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Quantification of urine 17-ketosteroid sulfates and glucuronides by high-performance liquid chromatography–ion trap mass spectroscopy

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Abstract

The measurement of androgen steroids has been utilized as a clinical indicator of adrenal function, androgen abuse, and as a prediction of general health or biological aging. An improved high-performance liquid chromatography–ion trap mass spectroscopic method with sonic spray ionization (SSI) technology for the quantification of individual urinary 17-ketosteroid sulfates and glucuronides was developed and validated. Sample preparation was simplified using a C_{18} cartridge followed by direct injection onto a reversed-phase HPLC column. Individual 17-ketosteroid from 63 urinary specimens collected in a 24-h period was measured. 17-Ketosteroid conjugates, total 17-KS-S and the ratio of total 17-KS-S to creatinine referred to herein as the Anabolic/Catabolic Index (ACI) showed statistically significant negative correlations with age.

Keywords: 17-Ketosteroid sulfate; 17-Ketosteroid glucuronide; Steroids

1. Introduction

Dehydroepiandrosterone (I, DHEA), also referred to as, dehydro-3-isoandrosterone or 3-β-hydroxyandrost-5-ene-17-one, is a steroid secreted primarily by the adrenal gland and plays an important role as the primary precursor of many important sex steroids [1]. The negative correlation between age and the secretion of DHEA (I) and its sulfate form (II, DHEA-S) has been documented in human studies [2,3]. Serum DHEA (I) and/or DHEA-S (II) levels have been reported as constructive associations with central nervous system function, immunity, cardiovascular functions, insulin sensitivity, and body composition. Acute physiological trauma such as burns and serious illness are associated with decreases in DHEA and DHEA-S concentrations [4]. In recent years, 'DHEA deficiency syndrome' has been introduced as a new term for old age and there has been an increasing interest in the potential anti-aging benefits from DHEA/DHEA-S replacement therapy [5]. The measurement of steroids in human serum and urine specimens has been utilized as a clinical indicator of adrenal function [6], androgen abuse in

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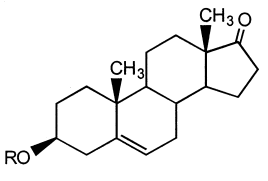
sports [7], and as a prediction of general health [8]. Recently, researchers at Hokkaido University in Japan discovered that total urinary 17-ketosteroid sulfate conjugates (17-KS-S) had a significant negative correlation with the health status of individuals [9]. The amount of 17-KS-S secreted over 24 h and adjusted by creatinine was inversely correlated to recovery from infection, illness, injury and psychosocial stress [10].

Urinary 17-ketosteroids consist primarily of 17-KS sulfates and 17-KS glucuronides (Fig. 1). Quantification of individual ketosteroid glucuronides and sulfates in biological fluids has been a great challenge due to the similarity of their structures and low concentrations. Radioimmunoassay has been widely utilized for quantification of DHEA/DHEA-S [11]. However, most of the ELISA kits have only been designed for DHEA and/or DHEA-S and not for other 17-KS conjugates. Quantification by classical enzymatic and chemical hydrolysis followed by Zimmermann color reaction has limited diagnostic value due to non-specificity [12]. Extensive column chromatographic separation of ketosteroid sulfates from glucuronides [13], or chemical labeling followed by fluorescence HPLC quantification [14] are time consuming and unsuitable for large numbers of samples. Recently, mass spectroscopic detection together with GC has been described for quantification of 17-ketosteroids with [15] and without derivatization [16]. HPLC–MS also has been reported in the quantification of 17-ketosteroid sulfate in serum [17] and estrogen sulfates in urine [18].

The aim of this study is to develop and validate a methodology quantifying individual 17-KS conjugate in human urine and to generate reference values of the indicated steroid sulfates and glucuronides which could be used in research regarding the relation between steroids and aging/disease, and in monitoring steroid abuse in sports and in dietary supplement consumption.



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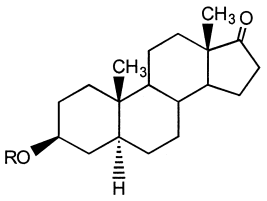
II R = SO_3Na
III R = $C_6H_9O_7$



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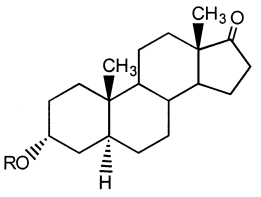
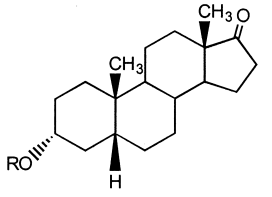


V R = SO_3Na
VI R = $C_6H_9O_7$



VII R = SO_3Na
VIII R = $C_6H_9O_7$

Fig. 1. Structure of dehydroepiandrosterone (I, DHEA), dehydroepiandrosterone-3-β-sulfate (II, DHEA-S), dehydroepiandrosterone-3-β-glucuronide (III, DHEA-G), epiandrosterone-3-β-sulfate (IV), androsterone-3-β-sulfate (V), androsterone-3-β-glucuronide (VI), etiocholanol-17-one-3-β-sulfate (VII), etiocholanol-17-one-3-β-glucuronide (VIII).

