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Review

Steroid analysis for medical diagnosis

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Abstract

Steroid assays are important for medical diagnosis of diseases related to steroid disturbances and abuse. This article reviews the recent progress in analytical methods for steroids in the clinical laboratory. The requirements for these methods are rapid, highly sensitive, specific, direct assay of conjugated steroids, the simultaneous analysis, identification of unknown steroids, and ultra-miniaturization of the separation system.

Keywords: Reviews; Clinical laboratory; Separation analysis; Steroids; Hormones

Contents

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1. Introduction

Steroid hormones are important in controlling human body functions as a part of the endocrine system together with neuronal systems and the immune system.

Steroids assay are used clinically for diagnosis of stress [1], Cushing's syndrome [2], hirsutism [3], congenital steroid enzyme deficiency [4], hypertension [5], and as a test for doping [6].

Determination of steroids has become important in the fields of analytical chemistry including medical, pharmaceutical, engineering and agricultural sciences. However, the methods for steroid assays in the clinical laboratory are different from those in other fields because the clinical laboratory must respond to clinical demands for rapid, highly sensitive, simple and automated assays, those are safe and do not produce toxic waste.

Although both immunoassay and separation assay methods have been employed for determination of steroids in the clinical laboratory, immunoassay methods are more frequently used than separation methods because immunoassay methods meet the requirements of clinical assays. However, separation methods for steroid assay have recently made technical advances, and the recent progresses in separation methods for steroid assay are reviewed in this paper.

2. Types and functions of steroids

There are many kinds of steroids in the human body and in different pharmaceutical preparations. Steroids are active at very low concentrations, and have a range of biological effects (e.g., homeostasis). Clinical functions of steroids are reviewed in the following sections.

2.1. Glucocorticoids

Glucocorticoids are excreted from the adrenocortex with a circadian rhythm, and the most important glucocorticoid is cortisol. Glucocorticoids are important in protecting against stress, shock, inflammation, etc.

2.2. Synthetic glucocorticoids

Synthetic glucocorticoids have functions like natural glucocorticoids, that is, anti inflammatory, anti shock, anti stress functions; representative of synthetic glucocorticoids is betamethazone.

2.3. Mineralocorticoids

Mineralocorticoids are excreted from the adrenocortex; representative of mineralocorticoids is corticosterone. Mineralocorticoids act to maintain blood pressure, and control water and electrolytes (sodium and potassium) homeostasis.

2.4. Sex hormones

There are three kinds of sex hormones; androgens, estrogens, and progestins.

2.4.1. Androgens

Androgens are excreted from the testis and adrenocortex, and the major androgen is testosterone. Androgens have a virilization effect and increase muscle volume. Androgens also promote growth of prostate cancer cells.

2.4.2. Estrogens

Estrogens are excreted from the ovary; representative of estrogens is estradiol. Estrogens work to make ovulatory phase of menstrual cycle and development of breast and bone. Besides, estrogens promote growth of breast cancer cells.

2.4.3. Progestins

Progestins are excreted from the ovary, and a representative example of a progestin is progesterone. Progestins act during the lutenizing phase of the menstrual cycle and are involved in maturation of the endometrium of the uterus.

2.4.4. Anabolic steroids

Anabolic steroids including natural testosterone and dehydroepiandrosterone have androgen like functions and increase volume of muscle. Therefore, anabolic steroids are assayed to detect doping and abuse.

3. Characteristics of steroids for separation analysis

3.1. Steroid analysis and its structure

Steroids in human body fluids are present in free and conjugated forms [7]. Free steroids are slightly hydrophobic, and the conjugated forms (glucuronide and sulfate) are hydrophilic. The solubility of steroids in water influences its extraction methods from specimens. Steroids have molecular sizes ranging from about 200–1000, and are non-volatile. This means that steroids need to be derivatized for assay by gas chromatography (GC). Steroids have UV absorption (254 nm) but no native fluorescence except for estrogens [8]. The major functional groups of steroids are hydroxyl and ketone groups those are weak in chemical reactivity, and this is a disadvantage of steroids for highly sensitive detection by fluorescence, chemiluminescence, or mass spectrometry (MS).

