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Simultaneous Analysis of Free and Sulfated Steroids by Liquid Chromatography/Mass Spectrometry with Selective Mass Spectrometric Scan Modes and Polarity Switching

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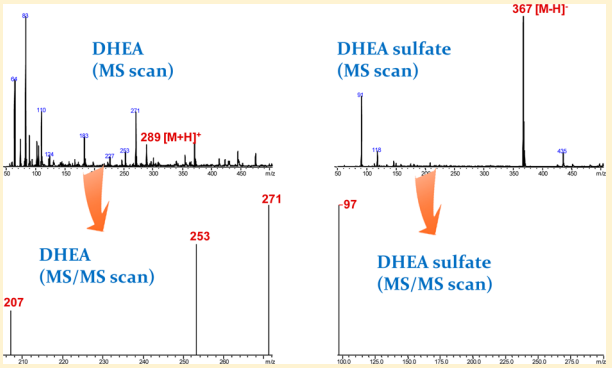
ABSTRACT: Sulfated steroids can act as a latent form of active free steroids, coexisting with them in biological specimens. To evaluate the metabolic significance of free and sulfated steroid species, a simultaneous analysis of eight free steroids [cholesterol, pregnenolone, 17α-hydroxypregnenolone, progesterone, 17α-hydroxyprogesterone, dehydroepiandrosterone (DHEA), testosterone, and 17β-estradiol] and four biologically relevant sulfated steroids was developed and validated, using selected-ion and multiple-reaction monitoring modes coupled to polarity-switching liquid chromatography/mass spectrometry (LC/MS). All steroids were separated on a reversed-phase phenyl column (50 mm × 2 mm, 3 μm) at a flow rate of 0.5 mL/min. The limits of quantification ranged from 0.1 to 50 ng/mL at extraction recoveries of 94.1−105.5%, while the precision and accuracy were 2.5−9.3% and 92.4−105.9%, respectively. Quantitative results obtained for samples from obese girls showed that the serum levels of DHEA sulfate were significantly increased (P = 0.004), along with the metabolic ratio representing DHEA sulfotransferase (P < 0.02). The developed novel LC/MS method can quantitatively profile both free and sulfated steroids in a single analytical run.



Metabolic processes convert molecules into lipophilic compounds for easy absorption into the body, whereas the body increases the hydrophilicity of molecules to excrete them through biotransformations in phase I and II metabolism. In contrast to phase I reactions, which can produce active metabolites, the addition of large anionic groups, such as sulfate, detoxifies and produces more polar metabolites that cannot diffuse across membranes but may be actively transported through them.[1,2] Steroid sulfates are considered to be biologically inactive metabolites; however, they play an important physiological role in steroid biosynthesis and feedback control mechanisms.[2−4]

Although methods based on liquid chromatography/mass spectrometry (LC/MS) have been used for analysis of free and sulfated steroids, two different instrumental protocols are generally required due to their different chemical and ionization properties. For accurate quantification, steroid sulfates need to be analyzed as intact molecules by only LC/MS,[5−8] while free steroids can be quantified by LC/MS[9,10] and gas chromatography/mass spectrometry (GC/MS).[11,12] However, both free and sulfated steroids can be analyzed with high analytical sensitivities through LC/MS analysis in positive- and negative-ionization modes, respectively.[5−10] In addition, free steroids produce many characteristic fragment ions during collision-induced dissociation, which makes the selection of quantitative ions easy and enables comprehensive analysis.[9,10] In contrast, steroid sulfates mainly show $[M - H - \text{sulfate}]^-$ and $[HSO_4]^-$ ions formed by dissociation of the corresponding precursor $[M - H]^-$ ions. Although the sulfate ion is detected as a base peak, it is not sufficiently selective because of its low molecular mass, which may result in interference from matrix backgrounds during trace quantification. Selected-ion monitoring (SIM) is a technique alternative to multiple-reaction monitoring (MRM) in tandem mass spectrometry (MS/MS) for the analysis of steroid sulfates.[7,8,13]

The biological activities of major endogenous steroids, such as cholesterol (Chol), dehydroepiandrosterone (DHEA), pregnenolone (Preg), testosterone (T), 17β-estradiol (E2), and their sulfate conjugates (Figure 1) in reproductive pathways can be monitored on the basis of their metabolic alteration in many physiological conditions, including metabolic syndromes.[14−16] To evaluate the metabolic functions of free and sulfated steroids, simultaneous LC/MS analysis in a single run was developed, utilizing both SIM and MRM modes in addition to polarity (positive and negative ionization) switching, which is useful for multicomponent analysis.[17,18] The validated method was successfully used to study childhood obesity of females, which affects the serum levels of free and sulfated steroids.[7,8,19]

