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Review

High-performance liquid chromatographic analysis of dehydroepiandrosterone

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Abstract

Qualitative and quantitative analysis of dehydroepiandrosterone and its conjugates in biological matrices and establishment of their relationships with physiological functions is a very active field. This review article discusses methods of separation and quantification of dehydroepiandrosterone and its conjugates using high-performance liquid chromatographic techniques.

Keywords: Reviews; Dehydroepiandrosterone; Dehydroepiandrosterone sulfate; Steroids

Contents

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1. Introduction

Steroids related to androgen biosynthesis regulate a variety of biological functions including reproductive and adaptive responses. Although principally synthesized by the adrenal gland and gonads [1], brain is a further site of steroid synthesis and metabolism [2,3].

Dehydroepiandrosterone (DHEA, I, Fig. 1) is a ubiquitous androgen, its sulfate conjugate (DHEAS, II) being the most abundant steroid in human plasma [4]. Man, with some other primates, is thus unique in having adrenals that secrete large amounts of the precursor steroids DHEA and DHEAS [5], which are converted, among others, into potent androgens and/or estrogens in peripheral tissues [6,7]. Interestingly, the circulating levels of DHEA and DHEAS decline progressively and markedly with ageing in distinct contrast to other adrenal steroids, such as glucocorticoids and mineralocorticoids, whose serum levels are relatively preserved with ageing. In addition to serving as a natural intermediate in the conversion of cholesterol to testosterone and estrogens, DHEA also acts independently of the sex hormones. It has been shown to be effective, albeit at doses higher than those expected for a true hormone type compound, in treatment of obesity in mice [8], rats [9] and, dogs [10], in increasing metabolic rate and thermogenesis [11] as well as in inducing the synthesis of thermogenic enzymes in rats [12,13]. It also depresses blood glucose in diabetic animals [14] decreases blood cholesterol [11,15,16], influences the activity of the immune system [17,18], reduces the incidence of tumors in mice [19,20] and enhances memory function in old mice [21]. Recently, ''DHEA deficiency syndrome'' has been introduced as a new term for old age [22]. DHEAS exhibits a variety of effects in the central nervous system, including interacting with γ-aminobutyric acid (GABA) type A receptors and sigma receptors, increasing memory and learning ability, and protecting neurons against excitatory amino acid-induced neurotoxicity [23]. DHEAS therapy may provide a beneficial effect on MID patients [24]. DHEA and DHEAS have been applied to the treatment of several disease conditions [25–28]. DHEA has been the subject of several conferences and review articles [29–36].

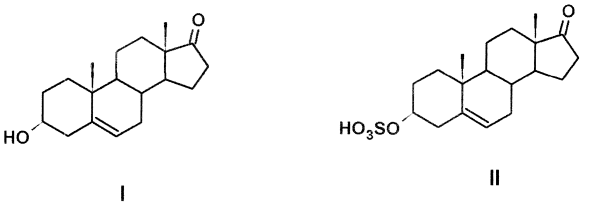

Fig. 1. Chemical structures of DHEA (I) and DHEAS (II).

In contrast to the very low concentration of DHEA in plasma, the concentration of its conjugate DHEAS is about 500-fold higher and does not reveal a circadian rhythm. Considered the leading marker of adrenal androgen secretion, plasma concentration of DHEAS is an important parameter for the evaluation of androgenic disorders [37]. It has been shown that DHEA and DHEAS levels in plasma are significantly increased in adrenal induced hirsutism [38,39]. Furthermore DHEA and DHEAS have been suggested to be indicators of ACTH secretion [40] and a tumor marker in women with breast cancer [41]. Consequently the importance of quantitative measurement of DHEA and DHEAS in body fluids and tissues cannot be over emphasized.

2. Dehydroepiandrosterone: biosynthesis and metabolism

The immediate precursor of DHEA is 17α-hydroxypregnenolone, a C_{21}-steroid, which is synthesized from cholesterol esters in a multi-step process via pregnenolone. The sulfation of DHEA is accomplished by steroid sulfotransferase, and the reverse reaction by steroid sulfatase. DHEAS can also be synthesized from sulfated precursors such as cholesterol sulfate and from pregnenolone sulfate. The biosynthesis and main metabolic pathways of DHEA are shown in Fig. 2. A relatively less understood metabolic pathway of DHEA is 7-hydroxylation followed by oxidation to 7-oxo-DHEA. Many of these conversions take place in organs other than the adrenal glands. Transformation of the adrenal precursor steroids DHEA and DHEAS into androgens, estrogens and other metabolites in peripheral target tissues depends on the level of expression of the

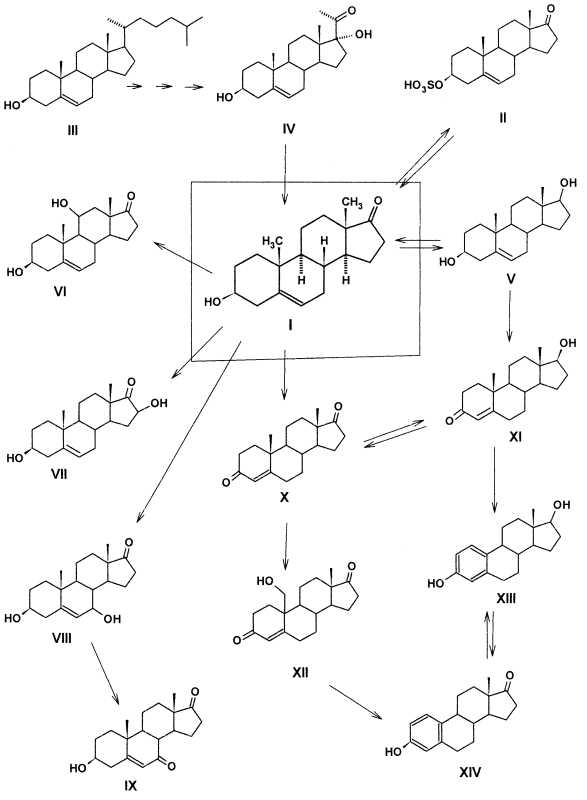

Fig. 2. Biosynthesis and main metabolic pathways of DHEA. I = DHEA; II = DHEAS; III = cholesterol; IV = 17α-hydroxy-pregnenolone; V = androstenediol; VI = 11-hydroxy-DHEA; VII = 16-hydroxy-DHEA; VIII = 7-hydroxy-DHEA; IX = 7-oxo-DHEA, X = androstenedione; XI = testosterone; XII = 19-hydroxyandrostenedione; XIII = estradiol; XIV = estrone.

various steroidogenic and metabolizing enzymes in each of these tissues [31,42].

3. Dehydroepiandrosterone sample preparation from biological matrices

It was realized long ago that some sort of purification of samples, particularly of those derived from biological matrices must be carried out before a satisfactory high-performance liquid chromatographic (HPLC) resolution can be achieved. The extent of sample clean up required, depends upon efficiency and the selectivity of HPLC technique, type of biological matrix and level of sensitivity required for the work. Use of a pre-column in combination with an analytical column using a column switching valve permits minimal sample work up [43] but the technique needs to be standardized before a large number of samples can be analyzed.

