally obtained with hexane and isopropanol. Most of the cortisol assay methods reported involve only liquid extraction.

The second emerging technique employing the solid-phase extraction (SPE) principle is becoming more popular for the extraction of both free and conjugated steroids from the physiological fluid. Excellent recoveries were reported for several endogenous and synthetic corticosteroids using this extraction technique [156]. Although the SE can provide a pure extract for the quantification of plasma cortisol, it is generally inadequate for urine samples. The utility of bonded-phase SPE cartridges is due to their specific interactions with the compound of interest, a mechanism quite different from solubility, the basis of SE techniques. The analyte can be bound to the solid-phase by a number of different mechanisms which are the same as for HPLC, i.e. hydrogen bonding, dipole–dipole interactions, hydrophobic dispersion forces and electrostatic (ionic) interaction. Examples of products employed for SPE are Sep-Pak C_{18}, Bond-Elut C_{18}, Extrelut, Tef Elutor, Serdolit AD-2 and LiChrosorb Si 60 columns. Chem Elut and Tox Elut columns contain diatomaceous earth as an adsorbent. These packings are characterized by high capacity and a high flow-rate of mobile phase and they can be used in a batch operation. The volume of eluting solvent is usually low (2–5 ml) so that a dry extract can be obtained rapidly by solvent evaporation. After phase separation, the portion of this second liquid phase can either be injected directly onto the LC, or more usually, the solvent evaporated and the residue reconstituted into a small amount of mobile phase or polar solvents that are compatible with the LC separation. The combination of SPE on cyclodextrin media, followed by a second SPE on the injector-mounted column has provided a relatively clean sample and necessitating injection of relatively large volumes of sample, thus enhancing sensitivity [37]. The SPE approach has the advantage that the precolumn can easily be switched on-line, which facilitates automation of the set-up if large series of samples have to be processed. The quantification of urinary free cortisol often involves on-line extraction or column switching techniques to yield a clean extract [133]. Recently, Santos-Montes et al. [156] compared SE and SPE for urine samples spiked with corticosteroids. It was found that the best recoveries were achieved using Serdolit AD-2 giving an acceptable clean-up of the samples.

Dialysis has been used as a purification technique in some reports. However, it is as such a slow process and it can only be used to prepare samples in which the analytes are at high concentration or with very sensitive detection, e.g. fluorescence detection. However, coupling dialysis with HPLC provides the ability to concentrate the analyte prior to analysis [18].

4.3. Automation of sample preparation

HPLC methods applied in bioanalysis usually require elaborate sample pretreatment, including deproteinization, removal of interferences and sample enrichment. Most studies referred to in this review have employed "off line techniques" for purification procedures. To fully justify automation, the laboratory must have a large throughput for the assay to be automated; to automate an assay with few samples would not be cost-effective. To automate the system, the various operations need to be coordinated so

that the system as a whole will function with precision and reliability, completely unattended [159]. SE has proved very difficult to automate efficiently. Most systems using SPE with disposable cartridges have employed off-line techniques. Some reports have appeared describing automatic column switching techniques using SPE [59,62,133]. When dialysis is used for on-line sample preparation, concentrations of the analytes have to be high because of low recoveries of this technique [18]. The problem of automation is of a technical nature and will be solved by manufacturers of HPLC equipment.