3.2. Behaviors of steroids in specimens

Specimens used for clinical analysis of steroids include urine, serum, and saliva, and have some of behaviors of steroids (free forms, conjugated forms, protein binding forms, etc.). Different behavior of steroids in human specimens requires different considerations for developing methods for steroids.

3.2.1. Urine

Human urine specimens contain steroids in conjugated and free forms. Conjugated steroids are the major fraction (e.g., about 99.7%) and free forms are present in minor amounts (e.g., about 0.3%). Urine specimens are collected for 24 h to avoid the influence of circadian rhythm of steroid and steroid metabolite excretion.

3.2.1.1. Conjugated form

Conjugated steroids are often hydrolyzed with a strong inorganic acid (concentrated sulfuric acid, hydrochloric acid, or nitric acid), or treated with sulfatase or glucuronidase enzymes (Helix Pomatia juice, etc.) to produce the free form prior to analysis. The conjugated forms of steroids are important and give information on conjugating capacity of conjuga-

tion enzymes (delta 4-hydrogenase, steroid dehydrogenase, hydroxylase, etc.), hence conjugated steroids are targets of direct analysis by high-performance liquid chromatography (HPLC)–MS which is described in more detail later. The most commonly used type of MS is the negative chemical ionization type.

3.2.1.2. Free form

Free forms of steroids in urine are often at too low a level (e.g., cortisol about 100 n*M*/day) to assay, but represent the active form of steroids in serum. Therefore, enrichment of free urinary steroids are carried out by extraction of urine specimens with octadecyl silica (ODS, C_{18}) or BSA-ODS (bovine serum albumin bound ODS) gel packed minicolumns prior to the assay of free steroids [9,10].

3.2.2. Serum

Steroids in serum specimens exist in three forms, (i) free form (about 4%), (ii) albumin bound form (about 5%) and (iii) globulin bound form (about 91%). Only free forms of steroids are active biologically, hence the albumin and globulin bound forms are inactive.

3.2.2.1. Free form

Steroids in free forms are under negative feedback regulation by pituitary (adrenocorticotropic hormone; ACTH, follicle stimulating hormone; FSH, lutenizing hormone; LH) and hypothalamic hormones (corticotrophin-releasing hormone; CRH, LH releasing hormone; LH-RH, FSH-releasing hormone; FSH-RH), and show circadian rhythms of excretion. To obtain information of serum-free steroids, serum specimens are ultra filtrated by dialysis [11] or membrane filtration.

3.2.2.2. Globulin bound form

A large part of unconjugated serum steroids are bound to serum globulin. Sex steroid hormones (androgens, estrogens and progestins) bind to globulin (sex hormone binding globulin, SBG) [12] and corticosteroid hormones also bind to globulin (corticosteroid binding globulin, CBG) [13]. These steroids bound to globulin have no biological activity as steroids. Binding force of steroids to SBG or CBG is strong, but the steroids are released by extraction with organic solvent or a C_{18} gel minicolumn [14].

3.2.2.3. Albumin bound form

Free steroids bind to albumin in serum [15], and steroids bound to albumin have no biological activity. The binding force of steroids to albumin is weak, and albumin bound steroids are easily released from albumin with organic solvents (e.g., dichloromethane, ethyl acetate, and diethyl ether) and C_{18} gels.

3.2.3. Saliva

Unconjugated steroids in saliva are present in the free form, and reflect level of free steroid in serum with good correlation. Therefore, free salivary steroids assays are carried out for the purpose of obtaining information of free steroids in serum [16,17]. Free salivary steroid assay is easier than free serum steroid assay because of non-invasive and convenient sampling and less interfering substances.

