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Review

Gas chromatography and high-performance liquid chromatography of natural steroids

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Abstract

This review article underlines the importance of gas chromatography (GC), high-performance liquid chromatography (HPLC) and their hyphenated techniques using mass spectrometry (MS) for the determination of natural steroids, especially in human biological fluids. Steroids are divided into eight categories based on their structures and functions, and recent references using the above methodologies for the analysis of these steroids are cited. GC and GC–MS are commonly used for the determination of volatile steroids. Although HPLC is a widely used analytical method for the determination of steroids including the conjugated type in biological fluids, LC–MS is considered to be the most promising one for this purpose because of its sensitivity, specificity and versatility.

Keywords: Reviews; Steroids; Hormones; Androgens; Corticoids; Estrogens; Sterols; Bile acids; Vitamin D; Phytosteroids

Contents

1. Introduction

The basic steroid molecular skeleton consists of four rings of carbon atoms, perhydro-1,2-cyclopentenophenanthrene. Almost all natural steroids possess either one or, more usually two, methyl groups at

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''angular'' positions where two rings meet. The steroids with which we shall be mainly concerned are of six skeletal types according to the number of C-atoms, gonane (C_{17}), estrane (C_{18}), androstane (C_{19}), pregnane (C_{21}), cholane (C_{24}) and cholestane (C_{27}) as shown in Fig. 1. These compounds are natural hormones or precursors with the exception of cholane. According to their functions, steroid hormones can be divided into androgens, corticoids and estrogens. The other steroids such as bile acids (cholane), vitamin D (9,10-secosteroids) and cardiac steroids having an α,β-unsaturated ring at the 17β-position also have biologically important activities [1,2]. The systematic names of steroids were defined by the IUPAC Commission on the Nomenclature of Organic Chemistry and the IUPAC-IUB Commission on Biochemical Nomenclatures, as published in 1969 and definitively in 1972 [3,4].

Steroids comprise a large group of natural substances that must frequently be monitored in various biological materials. Additionally, numerous synthetic steroids have been used as therapeutic agents. Due to the metabolic versatility of steroid molecules, extremely complex mixtures are often encountered, necessitating the use of a chromatographic procedure prior to measurement [2]. Gas chromatography (GC) and high-performance liquid chromatography (HPLC) are commonly used separation and determination procedures, but the former is applicable only to volatile steroids and not to non-volatile ones such as conjugates. GC–mass spectrometry (MS) is also widely used for the determination of steroids [5], but liquid chromatography (LC)–MS is recently considered to be the most promising analytical method for the determination of steroids including conjugated ones due to its sensitivity, specificity and versatility [6].

In this article the authors present an overview of GC, GC–MS, HPLC and LC–MS of steroids, which originate from natural sources as well as the medically applied natural steroids. Steroids are divided into eight categories based on their structures and functions, and no attention will be given to steroid hormones used for animals. Review articles covering the chromatographic analyses of steroids in man [5], testosterone [7], corticoids [8], sterols [9], bile acids [10–12] and vitamin D [13,14] have been published since 1995. An excellent book on steroid analysis was also published in 1995 [2]; therefore, this article covers references published after the above review articles and the book. Recently, electrochromatography has also been used for the separation of steroids but the review article of this has been published in 2000 [15]; thus separation using this chromatography is not cited in this article.

2. Androgens

The androgens are an important group of C_{19} steroids comprising principally testosterone (T) and its steroid A-ring saturated metabolites, 5α-dihydrotestosterone (DHT), and the isomeric androstane-3,17-diols. In addition, Δ^4-androstenedione (AD) and dehydroepiandrosterone (DHEA) are usually classed as androgens, although the androgenic activity of these compounds is low or less. The source of the androgens is the testis, in particular the Leydig cells of the interstitial tissue. The adrenals are also an important source, particularly in women. The ovaries in pre-menopausal women also have the capacity for biosynthesis of androgens. It is now generally accepted that the androgenic potency of T occurs as a result of its very active conversion to DHT in target tissues such as the prostate. There are many other tissues such as testes, adrenals, ovaries, cardiac muscle, salivary glands and gingival tissue, where quite extensive breakdown of T can

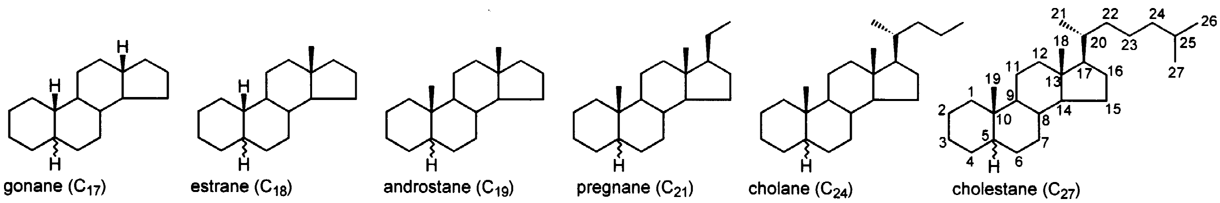

Fig. 1. Structures of steroids.

occur to AD and then to 17-oxosteroids, such as androsterone (A), epiandrosterone (epiA) and etiocholanolone (ET). The further metabolism of T and AD by hydroxylation at C-6, C-7 or C-16 is also known. The principal urinary 17-oxosteroids (A, epiA and ET) occur in urine as a mixture of glucuronides and sulfates. DHEA and its sulfated metabolite (DHEAS) are secreted by the adrenal cortex. In addition to these major urinary metabolites, T itself is excreted as the glucuronide, and 11-oxygenated 17-oxosteroids also occur as mixture of sulfates and glucuronides, which are the result of oxidative side-chain removal in the adrenals from corticosteroids such as cortisol and cortisone (Fig. 2). Analysis of these steroids in biological samples has been used to detect differential hormone action in human beings such as sexual function in men, hyperandrogenic disorders in women and puberty problems in children.

During the 1960s, many pharmaceutically licensed chemical analogues of T were released onto the market, these analogues being designed to enhance the anabolic effect for clinical purposes. However, athletes also desired these drugs, wanting the muscle building effect of T but without the androgenic effects. The International Olympic Committee re-established a medical commission, which banned the practice of doping in sports and introduced a drug testing program. The accepted test for T administration has been the determination of the urinary T/epitestosterone (epiT) ratio, a value of more than 6 being taken as the hallmark of drug misuse. However, proof of such administration is difficult to obtain, because T is an endogenous compound. Much effort has been made to detect anabolic steroids in biological fluids used for doping. These assays are usually performed by immunoassay because of its rapidity and high sensitivity, but several problems still exist: cross-reactions and interference with other substances. On the other hand, a number of chromatographic methods have been developed to overcome these problems. The reviews on steroids in man published in 1999 [5], on T published in 1995 [7] and in books [16,17] as described previously cover the above issue, and the following section reviews HPLC and GC–MS (or GC) analyses of androgens published after 1995 and 1998, respectively (Table 1).

The majority of analytical methods for endogenous and exogenous androgens uses GC–MS (or GC) which allows the identification of these steroids and metabolites with low detection limits, however requiring previous derivatization. The derivatization forms commonly used for GC or GC–MS have been the trimethylsilyl (TMS) and other alkylsilyl ethers for hydroxy functions and the *O*-methyloxime (MO) for ketonic functions. Juricskay and Telegdy reported [18] the determination of 28 different urinary steroid metabolites, including C_{19} and C_{21} steroids, of women with androgenic alopecia after enzymic hydrolysis and MO-TMS ether derivatization followed by capillary GC with flame ionization detection (FID). Kintz et al. determined DHEA [19] and T [20] in hair with silyl ether by GC–MS to confirm the physiological concentration range of these compounds. The concentrations of T, its glucuronide, A and ET in blood spots dried on filter paper were investigated by Peng et al. [21] using TMS ether derivatization and GC–MS. The same group also presented a determination method for T, nandrolone [19-nortestosterone (19-norT); a synthetic androgen] and their esters in hair using GC–MS [22]. Glucuronides of 19-norandrosterone (19-norA) and 19-noretiocholanolone (19-norET) are the principal urinary metabolites of 19-norT in human. However, it has been proven that enzymic conversion of androgens (T) to estrogens (estradiol) by aromatase is accompanied by 19-demethylation with production of small amounts of 19-norsteroids. Dehennin et al. [23] presented a GC–MS method for the determination of endogenous original 19-norA in urine, using deuterium-labeled 19-norET as internal standard (I.S.). The urine sample was extracted, hydrolyzed with β-glucuronidase and derivatized with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide–trimethylsilyliodosilane, and the resulting sample was analyzed by GC–MS. The determination limit was 0.02 ng/ml [signal/noise (S/N)=10] [23]. The determination method for urinary DHEA and DHEAS using GC–MS was reported by the same group [24]. The DHEAS was extracted, hydrolyzed, and then the liberated DHEA was derivatized to *tert*-butyldimethylsilyl ether and then analyzed by GC–MS with selected ion monitoring (SIM). The unconjugated DHEA was analyzed with the same method as used for the liberated one. They applied

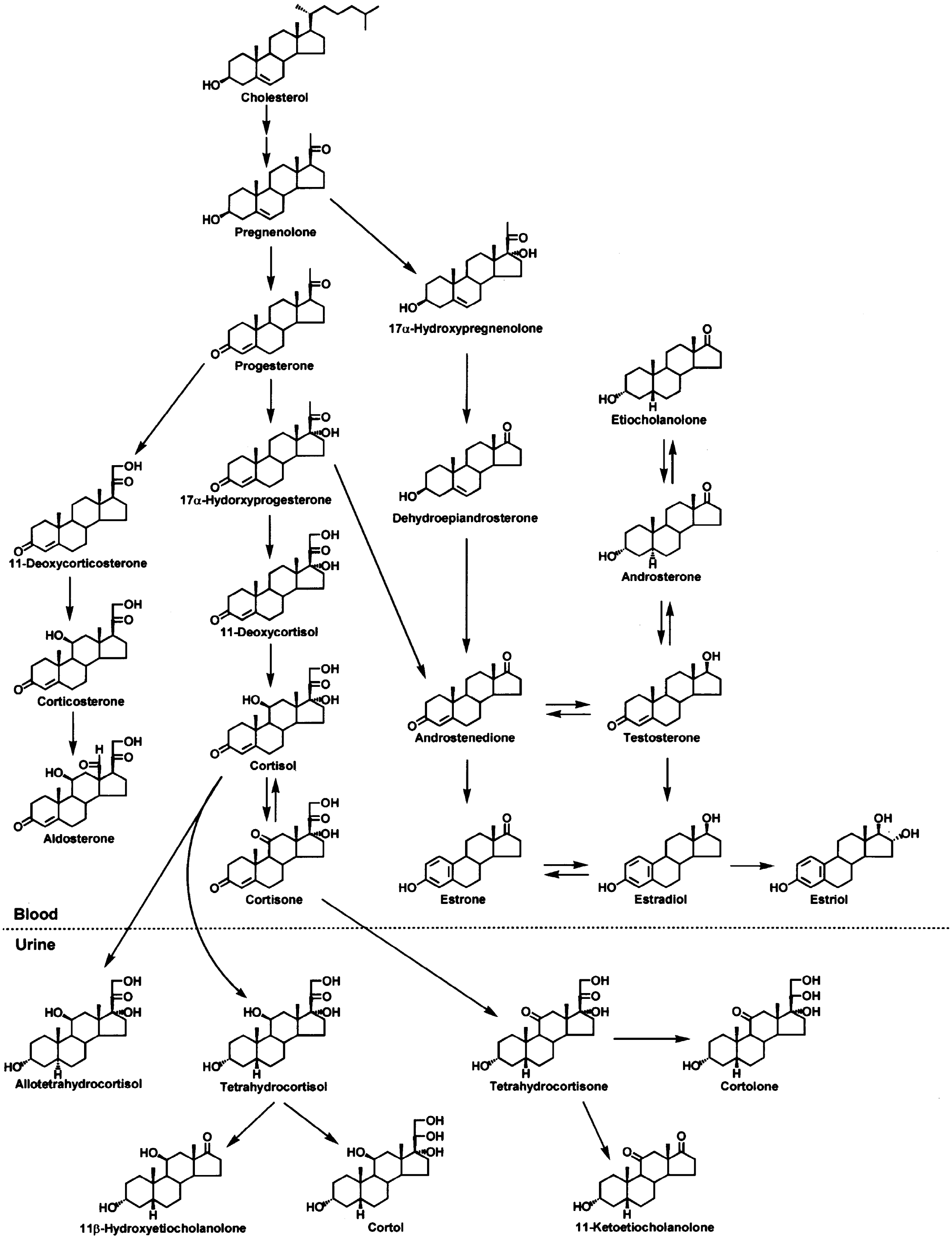

Fig. 2. Biosynthesis and metabolism of steroids.