3.1. Solvent extraction

Liquid–liquid extraction involving use of water-immiscible organic solvents such as dichloromethane, diethyl ether, ethyl acetate alone or in combination with hexane or diethyl ether to reduce water solubility, etc., has been used to recover free DHEA, unconjugated metabolites and fatty acid esters, and has the advantage that it eliminates proteins to a large extent. Earlier workers used benzene for the purpose [44,45]. Addition of sodium hydroxide to the samples prevents extraction of phenolic compounds, e.g., estrogens [45] and also prevents emulsion formation to a great extent provided vigorous shaking is avoided. Liquid–liquid extraction has also been used in combination with solid-phase extraction procedures. DHEA and DHEAS have been extracted from serum by diluting with excess of water miscible solvents such as acetonitrile [46], ethanol [44,47] or methanol [48,49] and repeating the process when necessary. Extraction of DHEAS from plasma and brain by diluting the matrix with tetrahydrofuran has been reported [50]. DHEA fatty acid esters were extracted from human plasma and from rat and guinea pig sera by diluting plasma/sera with ethanol, centrifugation, evaporation of the ethanol, and extracting the residue with hexane followed by solid-phase purification [51].

Liquid–liquid extraction is usually inadequate for the extraction of polar conjugates such as sulfates. However such conjugates can be paired with cations derived from tetraalkylammonium ions using the principles of phase transfer catalysis. The technique has been applied to extract DHEAS from serum using tetrapentylammonium ion with benzene. It was followed by derivatization with dansyl hydrazine [52]. Recently, an efficient method was developed for the extraction of DHEA and DHEAS based on a combinatorial approach employed to obtain the most efficient liquid–liquid protocol. This allowed free steroids and their sulfated analogues to be isolated separately in a two-step procedure using diethyl

ether–hexane (90:10, v/v) in the first step to extract free steroids and chloroform–2-butanol (50:50, v/v) in second step to extract steroid sulfates [53].

3.2. Solid-phase extraction

Historically, DHEA has been separated by applying one or more solid-phase techniques, e.g., alumina adsorption chromatography, Sephadex LH-20, Celite partition chromatography, etc. [44]. Partition column chromatography using Celite impregnated with ethylene glycol was used to divide a group of 14 steroids including DHEA, Δ^5-androstenediol, androgens and estrogens into five groups of steroids which were subsequently resolved by reversed-phase HPLC using a μBondapack C_{18} column and an acetonitrile–water system [54]. Nonionic resins, e.g., Amberlite XAD-2 and ion-exchange resins [55–58] have been superseded to a large extent by C_{18} cartridges. Convenience and ease-of-use are primary benefits of present day solid-phase extraction cartridges, which permit extraction of compounds of varying polarities. Like HPLC, there are three modes to choose from, i.e., normal-phase, reversed-phase and ion-exchange. This technique offers, among others, faster sample preparation with better recoveries and greater accuracy. A unified scheme for the differential extraction of conjugated and unconjugated C_{19}-steroids including DHEA on Sep-Pak C_{18} cartridges was developed using added radioactive steroid standards and detection of endogenous serum steroids by HPLC and enzymatic assays to provide separate profiles of unconjugated, glucuronidated and sulfated androgens in human serum [59]. In another study, the serum was treated with urea (60°C for 30 min), applied to Bond-Elut C_{18}, conjugated steroids were eluted with 47% methanol and free steroids with pure methanol [60]. Raeside et al. [61] subjected plasma samples to solid-phase extraction on Sep-Pak, and free DHEA, 7-dehydro-DHEA and conjugated steroids were eluted with diethyl ether and methanol, respectively, solvolyzed and analyzed by HPLC on a NovaPak C_{18} column using acetonitrile–water.

Although methanol is a common solvent used to elute free and conjugated compounds from C_{18} cartridges, various workers have used different strategies for better extraction and separation. Unbound nonmetabolized unconjugated hormones were extracted by a solid-phase extraction of urine and serum using Extrelut (C_{18}) and eluted with diethyl ether [55,62–65]. Extrelut was also used to extract DHEAS at pH 9.5 with ethyl acetate. The crude DHEAS thus obtained was subjected to HPLC on a Hypersil-SAS column using borate buffer (pH 7.0) saturated with pentanol-1 [66]. DHEA of porcine follicular fluid was concentrated by chromatography on Sep-Pak C_{18} cartridges and purified further by cation-exchange SP-Sephadex C_{25} [58]. The urine and plasma of pregnant women were analyzed for 16-hydroxy-DHEAS and DHEAS, after fractionation through Sep-Pak cartridges, to study placental sulfatase deficiency [54].

Since the discovery of DHEA in rat brain [67,68] significant interest has developed in its biological functions in this organ. Low concentrations of DHEA in rat brain (<70 ng/g) necessitate extra purification for satisfactory analysis. The analysis of neurosteroids has been reviewed [69]. In one study, the whole brain was extracted with ethyl acetate, applied to Bond-Elut C_{18} cartridges and crude extract purified by preparative HPLC [70]. In another study, Shimada et al. [71] extracted DHEA from rat brain by a complex procedure, which can be roughly described as follows: rat brain was extracted with ethyl acetate–chloroform (6:1, v/v) and subjected to silica gel chromatography. The fraction containing DHEA was treated with pyridine–acetic anhydride followed by *O*-methylhydroxylamine in hydrochloric acid. The obtained acetate–methoxime was twice chromatographed on silica gel and then analyzed by liquid chromatography–atmospheric pressure chemical ionization mass spectrometry (LC–APCI-MS) [71]. Free and sulfated steroids have been extracted from frog brain with dichloromethane, the solvent evaporated and the residue applied to Sep-Pak C_{18} cartridges pre-equilibrated with hexane. Sulfated steroids were extracted with 30% tetrahydrofuran in hexane [72]. Liere et al. [73] recently described extraction of neurosteroids including DHEA from a small sample of rat brain (10–40 mg) using solvent and solid-phase extractions followed by HPLC purification.

For an LC–MS study of human plasma steroids, the plasma proteins were denatured by dilution and heat (60°C for 20 min), applied to Sep-Pak and eluted with methanol [74]. DHEAS, DHEA glucuro-

nide and several 17-ketosteroids were purified by C_{18} cartridges followed by reversed-phase (RP) HPLC and detected by ion-trap MS [75]. In our laboratory we have found polymer-based cartridges such as Oasis (Waters) to be superior to silica-based cartridges for the extraction of DHEA and its various unconjugated metabolites for LC–MS studies. Crude liver extracts obtained by solvent extraction, were subjected to solid-phase extraction on Oasis. Cartridges were washed with water and water–methanol and DHEA and its derivatives eluted with methanol. Recoveries were high (>95%) and consistent [76].

3.3. Chemical and enzymatic hydrolysis

DHEAS in urine or plasma was traditionally hydrolyzed with sulfatase (Helicase) [45] by incubating overnight at 37°C, followed by extraction with organic solvents such as dichloromethane. Arylsulfatase [48] and β-glucuronidase [59,119] and mixture thereof [77] have been used. Enzymatic hydrolysis is usually time consuming but reaction may be speeded up by increasing the temperature. Alternatively DHEAS can be hydrolyzed by the ''solvolysis'' method of Burnstein and Liebermann [78] which involves treating the biological sample with an organic solvent such as ethyl acetate, diethyl ether, etc., at low pH (~1) overnight at ambient temperature. Sulfuric [52,73,79] and perchloric acids [79] are used generally. Elevated temperature (~35–40°C) lowers the required reaction time [79]. DHEAS has also been hydrolyzed by heating at 100°C with aqueous sodium acetate [80]. The subject matter has been reviewed [81,119] and pros and cons of enzymatic hydrolysis discussed [82–85].