5. HPLC as a separation method

5.1. Stationary phases

Columns for the HPLC of corticosteroids are typically 150–250 mm in length and have an internal diameter of 2–5 mm. The most widely used stationary phases are non-polar in nature, with octadecyl ligands bonded to microparticulate silicon [20,24,29,30]. A reversed-phase procedure on stationary phases with a shorter alkyl chain (C_6) has also been described [16]. HPLC assays for prednisolone have been developed utilizing either the reversed-phase or normal-phase modes. The reversed-phase assay described by Cannell et al. [31] compares favourably with the report of Rocci and Jusko [120], who used normal-phase HPLC to analyse prednisolone, prednisone and their 20β-reduced metabolites in samples from kidney perfusion studies. Cyano columns have some hydrophilic properties, thanks to the weakly polar cyano functionality, but they can be used for reversed-phase LC because of their short alkyl-chain length [102]. In comparison of bonded phases with non-polar C_{18}, 1,2-dihydroxypropyl ether (DIOL), nitro and cyano functionalities, the DIOL column provided superior separations of steroids [121,151]. An added advantage of DIOL phases was the reversal in elution pattern relative to reversed-phase packings. A high degree of reproducibility of retention times is one of the most important requirements if steroid amounts not detectable by absorbance have to be fractionated prior to immunoassay. As the eluted fractions after chromatography have to be evaporated to dryness prior to immunological quantitation, the volatile nature of the eluents makes the polar-coated stationary phases unequivocally superior to the reversed-phase system which requires time-consuming evaporation of aqueous solutions.

While bonded silica phases possess the desirable characteristics of well-controlled particle size, pore structure, and mechanical strength as well as a rich repertoire of bonding chemistry, they become undesirable from the standpoints of pH stability and residual chemical activity of the unprotected silica support. Manufacturers use various methods to reduce the unreacted-silanol content, such as special synthesis procedures for the silica gel base material, exhaustive endcapping, and the inclusion of high-coverage or polymeric coatings. If residual silanols are not minimized they can interact with polar compounds and cause tailing peaks and variable retention times.

One of the interesting novel reversed phases to emerge recently, porous graphitic carbon (PGC), provides unique selectivities in addition to improved chemical stability and surface homogeneity [122]. PGC is produced by the graphitization of a phenol-formaldehyde resin-impregnated silica gel. PGC exhibits the inherent pH stability and the chemical homogeneity that a bonded silica phase does not. PGC is uniquely selective to positional and stereoisomers. With PGC steroids that differ only by one additional double bond (prednisolone–prednisone and hydrocortisone–cortisone) resolve far better than solutes differing by functional group. Wade and Haegele [57] described an HPLC–UV method for simultaneous measurement of cortisone and cortisol in human saliva with sample preparation by solid-phase extraction on cyclodextrin (CD) media. CDs are cyclic, non-reducing oligosaccharides and have a broad versatility in their chromatographic applications [123].

Sample pretreatment using short precolumns coupled on-line with the analytical column provides an efficient means for trace enrichment of analytes and sample clean-up in HPLC. In recent

years, coupled column chromatography has been introduced also in steroid separations [133]. Efficiency and sensitivity are also being improved by replacing the older 10-μm materials with more efficient 5-μm and 3-μm packings, as well as by using minibore columns (2 mm I.D.) [45] and microbore columns with the internal diameters less than 1 mm [124]. Advantages of the microbore columns include a significant reduction in the consumption of mobile phase relative to conventional HPLC columns with sharp, symmetrical peaks with excellent resolution.

5.2. Mobile phases

Several different approaches have been described for optimizing HPLC separations, most of which emphasize the control of band spacing or selectivity. The use of systematic procedures for the determination of optimum solvent compositions for HPLC serves to eliminate time-consuming trial-and-error approaches for analyses of this nature. The versatility of HPLC is mainly based on the big variety of chemical equilibria known to be useful for tuning separation selectivity [139]. A few optimization procedures have been described, such as solvent triangle [125], window diagram [126] and factorial design techniques [127] and multi-criteria decision-making methods [128]. Valkó and Slégel [140] used molecular modelling for mobile-phase optimization in RP-HPLC. Chong et al. [141] achieved the optimization of the solvent composition by making use of the overlapping resolution mapping scheme.