Table 1
Analytical method for androgen using chromatography

Application	Analyte	Pretreatment protocol	Analytical method	Sensitivity	Ref.
Profile analysis of steroids in urine of women with androgenic alopecia	A, ET, DHEA, Δ^5-androstenediol, 5α-androstanediol, 11-ketoA, 11OHA,[a] 11OHET, 16OHDHEA, Δ^5-androstenetriol and other 18 steroids	Urine (20 ml)→Sep-Pak C_{18}→ hydrolysis [β-glucuronidase (β-G)]	GC–FID (MO-TMS)		[18]
Analysis of steroids in hair for doping test	DHEA, T, T ester I.S.: $[^2H_3]$T	Hair (100 mg)→treated with CH_2Cl_2→digestion (NaOH)→ neutralization→ISOLUTE C_{18}→ pentane extraction	GC–MS (silylation)	Linear range: 1–20 ng/mg Detection limit: 0.5 pg/mg	[19,20]
Analysis of steroids for sports drug test using blood spot filter paper	T, TG, AG, ETG, 17α-hydroxyprogesterone I.S.: $[^2H_3]$T, $[^2H_4]$ET	Blood spot→add KOH Free: hexane–Et_2O extraction G: KOH–water layer→hydrolysis (β-G)→*tert*-butyl methyl ether extraction	GC–MS (TMS)		[21]
Analysis of anabolic steroids in hair for doping test	Nandrolone, nandrolone-decanoate, T, T-propionate, -enanthate, -undecanoate I.S.: T-acetate	Hair (100 mg)→MeOH-TFA incubation hexane extraction	GC–MS–MS (TMS)	Determination limit: 0.02 ng/mg ($S/N=10$)	[22]
Analysis of steroids in urine for doping test	19-norA, 19-norET I.S.: $[^2H_3]$19-norET	Urine (10 ml)→Bond Elut C_{18}→ hydrolysis (β-G)→hexane–Et_2O extraction→DEAE-Sephadex A-25→ Sephadex LH-20	GC–MS (TMS)	Determination limit: 0.02 ng/ml ($S/N=10$)	[23]
Analysis of steroids in urine of men for doping test	DHEA, T, epiT, A, epiA, ET, androst-5-ene-3β,17α-diol, androst-5-ene-3β,17β-diol, 5α-androstane-3α,17β-diol, 5β-androstane-3α,17β-diol and their G, S	Urine (10 ml)→ODS cartridge G, S: methanolysis (TMSCl–MeOH)→ pentane-Et_2O extraction G: hydrolysis (β-G)→pentane–Et_2O extraction	GC–MS (*tert*-butyl-dimethylsilylation)		[24]
Analysis of steroids in hair	AD, A, ET, DHEA, DHT, T, progesterone, pregnenolone I.S.: $[^2H_3]$T	Hair (200 mg)→digestion (NaOH) →pentane extraction	GC–MS (SIM) (flophemesyl-TMS)	Detection limit: 0.02–0.5 ng/g ($S/N=3$) Calibration range: 0.1–10 or 0.5–50 ng/g	[25]
Analysis of steroids in hair for test of misuse of drug	T I.S.: $[^2H_3]$T	Hair (100 mg)→digestion (NaOH)→ Extrelut 3→Sephadex LH-20	GC–MS (SIM) (heptafluorobutyl ester)	Detection limit: 20 pg ($S/N=3.4$)	[26]
Analysis of steroids in serum of patients with hyperthyroidism or hypothyroidism	DHEAS, PREGS I.S.: androstenediol diacetate	Serum (0.2 ml)→Sep-Pak Vac tC_{18}→ Accell Plus QMA→hydrolysis (aryl sulfatase)→preparative HPLC	GC–MS (SIM) (heptafluorobutyl ester)	Measurable range: 0.01–1.8 μ*M* (DHEAS) 0.01–8.54 μ*M* (PREGS)	[27]
Metabolism of T by CYP450 3A	T, 6βOHT I.S.: 17α-methylT	Reaction mixture→CH_2Cl_2 extraction	GC–MS (SIM)	Calibration range: 0.25–100 μ*M*	[28]
Analysis of steroids in serum	DHEA, DHEAS I.S.: 5-androsten-3β-ol-16-one methyl ester	Serum (0.5 ml)→Oasis HLB	GC–MS–MS (SRM)		[29]

Table 1. Continued

Application	Analyte	Pretreatment protocol	Analytical method	Sensitivity	Ref.
Analysis of androgen glucuronides in urine	G of A, ET, 11-ketoA, 11βOHA, 11βOHET, DHT, DHEA, T I.S.: *n*-hexatetracontane	Urine (5 ml)→solid-phase extraction	High-temperature GC–MS (SIM) (methyl ester-TMS)	Detection limit: 15–20 pg ($S/N=3$) Calibration range: 20–3000 ng/ml	[30]
Optimization of HPLC conditions	T, epiT, 17α-methylT, 11-ketoT, 19-norT, 11βOHT, fluoxymesterone, boldenone, methandrostenolone, bolasterone, oximetolone, danazol, canrenone, norethindrone, androstenolone	Urine (3 ml)→CH_2Cl_2 extraction	HPLC with UV (190–360 nm)	Detection limit: 0.012–0.11 μg/ml	[31]
Optimization of HPLC conditions	T, epiT, 11βOHT, 11-ketoT, androstenolone, 19-norT, boldenone, bolasterone, cortisone, cortisol, corticosterone, deoxycorticosterone, 11α-hydroxyprogesterone	Urine (3 ml)→CH_2Cl_2 extraction	HPLC with UV (190–360 nm)	Detection limit: 0.012–0.107 μg/ml	[32]
Metabolism of T by cell microsome	T, A, 6βOHT, 16αOHT, 16βOHT, 2αOHT	Incubation mixture→AcOEt extraction	HPLC with UV (240 nm)	Linear range: 20–40 μg/ml (T) 280–4600 ng/ml (metabolites)	[33]
Analysis of steroids in plasma	7-oxoDHEA, 7-oxoDHEAS, 7-oxoDHEA3Ac I.S.: 17β-hydroxy-3β-methoxy-androst-5-en-7-one	S: plasma (1 ml)→dilution (water)→centrifugation→Sep-Pak C_{18}	HPLC with UV (240, 360 or 390 nm)	Linear range: 10–1000 ng/ml ($S/N=10$) Detection limit: 3 ng/ml ($S/N=3$)	[34]
Analysis of anabolic steroids in urine for doping test	T, epiT I.S.: 17α-methylT	Urine (5 ml)→Serdolit AD-2 resin→hydrolysis (β-G)→Et_2O extraction→pentane extraction	HPLC with UV (240 nm)	Determination limit: 20 ng/ml (T), 30 ng/ml (epiT) ($S/N=6$)	[35]
Analysis of steroids in serum	DHEAS	Serum (100 μl)→dilution (water)	Column switching LC–NI-APCI-MS	Detection limit: 10 ng ($S/N=3$) Linear range: 50–300 ng	[36]
Analysis of steroids in serum, brain, heart, liver, kidney, adrenal and testis	DHEAS I.S.: [$^2H_4$]DHES	Serum (100 μl)→Empore C_{18} Tissue→homogenization (MeOH)→hexane extraction→Bond Elut C_{18}	LC–NI-APCI-MS (SIM)	Detection limit: 0.5 ng ($S/N=3$) Linear range: 2–500 ng	[37]
Analysis of anabolic steroid conjugates in urine	TG, TS, epiTG, epiTS I.S.: [$^2H_3$]TG, [$^2H_3$]epiTS	Urine (2 ml)→solid-phase extraction	LC–ion-spray ionization-MS–MS (SRM)		[38]
Analysis of anabolic steroid conjugates in urine	TG, TS, epiTG, epiTS I.S.: $^2H_3$-labeled above steroids		LC–NI-ESI-MS–MS		[39]
Analysis of steroids in plasma for doping test	T fatty acid esters (enanthate, acetate, propionate, benzoate, isocaproate, cypionate, phenylpropionate, decanoate, undecanoate)	Plasma (1 ml)→precipitation (acetone–EtOH)→centrifugation→derivatization (Girard reagent)→Sep-Pak C_{18}	LC–PI-ESI-MS		[40]

[a] 11OHA: 11-hydroxyandrosterone; the rest is omitted.

the method to investigate the effect of a single dose of DHEA on the urinary androgen profile. Pentafluorophenyldimethylsilyl (flophemesyl)-TMS ether derivatives were introduced for simultaneous determination of eight steroids (AD, DHT, DHEA, T, A, ET, progesterone and pregnenolone) in human hair, which was done with GC–MS using [$^{2}H_{3}$]T as I.S. [25]. The method involved alkaline digestion, liquid–liquid extraction and subsequent conversion to the flophemesyl-TMS ethers, which formed an intense molecular ion and minimized background noise so that a sensitive analysis was achieved with SIM. The detection limits of the steroid varied in the range 0.02–0.5 ng/g ($S/N=3$). Scherer et al. [26] reported a method for the determination of T in human hair by GC–MS using [$^{2}H_{3}$]T as I.S.. They showed that the derivatization with heptafluorobutyric anhydride gave rise to high mass increments thus diminishing background noise and that these derivatives have shorter retention times than those of MO-TMS ethers. The detection limit was 20 pg ($S/N=3.4$). Tagawa et al. [27] presented a determination method for DHEAS and pregnenolone sulfate (PREGS) in serum of patients with hyperthyroidism and hypothyroidism using GC–MS after hydrolysis and derivatized with heptafluorobutyric anhydride (SIM: $[M-214]^{+}$). Testino et al. [28] presented a determination method for T and its metabolite, 6β-hydroxytestosterone, in liver microsomal incubates using GC–MS with SIM. The method did not require derivatization and molecular ions were selected for SIM. The method was used to characterize the cytochrome P450 3A activity of multiple preparations of human, rat and dog liver microsomes.

Although conjugated metabolites have usually been analyzed by GC or GC–MS after enzymic or alkaline hydrolysis and derivatization, some methods not requiring deconjugation were described. A simplified method for measurement of serum DHEA and DHEAS using GC–MS–MS in a single injection with selected reaction monitoring (SRM) was reported by Zemaitis and Kroboth [29]. It appeared that at the elevated temperature of the GC injector port, a reproducible decomposition occurred in which elements of sulfuric acid were removed and one of three isomers is formed with a different location of double bonds in the steroid A- or B-ring. A method for simultaneous determination of nine urinary androgen glucuronides by high temperature GC–MS after derivatization to methyl ester-TMS ethers was reported by Choi et al. [30]. They showed that high temperature GC–MS analysis using an MXT-1 stainless steel capillary column, which is thermally stable (leak-free at 400°C), with SIM detection provided complete separation of high boiling point intact glucuronide conjugates of nine androgens in symmetrical peak shape within 15 min, and that the detection limit was as low as 20 pg ($S/N=3$).

Many determination methods for androgens using HPLC with ultraviolet (UV) detection or LC–MS have been reported. The advantage of these methods is that they do not require hydrolysis of the conjugates prior to analysis. An HPLC optimization method for the separation of a mixture of urinary androgens and synthetic anabolic steroids was described by Gonzalo-Lumbreras and Izquierdo-Hornillos [31,32]. The effect of several variables (mobile phase, column packing material and temperature) on the separation was studied. The detection limits obtained by UV detection were in the range of around 0.01–0.1 μg/ml ($S/N=3$). The optimized methods were applied to the analysis, after liquid–liquid extraction, of human urine samples spiked with steroids. Baltes et al. [33] reported the separation of T and six of its hydroxylated metabolites in the incubation mixture with cell microsomes. Marwah et al. [34] developed a method using HPLC with UV (240 nm) detection for the determination of 7-oxodehydroepiandrosterone sulfate in human plasma after a single dose of 7-oxodehydroepiandrosterone 3β-acetate P.O. Using 1 ml of plasma for extraction, the detection limit of the assay was 3 ng/ml ($S/N=3$). T and epiT in urine were determined using an HPLC method with UV (240 nm) detection after enzymic hydrolysis and liquid–liquid extraction by Navajas et al. [35]. The determination limits at a S/N of 6 were 20 ng/ml for T and 30 ng/ml for epiT. Nakajima et al. [36] applied LC–atmospheric pressure chemical ionization (APCI) MS combined with a column switching technique to the determination of serum DHEAS. Human serum samples (100 μl) were directly injected into the sample preparation column, DHEAS was separated with an analytical column and analyzed by LC–APCI-MS in the negative-ion (NI) mode. The detection limit was 10 ng on-column at a S/N of 3. The same group also showed an application for the determination of DHEAS in biological samples, brain, liver, kidney, adrenal and testis [37]. The tissue homogenate containing [$^{2}H_{4}$]DHEA as I.S. was centrifuged, the supernatant was evaporated, extracted with an octadecylsilica (ODS) cartridge, and then subjected to LC–MS. The detection limit was 0.5 ng on-column (S/N=3). Direct determination of glucuronide and sulfate of T and epiT in urine has been studied using LC–MS–MS. Bean and Henion [38] presented a method using microbore HPLC combined on-line with ion-spray MS–MS in the positive-ion (PI) mode with SRM. SRM was based on the observation of product ions, representing losses of water and/or the conjugate moiety. Borts and Bowers [39] used LC–electrospray ionization (ESI) MS–MS with the NI mode. In both methods, the detection limits of analytes were in the low n*M* range in urine. Shackleton et al. [40] developed a method for characterization of nine kinds of T fatty acid esters using LC–ESI-MS in the PI mode. In the method, analytes were derivatized with Girard reagent, which converted the T fatty acid esters to polar, water soluble esters, and almost all compounds were separated by HPLC using a microbore C_4 column. Under ESI-MS conditions, providing optimal sensitivity, the spectra of the derivatives were essentially comprised of only a molecular ion $[M]^+$. $[M–59]^+$ and $[M–87]^+$ ions were observed as product ions in MS–MS analysis. The $[M–59]^+$ was explained by loss of $(CH_3)_3N$ from the Girard derivatives. This method was applied to the measurement of T fatty acid esters in the plasma of healthy volunteers after intramuscular administration of T fatty acid esters.

3. Corticoids

Corticoids are also called corticosteroids and are physiologically divided into mineralocorticoids acting in the metabolism of electrolytes and glucocorticoids acting in saccharometabolism. Because the adrenal cortex contains three types of cells each with its own characteristic enzyme system, the gland secretes a variety of steroid products (Fig. 2). The zona glomerulosa and the zona fasciculata secrete mineralocorticoids and glucocorticoids, respectively, and the other one is the zona reticularis that secretes adrenal androgens. A specific and sensitive method for the determination of these hormones in body tissue is necessary to elucidate the nature of many endocrine disease processes and is used for diagnosis and treatment. Because of the close structural similarity and their occurrence in low concentrations in body fluids and tissues, the development of reliable analytical methods is a challenging subject for analytical chemists.

Early methods for measuring corticoids were based upon the use of a fluorometric or colorimetric end-point, for example, the Porter-Silber reaction. However, they had several shortcomings, lacking specificity and requiring large volumes of sample. In clinical studies, nowadays, individual assays for most corticoids in plasma and urine are usually performed by immunoassay, but several problems as described in Section 2 still exist. On the other hand, a number of chromatographic methods employing various detection techniques, most of which are based on HPLC, GC–MS and LC–MS, have been developed and some of them have corroborated these negative portrayals of immunoassay. In general, chromatographic analysis of corticoids involves several steps, that is, solvent and/or solid-phase extraction, separation on a column and then determination. In the case where the steroid binds the serum protein, deproteinization with an organic solvent is necessary. Furthermore, solvolysis or enzymic hydrolysis of the conjugated ones is necessary prior to the determination of urinary corticoids. As I.S. for the analysis of these steroids, a synthetic analogue and a stable isotope-labeled compound have been most commonly utilized in HPLC and GC–MS, respectively. The analyses of these steroids which have been done up to 1994 have been described in a review article [8] and a book [41]. Clinical applications of GC and GC–MS of steroids have been reviewed in 1999 [5]. This section reviews the chromatographic methods for the analysis of endogenous corticoids in biological fluids published after the above article and the book.