3.4. Derivatization

3.4.1. Chemical derivatization

17-Oxosteroids including DHEA have no strong UV absorbing group and historically their detection limits were more than 1 μg using a refractive index detector [43,86]. In order to increase the sensitivity in the assay of 17-oxosteroids, 2,4-dinitrophenylhydrazine (2,4-DNPH) has been used as carbonyl derivatizing reagent by several workers [46,87–92]. Recently, 2,4-DNPhydrazones (Fig. 3) of 17-ketosteroid sulfates and glucuronides from human urine

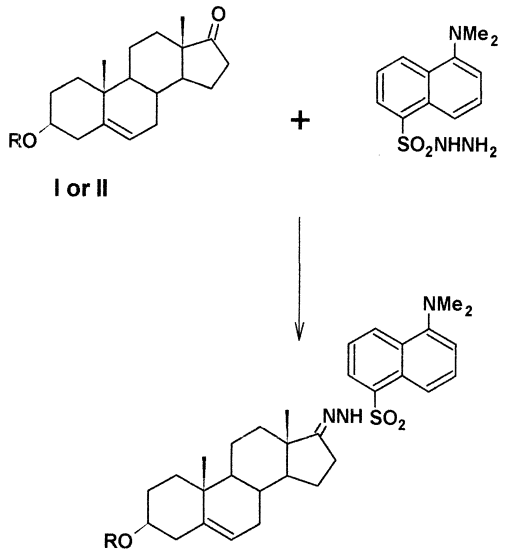

Fig. 3. Derivatization reaction of DHEA (I) and DHEAS (II) with dansyl hydrazine.

were studied by LC–MS [93]. Hydrochloric acid, trichloroacetic acid and trifluoroacetic acid have been used as catalysts in methanol, ethanol, acetonitrile, benzene and acetone as solvents [45,94–96]. Use of trifluoromethanesulfonic acid has also been reported [97,98].

Several fluorescence HPLC methods using dansyl hydrazine as derivatizing reagent have been reported [43,94,99–102]. The method is highly sensitive {detection limit for free 17-oxosteroids (Fig. 4) in urine and serum ~60 pg [45]}. The derivatization reaction was usually carried out in benzene in presence of trichloroacetic acid for 10 min at 60°C. The results obtained by radioimmunoassay (RIA) and this method, were found to be in good agreement for DHEAS [43]. The method was subsequently extended to direct estimation of sulfates and glucuronides [99] in urine [100].

3,4-Dihydro-6,7-dimethoxy-4-methyl-3-oxoquinoxaline-2-carbonyl chloride [103] and azide [104] were found to be highly sensitive fluorescence derivatization reagents for primary and secondary alcohols in HPLC. The detection limit for DHEA was 6.6 fmol using a YMC pack C_8 column with 70% aqueous methanol [103]. The use of benzene was later avoided by carrying out derivatization in

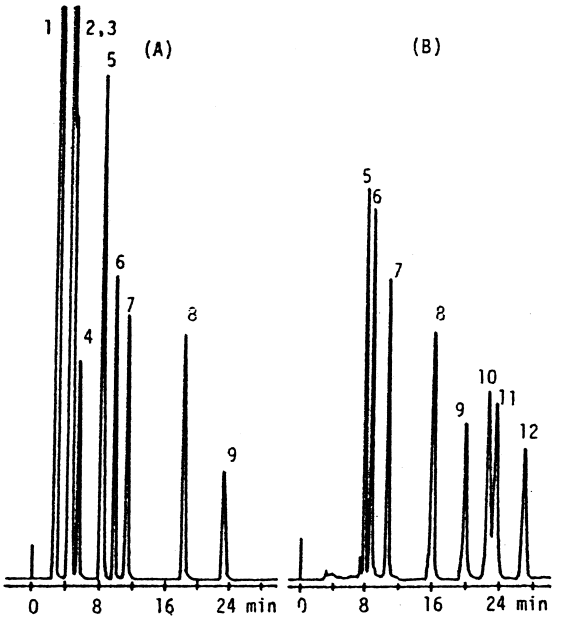

Fig. 4. Chromatograms of dansyl hydrazones of 17-oxosteroid standard mixture. Peaks: 1=androsta-3,5-diene-17-one; 2=androstanedione; 3=androstadienedione; 4=androst-4-ene-3,17-dione; 5=androsterone; 6=DHEA; 7=etiocholanolone; 8=11-oxoandrosterone; 9=11-oxoetiocholanolone; 10=16α-hydroxy-DHEA; 11=11β-hydroxyandrosterone; 12=16α-hydroxyandrosterone. (A) Hitachi gel (250×4 mm I.D.) column; mobile phase, dichloromethane–ethanol–water (400:1:2), 1 ml/min; Hitachi 204 fluorescence detector (excitation 350 nm, emission 505 nm). (B) Zorbax SIL (259×4.6 mm I.D.) column; Jasco FP-110 fluorescence detector (excitation 365 nm, emission 505 nm); other conditions as in (A). From Ref. [45], with permission.

acetonitrile [52,105] for the determination of DHEAS in urine along with 10 other sulfates and glucuronides [105] and for the estimation of DHEAS in serum [52]. 3-Chloroformyl-7-methoxycoumarin is another fluorescent derivatization reagent for alcoholic compounds, which was applied to the determination of DHEA in human urine. The conjugated 17-oxosteroids were enzymatically hydrolyzed and derivatized in acetone and/or benzene followed by HPLC on a C_{18} column using methanol–2% aqueous acetic acid. The detection limit was 0.3 pmol with a 20 μl injection [78]. The retention behavior of five 17-oxosteroids including DHEA derivatized with fluorogenic reagent 5-dimethylamino-1-naphthalenesulfonic hydrazide or 4-(*N*,*N*-dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole was examined on a C_{18} column and methanol–phosphate buffer or acetonitrile–phosphate buffer. Inclusion chromatography using cyclodextrin as a mobile phase additive is also used for this purpose and was found effective in separating the isomeric derivatized 17-oxosteroids [106]. DHEA was identified in rat brain after derivatization with 1-anthroyl cyanide [107] and 4-(*N*,*N*-dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole (DBDH) followed by HPLC on YMC C_8 or Wakosil normal-phase columns using acetonitrile–water and hexane–acetone or hexane–ethyl acetate mobile phases and fluorescence detection [69]. DHEA and five other oxosteroids were derivatized with 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionohydrazide (BODIPY) in ethanol using trifluoroacetic acid, and separated on a Wakosil 5C4 column using acetonitrile–water (7:3) as mobile phase. The limit of detection (LOD) for DHEA was 1.2 pmol ($S/N=5$) in human serum [108]. Benzoyl derivatives of urinary steroids were resolved on a slurry-packed fused-silica capillary column by micro RP-HPLC [109].