Method development for reversed-phase HPLC (RP-HPLC) procedures is most easily carried out by initial optimization of solvent strength (% organic in the mobile phase). This can be readily achieved by carrying out two initial experimental runs in either an isocratic or gradient mode, followed by computer simulation to map retention and resolution as a function of % organic [129]. West [130] utilized a 2-keto alkane system for calculating HPLC retention indices of a series of steroids, and then utilized the retention index to predict retention and resolution of the compounds as a function of solvent strength and selectivity. The ultimate goal was to utilize the capability to predict resolution for optimizing the separation of steroid mixtures or pairs of individual steroids.

The correct combination of stationary and mobile phases is essential. This is demonstrated by the reversed-phase chromatography of adrenal steroids on various ODS materials differing in the number of free silanol groups [131]. Many useful steroid separations have been achieved using isocratic elution with a binary solvent as mobile phase. In some cases, phosphate buffer is incorporated in the mobile phase. With these systems the buffer anion and pH exert a significant effect on the separation. The selection of the optimum mobile phase composition is difficult for multi-component separations by isocratic RP-HPLC. For a complex biological sample containing many hormones, the use of ternary mobile phases has permitted high selectivity [43]. The use of ternary mobile phases has simplified many otherwise difficult separations. In the case of normal-phase HPLC, the elution of highly polar steroids such as E and F takes a long time, and therefore a gradient system needs to be employed as the mobile phase. The success of a separation particularly for late eluting steroids often depends on the gradient shape. Non-linear, linear and stepped gradients have been used.

Shalaby and Shahjahan [30] tested the selectivity of different organic solvents: methanol, acetonitrile and tetrahydrofuran separately after mixing with phosphate buffer at different pH and with different ratios. All the tested analytes (dexamethasone, hydrocortisone, hydrocortisone acetate, prednisolone and prednisolone acetate) gave highly resolved, sharp peaks with acetonitrile–phosphate buffer (6:4) at pH 8 as mobile phase. Saisho et al. [26] separated several adrenal steroids without the use of any gradient system by using a mixture of methanol–acetonitrile–water (55:3:42, v/v/v) (Fig. 5).

Ionic surfactants have been widely used as pairing ions in RP-HPLC. They can be classified as non-ionic, anionic, cationic or zwitterionic on the ionisation characteristics of the polar group. Their use in HPLC has been at concentrations

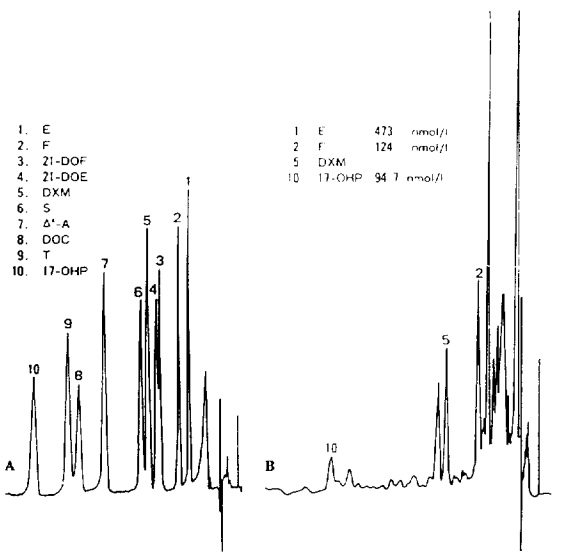

Fig. 5. (A) Chromatogram of the standard solution containing E, F, 21-DOF, 21-DOE, DXM, S, Δ^4-A, DOC, T and 17-OHP. Each peak corresponds to 20 ng. (B) Chromatogram of umbilical cord blood. A mixed solvent system composed of methanol–acetonitrile–water (55:3:42, v/v/v) was used as the mobile phase. An ERC-ODS-1161 and an ERC-ODS1652 were used for the main column and pre-column, respectively. (From Ref. [26] with permission.)