3.2.4. Hair

Steroid assay in hair has not been popular in the clinical laboratory until now. However, determination of steroids in hair has advantages of easy conservation of hair specimens, no circadian rhythm of steroid excretion, and retrospective measure of steroid excretion. There are some reports on anabolic steroid [18,19], dehydroepiandrosterone [18], estrogen [20,21], progesterone [20] and corticosteroid [22] assay in hair. The steroids in hair specimens were extracted after alkaline digestion, then determined by GC or HPLC coupled to MS [18,22].

4. Recent progresses in separation analysis of steroids

Steroid analysis has been carried out for medical diagnosis of stress, hypertension, Cushing syndrome, adrenogenital syndrome, amenorrhea, and infertility, etc. For assaying steroid in human body fluids, there are additional requirements in comparison with assays in other fields [23,24]. Practical considerations are listed as follows.

4.1. Rapid assay

Separation assay by GC, HPLC or capillary electrophoresis (CE) takes a long time (e.g., 40–60 min) per specimen, and preparation procedures for specimens such as extraction or derivatization add more time to the assay. For clinical assay of steroids, a few minutes of assay time per specimen are required.

4.1.1. Fast HPLC with a microbore column

Sizes of column and gel used influence analysis time of steroids. Recent developments of column and gel size tend to down sizing, e.g., a microbore and short column (3–5 cm long, 1–2 mm I.D.), gel size (1–2 μm) [25]. Smaller and shorter columns packed with smaller sized gels brought more rapid analysis times within 5 min per specimen with no loss of resolution capacity [26]. Reversed-phase HPLC using a C_{18} column is popular for steroid analysis.

4.2. Highly sensitive assay

As steroids in human fluids are sometimes at too low levels to detect, highly sensitive determination methods are required. Fluorescence and chemiluminescence detection methods for steroids were developed for highly sensitive assays.

4.2.1. Fluorescence

Fluorescence detection methods for steroid assay were employed to assay of steroids at low levels. As steroids have no native fluorescence except for estrogens, derivatization of steroids was required. Although the derivatization and its clean up procedure have problems of time consumption for clinical assay, some inventions to solve the problem were developed (e.g., on-line photochemical derivatization of estrogens, simple sulfuric–ethanol derivatization of corticosteroids). The fluorescent methods for steroids analysis reported were native fluorescence, photochemical derivatization, sulfuric acid–ethanol fluorescence, and fluorescent derivatization with labeling reagents.

4.2.1.1. Native fluorescence of estrogens

Only estrogens among steroids emit native fluorescence (emission 310 nm), and this allows rapid and relatively specific analysis of estrogens [8]. However, the native fluorescent intensity of estrogens is not strong because of its short emission wavelength of 310 nm.

4.2.1.2. Photochemical derivatization

Estrogens were converted to fluorophores by on-line UV radiation post-column, and assayed by fluorescence detection (excitation 280 nm, emission 312 or 410 nm) [27]. This photochemical derivatization of estrogens had no need of clean up procedures after derivatization, and enabled rapid and sensitive determination of estrogens.

4.2.1.3. Sulfuric acid–ethanol fluorescence derivatization

Cortisol, corticosterone and testosterone, which are non-fluorescent, were converted to fluorophores (excitation 365 nm, emission 520 nm) by incubation with a mixture of concentrated sulfuric acid and ethanol for a few minutes without heating. The intensity of fluorescence is strong, and detection limit of cortisol was 0.26 μg/dl ($S/N=3$). This sulfuric acid–ethanol derivatization method was relatively specific to cortisol, corticosterone, cortisone, dexamethasone and testosterone, and used originally for determination of 11-hydroxycorticosteroids (11-OHCS) in serum or urine [28,29] (Table 1). Then the sulfuric acid–ethanol derivatization method was applied to the assay of cortisol and corticosterone by HPLC with fluorescence detection [30–32] (Fig. 1). This enabled highly sensitive and simple detection of cortisol, corticosterone and testosterone, but needed careful dealing with concentrated sulfuric acid (Table 1).