Derivatization reagents possessing ferrocene as an electrophore were developed for electrochemical detection. Ferrocenoyl azide and 3-ferrocenylpropionyl azide proved to be satisfactory for use in derivatization of alcoholic hydroxyl compounds with respect to sensitivity and reactivity. Hydroxysteroids including DHEA were readily converted with these carboxylic acid azides under mild conditions to provide urethanes, exhibiting maximum sensitivity at +0.4 V vs. Ag/AgCl with a detection limit of 0.5 pmol. The method was used to characterize the products formed from digitoxigenin by in vitro bioconversion [110]. From a group of 11 aromatic hydrazine and hydrazide derivatives, 2,5-dihydroxybenzoylhydrazide was selected as the most appropriate derivatizing reagent for HPLC of keto steroids using electrochemical detection. DHEA was satisfactorily resolved in a group of seven ketosteroids (Fig. 5) on a reversed-phase system and detected at +0.20 V vs. Ag/AgCl [111]. Analytical and mechanistic aspects of the electrochemical oxidation of ketosteroids derivatized with 4-nitrophenylhydrazine and 2,4-DNPH were investigated for their use in amperometric detection in biological fluids [112]. 4-

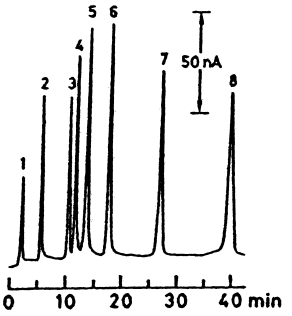

Fig. 5. Chromatogram of 2,5-dihydroxybenzohydrazones of a synthetic mixture of ketosteroids by HPLC with electrochemical detection. Peaks (concentration injected): 1=2,5-dihydroxybenzohydrazide (260 pmol); 2=corticosterone (650 pmol); 3=DHEA (570 pmol); 4=testosterone (830 pmol); 5=norethisterone (1330 pmol); 6=ethisterone (1280 pmol); 7=androsterone (1260 pmol); 8=pregnenolone (1710 pmol). HPLC conditions: column, Chemcosorb 5-ODS (150×4.6 mm I.D.); mobile phase, 0.05 *M* phosphate buffer (pH 7.0)–acetonitrile (64:36, v/v); flow-rate, 1.0 ml/min. Applied potential, +0.20 V vs. Ag/AgCl. From Ref. [111], with permission.

Nitrophenylhydrazones of ketosteroids were efficiently separated and estimated in human blood by electrochemical detection (LOD for DHEA 0.2 ng) [113].

3.4.2. Dehydroepiandrosterone analysis using immobilized enzymes as post column attachment

Lack of sensitivity was a major problem in the estimation of DHEA and DHEAS before the advent of bench top mass spectrometers with soft ionization techniques. This sent chromatographers scrambling for various alternatives. One such attempt led to the use of highly purified and active bacterial hydroxysteroid dehydrogenases (HSDs), which promote nicotinamide nucleotide-dependent stereospecific oxidations and reductions at specified positions of steroids. In the presence of catalytic amount of steroids, these enzymes promote the transfer of hydrogen (transhydrogenation) between nicotinamide–adenine dinucleotide (NAD) and reduced (dihydro) NAD (NADH) analogues. The method is highly sensitive and can be used to detect subpicomol quantities of steroids [114]. This technique led to the development of immobilized enzymes for the estimation of DHEAS in serum samples [47,115]. β-HSD was covalently bound to aminopropyl-CPG (controlled pore glass, pore diameter 5.44 Å) and used together with HPLC as a post column attachment. In this method on-line detection of individual Δ^5-3β-hydroxysteroids was performed using immobilized β-HSD and a fluorimeter to detect the resulting NAD transformation from the oxidized to the reduced form after separation by HPLC. β-HSD catalyses reaction to produce NADH [47]:

$$3\beta,17\beta\text{-Hydroxysteroids} + 2NAD^+ \text{—}(\beta\text{-HSD})\rightarrow$$
$$3,17\text{-oxosteroid} + 2NADH + 2H^+$$

The immobilized enzyme was sufficiently stable for 1 month or for 180 tests when used repeatedly. DHEA and DHEAS were separated from other steroids using a methanol–water (70:30 to 30:70) gradient. DHEAS and androstenediol could be measured simultaneously. Still other publications deal with an HPLC method to determine individual 3α- and 3β-hydroxysteroids (Fig. 6) in serum using 3α-

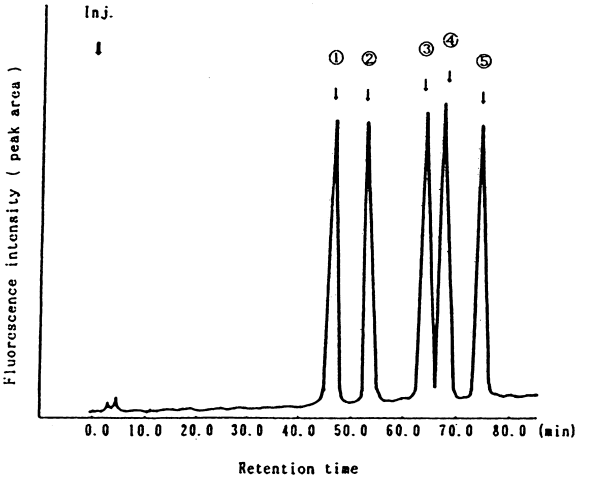

Fig. 6. Chromatogram of NADH produced by individual steroids. Peaks: 1=androstenediol (100 ng); 2=DHEA (300 ng); 3=etiocholanolone (50 ng); 4=androsterone (50 ng); 5=internal standard (5β-pregnane-3β,20α-diol, 400 ng). From Ref. [116], with permission.

HSD and 3β-HSD immobilized together on one column [116,117].

4. Qualitative and quantitative analysis of dehydroepiandrosterone and its conjugates with HPLC methods

4.1. Normal- and reversed-phase HPLC

There are two primary methods for determining steroid hormones: immunoassays mainly RIAs, to assay the specific hormones, mainly in serum [118]; and chromatography, e.g., GC and HPLC [119]. Difficulties in the measurement of DHEA by HPLC result essentially from (i) lack of a UV chromophore in the molecule, and (ii) low concentration in plasma (~15 to 30 nmol/l [4]). The strategies used for resolving and estimating DHEA and DHEAS, usually in biological matrices, are as varied as the technique of HPLC itself. Both normal-phase and reversed-phase techniques have been used extensively, very often after derivatization. Extraction, purification and estimation of androgens have been reviewed [32], and estimation of detection and quantitation limits in a model of HPLC analysis of a steroidal compound has been discussed [120].

A normal-phase silica gel column was used to resolve dansyl hydrazone derivatives [43] using a dichloromethane–ethanol–water (400:1:2, v/v) at 1 ml flow-rate followed by fluorescence detection (λ_{ex} 350 or 365 nm, λ_{em} 505 nm). The use of RP-HPLC on μBondapack C_{18} using 10 m*M* sodium acetate in methanol–water–acetic acid (65:35:1) for resolving dansyl hydrazones of 17-oxosteroid glucuronides and sulfates has been reported [121]. But the sensitivity achieved (~3–30 ng) was not as high as that obtained by normal-phase HPLC [43] of dansyl hydrazones.

The normal-phase resolution of 11 androgens including DHEA on two silica gel columns joined in series may be of historical interest only [122], but the system may serve as a guide to those interested in normal-phase studies. Because of tremendous advances made in column chemistry and packing, it may be possible to obtain still better resolution on short columns. HPLC of 19 hormonal steroids including DHEA was investigated on nitro, cyano and diol columns in normal-phase and compared with reversed-phase HPLC on a C_{18} column [123]. The diol column, run with a dioxane–hexane gradient system was shown to be superior to the other systems studied.