below the critical micellar concentration but at higher concentrations they tend to aggregate together, and above the critical micelle concentration they form micelles. The unique selectivity of such systems is due to the micellar core, which provides a hydrophobic site for identification with apolar solutes in the pure aqueous phase, and is due also to the combination of factors including the existence of adsorption, hydrophobic and electrostatic effects. Ionic surfactants strongly adsorb on chromatographic stationary phases. This adsorption can be modified by adding various organic solvents in the micellar mobile phase [132]. The use of micellar chromatography has been reported to allow a direct injection of biological fluids (serum, plasma and urine) [133]. In recent years column switching combined with the use of micellar clean-up prior to LC has been introduced. Sodium dodecyl sulphate (SDS) is added to the mobile phase for loading the untreated plasma/urine samples onto a precolumn. The proteins are solubilized by the SDS and washed out, whereas the analyte is retained [133]. Helboe [134] demonstrated nice separation of 12 common steroids by a reversed-phase column, dynamically modified by cetyltrimethylammonium bromide added to the eluent, and a mobile phase that is a mixture of an aqueous buffer and two organic solvents (e.g. methanol and tetrahydrofuran). When using this approach it has been shown that only minor brand-to-brand variations in selectivity occur. Hariharan et al. [45] added 5 ml of triethylamine per liter of water and adjusted the pH to 6.5 with citric acid; this minimizes the influence of residual silanol groups of the C_{18} packing material on the elution of the different steroids and simultaneously sharpens the peaks.

The chromatographic behavior of corticosteroids and its fluorescent derivatives with anthroyl cyanide was examined by the RP-HPLC by Shimada and Nonaka [135]. The separation of the compounds was much improved by the addition of the suitable cyclodextrin to the mobile phase. The presence of β-cyclodextrin in the mobile phase appears essential to enhance the detection of pregnenolone because of a poor UV absorption of this steroid [136]. This inclusion complexation of steroids with β-cyclodextrins (βCD) has been described by many authors [137,138]. Generally, the steroids form complexes with βCD in a 1:1 or 1:2 ratio.

5.3. Detection systems

The choice of detection system is governed largely by the expected concentration range of steroids and the sample type. The low levels of steroids normally encountered in biological samples require a detection of maximal sensitivity. Ultraviolet and fluorescence detection are usually part of HPLC analyses for steroid hormones. However, the limit of a quantitative determination of these methods is thought to be 0.5–1 ng, while RIA detects picogram quantities. Detection has been facilitated by pre-column or post-column derivatization of the steroids. Pre-column derivatisation is used to improve the sensitivity or selectivity of detection or to change the physical nature of the analytes so as to alter

their chromatographic mobility. In practice, post-column derivatisations are limited to those reactions that require simple conditions and only one or two reagents. Derivatizations must proceed rapidly and quantitatively to produce a single product under mild conditions which avoid undesirable alteration of the steroid. Furthermore, excess derivatizing reagent must be readily removed to avoid interference in the subsequent quantification. Simultaneous measurement with two complementary detectors offers considerable potential for the quantification of a steroid. For example, UV and fluorescence make a powerful combination. Further developments in coupled HPLC–mass spectrometric systems will undoubtedly enhance the role of HPLC in the determination of corticosteroids.

5.3.1. UV absorbance

The conjugated double bond found in most biologically active steroids shows maximum UV absorption around 240 nm. Detection sensitivities of 1–10 ng are found typically for the corticosteroids. The minimum detectability with HPLC–UV is determined by extraction methods as well as the sensitivity of the detector. The established standard among detectors in HPLC is the fixed wavelength (254 nm) UV detector. A variable wavelength or diode-array detector provides maximal sensitivity by monitoring the wavelength maximum [151]. The HPLC–UV measurement of urinary free cortisol, plasma cortisol and other glucocorticoids, especially 11-deoxycortisol, has been shown to facilitate the diagnosis of adrenal-associated glucocorticoid disorder such as Cushing's syndrome, adrenal hyperplasia and adrenal tumours. Patients with CAH have an abnormal elevation of serum levels of precursor steroids. All analytical problems involving corticosteroids in pharmaceutical preparations can be solved by HPLC–UV, because of the relatively high steroid contents. Several HPLC–UV methods have also been described for measuring synthetic corticosteroids in biofluids [20–24,30–33]. An HPLC–UV method is useful also for the follow-up of patients under treatment [8].