4.2.1.4. 9-Anthroylnitrile derivatization of corticosteroids

Corticosteroids are generally difficult compounds for derivatization because of its no active functional groups. However, 21-hydroxyl group of corticosteroids was targeted for fluorescent derivatization with 9-anthroylnitrile (9-AN) [33,34]. The pre-column derivatization of corticosteroids with 9-AN needed clean up procedures prior to HPLC separation.

4.2.1.5. Dansyl derivatization of estrogens

The phenolic group of estrogens was derivatized with dansyl chloride to fluorophores off-line at

Table 1
Retention times and relative fluorescence intensities of the steroids derivatized with sulfuric acid and ethanol

Steroid	Retention time (min)	Relative fluorescence intensity
Cortisol	12.5	100
All-tetrahydrocortisol	12.3	2.0
Prednisolone	12.5	0.4
Cortolone	13.2	0.2
Prednisone	–[a]	
Dexamethasone	–[a]	
Betamethasone	–[a]	
Cortol	–[a]	
Tetrahydrocortisone	–[a]	
Corticosterone	23.0	218
Androstenediol	9.75	0.5
Androstenedione	11.5	0.6
Testosterone	76.8	304
Dehydroepiandrosterone	–[a]	
β-Estradiol	11.8	2.7
Estriol	12.9	0.7
Estrone	–[a]	
Progesterone	–[a]	

Retention times and relative fluorescence of various steroids (5 μmol) in comparison with that of cortisol. Strong fluorescence was observed only for the cortisol, corticosterone and testosterone derivatives.

[a] No peak observed.

alkaline pH [35]. The dansylated estrogen emits much stronger fluorescence than the native fluorescence of estrogens. However, clean up of the dansyl estrogens was required prior to its separation.

4.2.2. Chemiluminescence

Chemiluminescent determination of steroid enables highly sensitive assay. The detection limit of chemiluminescence (CL) is estimated as the same or superior to the limit of radioimmunoassay. CL detections applied to steroid analysis were lucigenin CL and peroxyoxalate CL.

4.2.2.1. Lucigenin chemiluminescence of corticosteroids

Lucigenin CL was applied to assay of corticosteroids by using its reductive character. Corticosteroid was reacted on-line with lucigenin at alkaline pH after separation by reversed-phase HPLC [36].

4.2.2.2. Peroxyoxalate chemiluminescence of estradiol

Peroxyoxalate chemiluminescence (PO-CL) assays hydrogen peroxide or fluorophores with high sensitivity. Two kinds of peroxyoxalate chemiluminescence (PO-CL) were applied to detection of estradiol. Firstly, estradiol was labeled off-line with dansyl chloride to fluorophores (dansyl estradiol) and reacted with peroxyoxalate ether, hydrogen peroxide and imidazole after separation by HPLC [35,37] (Fig. 2). The second PO-CL detection of estradiol was proposed by conversion of estrogens to hydrogen peroxide with imidazole at alkaline pH precolumnly and detected by PO-CL with a fluorophore (rubrene) [38].

4.3. Automated extraction and assay

Extraction procedures for steroids analysis adversely influence on assay time and number of specimens assayed per day in clinical laboratories. For rapid assay of steroids in clinical laboratories, simple and easy extraction methods have been developed.

4.3.1. Off-line and on-line extraction

Steroids exist at low concentrations in human body fluids, and must be enriched and extracted from contaminants prior to its separation analysis. The pre-column extraction procedures using organic sol-