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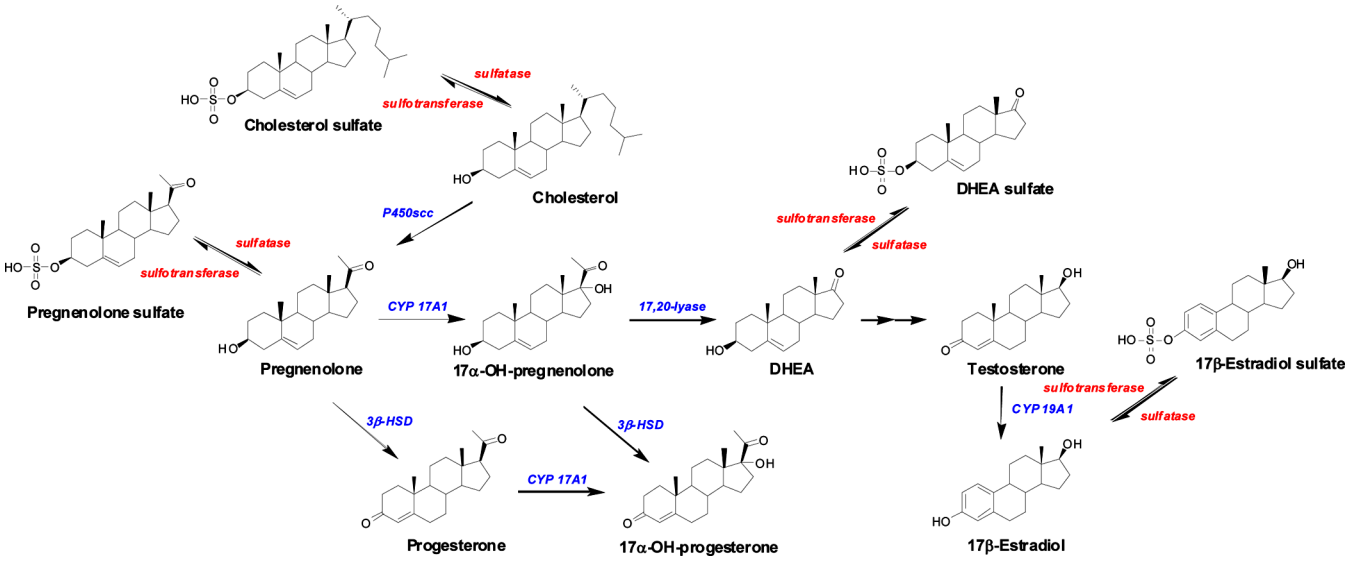

Figure 1. Overview of reproductive pathways in steroid metabolism. Cholesterol is converted to pregnenolone, catalyzed by P450scc. CYP17A1, 17,20-lyase, and 3β-HSD are enzymes for the production of 17α-hydroxypregnenolone, DHEA, and progesterone, respectively, from pregnenolone. DHEA is further metabolized into the sex steroids testosterone and 17β-estradiol.

Table 1. LC/MS Characteristics of Eight Free and Four Sulfated Steroids

compd	abbr	scan mode, polarity	mol mass, Da	precursor ion, m/z	product ion, m/z (CE)[a]	dwell time, ms	retention time, min
			Analytes				
cholesterol	Chol	MRM, +	386.65	369.30	**147.15** (25), 161.20 (21)	10.0	9.66
pregnenolone	Preg	MRM, +	316.48	317.10	**299.25** (11), 281.25 (15)	10.0	6.62
progesterone	Prog	MRM, +	314.46	314.95	**97.20** (23), 109.15 (25)	10.0	6.70
17-hydroxypregnenolone	17-OH-Preg	MRM, +	332.48	314.90	**297.30** (13), 159.25 (25)	10.0	5.56
17-hydroxyprogesterone	17-OH-Prog	MRM, +	330.46	330.95	**97.20** (24), 109.20 (27)	10.0	5.75
dehydroepiandrosterone	DHEA	MRM, +	288.42	289.10	**271.15** (10), 253.25 (13)	10.0	5.59
testosterone	T	MRM, +	288.42	288.90	**97.20** (23), 109.15 (25)	10.0	5.28
17β-estradiol	E2	MRM, +	272.38	273.20	**107.20** (27), 135.15 (16)	10.0	5.40
cholesterol sulfate	Chol-S	SIM, −	466.72	**465.50**	91.20[b]	24.0	7.57
dehydroepiandrosterone sulfate	DHEA-S	SIM, −	368.49	**367.20**	91.20[b]	24.0	4.54
pregnenolone sulfate	Preg-S	SIM, −	396.54	**395.30**	91.20[b]	24.0	5.29
17β-estradiol 3-sulfate	E2-S	SIM, −	352.45	**350.70**	91.20[b]	24.0	4.05
			Internal Standards				
cholesterol-d_6	Chol-d_6	SRM, +	392.69	375.40	**152.20** (25)	24.0	9.63
pregnenolone-d_4	Preg-d_4	SRM, +	320.27	321.30	**303.35** (11)	24.0	6.61
progesterone-d_9	Prog-d_9	SRM, +	323.52	323.90	**100.20** (23)	24.0	6.67
17-hydroxypregnenolone-d_3	17-OH-Preg-d_3	SRM, +	335.50	318.10	**300.30** (14)	24.0	5.55
17-hydroxyprogesterone-d_8	17-OH-Prog-d_8	SRM, +	338.51	338.80	**100.15** (27)	24.0	5.72
testosterone-d_3	T-d_3	SRM, +	291.44	291.90	**109.20** (24)	24.0	5.28
17β-estradiol-d_4	E2-d_4	SRM, +	276.41	277.20	**137.10** (17)	24.0	5.38
cholesterol-d_7 sulfate	Chol-d_7-S	SIM, −	473.74	**472.20**		24.0	7.56
dehydroepiandrosterone-d_6 sulfate	DHEA-d_6-S	SIM, −	374.51	**373.40**		24.0	4.52
pregnenolone-d_4 sulfate	Preg-d_4-S	SIM, −	400.55	**399.40**		24.0	5.27
17β-estradiol-d_4 3-sulfate	E2-d_4-S	SIM, −	355.45	**354.80**		24.0	4.11