Lin and Heftmann [124] studied retention behavior of 69 androstane derivatives on normal- and reversed-phase HPLC. The total number of hydroxyl and keto groups on the androstane molecule was observed to be the most important factor in determining their chromatographic behavior. In general, hydroxyl groups contributed more to the polarity of the steroid molecules than keto groups, but an α,β-unsaturated keto group makes the steroids about as polar as does a hydroxyl group. We have observed [76] similar behavior for DHEA derivatives in reversed-phase HPLC. The order of elution was androstenetriols followed by androstenediols, 7-oxo-DHEA and DHEA (Fig. 7), verifying the above mentioned observations of Heftmann. Usually, axial 3-hydroxyandrostane derivatives eluted first in normal-phase HPLC followed by equatorial isomers. 17β-hydroxy derivatives were generally more polar than their 17α-epimers [124]. Separation of 3α/3β- and 17α/17β- and 5α/5β-isomers when the conjugate was on the 3α-hydroxyl has been reported [125]. A normal-phase HPLC system applicable to steroid separations involved in the assay of steroid 17α-hydroxylase and C_{17-20} lyase activities was developed using a diol column and hexane and hexane–isopropanol (75:25) gradient. The fraction containing DHEA was re-chromatographed using hexane and a hexane–isopropanol (85:15) gradient [63]. A hexane–tetrahydrofuran gradient system was used with silica stationary phase and a flow cell radioactivity detector [126]. Methods of analysis of steroid metabolism with radioisotope detection using solid scintillator [127] and flow through radioactive detector [128] have been documented.

Originally, the hydrolysis of conjugated steroids was the first step in their assay [129–133] but hydrolysis invariably results in the loss of valuable information concerning type or site of conjugation. Lafosse et al. probably made the first laudable though not so highly successful attempt to separate conjugated 17-oxosteroids [134]. Group separation of conjugated steroids prior to hydrolysis based on Sephadex LH-20 [56] and DEAE-Sephadex [135]

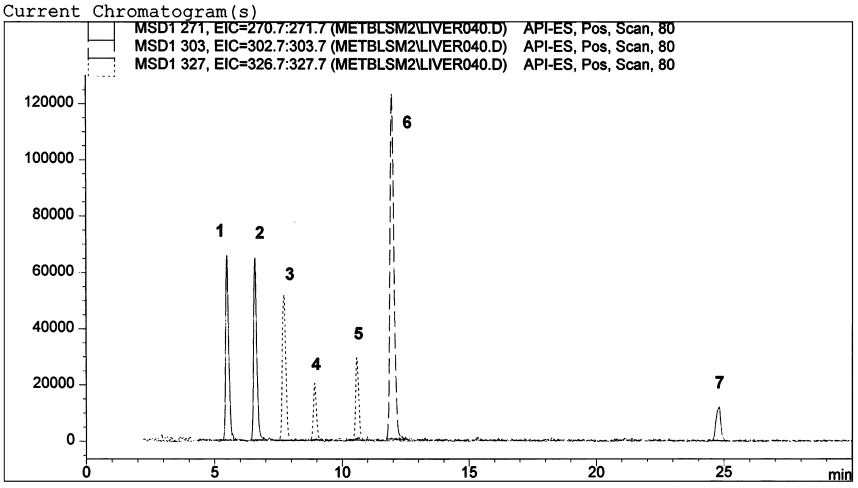

Fig. 7. Extracted ion chromatogram of 7-oxygenated metabolites of DHEA by LC–ESI-MS. Extracted from whole liver homogenate (200 ng/g). 1=3β,7β,17β-Androst-5-enetriol ($m/z=271$), 2=3β,7α,17β-androst-5-enetriol ($m/z=271$), 3=3β,17β-dihydroxyandrost-5-en-7-one ($m/z=327$), 4=7β-hydroxyDHEA ($m/z=327$), 5=7α-hydroxyDHEA ($m/z=327$), 6=7-oxo-DHEA ($m/z=303$), 7=DHEA ($m/z=271$). 25 ng each on-column. LC–MS conditions: column, Zorbax-C_{18}, 3.5 μm (75×4.6 mm); mobile phase, acetonitrile–water gradient, 20% to 50% acetonitrile in 30 min; flow-rate, 0.8 ml/min; drying gas, 12 l/min at 350°C; capillary voltage, 4500 V. From Ref. [76]; unpublished work of the authors.

has been developed. A method has been described for the determination of DHEAS in human serum by HPLC with electrochemical detection. The 17-ketosteroid sulfates were extracted with acetonitrile, derivatized with *p*-nitrophenylhydrazine [46] and resolved on a μBondapack C_{18} column using methanol–phosphate buffer (quantitation limit=80 ng/ml). Separation of DHEA, DHEAS and DHEA glucuronide on a Micropack CH column has been described. The retention data of these compounds allowed the comparison of the hydrophilic behavior of free and conjugated steroids [134].

A mixture of 13 steroids (urinary and synthetic) was separated in about 24 min using water–acetonitrile (60:40), a C_{18} column and UV–diode array detection (DAD) at 245 and 200 nm (DHEA) by applying Glajch's method with a possibility of application to urine analysis for different purposes such as clinical or doping control (see Refs. [136,137]). The use of a water–*n*-propanol gradient in resolution of DHEA from its biosynthetic precursors was found to be better than a methanol–water gradient [138]. Monocyte-derived macrophages converted [4-^{14}C]DHEA to significant amounts of Δ^5-derivatives such as 16-hydroxy-DHEA, androstenediol, and 3β,16α,17β-androstenetriol. However, the production of Δ^4-steroids (androstenedione, testosterone and 16-hydroxytestosterone) and estrogens was relatively low [139]. A combination of thin-layer chromatography (TLC) and HPLC was also used to study DHEA metabolism in gonads of turtle embryo [140].

Liebermann et al. studied the biosynthesis of DHEA in brain glial tumor cells using a combination of liquid–liquid extraction, solid-phase extraction on Sep-Pak, RP-HPLC followed by GC–MS analysis. A precursor of DHEA was proposed to be a steroid oxygenated at C-17 and C-20 [141,142]. 7α-Hydroxy-DHEA was shown to be a major metabolite in the metabolism of DHEA by cultured human adipose stromal cells using HPLC followed by GC–MS [143]. DHEA and other products formed from metabolism of digoxigenin were resolved on a TSK gel ODS column as ferrocene derivatives using electrochemical detection [110]. The concentrations of C_{19} steroids in guinea pig and rat adrenals before and after castration were measured by HPLC on a NovaPak C_{18} column using a gradient of methanol–water to acetonitrile–tetrahydrofuran [144]. An optimized isocratic RP-HPLC (acetonitrile–water, 45:55,

at 1 ml flow-rate on a C_{18} column) with on line radioactivity detection was used to study the conversion rates and metabolic patterns of the steroids in living cells [145]. Steroid metabolites synthesized by the gonads of teleosts were identified and quantitated using five systems of HPLC involving use of UV and refractive index detectors, and by TLC following HPLC purification [146].

We cite here the use of paper chromatography as a pre HPLC purification tool, lest we may forget this beautiful technique, which formed the backbone of many biochemical studies not long ago. Allenmark et al. were able to resolve and identify 13 testicular steroid metabolites including DHEA using a combination of paper chromatography and RP-HPLC [147].