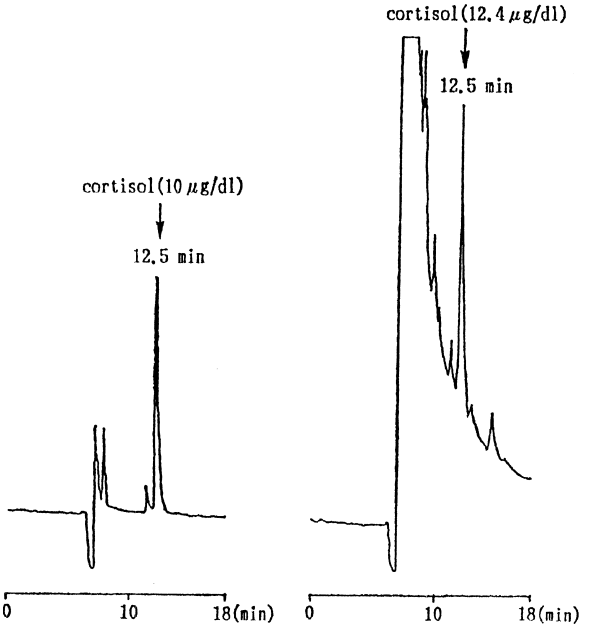

Fig. 1. Chromatograms of cortisol derivatized with sulfuric acid and ethanol and detected by fluorescence. Left: authentic cortisol. Right: serum cortisol. Concentrations of authentic cortisol and serum cortisol were 10 and 12.4 μg/dl, respectively. The retention time of sulfuric derivatized cortisol was 12.5 min. Pre-column: ODS-80 Tm, 5 μm I.D., TSK; 10 mm×4.6 mm I.D. Analytical column: ODS-80 Tm, 5 μm I.D., TSK; 150 mm×46 mm I.D. Mobile phase for the pre-column: 10 m*M* potassium hydrogen phthalate buffer (pH 1.85). Flow-rate: 1.0 ml/min. Mobile phase for the analytical column: acetonitrile–tetrahydrofuran–19 m*M* potassium hydrogenphthalate buffer (40:6:54, v/v, pH 1.85). Flow-rate 1.0 ml/min, Fluorescent wavelength: excitation 485 nm, emission 520 nm.

vents (dichloromethane, diethyl ether, ethyl acetate) [39–41], solid-phase cartridge packed with C_{18} or BSA-ODS gels [24,42–47] or affinity gels [48] were developed, but those were time consuming and the recovery rate was less than 100%. These results were major problems for medical analysis. To resolve the problems, a sample direct injection method [49] with a column switching technique was developed and then enabled automated HPLC analysis of steroids, where extraction of steroids was carried out automatically on-line.

4.3.2. Automated analysis

For automated analysis of steroids, fully automated procedures of sample injection, extraction of steroid, separation, detection and data processing are necessary [48,49]. Automated analysis of steroids by HPLC meets the requirements for clinical assays. Auto injection and extraction of steroids in specimens containing proteins became possible by using a BSA-ODS gel pre-column and a column switching technique. BSA-ODS gel is a C_{18} gel of which surface is covered with BSA [24,49] to have biphasic characters, and inside pores of BSA-ODS gel are hydrophobic and surface of the gel is hydrophilic. Steroid bound to serum protein (albumin, steroid binding globulin) is released on the surface of BSA-ODS gel, and extracted to C_{18} chain in the inside pore of the gel. Serum proteins bounded steroid are excluded out of surface of the gel. Both pre-column which contained BSA-ODS gels and an analytical column which contained C_{18} gels were switched automatically using an electric column switching valve and a system controller to transfer steroid from a pre-column to an analytical column (Fig. 3). This enabled fully automated HPLC analysis of steroids (Fig. 4) [49].

4.4. Pure determination of steroids

Colorimetric determination methods (e.g., Zimmermann, Porter–Silber reactions and enzyme immunoassay), are subject to interference from contaminants in specimen that produce erroneous data. However, separation assay methods can avoid the interference due to contaminants in specimens.

4.4.1. Zimmermann and Porter–Silber reactions

Urinary 17-ketosteroids (17-KS) and 17-hydroxycorticosteroids (17-OHCS) were originally assayed by Zimmermann reaction and Porter–Silber reaction [50,51] for testing excretion of androgens and glucocorticoids, respectively. These reactions were subject to interference by drugs and other contaminants in urine specimens. Prior solid-phase extraction of 17-KS [24] and 17-OHCS from urine specimens with a C_{18} gel minicolumn diminished the effect of interfering substances. Separation by HPLC of urinary 17-KS and 17-OHCS steroids completely eliminated the interferences [52].