[a]CE, collision energy. [b]Characteristic ions detected in SIM analysis for peak identification. Quantitative ions are shown in boldface type.

EXPERIMENTAL SECTION

Reagents and Materials. Free and sulfated steroids examined in this study are listed in Table 1. All endogenous steroid reference materials were supplied by Steraloids (Newport, RI) and Sigma (St. Louis, MO). Deuterated internal standards (IS) [testosterone-16,16,17-d_3 (T-d_3), dehydroepiandrosterone-2,2,-3,4,4,6-d_6 (DHEA-d_6), 17β-estradiol-2,4,16,16-d_4 (E2-d_4), pregnenolone-17α,21,21,21-d_4 (Preg-d_4), progesterone-2,2,4,6,6,17

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Table 2. Method Validation Results for Eight Free and Four Sulfated Steroids

analyte	LOQ (ng/mL)	approx LOQ calibration range (ng/mL)	intraday (n = 5) precision (% CV)	intraday (n = 5) accuracy (% bias)	interday (n = 5) precision (% CV)	interday (n = 5) accuracy (% bias)	recovery (%)	stability[a] (%)
Chol[b]	0.05	100	4.5	105.8	4.1	102.5	104.7	98.8
Preg	0.2	500	8.3	105.0	7.7	105.9	96.9	100.3
Prog	0.1	100	5.8	97.1	5.0	95.1	103.6	97.7
17-OH Preg	0.2	100	3.9	95.2	5.1	96.1	97.4	106.4
17-OH Prog	0.2	100	9.3	95.3	7.4	97.9	94.1	99.7
DHEA	0.5	1000	5.1	100.5	6.4	99.4	94.1	96.7
T	0.2	100	4.0	94.6	4.9	92.4	97.0	106.1
E2	1	50	7.5	102.4	7.6	98.1	98.2	100.9
Chol-S	5	1000	2.9	103.0	5.5	103.7	97.5	101.3
DHEA-S	0.1	1000	3.5	95.2	3.3	96.3	100.5	101.5
Preg-S	0.2	1000	2.5	100.7	3.9	101.1	103.3	100.6
E2-S	0.5	50	7.6	97.9	6.5	98.6	99.7	99.8

[a]Postpreparative stability after one batch. [b]Concentrations are presented in units of micrograms per milliliter for cholesterol.

α,21,21,21-d_9 (Prog-d_9), 17-hydroxypregnenolone-21,21,21-d_3 (17-OH-Preg-d_3), 17-hydroxyprogesterone-2,2,4,6,6,21,21,21-d_8 (17-OH-Prog-d_8), cholesterol-2,2,3,4,4,6-d_6 (Chol-d_6), dehydroepiandrosterone-2,2,3,4,4,6-d_6 sulfate (DHEA-d_6-S), 17β-estradiol-2,4,16,16-d_4 3-sulfate (E2-d_4-S), pregnenolone-17α,21,21,21-d_4 sulfate (Preg-d_4-S), and cholesterol-25,26,26,26,27,27,27-d_7 sulfate (Chol-d_7-S)] were purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada) and Sigma. Sodium acetate (reagent grade), acetic acid (glacial, >99.99%), and formic acid (mass spectrometry grade, 98%) were acquired from Sigma.