With some direct assays serious overestimation of serum cortisol levels can occur in patients with a disturbance of the normal cortisol biosynthetic pathway, either from an inherited defect, drug therapy or immaturity of the adrenal gland in newborns. Also deteriorating renal function leads to accumulation of steroid metabolites in blood in substantial concentrations, and antisera which cross-react with the conjugates will produce spurious results. HPLC techniques for measuring cortisol in serum/plasma and urine give good agreement with RIA values and are potentially free from interference. Besides, steroid analysis by HPLC offers the advantage of the simultaneous determination of several steroids with rapidity and specificity.

Since patients with enzyme deficiencies have elevated concentrations of corticosteroids, the sensitivity of UV detection is sufficient for accurate diagnosis. Such methods are rapid, specific, and can be of great importance for the determination of e.g. serum cortisol, 11-deoxycortisol and 17-hydroxyprogesterone needed for the rapid and accurate diagnosis of 21-hydroxylase deficiency and 11β-hydroxylase deficiency in neonates and infants with ambiguous genitalia or with unexplained salt-losing [152]. Since the serum levels of 17-hydroxyprogesterone and 11-deoxycortisol in healthy children are undetectable by this detection, the appearance of peak at the positions of these steroids is at once seen to be abnormal.

Recent work in several laboratories has shown that HPLC with UV detection can be used to determine low concentrations of several steroids simultaneously. Wade and Haegele [37] described a RP-HPLC–UV system provided with an injector-mounted reversed-phase extraction column for differential measurement of cortisol and cortisone in human saliva. Oka et al. [13] described a normal-phase method with a detection limit of 300 ng/l for corticosterone and 500 ng/l for cortisol. Hariharan et al. [42] developed a reversed-phase method for simultaneous analysis of corticosterone and cortisol in human plasma with a detection limit of 300 ng/l for both steroids. Chen et al. [39] described an HPLC–UV derivatization method for the simultaneous determination of BTM and DXM. The method is

based on the derivatization of the structural epimers of the drugs with a homochiral reagent, N-carbobenzoxy-L-phenylalanine, for better molecular recognition. De Beer [21] described an on-line HPLC diode-array spectroscopic method, which recorded on-line the UV spectrums, for the identification of more than 60 different steroidal compounds. By means of a BASIC search program, consulting a database with the retention parameters of all the steroids examined on the initial elution system, those steroids that had relative retention times close to the measured ones were selected for final identification. An HPLC retention index data base together with the UV spectral data base can provide a higher degree of confidence in results obtained from a library search routine [6]. As a peak recognition technique several statistics have been used [153].

5.3.2. Fluorescence

Although HPLC methods using UV detection offer simplicity, HPLC with fluorimetric detection provides the greatest sensitivity (typically 1 ng). Precisely controlled reaction conditions and the instability of fluorescence are limiting factors for the usefulness of fluorimetry.

Measurement of sulphuric acid-induced steroid fluorescence was introduced by Mattingly [48]. The basic HPLC fluorimetric detection method for corticosteroids, e.g. Gotelli et al. [50], involves extraction of aqueous samples with organic solvent, acid hydrolysis, with or without evaporating the extractant, isolating the acid sample and diluting with HPLC mobile phase. Mason et al. [60] added an ethyl acetate extraction step after acid hydrolysis of samples removing acid from the HPLC.