Aldosterone (ALDO) is a major mineralocorticoid synthesized and secreted from the zona glomerulosa. The production rate and blood concentration of ALDO are controlled by the renin–angiotensin system. Its primary physiological function is to regulate the metabolism of sodium and potassium ions, by stimulating the re-adsorption of sodium, in exchange for potassium and hydrogen ions. ALDO concentration affects extracellular fluid volume and blood pressure. Hyperaldosteronism is a well-defined chemical disorder and a recognized cause of hypertension. On the other hand, a low level of ALDO is observed in patients with Addison's disease. ALDO has a UV absorption based on its α,β-unsaturated ketone structure, but it is impossible to measure its plasma level by a method using HPLC with UV detection because of its low concentration (less than 0.5 pmol/ml), which has conventionally been measured by radioimmunoassay (RIA). Fredline et al. [42] reported an LC–APCI-MS–MS method for the determination of ALDO in serum and plasma. After solvent extraction, samples containing flumethasone (a synthetic corticoid) as I.S. were separated on a phenyl column and analyzed in the NI mode by SRM using the CHO eliminated ion, which provided a determination limit of 15 pg/ml. The method was applied to assess abnormal ALDO production in hypertensive patients, and the results obtained were compared with those obtained by RIA. The assays correlated well, but the RIA method showed an overestimation compared with the LC–MS–MS method at a concentration of less than 100 pg/ml, which may come from cross-reactions or interference with other substances.

Although corticosterone (B) belongs to the glucocorticoids, it is an intermediate of the biological synthesis of ALDO and also has significant mineralocorticoid activity. Secretion of B in man is markedly raised in 17α-hydroxylase deficiency patients and in some rare cases of adrenocortical carcinoma. B is the most typical glucocorticoid in rats, and its plasma concentration is much higher than that in man. Some determination methods for B in rat biological fluids using HPLC with UV detection have been reported. Hay and Mormède [43] simultaneously determined B and 11-dehydrocorticosterone in rat urine, the latter of which is produced from B by 11β-hydroxysteroid dehydrogenase (11β-HSD). Mehvar et al. [44] monitored the plasma concentration level of endogenous B in rat after intravenous administration of methylprednisolone succinate (a synthetic corticoid). Ghulam et al. [45] described the quantitative analysis of B in human serum using LC–MS. The method employed diethyl ether extraction, separation with an ODS microbore column and ESI-MS detection. The mass spectrometer was operated in the PI mode of SIM ($[M+H]^+$), triamcinolone (a synthetic corticoid) was used as I.S. and the detection limit was 4.5 pmol per injection ($S/N=3$). Mikšík et al. [46] have investigated the in vitro metabolism of B and 11-dehydrocorticosterone with LC–MS. Separation of sixteen standard corticosteroid metabolites was performed with a reversed-phase semi-micro column using a linear gradient elution and mass selection (the PI mode, SIM). The method was applied to the monitoring of the activity of steroid converting enzymes, 20β-hydroxysteroid dehydrogenase and 11β-HSD in an avian intestine, and the 20β-dihydrometabolites of B and 11-dehydrocorticosterone together with 5β-pregna-3α,20β,21-triol-11-one were determined.

Cortisol (F) is a major glucocorticoid and its production and secretion are controlled by adrenocorticotropic hormone (ACTH) secreted from the pituitary. F promotes gluconeogenesis and the metabolism of proteins and lipids in the liver and muscle. F also has anti-inflammatory and weak sodium pool activities. The levels of F and its precursor, 11-deoxycortisol, in human biological fluids are considered as good biochemical markers of the hypothalamic–pituitary–adrenocortical axis activity. In clinical studies, the plasma/serum level is utilized as a diagnostic index of a dexamethasone (a synthetic corticoid) suppression test. It has been demonstrated that F in plasma is largely bound to proteins (transcortin and albumin) and that the protein-bound F is biologically inactive, whereas the non-bound (unconjugated) fraction is active. Less than 1% of total F is excreted unchanged in urine; the rest is excreted as metabolites and conjugates. Because the level of urinary free F correlates with the fraction of plasma F and the F production rate (FPR), the measurement of urinary F is widely used for the assessment of suspected hypercortisolaemia, Cushing's syndrome. A large number of methods using HPLC with UV detection (ca. 250 nm) has been described for the quantitative measurement of F in urine [43,47–49]. In most of these methods, a simultaneous determination of F and its inactive metabolite, cortisone (E), was done after solid-phase extraction using ODS cartridges, and a synthetic corticosteroid (dexamethasone, methylprednisolone, and so on) was used as I.S..

Methods using HPLC with UV detection have also been applied to the determination of serum/plasma F level because of the relatively high level at which it circulates in blood (250–550 n*M*). Döppenschmitt et al. reported simultaneous determination methods for endogenous F and exogenous prednisolone acetate [50] or triamcinolone acetonide [51] in plasma following administration of the synthetic glucocorticoids to evaluate the suppression of F production by them. A simultaneous determination method using HPLC with fluorescence (FL) detection of F, E, prednisolone, prednisone, 6β-hydroxycortisol (6β-OHF) and 6β-hydroxyprednisolone in plasma and urine of renal transplant patients who received prednisolone was also reported [52]. In the method, 9-anthroyl cyanide was used as a fluorescent derivatization reagent and the detection limit of F and E was 0.1 ng/ml at a S/N of 3. A chemiluminescence (CL) detection of the authentic E and B was also attempted [53]. The method utilized the CL produced by the reaction of the α-hydroxycarbonyl group of the steroids with luminol and hexacyanoferrate (III and II) in alkaline solution, and the detection limit of E was reported as 1.1 pmol at a S/N of 3. Because the release of ACTH is influenced by stress, the level of circulating F is considered to be a marker of stress. Saliva is considered to be the most suitable source for monitoring human stress, because saliva sampling is stress-free (in contrast to blood sampling) and saliva reflects biological responses more directly than urine, which provides in turn a kind of cumulative or averaged response. Okumura et al. [54] developed a fully automated column-switching HPLC system for assay of salivary F. The column-switching system consisted of a polymer-coated mixed-functional silica column for deproteinization and a CN column for frontal concentration and separation. F was detected with a laser-induced FL detector after post-column derivatization using sulfuric acid. The detection limit was 8 fmol at a S/N of 3.

LC–MS is also utilized for F analysis. Kim et al. [55] examined the effect of organic acids in the mobile phase on the ionization of F and E in thermospray (TSP)–MS. They reported that the sensitivity of the determination of F and E in human urine was maximized when formic acid was added to the mobile phase. Shibasaki et al. [56] reported a stable isotope dilution LC–TSP-MS method of F and E in human plasma, in which $HCOONH_4$ was used as the mobile phase additive. When a mixture of tetrahydrofuran and methanol was used as an organic modifier of the LC eluent, a significantly large isotope effect of the deuterium-labeled compounds on the HPLC behavior was observed, which was not observed when acetonitrile was used. The detection limits of the steroids were ca. 0.5 ng per injection ($S/N=3$). An on-line coupling of solid-phase extraction based on a restricted-access support with LC–APCI-MS of F in human plasma has also been reported [57]. A pre-column packed with an ODS alkyl-diol support was used for the direct plasma injection. The sample pretreatment and LC–MS analysis were performed at the same time, which was done in a short time (5 min/analysis).

Brandon et al. [58] presented a stable isotope dilution GC–NI-chemical ionization (CI)–MS method for the measurement of FPR. F extracted from serum was derivatized to pentafluoropropionate, which is a highly electronegative derivative. The method provided 50–100-fold greater sensitivity than PI-MS and allowed estimation of FPR with use of small amounts of serum (0.5 ml). The mean 24 h FPR of healthy children receiving continuous infusion of deuterium-labeled F was reported.

The interconversion of the active form, F, and its inactive metabolite, E, is catalyzed by 11β-HSD. Two distinct isoforms of 11β-HSD, 11β-HSD1 (hepatic) acting predominantly as an 11-oxoreductase (i.e. conversion of E to F) and 11β-HSD2 (renal) catalyzing exclusively 11β-dehydrogenation (F to E), have been characterized. It has been reported that in in vitro experiments, a mineralocorticoid receptor has an equal affinity for F and ALDO; therefore, 11β-HSD2 is considered to act as a tissue-specific protector for a mineralocorticoid receptor in ALDO selective tissues by inactivating F to E. Therefore, defects in 11β-HSD2 result in apparent mineralocorticoid excess (AME) characterized by hypertension, hypokalemia and low renin. Kasuya et al. [59] developed a GC–electron ionization (EI)–MS method for the simultaneous determination of endogenous F and E and exogenous deuterium-labeled F and E in human plasma and applied the method to the kinetic studies of the interconversion of F and E in man following administration of [1,1,19,19,19-$^{2}H_5$]F or [1,1,19,19,19-$^{2}H_5$]E. The plasma samples containing endogenous and exogenous (labeled) F and E were analyzed by the double isotope dilution method, which required two series of plasma samples obtained at each time after the administration. The method, however, is time-consuming and often leads to a drawback in accurate and selective determination of low levels of these compounds in biological fluids. An improved method using [1,2,4,19-$^{13}C_4$, 1,1,19,19,19-$^{2}H_5$]F and [1,2,4,19-$^{13}C_4$, 1,1,19,19,19-$^{2}H_5$]E as analytical internal standards, syntheses of which were described in the literature [60], has also been developed by the same group [61]. The compounds [1,2,4,19-$^{13}C_4$]F and [1,2,4,19-$^{13}C_4$]E were used as tracers. The steroids were measured by the SIM mode of the molecular ions after the derivatization to bismethylenedioxy-pentafluoropropionate (BMD–PFP) (Fig. 3).

F and E are metabolized extensively to many compounds. The day-to-day variation in urinary excretion of F has to be ascribed to both the adrenal production and the metabolism. Tetrahydrocortisol (THF), allo-tetrahydrocortisol (allo-THF) and tetrahydrocortisone (THE) are produced from F and E by a two-step reduction of the steroid A-ring catalyzed by 5α- or 5β-reductase, followed by 3-oxoreductase. As mentioned above, the mineralocorticoid receptor has an equal affinity for F and ALDO; therefore, defective renal F metabolism with consequent access of F to the mineralocorticoid receptor thereby causing the mineralocorticoid effects explains the syndrome of AME. Furuta et al. [62] reported a stable isotope dilution GC–EI-MS method for the simultaneous determination of THF, allo-THF and THE in human plasma and urine. The conversion of the metabolites to BMD–PFP derivatives produced satisfactory chromatographic behavior of the three tetrahydrocorticoids. The determination was carried out by SIM using the characteristic fragment ions ($[M-30]^+$) of the derivatives, in which the detection limit was 25 pg per injection ($S/N \geq 2$). Kasuya et al. [63] also reported a new approach using a stable isotope methodology in evaluating 11β-HSD activities in vivo based on urinary excretion of F, E, THF, allo-THF and THE. The method involved the measurement of deuterium-labeled F and its metabolites by GC–EI-MS simultaneously with endogenous F, E and their steroid A-ring reduced metabolites after oral administration of deuterium-labeled F to healthy subjects. A method using HPLC with FL detection for the determination of urinary THF, allo-THF and THE has been reported by Neufeld et al. [64]. They used 9-anthroyl cyanide as a derivatization reagent and clarified the derivatization conditions to give only the 21-derivatized product (derivatized only at the primary hydroxy group). The method was applied to the assay of urine of a healthy subject and a patient suffering from 5α-reductase deficiency; in the latter, allo-THF was not detected. Yergey et al. [65] used LC–chemical reaction interface (CRI)-MS for the determination of FPR based on urinary excretion of F metabolites. After infusion of [9,12,12-$^{2}H_3$]F to men, the urine was subjected to solid-phase extraction, hydrolysis by β-glucuronidase/sulfatase and then LC–CRI-MS. The profile analysis of THF, THE and cortolone (mixture of 5α- and 5β-cortolone) was described. Dodds et al. [66] examined the HPLC separation of F, E, 20α-dihydrocortisol (20α-DHF), 20β-dihydrocortisol (20β-DHF), 20α-dihydrocortisone (20α-DHE) and 20β-dihydrocortisone (20β-DHE) in tissue culture media from in vitro perfusions of the human placental lobule. These six steroids and 6α-methylprednisolone (I.S.; a synthetic corticoid) were extracted with solid-phase extraction cartridges, separated by two ODS columns linked in series and monitored by UV absorption at 242 nm. Dodds et al. [67] modified the separation technique and reported an LC–ESI-MS–MS method for the determination of F, E, their 20-reduced metabolites (20α-DHF, 20β-DHF, 20α-DHE and 20β-DHE), THF and THE in placental perfusate. In this report, a semi-micro column with a flow-rate of 80 μl/min was used. Multiple reaction monitoring was employed in the NI mode, and the detection limit was 50 pg on column.