A Strata-X 33-μm polymeric reversed-phase cartridge (60 mg, 3 mL) used for solid-phase extraction (SPE) was obtained from Phenomenex (Milford, MA), and all organic solvents used (analytical and HPLC grade) were obtained from Burdick & Jackson (Muskegon, MI). Deionized water was prepared with a Milli-Q purification system (Millipore, Billerica, MA). Ultrafree-MC centrifugal filters [poly(vinylidene difluoride), 0.1 μm pore size; Millipore] and 3000 molecular weight cutoff (MWCO) filters were supplied by Millipore.

Standard Solutions and Quality Control Samples. Stock solutions of all reference standards were prepared at a concentration of 1 mg/mL in methanol, and working solutions were made up with methanol at 100 μg/mL. Calibration solutions were prepared by diluting each methanolic standard to various concentrations ranging from 0.01 to 10 μg/mL, while cholesterol was used at levels of up to 100 μg/mL. All standard solutions were stored at −20 °C until required and were stable for a minimum of 3 months.

For both calibration and quality control (QC) purposes, a steroid-free serum was prepared from a commercial one (SCIPAC, Sittingbourne, U.K.). Since Chol and Chol-S are present at higher concentrations even in the SCIPAC serum, three different techniques, including charcoal stripping, MWCO centrifugal filtering, and Strata-X cartridge purification, were tested. Additional charcoal-stripped serum samples still contained large amounts of lipidlike components, including Chol and Chol-S, while Strata-X purification resulted in small chromatographic peaks corresponding to both steroids in some samples (data not shown). After the SCIPAC serum samples were centrifuged on an Amicon ultrafilter (MWCO 3000; Millipore) for 20 min at 12 000 rpm, the lipophilic compounds were absorbed by membrane filters, and the filtrates did not contain any steroids or other potentially interfering compounds. The MWCO-filtered serum was used as a calibrant and for QC samples after checking for endogenous steroids with negative results. The QC samples were prepared at four different concentrations [limit of quantification (LOQ), low QC, medium QC, and high QC] in steroid-free serum spiked with eight free and four sulfated steroids.

Preparation of Serum Samples. Serum samples (100 μL) were spiked with IS mixtures (20 μL) [T-d_3, 20 ng/mL; E2-d_4, Preg-d_4, and E2-d_4-S, 200 ng/mL; DHEA-d_6, Prog-d_9, and 17-OH-Preg-d_3, 100 ng/mL; 17-OH-Prog-d_8 and Chol-d_6, 500 ng/mL; DHEA-d_6-S, Preg-d_4-S, and Chol-d_7-S, 40 ng/mL], and the spiked samples were added to an acetate buffer (2.9 mL, pH 5.2) solution. For SPE, the Strata-X 33-μm cartridges were preconditioned with 2 mL of methanol followed by 2 mL of deionized water. After the elution of all analytes with 2 mL of methanol (twice), the methanolic eluent was evaporated under N_2 at 40 °C. Finally, the dried residue was reconstituted with methanol (100 μL) and centrifuged on an ultrafree-MC centrifugal filter for 5 min at 14 000 rpm prior to injection into the LC/MS system (5 μL).

Instrumental Conditions. The LC/MS system (LC/MS 8050, Shimadzu Corp., Japan) was composed of a Shimadzu Nexera ultra-high-performance liquid chromatograph and an 8050 triple quadrupole mass spectrometer combined with a heated electrospray source. All steroids were separated on a reversed-phase Unison UK phenyl C18 column (3 μm particle size, 50 mm × 2 mm; Imtakt Corp., Japan) during a 25 min run at a flow rate of 0.5 mL/min. The eluent consisted of 0.1% formic acid in 95% water (mobile phase A) and 0.1% formic acid in 95% acetonitrile (mobile phase B). The following gradient was used: 0 min at 0% B; 0−6 min at 0−40% B; 6−7 min at 40−60% B (hold 3 min); 10−15 min at 60−100% B (hold 8 min). Subsequently, the initial conditions (0% B) were adopted for 2 min before the next sample was run. The autosampler temperature was maintained at 10 °C.