2. Experimental

2.1. Materials and reagents

Dehydroepiandrosterone-3-β-sulfate (II, DHEA-S), dehydroepiandrosterone-3-β-glucuronide (III, DHEA-G), epiandrosterone-3-β-sulfate (IV), androsterone-3-β-sulfate (V), androsterone-3-β-glucuronide (VI), etiocholanol-17-one-3-β-sulfate (VII), etiocholanol-17-one-3-β-glucuronide (VIII) were purchased from Sigma Chemicals with >99% purity and used as analytical standards. ACS grade ammonium acetate, sodium hydrogenphosphate and sodium dihydrogenphosphate were purchased from Sigma. Acetonitrile, HPLC grade, and methanol, ACS grade, were purchased from Fisher. C_{18} Sep-Pak cartridges (Vac RC 500 mg) were purchased from Waters. Creatinine quantification kit (555-A) was purchased from Sigma.

2.2. Instrumentation

A Hitachi M-8000 ion trap mass spectrometer (LC-3DQ-MS) with a sonic spray ionization source was utilized for quantification in a negative ionization mode. The LC–MS was calibrated by direct injection of 10 ng/μl of total 17-ketosteroids using a Harvard Apparatus model 11 syringe pump at a flow-rate of 100 μl/min. Unit mass resolution was established and maintained at ±0.3 *m/z*. Nitrogen gas was generated from a liquid nitrogen tank with output pressure of 60 p.s.i. and ion source inlet pressure at 3.6 kgf/cm^2; and helium gas was utilized as a buffer gas with output pressure of 60 p.s.i. and ion source inlet pressure at 3.0 kgf/cm^2. The sonic spray ion source plate had a temperature of 275°C with 1.0 kV voltage. Aperture temperatures were set up at 170°C and 120°C, respectively. The draft voltage was 75 V and the focus voltage was 35 V. The ion accumulation time was 300 ms with a scan range of 300 to 500 *m/z*. Negative-ion mass spectra of steroid sulfates and glucuronides are dominated by the anion $[M\text{-}H]^-$. This ion was used for selected-ion monitoring (SIM). A mass scan range at $[M\text{-}H]^- \pm 1$ *m/z* was used for quantification in centroid mode to secure the highest sensitivity and reproducibility. An electronic spray ionization (ESI) experiment was run at the same conditions as SSI except assistant gas and desolvator temperatures were 180°C with 3.0 kV voltage at the probe. Aperture temperatures were set up at 150°C and 120°C, respectively.

2.3. Chromatographic conditions

The mobile phase solvents were delivered by a Hitachi L-7100 gradient pump through a Hitachi inline degasser at a flow-rate of 0.4 ml/min. The mobile phase was composed of an isocratic solution of 0.1 *M* ammonium acetate–acetonitrile (75:25, v/v) at time $t=0$ to $t=12.5$; then the column was washed with DI water–acetonitrile (25:75, v/v) at time $t=12.6$ to $t=15.0$. A 5-min post run time was used to re-equilibrate the column. A C_{18} column (10.0×2.0 mm, I.D. Luna, Phenomenex) packed with 3-μm diameter particles and protected with a C_{18} guard column (ODS, 4.0×2.0 mm, I.D. cartridge, Phenomenex) was used in a Hitachi L-7300 column oven (50.0±0.2°C). A Hitachi L-7200 sequential autosampler (5±0.2°C) was utilized to inject 20.0-μl samples. The effluent from the column was transferred directly onto a Hitachi M-8000 ion trap mass detector connected with a D-7000 interface module.

2.4. Extraction procedure

A urine sample of 5 ml was mixed with 5 ml of 67 m*M* sodium phosphate buffer (pH=7.0) and loaded onto a Sep-Pak C_{18} cartridge (500 mg) activated with 5 ml of methanol and conditioned by 5 ml of 67 m*M* sodium phosphate buffer. The column was washed with 3 ml of DI water followed by 3 ml of 30% methanol, then dried under vacuum for 5 min. The 17-ketosteroids were eluted with 3 ml of methanol and collected in a test tube. The solvent in the eluent was removed by a RapidVap at a vortex speed of 40 rpm/min at 35±1°C. The residue was dissolved in 2.5 ml of the mobile phase, filtered through a 0.45-μm filter and a 20-μl aliquot was injected for LC–MS analysis.