F and E are also metabolized to 6β-OHF, 6α-hydroxycortisol (6α-OHF) and 6β-hydroxycortisone (6β-OHE) by cytochrome P450 (CYP) 3A4 in the endoplasmic reticulum of hepatocytes. The endogenous hormonal ratio of 6β-OHF to F in the urine is an index for the activity of this enzyme induced by many xenobiotics including drugs. The 6β-hydroxylation has recently drawn attention, due to the physiological significance in hypertension. Therefore, simultaneous assay methods of 6β-OHF and F in urine using various techniques, HPLC with UV detection [68,69], LC–MS [70,71] and GC–MS [72],

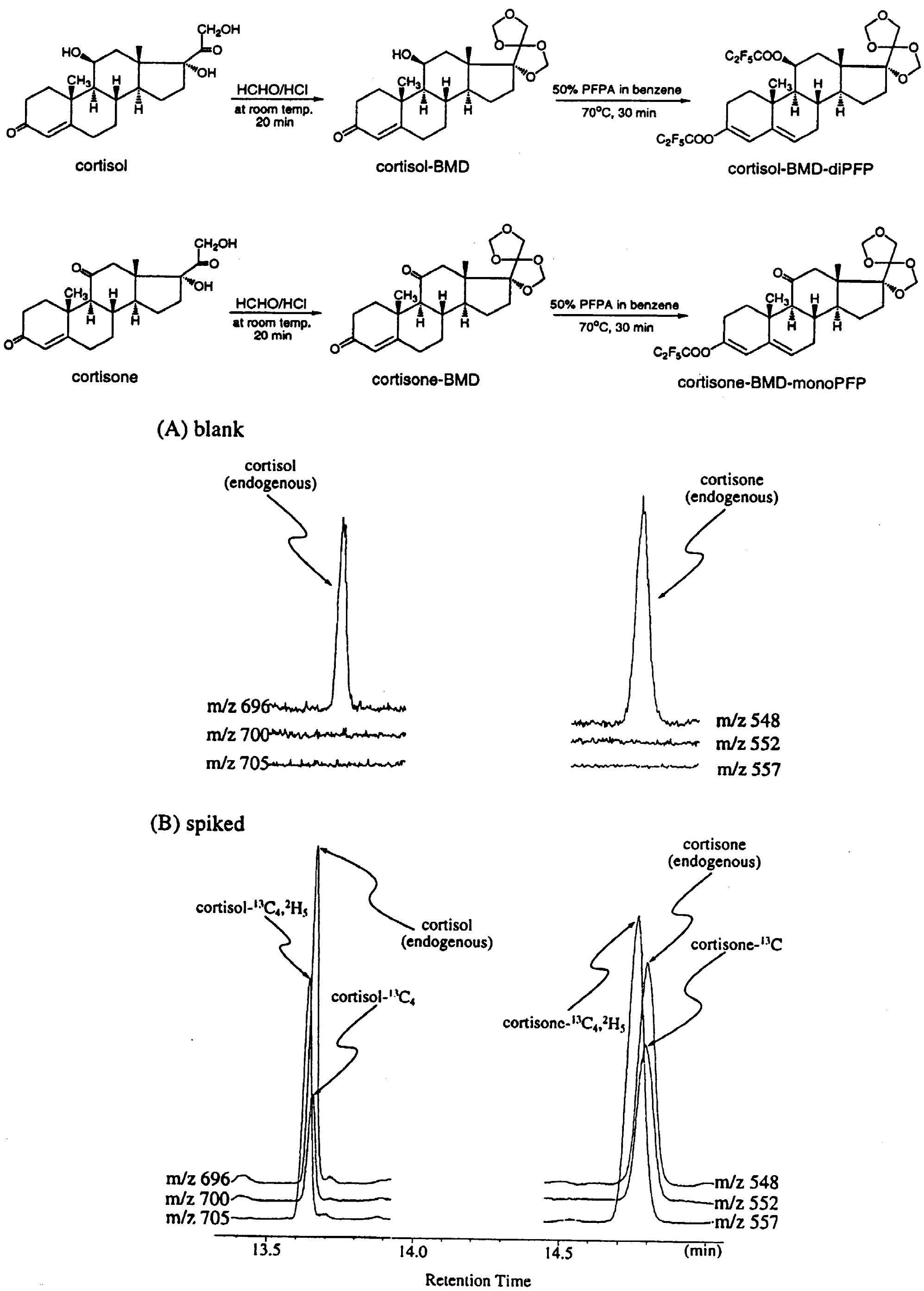

Fig. 3. GC–MS chromatograms of BMD–PFP derivatives of cortisol/[$^{13}C_4$]cortisol/[$^{13}C_4$,$^2H_5$]cortisol (m/z 696, 700 and 705) and cortisone/[$^{13}C_4$]cortisone/[$^{13}C_4$,$^2H_5$]cortisone (m/z 548, 552 and 557) after processing from 0.5 ml of blank (A) and spiked plasma (B) with [$^{13}C_4$]cortisol/[$^{13}C_4$,$^2H_5$]cortisol (32.60 and 80.42 ng) and [$^{13}C_4$]cortisone/[$^{13}C_4$,$^2H_5$]cortisone (24.12 and 48.04 ng) [61].

have been developed. Bidart and Lesgards [69] reported an automated HPLC with UV detection system that employed the column-switching technique with internal surface reversed-phase pre-column for pretreatment of urine. The method of Tang et al. [71] employed a tandem MS which was so specific that the pretreatment was very simple (ethyl acetate extraction). The chromatographic run time was short (8 min), which created a high run capacity. Furuta et al. [72] described a stable isotope dilution method using a capillary GC–EI-MS for the simultaneous determination of 6β-OHF, 6α-OHF and 6β-OHE in human urine. The determination was carried out with the SIM of the characteristic fragment ions ($[M-31]^+$) of the MO-TMS derivatives.

18-Hydroxycortisol (18-OHF) is probably a zona fasciculata product and is secreted in small quantities by healthy subjects. However, in Conn's syndrome and particularly in dexamethasone-suppressible hyperaldosteronism, it is secreted in much larger quantities, providing a valuable quantitative diagnostic index. 18-Oxocortisol (18-oxoF) has minor mineralocorticoid activity and its level is also raised in Conn's syndrome and dexamethasone-suppressible hyperaldosteronism. These steroids are often called hybrid corticoids as they have the characteristic structures of glucocorticoids and mineralocorticoids. Kurosawa et al. [73] described a method for measuring 18-OHF, 18-hydroxycortisone and 18-oxoF in human urine by HPLC with FL detection. The method involved the derivatization of the 18-oxygenated corticosteroids to 21-anthroyl esters with 1-anthroyl cyanide. The method was applied to the assay of urine of a patient with primary aldosteronism, and it was reported that all the 18-oxygenated corticoids levels of the same patient showed significant decreases after adrenalectomy. The identification of 19-oxo-11-deoxycortisol produced in the reaction mixture of 11-deoxycortisol with bovine CYP450 (11β) using LC–APCI-MS has also been reported [74].

4. Estrogens

Estrogens influence the growth, development differentiation and function of peripheral tissues of the female and male reproductive system such as the mammary gland, uterus, vagina, ovary, testis, epididymis and prostate. It has also been known that estrogens play an important role in bone maintenance, in the cardiovascular system and in the central nervous system. Estrogens are mainly produced in the ovaries and testes, but also other tissues such as the adrenals. Representative estrogens, estrone (E_1), estradiol (E_2) are synthesized from androgenic precursor (Δ^4-androstenedione and testosterone) by demethylation and aromatization, and these are transformed to each other by the action of 17β-hydroxysteroid dehydrogenase (Fig. 2). Estriol (E_3) is formed via 16α-hydroxylation of E_1 and is also formed from the principal precursor, 16α-hydroxydehydroepiandrosterone, in the placenta in pregnant women. Among them, E_2 is functionally the most potent estrogen. Estrogens are further metabolized by conjugation to sulfate and glucuronide and by hydroxylation at the steroid A-ring to catechol estrogens (2- or 4-hydroxyestrogens). The chromatographic analysis of these estrogens have been reviewed by Wolthers and Kraan [5] and a book [75]. The following section reviews the chromatographic analysis of estrogens published after that.

Estrogen metabolism has been implicated in the risk of hormone-dependent diseases and has demonstrated physiological significance. Katayama et al. [76] reported a sensitive HPLC method for the determination of unconjugated and conjugated estrogens (E_1, E_2 and E_3) using a fluorescent prelabeling reagent, 2-(4-carboxy)-5,6-dimethylbenzimidazole. The conjugated estrogens were digested by enzymic hydrolysis, and the method was applied to monitoring of unconjugated and conjugated estrogens in serum from patients who attend in vitro fertilization embryo transfer. A more sensitive FL detection method was reported by De Silva [77]. E_2 was derivatized with pyrenesulfonyl chloride and detected by laser-induced FL detection. The detection limit of derivatized E_2 was 0.2 fmol, which was achieved using a He–Cd laser ($\lambda_{em.}$ 325 nm). CL detection was introduced for the determination of E_2 in plasma using HPLC by Yamada et al. [78]. E_2 was derivatized with dansyl chloride and analyzed by an HPLC–CL system using bis(2,4-dinitrophenyl)oxalate-H_2O_2. The detection limit of dansyl-E_2 was 15 fmol in the standard solution and 44 fmol in the rat plasma sample ($S/N=3$). Choi et al. [79] reported the determination of E_1 and E_2 in man hair by GC–EI-MS with SIM. The method involves alkaline digestion of a hair sample, alkyloxycarbonylation of the phenolic hydroxy groups with isobutyl chloroformate (isoBOC), extraction with an organic solvent and chlorodifluoroacetyl derivatization in order to protect the remaining alcoholic hydroxy group. In SIM, the most intense ions, $[M–100]^+$ resulting from the cleavage of isoBOC, were selected as quantitative ions, and the detection limits of E_1 and E_2 were 1 and 2 pg ($S/N=3$), respectively. Although the investigation was not used for the biological sample, it was shown that the derivatization with pentafluorobenzyl (PFB) bromide was useful for more sensitive determination of estrogens using GC–NI-CI-MS [80] or LC–NI-APCI-MS [81]. PFB derivatives were used as electron-capturing derivatives to produce an intense $[M–PFB]^-$ ion in both ionization systems.

Estrogens are present in biological tissues and fluids mainly as water soluble conjugates with high polarity, i.e. sulfate and glucuronide. HPLC or LC–MS analyzes the low volatility and polar conjugated estrogens without hydrolysis and derivatization, which are necessary prior to GC–MS analysis. Zhang and Henion [82] developed a sensitive and robust method to determine five estrogen sulfates in man urine employing high-throughput solid-phase extraction with 96-well technology, and HPLC coupled with a negative turbo ion spray tandem MS in the SRM. E_3 and E_3 3-sulfate (S) are the most abundant estrogens circulating in the serum of the fetus and pregnant women. In order to investigate the feto-placental function during pregnancy, E_3 and $E_3$3S levels in urine, serum and amniotic fluid have been measured. Tagawa et al. [83] reported a simultaneous determination method for E_3 and $E_3$3S in human serum by column-switching semi-micro HPLC with electrochemical detection (ED; for E_3) and UV detection (for $E_3$3S). In this method, tetra-*n*-butylammonium ion was added in the mobile phase as a counter ion for $E_3$3S. The detection limits of E_3 and $E_3$3S in human serum were 2.5 and 295 ng/ml ($S/N=3$), respectively. The method was applied for the measurement of E_3 and $E_3$3S in serum from umbilical arteries from 18 full-term healthy neonates. E_3 3-glucuronide (G) and $E_3$16G are also the main metabolites of E_3 in urine, so that the relationships between the concentration of these glucuronides in urine and the pathological balances of pregnancy have been widely investigated. Iwata et al. [84] developed a fluorescent derivatization reagent for carboxylic acid, 6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone-3-propionylcarboxylic acid hydrazide (DMEQ-hydrazide) and applied it for the detection of $E_3$3G and $E_3$16G, which were the main metabolites of E_3 and excreted in urine (Fig. 4). The detection limits of E_3-3G and -16G were 150 and 180 fmol in 5 μl of urine ($S/N=3$), respectively. Preeclampsia, a serious disease that affects many women during pregnancy, is now recognized pathophysiologically as an endothelial dysfunction that is probably related to the accumulation of lipid peroxides produced in the placenta. Takanashi and co-workers [85,86] hypothesized that 2-hydroxyestradiol 17-sulfate (2OHE$_2$17S) is the placental antioxidant during pregnancy and established the analytical method for 11 kinds of steroid D-ring conjugated estrogens including catechol- and guaiacol-estrogens in pregnancy urine using HPLC with ED. Conjugates having a catechol- or guaiacol-structure were separately detected with adequate sensitivity by controlling the detector voltage even in the presence of a large amount of compounds having a phenolic structure, such as $E_3$16G. The detection limits of the catechol- and guaiacol- and classical-estrogen conjugates were about 2, 100 and 50 pg ($S/N=3$), respectively. They have also developed a determination method for 2OHE$_2$17S by RIA [86].

Epidemiology studies indicated that estrogen replacement therapy for postmenopausal women with conjugated equine estrogen preparation has a protective effect on coronary heart disease and recent studies clearly show that Premarin favorably alters the high density lipoprotein/low density lipoprotein ratio in postmenopausal women. Chandrasekaran et al. [87] reported a method using HPLC with FL detection for the determination of total (conjugated and unconjugated) 17α-dihydroequilenin in plasma. In this method, the plasma sample was subjected to HPLC using a C_6 column after pretreatment involving enzymic hydrolysis and solvent extraction, and detection was accomplished with an FL ($\lambda_{ex.}$ 210 nm, $\lambda_{em.}$ 370 nm) detector. The method was applied to determine the pharmacokinetic and pharmacodynamic relationships of 17α-dihydroequilenin 3-

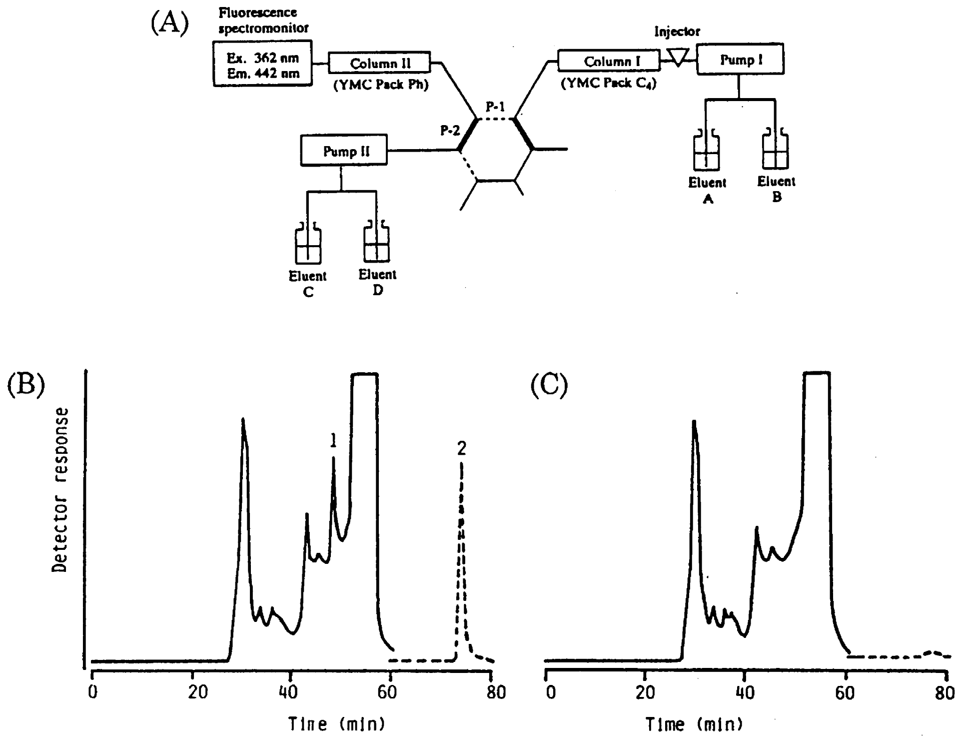

Fig. 4. Flow diagram of the column-switching HPLC system (A) and chromatograms of the DMEQ derivatives of $E_3$3G and $E_3$16G obtained with 5 μl of human urine from pregnant woman (B) and normal male (C). Peaks: 1, $E_3$3G; 2, $E_3$16G; others, endogenous urinary substances. Detector sensitivity: solid line: ×4; dotted line: ×1 [84].

sulfate in model animals. Another HPLC method with FL detection in combination with a post-column on-line photochemical derivatization was described for the determination of equilin and equilenin in urine from postmenopausal women after estrogen replacement therapy by Gatti et al. [88]. The detection limits of equilin and equilenin were 50 and 10 fmol, respectively. An immunoaffinity extraction for the determination of equilin and its metabolites in plasma using HPLC was reported by Ikegawa et al. [89].