All analytes were monitored in a single run at the high-speed scan rate of 30 000 u/s in SIM and MRM modes coupled to polarity switching (5 ms). Among the steroids tested, eight free steroids were efficiently detected in the positive-ionization mode by a MRM method based on $[M + H]^+$ precursor ions, and the four sulfated steroids were detected in the negative-ionization mode by a SIM method, with the base peak of $[M - H]^-$ ions used for quantification. The mass spectrometer was operated under the following optimized conditions: interface temperature 300 °C, desolvation line temperature 250 °C, nebulizing gas

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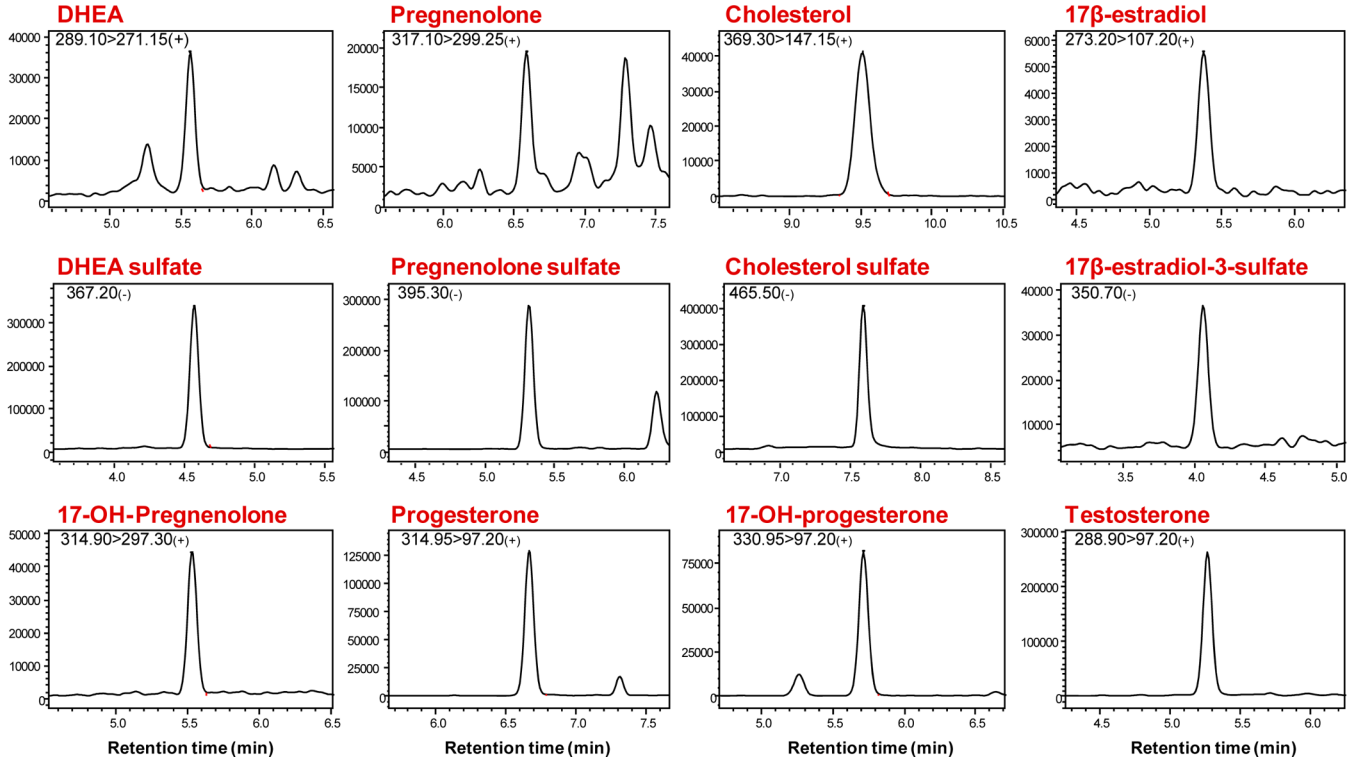

Figure 2. LC/MS chromatograms of eight free steroids and four sulfated steroids analyzed in SIM and MRM modes. All steroids were separated on a reversed-phase Unison UK phenyl C18 column (3 μm particle size, 50 mm × 2 mm) at a flow rate of 0.5 mL/min. The eluent consisted of 0.1% formic acid in 95% water (mobile phase A) and 0.1% formic acid in 95% acetonitrile (mobile phase B). The following gradient was used: 0 min at 0% B; 0−6 min at 0−40% B; 6−7 min at 40−60% B (hold 3 min), 10−15 min at 60−100% B (hold 8 min).

(argon) flow 3.00 L/min, heating gas flow 10.00 L/min, heat block temperature 400 °C, and drying gas (nitrogen) flow 10 L/min.