Several fatty acid esters of DHEA, used in cell culture experiments (ZR-75-1 breast cancer cells) were resolved on a NovaPak C_{18} column and eluted with acetonitrile–2-propanol (68:32), and detected with on line radioactivity detection [148]. Shimada et al. resolved fatty esters of DHEA in rat brain on normal- as well as reversed-phase HPLC after converting them into 17*O*-methyloxime derivatives [149]. In another study involving analysis of DHEA fatty acid esters (palmitic, oleic, stearic, etc.) in human and rat plasma [51], choice of solvent system (hexane–isopropanol on a NovaPak C_{18} column) was a little unusual but perhaps the extreme nonpolar behavior of these derivatives justifies it. There is also a report on Sep-Pak purified frog brain tissue extracts being analyzed by a NovaPak C_{18} column equilibrated with 100% hexane. Sulfated steroids were eluted using a gradient of tetrahydrofuran (0–100% in 45 min) [72,150].

4.2. Paired-ion chromatography

Simultaneous separation of steroid-3-sulfates and the corresponding free steroids was accomplished in RP-HPLC by use of aqueous ammonium sulfate, which resulted in retention of the steroid sulfates to C_{18} silica packing by an apparent ion-pairing mechanism without affecting the retention of the free steroids [79]. The increase in retention of the steroid sulfate esters was attributed to ion pairing of the ionized steroid sulfate and the cation of the mobile phase. With increasing counter-ion concentration, their appears to be a greater neutralization of the steroid sulfate charge which increases the interaction of the hydrophobic portion of the steroid conjugate with the bonded phase and this results in greater k' values. Therefore, the steroid sulfates and the corresponding free steroids could be resolved simultaneously by RP-HPLC with the addition of a simple counter ion to the aqueous portion of the mobile phase [79]. A method to prevent co-elution of steroid sulfates (DHEAS) with proteins in serum, from the pre-column in column switching HPLC was developed by adding tetra-*n*-butylammnium ion to the mobile phase. Human serum was diluted with mobile phase and analyzed using polymer coated mixed function pre-column and an analytical column [43]. The resolution by isocratic RP-HPLC of a mixture of DHEAS, 16α- and 16β-hydroxyDHEA-3-sulfate in presence of several steroid sulfates was achieved using methanol–water eluent with addition of triethylammonium sulfate as paired ion reagent [151]. The effect of cetyltrimethylammonium ion on the elution pattern of DHEAS and 16-hydroxy-DHEAS in presence of estriol conjugates using a Hypersil ODS column has been studied [54].

4.3. HPLC followed by quantification using radioimmunassays

RIA has been widely used for the quantification of DHEA/DHEAS [152] perhaps because of its simplicity and sensitivity, but quantitative data vary widely. The reliability of RIAs has been debated repeatedly because of the cross reactivity of antisera and because normal ranges reported in the literature vary considerably. Previous reports have stressed that different RIA kits lead to widely divergent concentrations when the same plasma was analyzed for DHEAS, the origin of the antisera used, was the feature that contributed most to the divergent results. Furthermore the pattern of these differences varies when plasma from patients with different androgenic disorders is analyzed [153,154]. The American College of Pathologists submitted the same four samples to more than 200 laboratories for DHEAS analysis, and the results were shown to differ by a factor of four between the high and low values [74]. When the same samples were analyzed both by LC–MS and RIA, the RIA values were generally double of those

obtained by LC–MS [74]. However, there are conflicting reports regarding the values obtained by HPLC studies and RIA. Good correlation between the values obtained by the HPLC methods and by RIA in serum has been reported [46]. A comparative study of DHEAS analysis using RIA and enzymatic analysis was published [80].

The selectivity of RIAs has been improved by HPLC [155–158] of biological matrices of interest, collection of fractions corresponding to elution pattern of DHEA, followed by RIA of collected fractions. This avoids the problem of cross reactivity to a great extent, but not the effect of different RIA kits, the limitation of the technique being the possible interferences from co-eluting compounds and efficiency of the fraction collection device. Urinary unconjugated DHEA and several other androgens [55], conjugated and free steroids [60] were thus estimated by methods involving solid-phase extraction, HPLC and subsequent RIA of individual steroids. Levels of androgens including DHEA and Δ^5-androstenediol in rat prostate and serum after castration were measured by HPLC–RIA [159]. Using a combination of HPLC and RIA, DHEA along with 12 other naturally occurring hormones was estimated in small volumes of serum (0.1 ml). The steroids were resolved on a C_{18} column using a combination of gradient and isocratic HPLC [160]. DHEA along with fourteen steroid hormones was estimated in a single human serum sample using normal-phase HPLC followed by RIA [161].

4.4. LC–MS

The LC–MS instruments involving soft ionization techniques in general do not depend on particular functional groups and can be used to confirm rapidly the identity of a wide range of organic compounds including large and small, polar and nonpolar molecules. LC–MS provides molecular mass and structural information depending upon fragmentation patterns, selectivity and sensitivity needed for quick identification of compounds, and quantification of trace-level components in complex matrices. It complements traditional HPLC detectors and permits direct analysis of polar nonvolatile conjugates without derivatization and/or hydrolysis; a major drawback of GC–MS.

Interfacing an HPLC with a mass spectrometer requires the use of volatile mobile phase additives when necessary. Acetic acid, formic acid, trifluoroacetic acid, ammonium acetate and formate are commonly used. Introduction of HPLC columns, which can withstand onslaught of high pH values (up to 11 pH) has enabled the use of volatile bases such as ammonia, *tert*.-ethylamine, etc. in the mobile phase.

The measurement of DHEA, DHEAS and related compounds by mass spectrometry has a long history. Plasma steroid sulfates were the first steroids to be analyzed by GC–MS as a result of development of efficient liquid chromatographic techniques (Sephadex LH-20) for their specific isolation [161,162]. DHEAS was also the first steroid to be quantified by GC–MS in the selected ion monitoring (SIM) mode [163]. However in all these earlier studies, DHEAS and other steroid conjugates have had to be hydrolyzed prior to analysis and derivatization was generally necessary [74]. This approach has a number of limitations. The enzymatic hydrolysis of some steroid conjugates may be incomplete in a particular matrix due to competitive or noncompetitive inhibition of enzymes. The inability of aryl sulfatase to cleave sulfate from the 17-position has been documented [82] and variation in hydrolysis with different β-glucuronidase preparations has been documented for other drugs [83,84]. Last but not least, it has been suggested that contaminants in some enzyme preparations used for combined hydrolysis may convert one steroid into another depending upon reaction conditions [85]. Use of GC–MS and LC–MS in steroid analysis has been reviewed [164–166].