4.4.2. Cross reaction by immunoassay

Enzyme- and radioimmunoassay methods for ster-

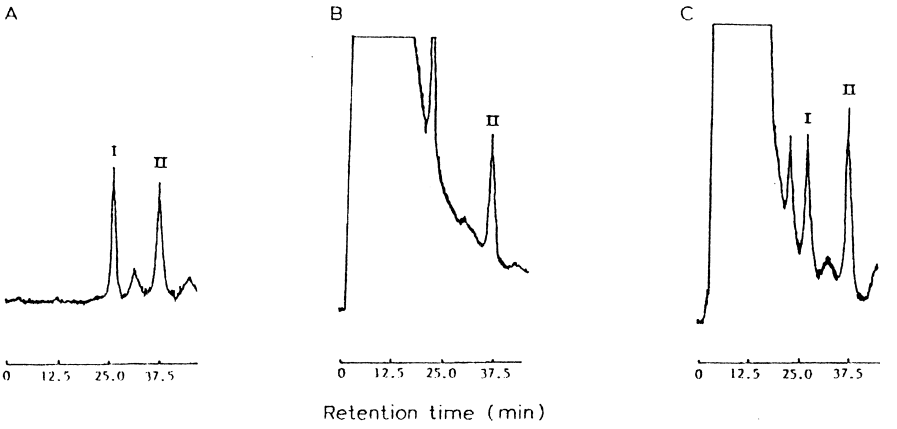

Fig. 2. Chromatograms of dansylated estradiol by peroxyoxalate chemiluminescence. Chromatograms of dansyl-17α-estradiol (I) and dansyl-17β-estradiol (II): (A) authentic dansyl estradiol (150 pg of each); (B) serum sample (500 pl); (C) pooled serum sample spiked with 17α-estradiol (200 pg) and 17β-estradiol (300 pg).

oids [53] are widely employed in clinical laboratories because they are rapid, highly sensitive and can be automated. However, immunoassays of steroids are subject to interference due to cross reacting antibodies [54,55]. Comparison of values for steroids obtained revealed lower values by HPLC than values by immunoassay. This means that the values by immunoassay had possibility of interferences by immuno cross-reactions with contaminants in the specimens. There has been extensive work on improving the specificity of antibody to steroids in order to avoid cross reactivity [56], but the problem still remains because hapten antigens of steroids could not produce completely specific antibody to each steroid. For the complete resolution of immuno cross-reaction, HPLC separation was used after extraction of steroids with antibodies (immunoaffinity extraction of steroids) [48,57].

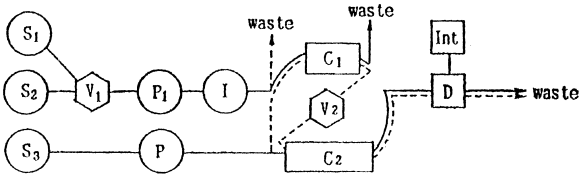

Fig. 3. Flow diagram of the HPLC system with sample direct injection and column switching technology. Sl: Water, S2: 10% aq. methanol, S3: 43% aq. methanol, Vl: valve (PTFE Rotary Valve 50, Rheodyne), V2: valve (Switching valve 7000, Rheodyne), I: injector (VL-614, Jasco), P1: HPLC pump (Twincle, Jasco), P2: HPLC pump (Trirotar-III, Jasco), C1: pre-column (BSA-ODS, 20 μm I.D., TSK; 150 mm×4.6 mm I.D.), D: UV monitor (638-41, Hitachi).