The separation of cortisol and prednisolone in the serum specimens has been quite difficult by isocratic RP-HPLC–UV methods. The method by Nozaki et al. [59] using precolumn sulphuric acid–ethanol fluorescence derivatization enabled the determination of serum cortisol with the detection limit of 3 ng/ml even though the serum contained both cortisol and prednisolone. The specificity resulted from the strong fluorescence of cortisol derivative while prednisolone emitted virtually no fluorescence. Use of the fully automated column-switching system contributed to good reproducibility and recovery. Many other methods based on HPLC coupled with pre-column fluorescence derivatization, have been developed for simultaneous assay of corticosteroids in biological samples [49,52,53,58]. Pre-column fluorescence labelling reagents, such as dansyl hydrazine [154] and 9-anthroyl nitrile [51], have been used for sensitive detection of cortisol. Sulphuric acid–ethanol fluorescence derivatization [46,47] of cortisol has the advantage of not requiring the elimination of labelling reagents, because they are not used. Katayama et al. [61] used pre-column derivatization with 2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole (CDB) for the determination of eight derivatives of corticosteroids. Detection limits of the steroids were 0.2–10.0 pg per 20 μl of acetonitrile (signal-to-noise ratio of 3), the method being 5–5000 times more sensitive than other HPLC methods using photometric detection [4–45] and HPLC methods using pre-column fluorimetric derivatization [49,52,53,58].

Yoshitake et al. [54] developed a sensitive and simple HPLC method with fluorimetric detection for the determination of 21-hydroxycorticosteroids, based on the conversion of the corticosteroids into the corresponding glyoxal compounds followed by reaction with 1,2-diamino-4,5-methylenedioxybenzene (DMB). The limits of detection (pmol) were 0.51 (Aldo), 0.71 (F), 0.42 (Pred), 0.45 (E), 9.2 (18-OHB), 0.77 (DXM), 29.4 (18-OHDOC), 0.14 (B), 0.69 (S) and 0.21 (DOC) in a 50-μl injection volume at a signal-to-noise ratio of 3.

Many recent adaptations of the acid-induced fluorescence method use more complicated equipment which may be unavailable to most laboratories. These include post-column hydrolysis [56] (0.5 pmol of corticosterone was detectable at a signal-to-noise ratio of 5) and injector-mounted extraction columns [57].

5.3.3. Mass spectrometry

Mass spectrometry (MS) affords specificity and sensitivity to steroid detection. Since the

mass spectrometer only detects ionizable components in the mass range indicated, many interferences can be avoided by choosing a mass range or "window" applicable to the compounds of interest but still above the majority of ionizable endogenous material. Because the corticosteroids are thermally labile, HPLC offers the advantage that the compounds can be analyzed without derivatization.

The greatest disadvantage to HPLC has been the difficulty with which it is interfaced with mass spectrometers. Many interfaces for the direct coupling of HPLC to MS have been described [65]. When direct liquid introduction with chemical ionization (CI) or moving belt interface with CI were used for measurements, significant degrees of thermal degradation occurred at lower concentrations [66,155]. Since the introduction of thermospray liquid chromatography–mass spectrometry (LC–MS) in 1983 by Blakely and Vestal [64], the combination of HPLC separations with specific mass spectrometric identification has become a practical technique. This methodology has increasingly been applied to the analysis of various non-volatile and thermally labile compounds, giving molecular mass information and limited fragmentation [75]. Thermospray response varies widely between different compounds, but derivatization has not been frequently used to overcome this problem. Compounds such as 18-hydroxycortisol are particularly troublesome in minimizing the number of derivatives formed because of interaction between the C_{18} hydroxyl and the side-chain. Detection limits of 5 pmol [74] and 14 pmol (5 ng) [76] have been reported for cortisol by LC–TSP–MS. However, Gaskell et al. [73] found LC–MS inferior to GC–MS in terms of sensitivity and precision and concluded that the magnitude and stability of the LC–MS response for cortisol had to be improved to make this method competitive. Nevertheless, quantitative data for a series of human sera showed satisfactory agreement with the GC–MS reference method. The use of the isotope-labelled internal standards has proved to be particularly important in LC–MS to compensate for the substantial variations in ionization conditions [70]. The technique by Esteban and Yergey [77] for measurement of plasma cortisol using a stable isotope dilution mass spectrometry (IDMS) is accurate and reliable providing the exact control of sample recovery. Paulson and Lindberg [79] used acetylation of the 21-hydroxyl group of cortisol and found that this rapid procedure increases thermospray response and decreases the detection limit of cortisol in biological samples. This group can be selectively acetylated under mild conditions without affecting the more sterically hindered 11β- and 17α-hydroxyl groups. Comparison between cortisol and acetylated cortisol showed that derivatization of cortisol increased the signal-to-noise ratio by a factor of about 4. The limit of detection (0.24 pmol) is twenty times lower than that reported by Esteban et al. [74], making LC–TSP–MS a viable alternative to GC–MS for the determination of cortisol in biological samples. Liberato et al. [72] recently presented data on the thermospray HPLC–MS of steroid glucuronides and Watson et al. [71] have discussed the negative-ion thermospray HPLC–MS of steroid sulphates and glucuronides. Shackleton et al. [68] used this technique for steroid profiling and carried out preliminary investigations on the separation of steroid glucuronides. Their promising results have been shown for the selected ion monitoring (SIM) of isomeric steroid glucuronides. The analysis of intact steroid conjugates would eliminate some of the problems associated with incomplete enzymatic hydrolysis of conjugated steroids. Micro LC–MS has been implemented into several limited corticosteroid determinations and proven successful [67].