A thermospray HPLC–MS method for the determination of serum DHEAS was reported using [7,7-$^2H_2$]DHEAS as an internal standard. Plasma steroid sulfates were separated on a C_{18} column using methanol–200 m*M* ammonium acetate (60:40). The method was used to determine DHEAS in adult human serum and the following values were obtained: males, 2.72 ± 0.25 μg/ml and females 2.15 ± 0.67 μg/ml [74]. Combined microbore HPLC–electrospray ionization (ESI) MS was used for the separation and identification of the individual components of a mixture of seven steroid sulfates including DHEAS [125,167] and detection of these

compounds by scanning and multiple reaction monitoring was reported [167]. Isocratic separation of steroid sulfates was carried out on a 1 mm I.D. C_{18} column using acetonitrile–water (15:85) at a flow-rate of 50 μl/min. The base peaks in mass spectra were due to the intact anion $[M-H]^-$, and usually these were the only ions present [167]. In the other study, HPLC was carried out on 1 mm I.D. C_{18} column (50 μl/min) or 300 μm I.D. packed fused-silica capillary with 3 μm C_{18} stationary phase (5 μl/min) using gradient elution with acetonitrile or methanol buffered with ammonium acetate. Triple quadrupole MS equipped with an ion spray interphase was used for detection. DHEA glucuronide showed predominantly $[M+NH_4]^+$ adduct while DHEAS formed a diammonium adduct $[M+NH_3.NH_4]^+$. Both, DHEAS and DHEA glucuronide exhibited a strong intense $[M-H]^-$ signal in the negative ion mode [125]. LC–MS of 2,4-DNPhydrazones of DHEAS has been reported [93]. Shimada et al. have reported separation and characterization of DHEA in rat brain by APCI-MS of the acetyl-methyloxime derivative [71]. The method was subsequently applied to the characterization of DHEA fatty acid esters (stearate and palmitate) in rat brain [149]. HPLC coupled with APCI-MS has also been used for the estimation of DHEAS in serum and tissues (brain, heart, adrenal, etc., Fig. 8) [168,169].

An extremely sensitive nanoESI-MS has recently been described [53] for DHEA, DHEAS and other steroids. The limit of detection for steroid sulfates from the biological matrix was 200 amol/μl (~80 fg/μl) with only 1 μl of sample being injected. Precursor ion scanning performed with a triple quadrupole MS was used to examine quantitatively the extracted free and conjugated steroids. Endogenous levels of free and conjugated steroids including DHEA were detected and quantified from physiological samples including urine and blood using $[^2H_2]$DHEA as the internal standard. DHEAS was detected in cerebrospinal fluid by LC–MS on a 2.1 mm Zorbax C_{18} column using acetonitrile–water (30:70) [53]. The sulfonation of DHEA has been studied in intact cultures as well as subcellular preparations using LC–MS. Additional structure information could be obtained by applying higher cone voltages or MS–MS analysis [170]. Analysis of DHEAS in serum of normal and premature babies has been reported [171].

A recent publication [75] deals with quantification of urine 17-ketosteroid sulfates (Fig. 8) and glucuronides by LC–ion trap MS with sonic spray ionization technology. The sonic spray technique, in which charged droplets are produced by spraying the analyte solution at a gas velocity higher than the sonic velocity so that gaseous ions are emitted from

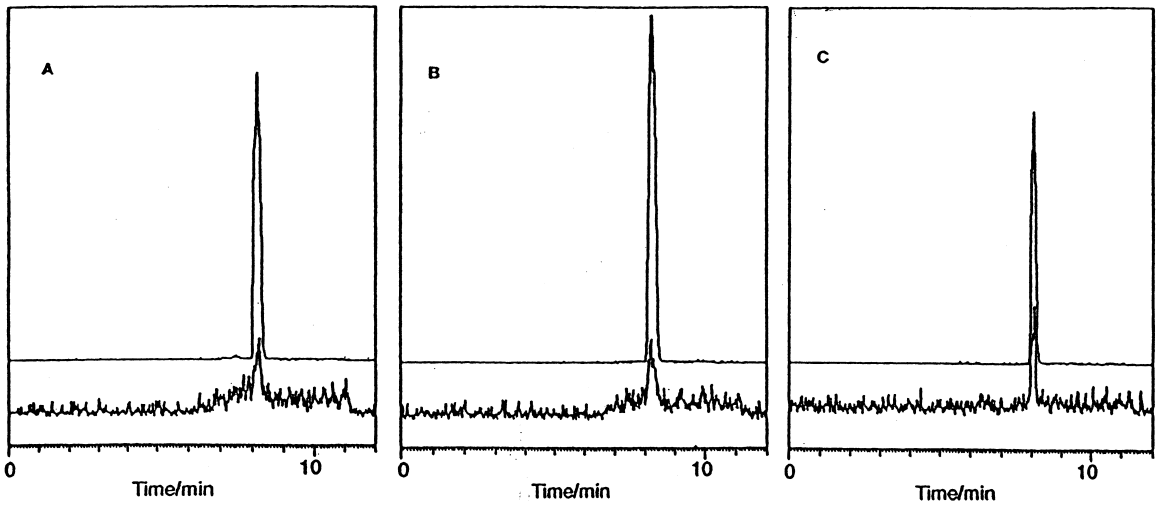

Fig. 8. Selected ion recording of DHEAS (*m/z* 367) and [7,7,16,16-2H4]-DHEAS (*m/z* 371) obtained with rat tissues, A = brain, B = heart, C = adrenal. From Ref. [168], with permission.

droplets without heat or application of an electric field. It was shown that under direct injection of 17-ketosteroid in 100 m*M* buffer solution, sonic spray generated a signal ~20 times stronger than that obtained using electrospray technique, thereby enabling use of high buffer concentration without any apparent loss of sensitivity. Using this technique DHEAS and DHEA glucuronide could be quantified up to 10 and 3 pg/μl, respectively [75]. Prough et al. have studied the metabolism of DHEA in rat and human liver microsomal fractions using LC–APCI-MS. Metabolites identified in both species included 16α-hydroxy-DHEA, 7α-hydroxy-DHEA and 7-oxo-DHEA [172].

5. Biological studies and applications

5.1. Dehydroepiandrosterone in body fluids

The measurement of DHEAS has proved to be valuable for assessing adrenocortical disorders [173,174]. A study of urinary excretion rates of nonmetabolized, unbound steroid hormones indicated that simultaneous estimation of urinary free cortisol and DHEA might be useful in differential diagnosis of hypercorticoid states due to adrenal adenoma and Cushing's disease [62]. Use of urinary free cortisol as an index of adrenal function [56] prompted a study involving estimation of free DHEA, androstenedione, testosterone, cortisol, aldosterone and 18-hydroxy DOC (DOC = 11-deoxycorticosterone) as a potential tool for assessing adrenal status in patients with Cushing's disease, Addison's disease, ectopic corticotropin syndrome and hirsutism [55]. There were no significant differences in excretion rates, although both urinary free DHEA and testosterone were distinctly lower in females than in males. Studies on potential clinical use of serum DHEAS assay in anovulatory women suggested that adrenal androgen excess is a cause of anovulation, a finding that was reported by several other investigators as well [47] (and references cited therein). The steroids profile, associated with Cushing syndrome caused by adrenal adenoma was quite distinct from that associated with the syndrome caused by adrenal hyperplasia. Serum concentrations of DHEA were significantly higher in patients with adrenal hyperplasia than with adenoma [160].

DHEA was isolated and identified in the extracts of fetal ovarian blood. This finding added substantial support to suggestion of fetal gonadal secretion of DHEA as a precursor in a feto-placental unit for estrogen production in the mare. In the eighth month of pregnancy the principal steroid secreted was apparently DHEA [175,176]. Using HPLC and RIA, Raeside et al. further showed that DHEA and 3β-hydroxyandrosta-5,7-dien-17-one were secreted in large quantities by the remarkably hypertrophied fetal gonads of both sexes of in-utero foals, but showed a decline in late pregnancy when fetal gonads regress, and declined rapidly within a few days after birth. The mechanism and significance of this abrupt decline in gonadal steroidogenesis is still unknown [61].