2.5. Standard curve and stability

Approximately 13 mg of DHEA-S (II), 4 mg of DHEA-G (III), 0.5 mg of epiandrosterone-3-β-sul-

fate (IV), 25 mg of androsterone-3-β-sulfate (V), 20 mg of androsterone-3-β-glucuronide (VI), 22 mg of etiocholanol-17-one-3-β-sulfate (VII), and 20 mg of etiocholanol-17-one-3-β-glucuronide (VIII), were mixed with 100 mg of boric acid, added to a 100-ml volumetric flask, and dissolved in HPLC mobile phase to yield a 1045 ng/μl stock solution of total 17-ketosteroids. The stock solution was diluted 1:100 with the mobile phase to generate an external standard at a concentration of 10 ng/μl. Calibration standards at concentrations of 60, 30, 18, 12, 3, 1, 0.6, 0.2 ng/μl of total 17-KS were obtained by serial dilution of the stock solution. The stability of the standards was measured after storage at -10 ± 1°C for 2 months. Each determination was performed in duplicate.

2.6. Standard recovery

The recovery yields of 17-ketosteroid glucuronides and sulfates were measured by comparing 17-KS peak area ratios from an urine specimen spiked with and without known amounts of standards at two concentrations (1.4 and 4.7 ng/μl) of total 17-ketosteroids. The urine samples were processed in triplicate and analyzed as described earlier.

2.7. System suitability and data analysis

The sample analysis started with one blank injection for background subtraction. Then an external standard at a concentration of 10 ng/μl total 17-ketosteroids was injected in quintuplet. The LC–MS system was judged suitable for quantification analysis if the mean peak areas and retention times for each individual 17-ketosteroid had less than 5% coefficient of variation (C.V.). The sample 17-KS concentration was calculated from the ratio of the peak area of the compound of interest to that of the external standard multiplied by its concentration. The concentration of creatinine in urine was measured by a colorimetric method [19]. The urinary Anabolic/Catabolic Index (ACI=X/Y) was calculated as a ratio of total 17-ketosteroid sulfate concentration (X, ng/μl) to creatinine concentration (Y, μg/μl).

2.8. Urine specimen

Urine specimens were collected in a period of 24-h from 66 normal human volunteers including 49 females and 17 males, aged 40 to 65. Boric acid and thymol were used as preservatives, 200 mg, respectively, in each urine container. Total volume of each urine specimen was recorded. The urine specimens were quickly aliquoted, frozen, and immediately shipped on dry ice by overnight service. These specimens were thawed at room temperature and processed as described on the same day of receipt.

Pearson correlation coefficient is used to quantify the relation between age and individual 17-KS and total 17-KS-S. Two-tailed t-test was used to test whether a correlation coefficient was significant from zero [20].

3. Results and discussion

3.1. Mass detector condition

Separation of 17-ketosteroid conjugates can only be achieved by utilizing a high concentration of ammonium acetate buffer (0.1 M). Unfortunately, LC–MS detection of the conjugates is greatly reduced by the presence of ammonium acetate and the sensitivity of Electronic Spray Ionization (ESI) in particular has been reported at more than ten-fold reduction [18]. Sonic Spray Ionization (SSI) has been established as a new technology to generate molecular ions under a very wide range of solvent systems and liquid flow-rates [21] and to produce highly charged ions from protein solutions under much milder conditions [22]. Charged droplets are produced by spraying the analyte solution at a gas velocity higher than the sonic velocity so that gaseous ions are emitted from the droplets without heat or application of an electric field [23]. Our experiments showed that under direct injection of 17-ketosteroid in 0.1 M buffer solution, SSI generated no significant signal loss vs. a 95% signal loss if ESI was used. Under ESI conditions, the limit of quantification (LOQ) for 17-KS-S was between 250 and 400 pg/μl and for 17-KS-G was 1450–2000

pg/μl, which was more than 20 times less sensitive than SSI. It demonstrated that Sonic Spray Ionization (SSI), had superior ionization efficiency compared to ESI while using high concentrations of buffer as an eluent. High concentration of ammonium acetate did not cause significant overflow of interested ions in ion trap at the tested concentrations up to 50 ng/μl of total 17-KS. The potential reason is that ammonium acetate is not stable and will be decomposed at high temperature, especially when water exists. Since the temperature of SSI ion source was set at 275°C. We can expect that the majority amount of ammonium acetate has been evaporated before ionization and passed through several apertures. Overflow of the ion trap due to the limitation of ion storage capacity has only been observed at high concentration of total 17-KS. The linearity response was lost when the concentration of total 17-KS reached to 50 ng/μl, which was more than ten times higher than 17-KS concentration in normal urine specimen.