For quantification, the following MRM transitions were used: 288.90 → 97.20 and 288.90 → 109.25 for T, 369.30 → 147.15 and 369.30 → 161.20 for Chol, 317.10 → 299.25 and 317.10 → 281.25 for Preg, 314.95 → 97.20 and 314.95 → 109.15 for Prog, 314.90 → 297.30 and 314.90 → 159.25 for 17-OH-Preg, 330.95 → 97.20 and 330.95 → 109.20 for 17-OH-Prog, 289.10 → 271.15 and 289.10 → 253.25 for DHEA, and 273.20 → 107.20 and 135.15 for E2. Base peaks at m/z 465.50, 367.20, 395.30, and 350.70 were used for SIM of Chol-S, DHEA-S, Preg-S, and E2-S, respectively. The corresponding internal standards of four sulfated steroids used $[M - H]^-$ as quantitative ions (Table 1). The dwell time was 10 ms for the eight free steroids and 24 ms for the four sulfated steroids and the 12 corresponding deuterated internal standards. Peaks were identified by comparison of retention times and matching the area ratios of characteristic ions. Data acquisition was performed by use of Lab Solutions LCMS version 5.6 software (Shimadzu Corp.).

Method Validation. In validation processes, the LOQ, calibration linearity (r^2), precision expressed as coefficient of variation (% CV), and accuracy as relative error percentage (% bias) were evaluated for intra- and interday assays over five replicates on five different days, respectively, for quality control (QC) samples. QC samples were prepared at four different concentrations, depending on the individual sensitivity and reference values of serum steroids. The corresponding QC concentrations, labeled LOQ, low QC (3- or 5-fold excess of LOQ), medium QC, and high QC, are listed in Table 2. Calibration samples were made up at 15 different concentrations, ranging from individual LOQs to 1000 ng/mL, while Chol calibration samples were in the range from LOQ to 100 μg/mL. Extraction recoveries were determined by addition of known amounts of the mixed working solutions, using QC samples at four concentrations in three replicates for all steroids. Absolute recoveries were calculated by comparing the results of sample analysis in the course of overall sample preparation.

Postpreparative Stability Test. The stabilities of serum steroids were evaluated by reinjecting samples after one batch analysis of validation samples being placed in the sample tray of the autoinjector. These samples were quantified by use of a calibration curve obtained from freshly prepared solutions, and the obtained concentrations were compared to the nominal ones.

Subjects and Sample Collection. Serum samples were obtained from 31 obese (8.5 years, 6.1−9.9) and 42 age-matched normal girls (8.2 years, 5.9−9.9) enrolled at the Inje University Sanggye Paik Hospital (Seoul, Korea). The study protocol was approved by the Institutional Review Board of the Inje University Hospital (SPIRB13-094), and written informed consent was obtained in compliance with the Declaration of Helsinki Principles. Bioelectrical impedance analysis (Inbody 720 instrument) was used to calculate body mass index [BMI; weight divided by height squared (kg/m^2)] and body composition. All subjects were assigned to one of two groups: obese (BMI ≥ 95th percentile) and normal (BMI < 85th percentile) girls. Blood samples for the measurement of steroid and lipid profiles were collected after a 10-h overnight fast. All samples were stored at −80 °C until used.

Statistical Analysis. Data manipulation was performed by use of SPSS (v. 22; SPSS Inc., Chicago, IL). Statistical analysis was preceded by the Shapiro−Wilk test for normality. The concentrations of free and sulfated steroids in serum samples obtained from normal and obese females were compared in three groups by the Kruskal−Wallis test, while the Mann−Whitney test was used

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for comparison between groups. Quantitative results and group differences were compared by the unpaired Student's *t*-test. *P* values of less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Development of the LC/MS Method. To achieve simultaneous analysis of both free and sulfated steroids in positive- and negative-ionization modes, an LC/MS method with no ion loss during polarity switching in the MS scan was required. Although previous studies using traditional LC/MS-based methods allowed for reproducible and selective analysis, two separate instruments were generally used due to the different chemical and ionization properties of free and sulfated steroids.[8,20,21] The aim of this study was to develop a novel LC/MS method for simultaneous analysis of the eight free and four sulfated steroids in a single run and apply the validated method clinically. Due to its ability to differentiate between aromatic compounds with similar structure and identical mass, such as anxiolytic drugs or other steroids, a phenyl stationary phase can achieve higher selectivity with respect to their different degree and position of unsaturation on the basis of $\pi-\pi$ interaction characteristics.[22,23] In this study, chromatographic separation was achieved with good peak selectivity in high signal-to-noise (S/N) ratios by use of a phenyl column, with retention times ranging from 4.05 min for E2-S to 9.66 min for cholesterol (Figure 2).