Total 17-ketosteroid sulfate concentration, measured by LC–ion trap MS, and normalized by creatinine showed negative correlations with age, and it could be utilized as a convenient and sensitive biomarker of aging-related DHEA/DHEAS deficiency [75]. Serum concentration of DHEA and DHEAS were significantly lower in patients with hypothyroidism than in age- and sex-matched controls. By contrast, in patients with hyperthyroidism, serum DHEAS was significantly higher, but serum DHEA concentration was within the reference range [48]. The plasma concentrations of several neuroactive steroids were measured in menopausal women by HPLC–RIA. Only DHEA and its metabolite 5α-androstane-3α,17β-diol, a possible allosteric modulator of GABA receptors, were significantly higher in the asymptomatic group indicating that this endogenous steroid modulates the central GABAergic tone and it may have a role in preventing the expression of anxiety in the asymptomatic women [177].

The transport characteristics of neurosteroid DHEAS, investigated at the blood–brain barrier in a series of functional in vivo and in vitro studies, revealed that DHEAS efflux transport takes place across the blood–brain barrier, and studies involving in vitro DHEAS uptake, and reverse transcriptase polymerase chain reaction (RT-PCR) suggest that there is oatp2-mediated DHEAS transport at the blood–brain barrier [50].

16-Hydroxy-DHEAS, the intermediary precursor in the biosynthesis of estriol, assumes importance in the genetic variant ''placental sulfatase deficiency'', and there is a strong tendency for the children of such pregnancies to develop ichthyosis. A reliable antenatal diagnosis can be made by determining steroid precursors, with 16-OH-DHEAS having the greatest discriminating power [178]. All cases of placental sulfatase deficiency showed substantial 16-OH-DHEAS peaks in the urine of pregnant women [179].

HPLC fractionation of plasma digitalis like factor (DLF) of adult plasma showed that DHEAS [180] and DHEA glucuronide [156] formed a major portion of DLF as it inhibited Na,K-ATPase and displaced [^{3}H]oubain from Na,K-ATPase. It has been reported that endogenous DLF or digoxin cause inhibition of Na,K-ATPase, displacement of [^{3}H]oubain from Na,K-ATPase, natriuretic activity, etc. [180].

5.2. Dehydroepiandrosterone analysis in body tissues

Early stages in the sequence of pregnenolone metabolism to testosterone were studied in homogenates of human and rat testes using HPLC followed by scintillation counting. In rat, metabolism of pregnenolone to testosterone proceeds via the Δ^4 pathway whereas in human it takes place almost exclusively via the Δ^5 intermediates, DHEA and androstenediol [181]. In the incubation of definitive fetal zone tissue using [^{3}H]pregnenolone as precursor, the Δ^5-hydroxysteroids 17-hydroxypregnenolone and DHEA and their sulfates comprised 85–90% of the metabolic tracer [182]. A human ovarian thecal-like tumor cell culture model system was developed to study C_{19} steroidogenesis. Analysis of steroids was accomplished using HPLC and RIA [183].

ZR-75-1 breast cancer cells generated nonconjugated steroids from low density lipoprotein-incorporated lipoidal DHEA, suggesting that lipoidal DHEA may indeed act as substrate for potent steroid formation after entry into steroid target cells. HPLC analysis of the culture medium indicated the presence of DHEA and androst-5-ene-3β,17β-diol [148]. Formation of DHEA fatty acid esters by lecithin-cholesterol acyltransferase (LCAT) in human plasma high density lipoproteins has been investigated and it was suggested that LCAT was the sole circulating enzyme that has DHEA esterifying activities [51]. This subject has been reviewed [184].

Conversion of DHEA to physiologically relevant amounts of Δ^4- and other Δ^5-steroids and estrogens was demonstrated in monocyte-derived macrophages. The conversion leads to an increase of downstream hormones in target macrophages, which may be an important factor for local immunomodulation [139]. In vitro metabolism of pregnenolone was studied in uteri of untreated and luteinizing hormone treated mice that had been ovariectomized at late-diestrus stage. In the uteri of untreated animals, DHEA was identified, indicating that mouse uterus behaves like steroidogenic tissue [185].

RP-HPLC analysis of frog telencephalon and hypothalamus extracts showed the presence of substantial amounts of DHEAS-immunoreactive material which coeluted with synthetic DHEAS. The concentration of DHEAS detected in the telencephalon and hypothalamus were, respectively, eight and five times higher than in serum thereby demonstrating the occurrence of HST-immunoreactive material in neurons of the frog telencephalon and diencephalons [72]. The telencephalic cells have an active form of 17β-HSD [150] and are capable of synthesizing DHEA and other hormones [72,186].

5.3. Dehydroepiandrosterone in pharmaceuticals and dietary supplements

Analysis of DHEA in bulk pharmaceuticals using light-scattering detection [187] has been reported. The detection limit was ~0.5 μg. The detector response varied with solvent composition. This detector was useful for determining impurities in bulk pharmaceuticals [188]. Analysis of DHEA in over-the-counter dietary supplements has been reported using HPLC with diode array UV detection [189]. A HPLC method using a Zorbax C_{18} column and methanol or acetonitrile with phosphate buffer as mobile phase, and UV detection at 292 nm was used to isolate and analyze DHEA from complex product mixtures [190]. DHEA in injections was determined using an ODS column, a mobile phase of acetoni-

trile–water buffered with tetrabutylammonium phosphate and refractive index detection [191].

6. Conclusions

High-performance liquid chromatographic techniques used in the profiling of dehydroepiandrosterone in biological matrices have been reviewed. The recent availability of bench-top mass spectrometers incorporating soft ionization techniques, which can be coupled to HPLC equipment, has made the accurate analysis of DHEA and its intact conjugates a bench-top reality. The technique of HPLC, especially, LC–MS adds significant value in the field of biochemical and clinical research by offering a highly reliable, specific and accurate yet sensitive alternative with a lot more potentials of profiling DHEA and its conjugates in complex biological matrices. This is likely to open new vistas in our understanding of biological functions of DHEA, once termed ''the fountain of youth'' [35].

7. Steroid nomenclature: abbreviations, trivial names and systematic names

ACTH	adrenocorticotropic hormone
Aldosterone	18,11-Hemiacetal of 11β,21-dihydroxy-3,20-dioxo-pregn-4-ene-18-al
Androstanediol	5α-Androstane-3α-17β-diol
Androstenediol	Androst-5-en-3β,17β-diol
Androstenedione	Androst-4-ene-3,17-dione
Androsterone	3α-Hydroxy-5α-androstan-17-one
Corticosterone	11β,21-Dihydroxy-preg-4-ene-3,20-dione
Cortisol	11β,17α,21-Trihydroxy-preg-4-ene-3,20-dione
Dehydroepiandrosterone, DHEA	3β-Hydroxy-androst-5-en-17-one
Dihydrotestosterone	17β-Hydroxy-5α-androstan-3-one
11-Deoxycorticosterone, DOC	21-Hydroxy-pregn-4-ene-3,20-dione
Estradiol	1,3,5(10)-Estratriene-3,17β-diol
Estriol	1,3,5(10)-Estratriene-3,16α,17β-triol
Estrone	3-Hydroxy-1,3,5(10)-estratrien-17-one
Etiocholanolone	3α-Hydroxy-5β-androstan-17-one
HSD	hydroxysteroid dehydrogenase
HST	hydroxysteroid sulfotransferase
MID	multi−infarct dimentia
Pregnenolone	3β-Hydroxypregn-5-en-20-one
Progesterone	4-Pregnene-3,20-dione
Testosterone	17β-Hydroxyandrost-4-en-3-one

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