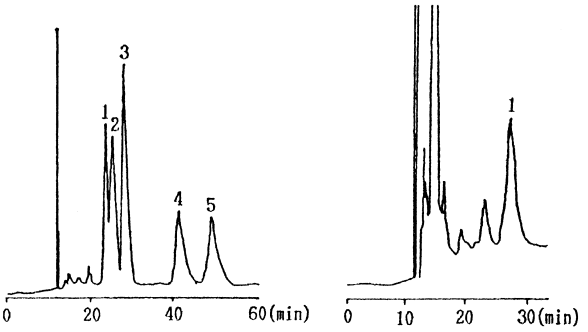

Fig. 4. Chromatograms of cortisol by fully automated HPLC. Left: chromatogram of authentic glucocorticoids; peaks: 1=prednisone (retention time, 23.5 min), 2=cortisone (25.1 min), 3=cortisol and prednisolone (28.2 min), 4=dexamethasone (41.4 min), 5=corticosterone (49.2 min). Chromatographic condition: pre-column, BSA-ODS, 20 μm I.D., TSK, 10 mm×4.6 mm I.D., analytical column; BSA-ODS, 5 μm TSK, 150 mm×4.6 mm I.D. Mobile phase; stepwise gradient between water and 10% methanol for the pre-column, 45% methanol for the analytical column, flow-rate 1.0 ml/min, respectively, column temperature; ambient, UV wavelength 245 nm, attenuation of the integrator; 8. Right: chromatogram of cortisol in serum. Serum volume; 200 pl (the serum cortisol 21.5 pg/dl assayed by radioimmunoassay). Chromatographic conditions were same as those of the left chromatogram; peak I=cortisol.

4.5. Direct assay of conjugated steroids

Steroid conjugates (steroidal glucronides and sulfates) are usually assayed after hydrolyzing the conjugated forms with strong inorganic acid, glucuronidase or sulfatase. However, the conjugated fraction has information of activity of conjugating enzymes and detoxification in liver [58,59]. Therefore, direct assay of conjugated steroids gives significant information on the detoxification capacity of liver. Conjugated steroids were extracted with BSA-ODS (Fig. 5), or a C_{18} gel minicolumn, and then assayed by colorimetry [60] or HPLC–MS [61,62].

4.6. Simultaneous analysis of steroids

Adrenogenital syndrome is a congenital disease lacking of enzymes (C-11 hydroxylase, C-17 hydroxylase, C-18 hydroxylase, C-21 hydroxylase and 3β-hydroxysteroid dehydrogenase) for steroid metabolism, and has some types of the symptoms. The patients have disturbances caused by abnormal excretion of sex hormones, mineralo- and glucocorticoids. Urinary steroid profiles are used to determine the type of the adrenogenital syndrome. Pfaffenberger and Horning [63] demonstrated the urinary abnormal steroid profile from the patients of adrenogenital syndrome giving strong impact of efficiency of profile analysis by GC after methoxime-trimethylsilyl (MO-TMS) derivatization. The profile analysis can identify enzyme responsible for the adrenogenital syndrome. MS detection coupled to GC [64], and HPLC [65] makes identification of the steroidal peaks easy. Simultaneous assay of steroids in medical specimens is helpful for diagnosis of not only adrenogenital syndrome, steroids excreting tumor, but also liver dysfunction for detoxification.

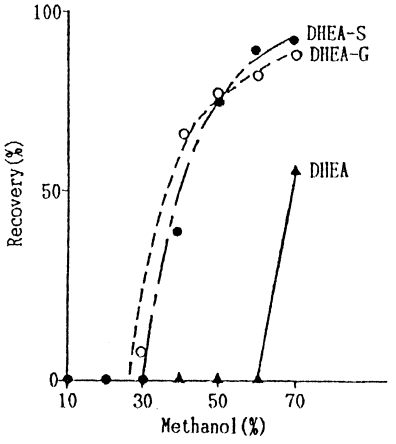

Fig. 5. Extraction of conjugated 17-KS with BSA-ODS minicolumn. Effects of concentration of methanol in the mobile phase on elution of 17-KS. After loading 3.0 ml of 50 pmol/l DHEA. DHEA-S and DHEA-G solution onto a BSA-ODS minicolumn and washing with 3.0 ml of phosphate buffer (pH 7.0), free and conjugated 17-KS were eluted.

4.7. Identification of unknown steroids

Identification of unknown steroids in clinical specimens is often requested for diagnosis of doping, abuse of anabolic steroids, metabolic disorders and tumorous excretion of steroids. HPLC–MS and capillary GC–MS are advantageous for identification of unknown steroids because mass spectrometry enables on-line identification of steroids after separation.