Human breast cancer and endometrium cancer is promoted by prolonged exposure to endogenous and/or synthetic estrogens or their metabolites. Although the mechanism of estrogenic carcinogenesis has remained unknown, covalent modification of estrogen-DNA bases is thought to be a crucial initiating event. Van Aerden et al. [90] used LC–ESI-MS–MS for the assessment of the reactivity of estrogen-2,3-quinone toward deoxyribonucleosides in crude reaction mixtures. Multisequential MS^n performed on a quadrupole ion-trap mass spectrometer enabled the discrimination of isomeric adducts present in the reaction mixture. Itoh et al. [91] synthesized two estrogen sulfates, pyridinium 3-methoxyestra-1,3,5(10)-trien-6α-yl sulfate and its 6β isomer as model compounds to demonstrate the carcinogenesis of estrogen, reacted with calf thymus DNA, and the produced steroid DNA adducts were identified using a method using HPLC with UV (272 nm) detection.

5. Cholesterol and related sterols

Cholesterol is an important lipid in many vital processes. It is a structural constituent of cell membranes and a substrate for the synthesis of bile acids and steroid hormones (Fig. 2). On the other hand, cholesterol is also one of the major risk factors for arteriosclerotic diseases, such as hypertension and cerebrovascular disease. In normal plasma, about 70% of total cholesterol is esterified with long-chain fatty acids. Although plasma cholesteryl ester species are parameters reflecting dietary fat intake, analysis of cholesterol generally involves hydrolysis. A high value of total serum cholesterol (unconjugated and ester forms) can be found in subjects with familial hypercholesterolemia, coronary heart disease and atherosclerosis. Total cholesterol in the serum is conventionally measured by enzymic/colorimetric methods. However, phytosterol, 7-dehydrocholesterol (7-DHC) and so on are not distinguished from cholesterol in these methods. Sterols show absorbance generally in the 190–210 nm range, which is not convenient for the analysis of these compounds with HPLC using UV detection in its specificity and sensitivity. Therefore, their analyses have usually been done with GC rather than HPLC. As detection systems of GC, FID and MS have been most commonly used; whereas many different detectors have been used with HPLC. Most sterols do not have specific UV-absorptive properties as described above; therefore, a UV detector has been used for only some sterols, such as oxosterols based on their carbonyl groups, or after derivatization. In addition, the light scattering evaporation detector, which measures the intensity of the light scattering by the fine droplets formed by the solute upon evaporation of the column effluent, though it is less sensitive than the UV detector, has been normally used for the assay of sterols with high concentration. The application of LC–MS has also been investigated. Because sterols are highly lipophilic and have a few polar functional groups, it is difficult to use ESI and APCI as ionization methods, and a particle-beam (PB) interface employing EI as ionization method has recently been used for coupling LC and MS. The chromatographic analysis of these sterols published up to 1994 has been reviewed by Hoving [9] as previously described, and those done after 1995 are cited below.

Beaumier-Gallon et al. [92] presented a GC–MS method for the simultaneous measurement of cholesterol and exogenous [25,26,26,26,27,27,27-$^{2}H_7$]-cholesterol in plasma and lipoprotein fractions. Using this method, they studied the kinetics of cholesterol in humans following the administration of labeled cholesterol as a tracer to healthy subjects. Cholesterol can be analyzed with GC without derivatization since many years, but the peak shape is not optimal. Therefore, GC and GC–MS methods require a time-consuming derivatization step. Kock et al. [93] developed an LC–PB-MS method of total cholesterol in serum, which required no derivatization procedure. The serum was subjected to alkaline hydrolysis and cholesterol was extracted with cyclohexane. The determination was carried out by SIM of the molecular ions formed by EI and [25,26,27-$^{13}C_3$]cholesterol was used as I.S.. The results obtained by the method and those by the stable isotope dilution GC–MS method correlated well, but in the LC–MS method, some steroids (lathosterol and so on) interfered with the determination of cholesterol.

Based on flow injection–MS–MS, an interesting analytical method of cholesterol using nano-ESI has been presented by Sandhoff et al. [94]. In the method, cholesterol was derivatized to cholesterol sulfate by a sulfur trioxide–pyridine complex, introduced into the mass spectrometer operated in the NI mode at a flow-rate of 50–100 μl/min and analyzed with a precursor ion scan for the sulfated ion (HSO_4^-). Linearity was obtained in the range of 1–300 pmol of cholesterol using 200 pmol of [3,4-$^{13}C_2$]cholesterol as I.S.. Unconjugated cholesterol in the cells and subcellular membranes of Chinese hamster was quantified. In addition to the above methods, HPLC with a light-scattering detection for cholesterol in human spermatozoa and seminal plasma has been reported [95].

The concentration ratio of cholesterol and cholestanol is increased in the serum of patients with cerebrotendious xanthomatosis (CTX) and liver and biliary tract diseases. Therefore, the simultaneous determination of cholesterol and cholestanol is helpful in diagnosis of these diseases. A determination method for cholesterol, cholestanol, coprostanol and epicoprostanol, the latter two sterols are being products of intestinal bacteria, in urine using GC–MS has been presented [96]. The method involved methyl *tert*-butyl ether and methanol extraction, silylation and then determination by FID. Nakajima et al. [97] reported a method using HPLC with ED for the determination of cholesterol and cholestanol in human serum. The method employed pre-column derivatization with 2-[2-(isocyano)ethyl]-3-methyl-1,4-naphthoquinone combined with a platinum catalyst. The detection limits at a S/N of 3 were 6.6 and

7.4 pg for cholesterol and cholestanol, respectively. A method using HPLC with UV detection of serum cholestanol has been reported by Halperin et al. [98]. The sterols, including cholesterol, cholestanol and so on, were derivatized to 4′-bromobenzenesulfonyl esters and heated in isopropanol. The derivatized cholesterol was solvolyzed to cholesteryl isopropyl ether having absorption at 208 nm, but the derivatized cholestanol did not change and was measured using absorption at 235 nm. The method was applied to measuring cholestanol levels in serum of patients with CTX and liver cirrhosis.

Smith–Lemli–Opitz syndrome (SLOS) is an autosomal recessive disorder caused by 7-DHC Δ^7-reductase deficiency. This enzyme converts 7-DHC to cholesterol. Patients with the disease show a combination of abnormally low plasma cholesterol levels with a markedly elevated concentration of 7-DHC. Although 7-DHC has also been conventionally measured by GC–MS, Rizzo et al. [99] presented a method using HPLC with diode array detection for the determination of 7-DHC in plasma. The method was based on absorption of the conjugated diene of 7-DHC at 280 nm. In this report, cholesta-5,7,9(11)-trien-3β-ol, which is a further characteristic metabolite of SLOS [100], was also detected. LC–PB-MS has also been applied to the determination of 7-DHC in plasma [101].

7α-Hydroxycholesterol (7α-HC) levels in human serum reflect bile acid synthesis and have been recognized as indicators of the rate of cholesterol catabolism regulated by cholesterol 7α-hydroxylase. Saisho et al. [102] developed a method using HPLC with FL detection for the determination of 7α-HC in dog plasma. In the method, 7α-HC was derivatized to a fluorescent derivative by 7-methoxycoumarin-3-carbonyl azide, which reacted with both 3β- and 7α-hydroxy groups. The method was used to investigate the effects of oral administration of cholestyramine on 7α-HC levels in dog plasma.

Lipid peroxidation has been suggested to play a significant role in the etiology of a number of diseases and in the aging process. In the analysis of oxysterols, the extraction solvent must be selected cautiously so that the oxidation reaction during the sample preparation does not occur: ethers are not suitable for this purpose. Yasuda and Narita [103] measured cholesteryl ester hyperoxides in human plasma by HPLC. The hyperoxides were extracted with a mixture of hexane and ethyl acetate, separated by a column-switching HPLC using aminopropyl- and octyl-silica columns followed by CL detection based on isoluminol and microperoxidase. A coulometric ED analysis of cholesteryl ester hydroperoxides has also been attempted [104]. Teng and Smith [105] reported an HPLC analysis of human erythrocyte oxysterols. The oxysterols [7α-HC, 7β-hydroxycholesterol, 19-hydroxycholesterol, (20*S*)-20-hydroxycholesterol, 25-hydroxycholesterol and (25*R*)-26-hydroxycholesterol] were oxidized to the corresponding hydroxy-Δ^4-3-ketones by cholesterol oxidase and analyzed by HPLC with monitoring at 235 nm. 7-Oxocholesterol was measured simultaneously. The method was applied to the analysis of these sterols in erythrocyte membranes of patients with sickle cell anemia or sickle cell trait. LC–PB-MS was also applied to the analysis of cholesterol oxides [106]. The report stated that in terms of ion abundance, the EI mode was superior to the PI- and NI-CI modes. Using reversed-phase LC–PB-EI-MS, the determination limits of cholesterol oxides were in the range of 2–4 ng/injection. A GC–MS method for 7-oxocholesterol in low density lipoprotein has also been developed [107]. The method was applied to the evaluation of pathological conditions in uremic patients with hemodialysis and it was found that the concentrations of 7-oxocholesterol/cholesterol in low density lipoprotein were increased in patients compared to healthy subjects.

Cholesterol sulfate is a minor constituent of various mammalian tissues and is highly polar and non-volatile, so LC–MS is considered to be suitable for analysis. Raith et al. [108] presented a method using LC–ESI-MS for determination of cholesterol sulfate in the human stratum corneum. In this method, cholesterol sulfate was separated from other lipids by high-performance thin layer-chromatography and HPLC with a narrow bore column and introduced to the mass spectrometer operating in the NI mode.

Sterols other than cholesterol are usually poorly adsorbed from the intestines. In subjects with phytosterolemia, plasma levels of phytosterols are elevated, which is caused by the loss of normal intestinal selectivity for cholesterol adsorption. Analysis of phytosterols in biological samples has been conventionally performed with a capillary GC in the same way as cholesterol, that is, solvent extraction, saponification, derivatization to TMS ether derivatives and determination. Dutta and Normén [109] examined the separation of Δ^5-unsaturated and saturated phytosterols using a capillary GC. Phytosterols are also important indicators of lipoprotein metabolism. Phillips et al. [110] reported a profile analysis of phytosterols, stanols and cholesterol precursors in human serum using a capillary GC. Sitosterol, campesterol, stigmasterol, sitostanol, campestanol, lathosterol, desmosterol, lathostanol and epicholesterol (I.S.) were separated on the GC column and quantified with FID.

6. Bile acids

The naturally occurring common bile acids are saturated C_{24} steroid carboxylic acids (cholanes). The primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA), are formed via 5β-saturation of the cholesterol double bond by hepatic enzymes, epimerization of the 3β-hydroxy group to the 3α-configuration and further insertion of a 7α-hydroxy group with or without a 12α-hydroxy group, and shortening of the side chain by three carbons and oxidation of the terminal carbon to a carboxy group. The secondary bile acids, deoxycholic acid (DCA) and lithocholic acid (LCA), are formed from the primary bile acids by the action of intestinal organisms. Bile acids are conjugated in the human liver with taurine or glycine before they are secreted via the biliary canaliculi into the bile. Ursodeoxycholic acid (UDCA), 7β-epimer of CDCA, is formed by bacterial action. These five bile acids are known as ''common'' bile acids (Fig. 5). In addition, bile acids are metabolized to sulfate or glucuronide in liver or kidney. In the normal situation in humans, bile acids are largely confined to the enterohepatic circulation, but in disease, the concentration of bile acids present in blood, urine and feces may dramatically increase. The chromatographic analysis, such as GC, HPLC or their combined MS, has been widely used because it enables a study on the profile of bile acids in biological materials. The reviews on bile acids published in 1998 [11], 1999 [12] and the book [111] as described previously cover the above issue, and the following section reviews the chromatographic analysis of bile acids published after 1998.

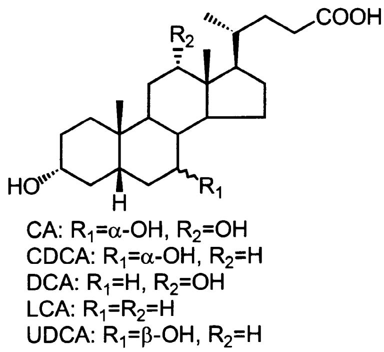

Fig. 5. Structures of common bile acids.

Chaudhury and Chaplin [112] presented a rapid method for the extraction of fecal bile acids using a supercritical fluid as an alternative to the tedious solvent extraction method. The method enabled fast determination of fecal bile acids using purification by ODS solid-phase extraction and HPLC analysis with pulsed amperometric detection. Batta et al. [113] reported a GC method for the determination of common bile acids as *n*-butyl ester-TMS ether derivatives. These bile acid derivatives showed longer retention times, were completely resolved and eluted later than the TMS ethers of common plasma sterols including sitosterol. This allows simplification of the pretreatment of plasma, and the method was applied for the determination of bile acids in human plasma. Scalia and co-workers developed a procedure for the determination of UDCA in human plasma and bile after administration of UDCA-containing dosage forms, which are widely used for treatment of cholesterol gall-stones and the therapy of cholestatic liver diseases, using GC–MS [114,115] and HPLC [114], and applied the method to determine the bioavailability of UDCA. The hexafluoroisopropyl trifluoroacetyl ester derivative was selected for GC–MS analysis of plasma UDCA levels, because it was easily and rapidly prepared by a one-step reaction. Biliary UDCA levels were determined by a rapid and simple HPLC with on-line sample purification.