The MS and MS/MS data for free and sulfated steroids were obtained by flow injection in 50% mobile phase B at 0.3 mL/min in positive- and negative-ionization modes. Full-scan MS of individual steroids showed $[M + H]^+$ ion as the base peak for free steroids and $[M - H]^-$ ion as the base peak for sulfated steroids. To quantify the steroids, two MRM transitions as quantitative and qualitative ions with their $[M + H]^+$ ions in the positive-ionization mode were used for the eight free steroids. Since only individual $[M - H]^-$ and sulfate ions were detected for all four sulfated steroids, they were effectively analyzed in the SIM mode, based on their corresponding $[M - H]^-$ ions in the negative-ionization mode. All steroids were successfully quantified by both SIM and MRM modes coupled to polarity switching in a single run (Table 1).

Solid-Phase Extraction. To simultaneously analyze free and sulfated steroids, modified SPE procedures from our previous studies were used for sample purification and concentration.[7,11] Strata-X SPE cartridges were used for selective and simultaneous analysis of free and sulfated serum steroids with good selectivity and extraction yields (>85% of overall concentration). Due to enhanced retention of polar and aromatic analytes by the functionalized polymeric sorbent by virtue of hydrophobic and $\pi-\pi$ bonding, the Strata-X reversed-phase cartridge was finally selected.

Method Validation. Quantitative free and sulfated steroid analysis based on the devised LC/MS method showed good chromatographic separation under the LC conditions used (Figure 2). Method validation was conducted by evaluating LOQ, linearity, accuracy, precision, reproducibility, and stability by use of spiked samples prepared from QC samples (Table 2). LOQ was defined as S/N ratio >10. T, Preg, 17-OH-Preg, 17-OH-Prog, and Preg-S had LOQs of 0.2 ng/mL, whereas Prog and DHEA-S exhibited values of 0.1 ng/mL. DHEA and E2-S had LOQs of 0.5 ng/mL, while E2, Chol-S, and Chol had LOQs of 1, 5, and 50 ng/mL, respectively. The devised methods were found to be linear ($R^2 > 0.997$) over the calibration ranges, with extraction efficiencies of all steroids at four different QC concentrations being between 94.1% and 105.5%.

Accuracy and precision were determined by analyzing QC samples at four different ranges, LOQ (0.1, 0.2, 0.5, 1, 5, and 50 ng/mL), low (0.5, 1, 2, 5, 20, and 200 ng/mL), medium (5, 10, 20, 50, and 100 ng/mL and 10 μg/mL), and high (20, 50, 200, and 500 ng/mL and 100 μg/mL) concentrations, according to individual sensitivity and calibration range. The intraday ($n = 5$) precision (expressed as % CV) ranged from 2.5% to 9.3%, whereas intraday accuracy (expressed as % bias) ranged from 94.6% to 105.8%; interday ($n = 5$) precision (% CV) ranged from 3.3% to 7.7%, and interday accuracy (% bias) ranged from 92.4% to 105.9%. The overall QC concentrations corresponded to the calculated amounts with an acceptable CV and bias of 15% for most steroids.

Postpreparative Stabilities. Instability can occur not only in the sample matrix but also in the prepared samples. Hence, it is important to test postpreparative stability under analytical conditions, including autoinjector conditions for the expected maximum run time, to determine if the analytical run could be repeated in the case of instrumental failure. All of these conditions did not affect analyte stability (96.7−106.4%), and high stabilities were obtained when the prepared samples were injected 1 day after being subjected to LC/MS analysis (Table 2).

Altered Steroid Metabolism in Obese Girls. The validated method was successfully applied to serum samples obtained from girls with childhood obesity. The clinical characteristics (BMI, body fat, and muscle mass) varied significantly between different groups, but other clinical parameters including total and lipoprotein-bound cholesterol and triglyceride, were not significant (Table 3). Overall serum steroids were measured simultaneously in a single run, with all steroids quantitatively detected in the serum, except for the low levels of E2 in prepuberty girls. The profiling analysis showed that obesity was associated with the concomitant alteration of most steroids; however, this was not statistically significant. The levels of testosterone tended to decrease in obese girls, which is not in accordance with the increased androgen levels in obese children (when gender differences and the Tanner stage are not taken

Table 3. Clinical Characteristics and Serum Steroid Levels of Girls with Childhood Obesity[a]