Mass spectrometric detection with HPLC puts some restrictions on the mobile-phase composition that can be used. The mass spectra will depend on the reagent gas used, which in this case is the gaseous mobile phase. The mass spectra will therefore depend on the mobile-phase composition. Non-volatile modifiers have a tendency to restrict the orifice of the interface, requiring frequent cleaning. The gradual improvement of the thermospray interface and greater appreciation of the temperature and pressure factors which influence fragmentation and ion transmission will increase the sensitivity.

5.3.4. RIA

Currently, RIA is the technique most widely used to determine corticosteroids in human biofluids. A common problem with RIA techniques is specific and non-specific interference with antibody–analyte binding [142–145]. Although low cross-reactivities are frequently regarded as insignificant, the concentration of different steroids varies over a wide range. Hence, apparently small cross-reactivities can become significant. The specificity of RIA varies with antisera and commercially available kits [146]. Comparisons of HPLC to an RIA method have been reported for cortisol, prednisolone, corticosterone, progesterone patients with CAH, 18-hydroxycortisol and 19-nor-deoxycorticosterone [7,12,14,81,84, 147–149]. The resolving power of HPLC combined with the selectivity of the antisera provide a high degree of specificity. HPLC is used as a chromatographic technique for the purification step. The localization of steroid fractions during a chromatographic run is triggered by the retention times established by chromatography of UV-detectable amounts of steroids prior to each batch. The reproducibility of retention times must be excellent for the preprogrammed HPLC fractionation to be performed. Each peak may be collected individually to be analyzed by RIA, leading to qualitative and quantitative analysis of the material that can be detected by RIA. The predominant advantage of this technique arises in the feasibility of its automation, thus eliminating the laborious processing of the chromatographic step. Numerous methods have been reported [80,83]. Recently, Ueshiba et al. [87] described a method for simultaneously measuring steroid hormones in a 100-μl aliquot of serum, using a combination of HPLC and RIA. The problems associated with handling of a radiolabelled substrate may be eliminated by using a chemiluminescent marker [150,162].

6. Conclusions

This paper reviews LC techniques utilized in the profiling of corticosteroids in human body fluids. The recent emergence of commercial HPLC equipment coupled with sensitive detectors has made available an analytical approach for the assay of corticosteroids with low levels of concentration. HPLC offers the advantage that non-volatile steroid conjugates and thermally labile corticosteroids can be easily analyzed. Specific methods help to achieve objective and unambiguous conclusions about the presence or absence of an analyte, and to avoid false-positive results.

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