Sakaki et al. [116] reported a simple and efficient method for the separation of a mixture of different series of ionic, highly polar, and hydrophilic conjugates of bile acids by ion-pair HPLC with a new volatile ion-pair reagent, di-*n*-butylamine acetate, as the mobile phase additive. The examined substrates included C-24 glycine- or taurine-amidated, 3-sulfated, 3-glucosylated, 3-*N*-acetylglucosaminidated, and glucuronidated conjugates of CA, CDCA, DCA and UDCA, as well as their double-conjugated forms. This method may be applied to the LC–MS analysis of these biologically important bile acid conjugates. Simultaneous analysis and structure–retention correlation were studied for various glycosidic-conjugated bile acids, including β-glucuronide, β-glucoside and β-*N*-acetylglucosaminide conjugates of bile acids, using HPLC with FL detection by Momose et al. [117] (Fig. 6). The bile acids as their C-24 pyrenacyl ester derivatives were measured on an ODS column, eluting with the mobile phase in the absence or presence of heptakis-(2,6-di-*O*-methyl)-β-cyclodextrin (Me-β-CD). The obtained data provided important information for characterizing the structures of bile acid glycosides. In connection with hepatobiliary diseases, the metabolic conjugation of bile acids into the corresponding carboxy-linked 24-glucuronides is of interest. These are ester glucuronides and are labile to acid or base treatment. Goto et al. [118] presented a method for identification of 24-glucuronides of common bile acids as their acetate-methyl ester derivatives, using LC–APCI-MS, in an incubation mixture with a male rat liver microsomal fraction (Fig. 7). Resolution of the α- and β-isomers of CA-, DCA-, UDCA-24-glucuronide was attended by Sumichiral OA-2500, and that of CDCA- and LCA-24-glucuronide by Cosmosil $5C_8$ column, and the mobile phase containing $AcONH_4$. The detection limit for glucuronide acetate-methyl ester was about 1 pmol ($S/N=5$) with the SIM mode monitoring characteristic ions $[M+NH_4]^+$. Ikegawa et al. [119] developed a method for

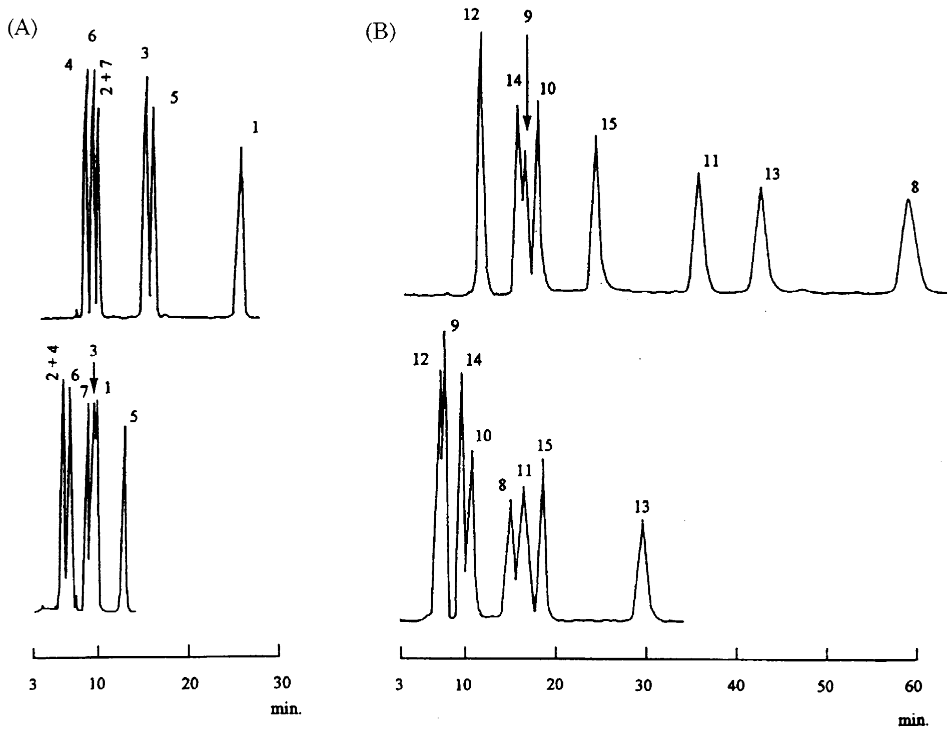

Fig. 6. Changes in the HPLC chromatograms of a mixture of unconjugated and glucuronic acid conjugated bile acids in the absence (upper) or presence (lower) of 5 m*M* Me-β-CD in a mobile phase. (A) Unconjugates, (B) glucuronides. Column, Capcell Pak C_{18}; detection, FL; mobile phase, MeOH–water (9:1, v/v). Peaks: 1, LCA; 2, hyodeoxycholic acid (HDCA); 3, CDCA; 4, UDCA; 5, DCA; 6, hyocholic acid (HCA); 7, CA; 8, LCA 3-glucuronide (G); 9, HDCA3G; 10, HDCA6G; 11, CDCA3G; 12, UDCA3G; 13, DCA3G, 14; HCA3G; 15, CA3G [117].

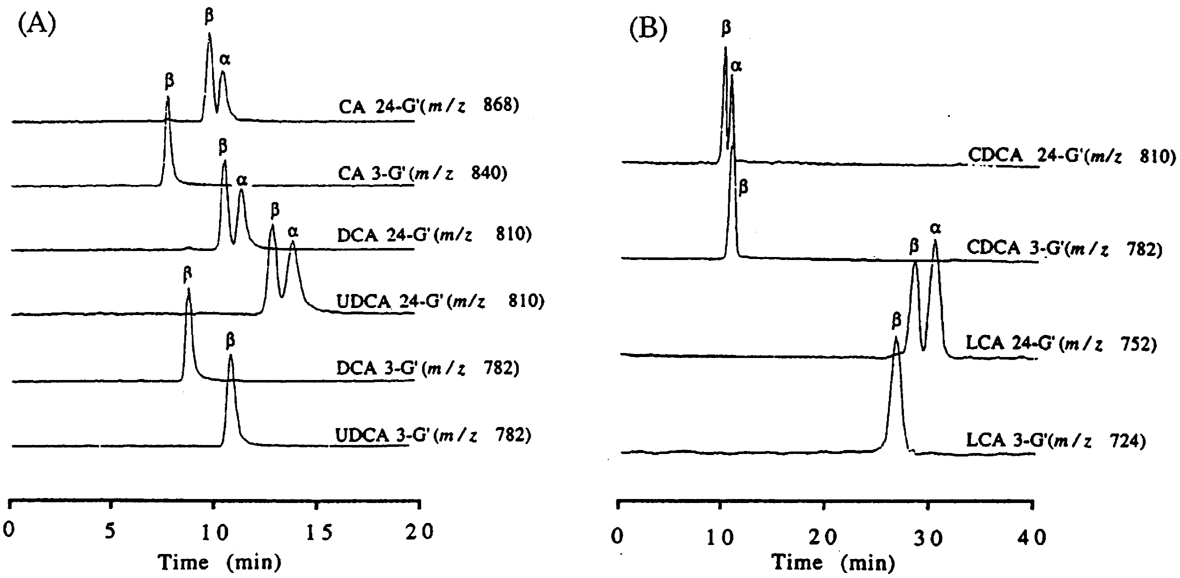

Fig. 7. LC–MS chromatograms of bile acid 24- and 3-glucuronide acetate-methyl esters (G′) monitored with corresponding adduct ions $[M+NH_4]^+$. Mobile phase, 200 m*M* $AcONH_4$ (pH 7.0)–MeOH (1:4, v/v); column, (A) Sumichiral OA-2500, (B) Cosmosil $5C_8$ [118].

the determination of the activity of hepatic glucuronosyltransferase catalyzing the formation of bile acid 24-glucuronides using HPLC with pulsed amperometric detection, and the detection limit was 10 ng. The characterization of cholyl-adenylate, covalently bound CA with 5′-monophosphate having an acid anhydride linkage, in an incubation mixture with a rat liver microsomal fraction using LC–ESI-MS in the NI mode has also been reported by Ikegawa et al. [120].

An inborn error that affects bile acid synthesis may lead to a deficiency of bile acids or to the accumulation of bile acid intermediates and their metabolites. Kimura and co-workers measured the concentration of urinary bile acids in infants, newborns and children, either without or with hepatic diseases [121–124]. Bile acids including unusual bile acids, such as 3-oxo-4-ene-, 3-oxo-4,6-diene- [121,122], 3β-hydroxy-5-ene- [123], other unsaturated ketonic and 7β-hydroxylated bile acids [124], were analyzed using GC–MS with SIM after derivatization to methyl ester-dimethylethylsilyl (DMES) ether and MO (in the case of 3-oxo bile acids). Setchell et al. [125] determined bile acids in urine, serum and liver tissue from patients with an oxysterol 7α-hydroxylase deficiency using GC–MS after derivatization to methyl ester–TMS ether, and confirmed that 3β-hydroxy-5-cholenoic acid and 3β-hydroxy-5-cholestenoic acid were the major products of hepatic synthesis.

CoA esters of 3α,7α,12α-trihydroxy- and 3α,7α-dihydroxy-5β-cholestan-26-oic acids and their 24-hydroxy or 24-ene forms are the intermediates in the oxidative shortening of the side chain (β-oxidation) in the biosynthesis of bile acids. Kurosawa et al. [126] and Bun-ya et al. [127] reported the analysis of steroisomers of C_{27}-bile acid CoA esters using HPLC (UV) and GC–MS (methyl ester-DMES ether derivative), respectively, and applied the method to the study of the stereochemistry of the products by enzymic reaction related to the β-oxidation. Vreken et al. [128] established the method for determination of 3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oic acid (varanic acid) by GC–MS. The separation of four varanic acid diastereomers was enabled by use of 2*R*-butyl ester–TMS ether derivatives. Using this method, they showed the specific accumulation of varanic acid diastereomers in the serum of a patient with a deficiency of peroxisomal D-bifunctional protein, which catalyzes β-oxidation.

7. Vitamin D and its metabolites

The so-called ''Vitamin D'' actually consists of two different compounds, vitamin D_3 (D_3) and vitamin D_2 (D_2), which differ in their side-chain structure at the 17β-position of the secosteroid. Although D_3 and D_2 are both adsorbed from the diet, only D_3 is biosynthesized in the skin upon irradiation of provitamin D_3, 7-dehydrocholesterol, with UV light. The levels of D_2 and its metabolites in biological fluids are usually less than one-tenth of those of D_3 and its metabolites. Vitamin D is transported with vitamin D binding protein (DBP), hydroxylated in the liver to 25-hydroxyvitamin D [25(OH)D], which is further metabolized in the kidney to the active form, 1α,25-dihydroxyvitamin D [1,25$(OH)_2$D], and has important functions, such as the regulation of calcium metabolism and bone formation. The induction of cell differentiation and immunoregulation by 1,25$(OH)_2$D were also reported, and much interest has been focused on this metabolite and its analogue as potential antileukemia agents. On the other hand, when the serum calcium level is greater than 90 μg/ml, 25(OH)D is mainly metabolized to 24*R*,25-dihydroxyvitamin D [24,25$(OH)_2$D], but also hydroxylated at the 23- and 26-positions to a certain extent (Fig. 8). These metabolites are further oxidized with the result that a large number of metabolites coexist in the blood.

The measurements of the serum/plasma concentration of the D_3 metabolites, especially 25(OH)D_3 (10–40 ng/ml) and 1,25(OH)D_3 (30–65 pg/ml), are widely used for the assessment of the vitamin D status and the follow-up of several diseases (parathyroid gland disorders, renal failure, rickets and sarcoidosis). 24,25$(OH)_2D_3$ at pharmacological doses was reported to cause a marked increase in bone volume and mechanical strength in animals without hypercalcemia, and it is expected to be an anti-osteoporotic drug. Therefore, the determination of the serum/plasma 24,25$(OH)_2D_3$ levels (0.5–4 ng/ml) is necessary to clarify its physiological significance and to develop new drugs.

GC–MS has a high ability for structural elucidation and plays an active role in the characterization of vitamin D metabolites. However, vitamin D compounds undergo thermal isomerization to give two isomers, pyro- and isopyro-forms, when the temperature rises to about 100°C, which is not always suitable for the quantitative analysis of these compounds using GC and GC–MS. The thermal isomerization is negligible in the HPLC analysis, and a number of applications using this methodology have been reported. Because of the relatively high level at which it circulates in serum, 25(OH)D_3 has been measured by HPLC with UV detection. In contrast the measurement of 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$ has been usually carried out in clinical applications by competitive protein binding assay using DBP and radioreceptor assay using a vitamin D receptor, respectively, but complicated pretreatment steps are required to remove some cross-reactive and interfering endogenous substances. To overcome this problem, sensitive immunoassays for 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$ have recently been developed. Furthermore, due to the development of derivatization methods and the advances in detection techniques, assay methods for 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$ based upon HPLC have been reported. All the previously described analytical methods, which were published before 1994, were presented in a couple of review articles [13,14] and a book [129].

In the first part of this section, methods using HPLC for the analysis of unconjugated vitamin D metabolites are reviewed. In the second part, an overview of chromatographic analysis, especially LC–MS, of conjugated vitamin D metabolites in biological fluids is presented. That is, the phase II reaction products (conjugated metabolites) of vitamin D, which are poorly understood compared to the unconjugated metabolites, have recently been found in animals, and much interest is focused on their physiological roles.