4.7.1. Abnormal excretion of natural steroids

Identification of natural steroids in urine and serum specimens submitted is the target for diagnosis of adrenogenital syndrome, tumor of adrenal gland. The responsible enzymes of abnormal metabolism of steroids were identified from analysis of abnormal excretion of steroid by GC–MS [66]. Free 17-oxosteroids and pregnanediol from urine specimens were derivatized with *N*-methyl *N*-trimethylsilyl trifluoroacetamide and identified by MS after separation by GC on-line.

4.7.2. Doping and steroidal abuse

Anabolic steroids used for increasing muscle mass are targets for the investigation for abuse [67] and doping test at athletic meets [6]. For testing use of anabolic steroids, GC–MS and HPLC–MS were usually employed to identify the kinds of anabolic steroids used [68–70].

4.8. Ultra-miniaturization of a separation system

Ultra-miniaturization of a separation system has been a recent trend, and is beneficent in the clinical laboratory. Advantages include faster assays, low

cost, and small space occupancy. Fused-silica capillaries and microchips with micro fabricated channels are tools for the ultra-miniatualization of a separation system [71–74].

4.8.1. Capillary GC

Fused silica capillaries which has narrow bore (25–100 μm I.D.) and long length (10–30 m) were used for GC and GC–MS. Capillary GC is characteristic for its high resolution and used for profile assay of steroids in clinical specimens [71,72]. Capillary GC coupled with MS became a more powerful tool to identify unknown peaks of steroid for doping tests and diagnosis of adrenogenital syndrome because MS analyzes the chemical structures of the volatile steroids after separation by capillary GC on-line.

4.8.2. Capillary electrophoresis

Fused-silica capillaries have been used for CE and applied to steroid analysis [73]. CE (micellar electrokinetic capillary chromatography) requires a small sample volume (e.g., 1–2 μl), enables rapid separation and high resolution of analytes. There are two kinds of capillary tubes used for CE those are gel packed type and hollow type. Steroids form micelles with a cationic surfactant in a running buffer, and migrate by electricity. Steroids are detectable with UV detection (254 nm), and photodiode array detection after separation by CE on-line.

4.8.3. Capillary HPLC

The fused-silica capillary is a recent innovation as an ultra narrow bore column (300 μm I.D.) for HPLC separation of steroid conjugates or anabolic steroids [5,62,67]. Functional groups such as ODS were introduced to the inner face of a capillary fused-silica tube or on gels packed in the capillary fused-silica tube. HPLC with a capillary column utilizes mobile phases at slow flow-rate such as 1 μl/min, and needs small sample volume (e.g., 1–2 μl). Capillary HPLC is very suitable for MS detection, and capillary HPLC coupled to MS with an electrospray ionization source is used for identification of unknown compounds, highly sensitive detection and profiling of metabolites. Another advantage of capillary HPLC coupled to MS for steroid analysis is that there is no need for pre-column derivatization of steroids.

4.8.4. Microchip separation

A microchip with a microfabricated channel has been tested for assay of steroids in a serum specimen [74]. Separation of free, fluorescein labeled cortisol and bound cortisol to antibody was performed in a competitive manner using a microchip electrophoretic system. The micro-channel (depth of 28 μm, width of 66 μm and effective length of 20 cm) enables fast separation within a few minutes (less than 30 s). Sample volume required is very small (e.g., 0.75 μl). The detection of fluorescent-labeled cortisol (1–60 mg/dl) is carried out by fluorescence (excitation 488, emission 520 nm).

5. Conclusion

Determination methods of steroids by separation assay for medical diagnosis have special considerations in order to meet requests from clinicians. Epoch-making technologies for steroids analysis to solve the technical problems were described in this paper. Technologies using a capillary fused column for GC, HPLC and CE, and a microchip with a micro fabricated channel are expected to significantly advance steroid analysis for medical diagnosis in the near future.

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