	normal ($n = 42$)		obese ($n = 31$)		
compd	mean	SD	mean	SD	*P*-value
BMI percentile	**44**	**21**	**98**	**1.6**	**<0.001**
BMI (kg/m^2)	**16**	**1.3**	**23**	**1.7**	**<0.001**
fat mass (kg)	**6.5**	**2.4**	**15**	**4.1**	**<0.001**
muscle mass (kg)	**22**	**2.2**	**25**	**4.5**	**0.003**
total C (mg/dL)	170	26	180	25	0.204
TG (mg/dL)	100	43	110	48	0.977
HDL-C (mg/dL)	54	11	52	11	0.085
LDL-C (mg/dL)	100	17	110	19	0.058
T (ng/mL)	0.51	0.32	0.41	0.20	0.080
DHEA (ng/mL)	7.8	13	5.2	13	0.400
Preg (ng/mL)	0.73	0.61	0.81	0.62	0.273
Chol (μg/mL)	710	440	610	370	0.290
17-OH Preg (ng/mL)	0.93	0.82	1.2	0.91	0.145
17-OH Prog (ng/mL)	0.42	0.21	0.31	0.23	0.335
Prog (ng/mL)	1.22	0.82	1.40	0.91	0.344
Chol-S (ng/mL)	**820**	**420**	**650**	**310**	**0.049**
DHEA-S (ng/mL)	**280**	**170**	**430**	**230**	**0.004**
Preg-S (ng/mL)	22	11	27	17	0.122
E2-S (ng/mL)	0.64	0.60	0.42	0.31	0.253

[a]Entries in boldface type indicate significant changes.

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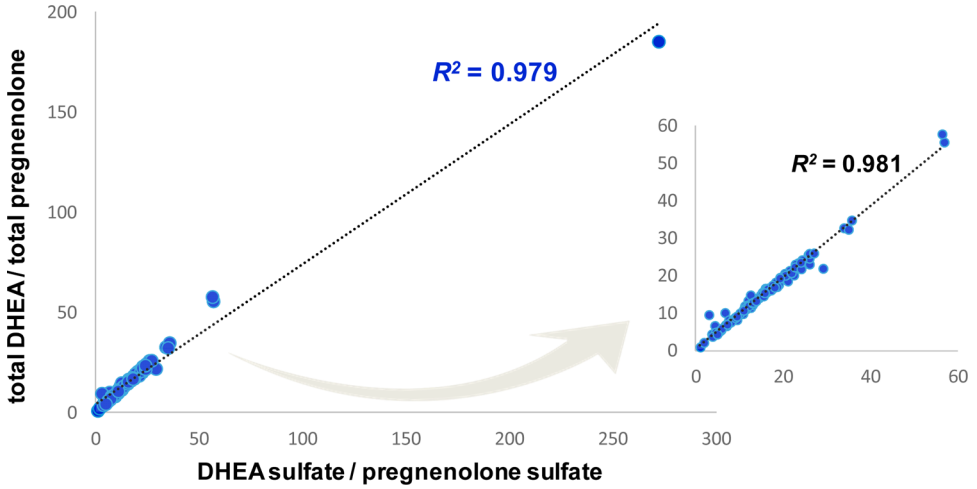

Figure 3. Association of metabolic ratios representing CYP17A1 activities. The metabolic ratios DHEA sulfate/pregnenolone sulfate and total DHEA/total pregnenolone showed good correlation. The highest intensity value in strengths of the association (R^2) is included and excluded (inset) for two linear fit models.

into account).[16,24] The level of Chol-S was decreased and that of DHEA-S was increased in obese girls (P = 0.004, t-test). This phenomenon was observed both in our gender-specific study[7] and in other works.[16,24,25] In our recent study, the serum levels of free cholesterol were slightly increased in obese girls,[26] which does not agree with the results in this study. It may be because pubertal conditions of subjects were not taken into account in a previous study.

The developed method also provides the metabolic ratios of free and sulfated steroids in a single run, which can indicate the enzymatic activity of steroid sulfotransferase. The activity of DHEA sulfotransferase was significantly higher in obese girls than normal ones ($P < 0.05$), in accordance with previous reports.[7] Preg and Chol sulfotransferase activities were statistically insignificant. Other metabolic ratios representing CYP17A1 activities were affected by obesity ($P < 0.05$), while the significantly increased metabolic ratios in obese girls were evaluated by use of levels of total and sulfated DHEA and Preg rather than levels of free DHEA and Preg. The body fat index in obese children is significantly associated with serum DHEA-S,[27] and it may reflect an increased CYP17A1 activity. Both metabolic ratios of total DHEA to total Preg and sulfated DHEA to sulfated Preg may be good indicators of CYP17A1 activity, and they showed good correlation linearity (Figure 3). The results may be supported by the association of gene expression of CYP17A1 with childhood obesity.[28,29]

CONCLUSIONS

A novel LC/MS-based analytical method was developed for simple, rapid, and reproducible quantification of serum steroids by selective MS scanning (SIM and MRM experiments) coupled with polarity switching (positive- and negative-ionization modes). The devised MS scan method for simultaneous analysis of free and sulfated steroids was successfully validated and clinically applied to evaluate the metabolism of steroids in obese girls. The obtained results provide experimental support for the importance of biologically active and inactive steroids as major reproductive factors.

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Notes

The authors declare no competing financial interest.

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