The methods using HPLC for the determination of unconjugated vitamin D and its metabolites in human serum/plasma reported after 1995 are summarized in Table 2. As mentioned above, 25(OH)D_3 can be measured with a UV detector based on its absorption at 265 nm (molar absorptivity, ϵ=ca. 18 000). Assay of vitamin D metabolites in serum/plasma usually involves extraction with organic solvents, accompanied by dissociation of the metabolites and DBP, and purification by several steps of chromatography followed by determination. Although early developed 25(OH)D_3 assays required a preparative HPLC step to remove interference, especially lipids,

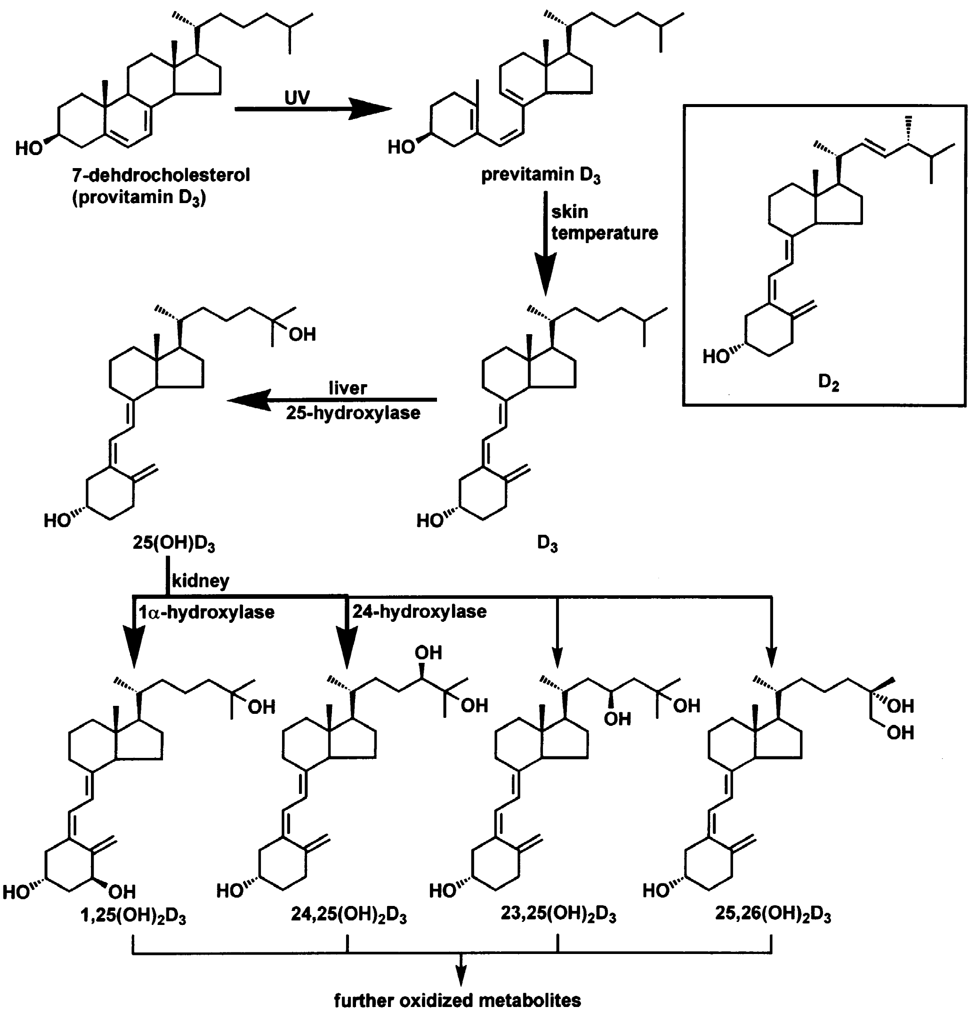

Fig. 8. Biosynthesis and metabolism of vitamin D_3 in human.

prior to quantitative analysis, this step has been recently replaced by solid-phase extraction using disposable cartridges. To account for recovery during the pretreatment procedure, ^{3}H-labeled compounds have been used, but they are not always favorable because of problems concerning disposal. A method reported by Takeuchi et al. [130] also used [^{3}H]25(OH)D_3 for the determination of the recovery. Whereas Shimizu et al. [131] reported a non-radioactive method using 22-hydroxytetranorvitamin D_3 as I.S. The two methods have used a normal-phase column, which is not always sufficiently versatile for routine analysis because serum/plasma contains polar components. In order to overcome these problems, a method employing a reversed-phase column and a non-radioisotope I.S. was developed by the authors [132]. Ortiz-Boyer and co-workers [133–136] reported a method for the determination of D_3 metabolites based on a continuous clean up/preconcentration procedure coupled with HPLC with UV detection. Although they first used an ODS mini-column as a pretreatment column [133–135], finally an aminopropylsilica column was used for that purpose [136]. The method exhibits a linear range between 0.05 and 100 ng/ml for a 2-ml plasma aliquot. The plasma concentrations of

Table 2
HPLC for the determination of unconjugated vitamin D and its metabolites in human serum/plasma

Analyte	Sample preparation	Analytical HPLC system (column, mobile phase and detection)	Determination limit (volume used)	I.S.	Ref.
$25(OH)D_2$, $25(OH)D_3$	MeCN extraction, Bond Elut C18OH and Bond Elut C18	Zorbax SIL, hexane–IPA, UV (265 nm)	0.5 ng/injection	$[^3H]25(OH)D_3$	[130]
$25(OH)D_3$	CH_2Cl_2–MeOH extraction and Sep-Pak silica	Zorbax SIL, hexane–$CHCl_3$–MeOH, UV (265 nm)	Not mentioned (1–2 ml)	22-Hydroxy-24,25, 26,27-tetranor-vitamin D_3	[131]
$25(OH)D_3$	EtOH extraction, Et_2O extraction and silica-gel column	J'sphere ODS-H80, MeCN–water, UV (265 nm)	5 ng/ml (0.5 ml)	25-Hydroxy-ergosterol	[132]
D_2, D_3, $25(OH)D_3$, $24,25(OH)_2D_3$, $1,25(OH)_2D_3$	IPA extraction, hexane extraction and column switching technique (amino-propylsilica column)	Ultrabase C_{18}, linear gradient MeCN–phosphate buffer to MeCN–MeOH, UV (270 nm)	50 pg/ml (2 ml)	Not used	[136]
$25(OH)D_3$, $24,25(OH)_2D_3$	CH_2Cl_2–MeOH extraction and NP-HPLC	Nucleosil $5C_{18}$, MeOH–MeCN–$HClO_4$ aq., ED (coulometric)	250 pg/ml (0.1 ml)	Not used	[138]
$25(OH)D_3$, $24,25(OH)_2D_3$, $1,25(OH)_2D_3$	IPA extraction, hexane extraction, column switching technique (amino-propylsilica column) and H_2SO_4 treatment	Ultrabase C_{18}, linear gradient MeCN–phosphate buffer to IPA–MeOH, FL ($\lambda_{ex.}$ 355 nm, $\lambda_{em.}$ 482 nm)	0.05 pg/ml (2 ml)	Not used	[140]
$25(OH)D_3$, $24,25(OH)_2D_3$, $1,25(OH)_2D_3$	CH_2Cl_2–MeOH extraction, Sep-Pak silica (A) and derivatization with DMEQTAD for $25(OH)D_3$ (A) plus NP-HPLC, derivatization and Bond Elut PSA (B) for $24,25(OH)_2D_3$ (B) plus NP-HPLC for $1,25(OH)_2D_3$	YMC-Pack ODS-AM, linear gradient MeOH–water to MeOH, FL ($\lambda_{ex.}$ 370 nm, $\lambda_{em.}$ 440 nm)	10 pg of derivatives/injection (2.5 ml)	22-Hydroxy-24,25, 26,27-tertanor-vitamin D_3 for $25(OH)D_3$ $[^3H]24,25(OH)_2D_3$ $[^3H]1,25(OH)_2D_3$	[142]
$24,25(OH)_2D_3$	MeCN extraction, Oasis HLB, silica-gel column and derivatization with MBOTAD	J'sphere ODS H-80, MeCN–water, APCI-MS ($[M+H]^+$)	230 pg/ml (0.3 ml)	$[^2H_6]24,25(OH)_2D_3$	[143]

IPA, isopropanol; NP, normal-phase.

$24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ obtained by the method were considerably higher than those previously reported, which is possibly due to contamination by other substances in the peaks corresponding to $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$. In addition to these methods, a capillary liquid chromatography of D_3 in bovine blood plasma has also been reported [137], in which 1 pg of D_3 was determined with UV detection ($S/N=3$).

In order to obtain greater sensitivity, other detection methods have been employed. A method using HPLC with coulometric ED for assay of $25(OH)D_3$ and $24,25(OH)_2D_3$ in serum has been published [138]. The method was highly sensitive with a determination limit of 250 pg/ml for a 0.1 ml plasma aliquot. A disadvantage of the method is the necessity of a preparative HPLC step in sample pretreatment and the absence of I.S., which should be used because of insufficient reproducibility of ED.

The FL detection of vitamin D metabolites has also been investigated. Vitamin D metabolites themselves are not fluorescent, and in order to utilize this methodology, some derivatization methods are required. Ortiz-Boyer and co-workers [139,140] converted D_3 metabolites [25(OH)D_3, 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$] to fluorescent substances by treatment with sulfuric acid after separation on a reversed-phase column. The method also employed the clean up/pre-concentration procedure mentioned above, and 0.05 pg/ml of the metabolites was measurable ($S/N=3$), when an Nd–Yag laser was used as an excitation source [140].

The Cookson-type reagent is a substituted 1,2,4-triazoline-3,5-dione (TAD), and TAD is a powerful dienophile which reacts with *s-cis*-diene of the vitamin D compound to form a Diels–Alder adduct (Fig. 9). By linking TAD with fluorescent moieties, several fluorescent Cookson-type reagents have been developed and applied to the FL detection of D_3 metabolites as a pre-column labeling reagent [13,14,129]. Shimizu and co-workers [141,142] synthesized 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalinyl)ethyl]-TAD (DMEQTAD) and applied the reagent to fluorometric assay of D_3 metabolites in human plasma. Although the method required complicated and time-consuming pretreatment steps including preparative HPLC (see Table 2), 10 pg of derivatives (equivalent to 5 pg of intact D_3 metabolites) was quantified.

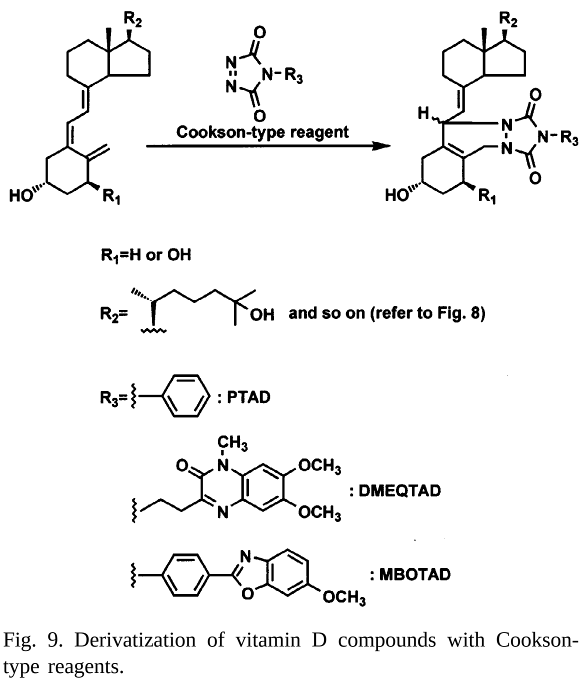

Fig. 9. Derivatization of vitamin D compounds with Cookson-type reagents.

LC–MS is considered to be a rapid and convenient method for the determination of vitamin D compounds in biological fluids. However, except for some synthetic analogues, the ionization efficiency of vitamin D compounds is very low; therefore, practical LC–MS methods for the determination of endogenous vitamin D metabolites in biological fluids had not yet been reported. Derivatives with Cookson-type reagents have also been examined in various ionization methods to enhance the sensitivity. The authors observed that compounds being rich in proton-affinitive atoms, such as oxygen and nitrogen, are excellent substrates for detection with APCI-MS operating in the PI mode. The authors developed one of the Cookson-type reagents called MBOTAD {4-[4-(6-methoxy-2-benzoxazolyl)-phenyl]TAD} as an FL labeling reagent for the HPLC analysis, which was applied to an assay of 24,25$(OH)_2D_3$ in plasma using LC–APCI-MS [143]. In the developed method, derivatized 24,25$(OH)_2D_3$ was subjected to LC–MS without removing the excess reagent, which is inevitable for the HPLC analysis using FL detection. The sensitivity increased twenty times due to the derivatization to give a detection limit of 18 fmol [equivalent to 7.5 pg of 24,25$(OH)_2D_3$; $S/N=3$]. The method was also rapid with an analysis time of 10 min from one injection to the next injection. Incidentally, a review article on mass spectrometric analysis of vitamin D compounds has also been published [144].

Like all steroids, vitamin D undergoes metabolism that in general renders the molecule more hydrophilic, and in the case of vitamin D, usually involves the introduction of an extra hydroxy function in the side chain. Inouye and co-workers have investigated the metabolism of D_3 using the recombinant vitamin D 25-hydroxylase expressed in *Escherichia coli* [145]. They have also reported the metabolism of

$25(OH)D_3$ by recombinant 25(OH)D 1α-hydroxylase [146] and 24-hydroxylase [147,148]. The formed metabolites were separated on a reversed-phase HPLC with a linear gradient elution and identified using APCI-MS operating in the PI mode, except for calcitroic acid, which was analyzed by ESI-MS operating in the NI mode.

Recently, a new metabolic pathway of vitamin D has been clarified by several investigators including the authors, which is the epimerization of the 3-hydroxy group on the steroid A-ring. Sekimoto et al. [149] isolated 3-epi-$1,25(OH)_2D_3$ from serum of rats treated with pharmacological doses of $1,25(OH)_2D_3$. This metabolite has also been detected in several cell lines incubated with $1,25(OH)_2D_3$ [150]. The separation of $1,25(OH)_2D_3$ and its 3-epimer was performed by a normal-phase HPLC with a mixture of hexane and isopropanol as eluent [149,150]. 3-Epi-$24,25(OH)_2D_3$ was first isolated as its 24-glucuronide (G) from the bile of rats administered $24,25(OH)_2D_3$ [151,152], and then it was also detected as the unconjugated form in plasma of rats dosed with $24,25(OH)_2D_3$ [153] or $25(OH)D_3$ [154]. Although $24,25(OH)_2D_3$ and its 3-epimer could hardly be separated in the usual reversed- and normal-phase HPLC, their separation was achieved by an inclusion reversed-phase HPLC using γ-cyclodextrin as the mobile phase additive [153,154]. The characterization of 3-epi-$24,25(OH)_2D_3$ was also carried out by LC–APCI-MS after derivatization with 4-phenyl-TAD (PTAD), giving rise to characteristic product ions by MS–MS and satisfactory separation of $24,25(OH)_2D_3$ and 3-epi-$24,25(OH)_2D_3$. The authors also investigated the epimerization mechanism in rats using LC–MS [153]. We first found that the enzyme controlling the epimerization existed in the cytosol fraction in the liver and required NAD and NADPH as coenzymes, and then the in vitro system for production of the 3-epimer from $24,25(OH)_2D_3$ was established. Using this in vitro system, the disappearance of deuterium of [3α-^{2}H]$24,25(OH)_2D_3$ was monitored by LC–MS. As a result, the deuterium was eliminated and displaced by a proton during the 3-epimerization, which meant that the 3-epimer was formed via the 3-oxo-form.

Due to the advances in analytical instruments, especially LC–MS, several conjugated vitamin D metabolites have been isolated and characterized. $25(OH)D_3$ 3-sulfate (S) was first identified and detected in human plasma by Axelson and Christensen [155,156]. The method relied on an indirect method, that is, $25(OH)D_3$ formed by hydrolysis was detected by HPLC and GC–MS, in which radiolabeled compound was used as I.S.. The authors developed a method using HPLC for the determination of $25(OH)D_3$3S in human plasma without hydrolysis [157,158]. The method employed solid-phase extraction, lipophilic ion-exchange chromatography (piperidinohydroxypropyl Sephadex LH-20; PHP-LH-20), and then separation on a reversed-phase HPLC with UV detection. The MBOTAD adduct of 25-hydroxy-7-dehydrocholesterol 3S was used as I.S. and the determination limit was 5 ng/ml. The method was applied to the determination of $25(OH)D_3$3S in plasma of patients with chronic renal failure and clarified that this sulfate was not detected in about one-third of plasma of patients [159].

The authors have reported the separation and characterization of monoglucuronides of D_3, D_2, $25(OH)D_3$ and $25(OH)D_2$ in the bile of rats orally administered each unconjugated form [160,161] and showed that the glucuronidation occurred not only at the 3- but also at the highly hindered 25-position (*tert*-hydroxy group) of the secosteroid [in $25(OH)D_3$, 3G:25G=ca. 5:2, in $25(OH)D_2$, 3G:25G=ca. 1:1]. The monoglucuronides were isolated by solid-phase extraction, PHP-LH-20 chromatography and preparative HPLC, and identified by comparison with authentic samples based on their chromatographic behavior and data obtained from enzymic hydrolysis using β-glucuronidase. In addition, the characterization of monoglucuronides of D_2 and $25(OH)D_2$ was done by LC–APCI-MS with a mobile phase containing $AcONH_4$ [161], in which molecular related ions ($[M-H]^-$) were observed in the NI mode. In addition, when the glucuronides were converted to their methyl esters, adduct ions ($[M+NH_4]^+$) were also observed in the PI mode.

Shimoyamada et al. [162] identified 23,25-dihydroxy-24-oxovitamin D_3 23G [$23,25(OH)_2$-24-oxo$D_3$23G] as a major biliary metabolite of rats dosed with $24,25(OH)_2D_3$ by LC–frit-fast atom bombardment–MS after methylation. The authors investigated the in vitro and in vivo glucuronidation of 24,25$(OH)_2D_3$ [163,164]. The formed monoglucuronides of 24,25$(OH)_2D_3$ were identified by LC–ESI-MS–MS, in which the PI mode was useful for the structural elucidation, though it was inferior to the NI mode in terms of sensitivity.

The conjugated metabolites in the bile of rats administered 24,25$(OH)_2D_3$ or 25$(OH)D_3$ were further studied using LC–MS by the authors [151,152]. The metabolites showing typical UV absorption spectra ($\lambda_{max.}$ 268 nm, $\lambda_{min.}$ 230 nm) of the vitamin D-triene structure on a photodiode array detector were isolated by several chromatographic steps, and then subjected to hydrolysis. The obtained aglycons of the metabolites were elucidated based on the chromatographic behavior and the mass spectral data (GC–MS and LC–APCI-MS). The conjugation positions were determined with LC–ESI-MS–MS–MS using the derivatization as described below [165]. Collision-induced dissociation used in LC–MS usually gives fragmentations based on the cleavage of carbon–hetero atom bonds or loss of water and alcohol, and cleavage of carbon–carbon bonds hardly occurs because of the low collision energy. Therefore, it is not easy to discriminate the positional isomers by LC–MS: indeed, no remarkable difference was observed in the product ion mass spectra of 24,25$(OH)_2D_3$3G and 24,25$(OH)_2D_3$24G, which were used as model compounds. On the contrary, the PTAD adducts of conjugated vitamin D metabolites were cleaved at the C-6–7 bond of the vitamin D skeleton and provided a product ion with the conjugation position indicated by a PI-ESI-MS–MS–MS. That is, in the case where the conjugation position exists at the steroid A-ring, a characteristic product ion at m/z 474 for the glucuronide or 378 for the sulfate is observed, whereas in the case where the conjugation position is on the side chain, only the corresponding ion (m/z 298) of the steroid A-ring is observed. Furthermore, whether the conjugation occurs at the 25-position or not can be established by acetylation with acetic anhydride and pyridine, because the reaction usually does not occur at the *tert*-hydroxy group. Using these methods, 24,25$(OH)_2D_3$3G, 24,25$(OH)_2D_3$24G, 24,25$(OH)_2$-$D_3$3S, 3-epi-24,25$(OH)_2D_3$24G and 23,25$(OH)_2$-24-oxo$D_3$23G were identified in the bile of rats administered 24,25$(OH)_2D_3$. In addition, 25$(OH)D_3$3G, 25$(OH)D_3$25G, 25$(OH)D_3$3S, 24,25$(OH)_2D_3$24G, 24,25$(OH)_2D_3$3S, 3-epi-24,25$(OH)_2D_3$24G, 23,25$(OH)_2$-24-oxo$D_3$23G and 23,25-dihydroxyvitamin D_3 23G [23,25$(OH)_2D_3$23G] were identified in the bile of rats administered 25$(OH)D_3$. Furthermore, based on the data in which significant amounts of the glucuronides of 23,25$(OH)_2D_3$ and 23,25$(OH)_2$-24-oxoD_3 were determined, the authors inferred that the hydroxylation at the 23-position plays an important role in the excretion of vitamin D.

8. Phytosteroids

The analysis of phytosteroids except for phytosterols is reviewed in this section; the analysis of the latter compounds was cited in Section 5. In plants, steroids play an important role as constituents of cell membranes, insect deterrents, and growth hormones. Steroids extracted from plants have been used in traditional medicine and some of these, like cardiac glycosides such as digoxin and digitoxin originated from *Digitalis*, are important and valuable drugs [166].

Although the value of monitoring the serum/plasma concentration of cardiac glycosides in clinical practice remains controversial (effective concentration: 0.5–2.0 ng/ml), the determinations of serum/plasma levels of these drugs are among the most frequent tests in the clinical laboratory. Although commercial kits based upon immunoassays are widely used to determine the serum/plasma concentration, these methods interact with an immunoreactive factor called endogenous digoxin [166]. The sensitivity of HPLC with UV detection was not sufficient to determine serum/plasma levels of *Digitalis* glycosides. Pulsed amperometric ED of these glycosides and their metabolites using authentic samples has been attempted to provide greater sensitivity (linear peak response; 10–70 ng) and selectivity [167]. Different approaches using pre-column [168] or post-column [169] derivatization of these glycosides in human serum/plasma followed by HPLC with FL detection have been done and gave satisfactory results.

The analysis of authentic cardiac glycosides (such as digoxin) and related compounds have been attempted with LC–MS^n using ion-trap MS, and different ionization methods, ESI and APCI (the PI and NI modes), gave different results with respect to sensitivity and structural information [170]. A method based on HPLC coupled with ion-spray MS has been developed for the specific determination of digoxin and related glycosides in human plasma. After a single-step liquid–liquid extraction, the solutes are separated on a reversed-phase column using $HCOONH_4$ as mobile phase additive and then detected with post-column split method followed by ion-spray MS. The major ion observed is $[M+NH_4]^+$ and the detection limit ($S/N=2$) in the SIM mode ranges from 0.15 to 0.60 ng/ml, making the method less sensitive than RIA, whereas it is much more specific [171].

The identification and determination of cardiac glycosides (such as digoxin and digitoxin) in blood and urine by LC–tandem MS (MS–MS) using ESI and $HCOONH_4$ buffer have been done. Under mild conditions for collision-induced dissociation, each $[M+NH_4]^+$ ion fragmented to produce a dominant daughter ion, which was essential to a sensitive method of SIM for the determination of cardiac glycosides. With blood or urine samples, a detection limit for digoxin and digitoxin as low as 0.05–0.1 ng/ml was achieved at a S/N of more than 3 [172].

The on-line chromatography with immunochemical post-column FL energy transfer detection for digoxin and its metabolites has been developed, which resulted in enhanced selectivity and sensitivity. *R*-Phycoerythrin (PE) was used as the donor and an indodicarbocyanine dye (Cy5) as the acceptor label. These labels allow the detection in the far-red spectral region, which is more selective for biological samples. Hence, digoxin was labeled with PE and the monoclonal antibody was labeled with Cy5. Digoxin and its metabolites were injected into the HPLC system followed by post-column injection of PE-labeled digoxin and by Cy5 labeled anti-digoxin antibody. The dynamic range of digoxin spiked in buffer was 0.05–10 ng/ml [173].

Brassinosteroids are polyhydroxylated phytosteroids and serve as plant regulators. These were analyzed using LC–MS with APCI (the PI mode) as their naphthaleneboronates, which were stable under reversed-phase HPLC conditions and provided high sensitivity (detection limit of 2 ng; $S/N=3$) in SIM [174–176].

The simultaneous profiling of steroidal glycoalkaloids and their aglycons [177] and monitoring of the seasonal dependence of 20-hydroxyecdysone, the content of *Serratulas wolffii,* using reversed-phase HPLC have been reported [178]. The direct determination of seven major bioactive isosteroidal alkaloids in bulbs of *Fritillaria* species, a commonly available traditional Chinese medicinal herb, has been done using capillary GC [179].

9. Others

The term neurosteroids applies to those steroids that are both synthesized in the nervous system, either de novo from cholesterol or from steroid hormone precursors, and accumulate in the nervous system to levels that are at least in part independent of steroidogenic gland secretion rates. Neurosteroids mainly consist of 17- or 20-oxosteroids and accumulate in the brain as the unconjugated form and their sulfates, fatty acid esters and sulfolipids. Recently, much interest has been focused on neurosteroids and neuroactive steroids for their physiological significance in the brain, in particular, the central nervous system [180]. The authors characterized and determined neurosteroids including conjugates in rat brains using GC–MS and LC–MS [180–188] (Fig. 10). The LC–nano-ESI tandem MS (the NI mode) was applied to the structural analysis and detection

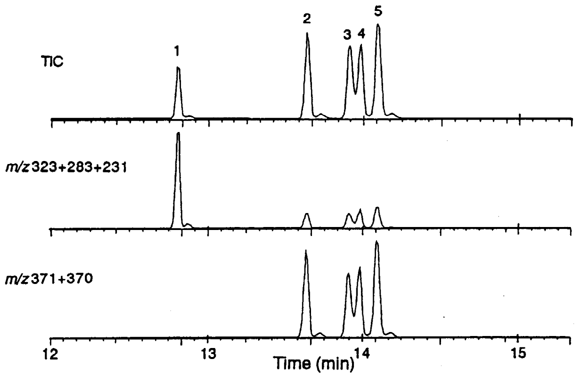

Fig. 10. GC–MS–MS chromatograms of authentic E_1 and guaiacol estrogens as TMS-MO derivatives. Peaks: 1, E_1; 2, $4OHE_1$ 4-methyl ether (Me); 3, $2OHE_1$3Me; 4, $2OHE_1$2Me; 5, $4OHE_1$3Me. Precursor ion, m/z 340 for E_1 (12.00–13.25 min) and m/z 401 for guaiacol estrogens (13.25–16.00 min); scan range: m/z 200–450 (TIC) [185].

of neurosteroids, in particular, steroid sulfates found in brains. A fused-silica reversed-phase capillary column was used for the separation. Complete structural information can be obtained from 1 ng of steroid sulfate, and the fragment ions characteristic of the sulfate ester group can be obtained from only 3 pg of sample [189]. GC–electron capture NI-CI-MS has been applied to the profile analysis of neurosteroids in cerebrospinal fluids and plasma. Neurosteroids were converted to carboxymethyloxime, pentafluorobenzyl and TMS derivatives, and the response curve showed linearity in the concentration range of 10–1000 pg/ml [190]. The validation of an analytical procedure to measure trace amounts of neurosteroids in brain tissue by GC–MS has been reported [191]. The analytical method for the determination of the neuroactive steroids, alphaxalone (a synthetic steroid) and pregnanolone, in rat plasma has been developed using reversed-phase HPLC with dansyl hydrazine as the fluorescent derivatization reagent. The method can be used on a routine basis for pharmacokinetic analysis of neuroactive steroids [192].

Direct detection of several authentic steroid sulfates and glucuronides, such as testosterone glucuronide and sulfate, was achieved with LC–ESI-MS. The PI mode MS and MS–MS spectra were employed to obtain both quantitative and structural information. Using a packed capillary column (300

Cookson type reagent provided satisfactory sensitivity and structural information [196].

10. Conclusions

Steroids show biologically important activities and some are used for medicines. Their analysis in biological fluids is necessary not only for drug monitoring but also to clarify their function in the human body. This review article underlines the importance of GC, HPLC, GC–MS and LC–MS for the determination of natural steroids, especially in biological fluids. GC and GC–MS are commonly used for the determination of volatile steroids. Although HPLC is a widely used analytical method for the determination of steroids including conjugated ones, LC–MS is considered to be the most promising method for this purpose due to its sensitivity, specificity and versatility. Skillful derivatization techniques are also necessary in utilizing these analytical methods.

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