3.2. LC–MS chromatogram of standards

Fig. 2A showed total ion chromatogram of seven 17-KS standards, which were separated within 20 min using a C_{18} HPLC column. Androsterone sulfate (V) and etiocholanolone sulfate (VII) are 5α and 5β isomers that were separated only with a high concentration of ammonium acetate buffer (0.1 *M*). However, androsterone-3-β-glucuronide (VI) and etiocholanol-17-one-3-β-glucuronide (VIII) were unseparable at this condition. Fig. 2B is a total ion chromatogram of a representative urine specimen. The individual 17-KS was identified based on retention time from the TIC as indicated. However, quantification of individual 17-ketosteroid was based on selected ion monitoring. Figs. 3–6 show the mass spectra and selective ion chromatograms of 17-KS conjugates. It is true for every 17-KS conjugate that $[M\text{-}1]^-$ ion is the base peak in the full scan mode without significant fragmentation. Therefore, the intensity of the $[M\text{-}1]^-$ peak was utilized for quanti-

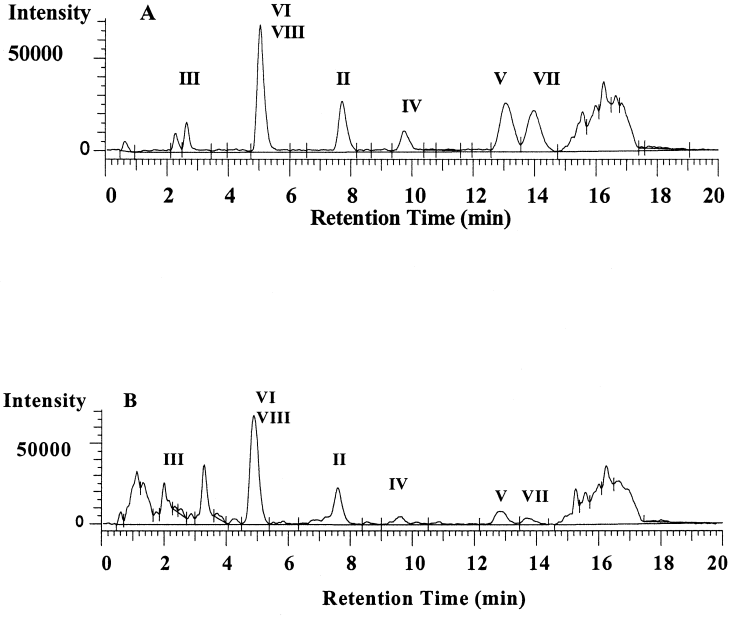


Fig. 2. (A) Total Ion Chromatogram (TIC) of seven 17-KS standards; Dehydroepiandrosterone-3-β-sulfate (II, DHEA-S), dehydroepiandrosterone-3-β-glucuronide (III, DHEA-G), epiandrosterone-3-β-sulfate (IV), androsterone-3-β-sulfate (V), androsterone-3-β-glucuronide (VI), etiocholanol-17-one-3-β-sulfate (VII), etiocholanol-17-one-3-β-glucuronide (VIII); and (B) Total ion chromatogram of a representative human urine sample.

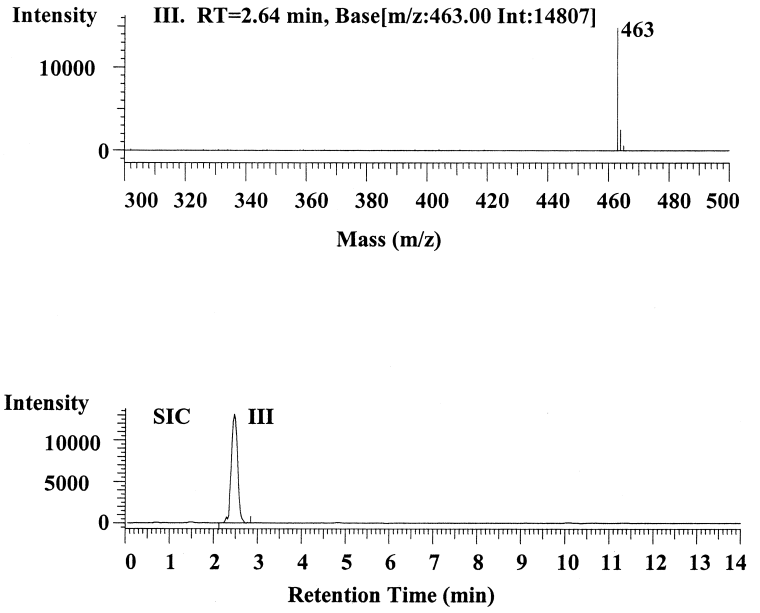


Fig. 3. Mass spectrum and selected ion chromatogram (SIC) for DHEA-G (III) in urine.

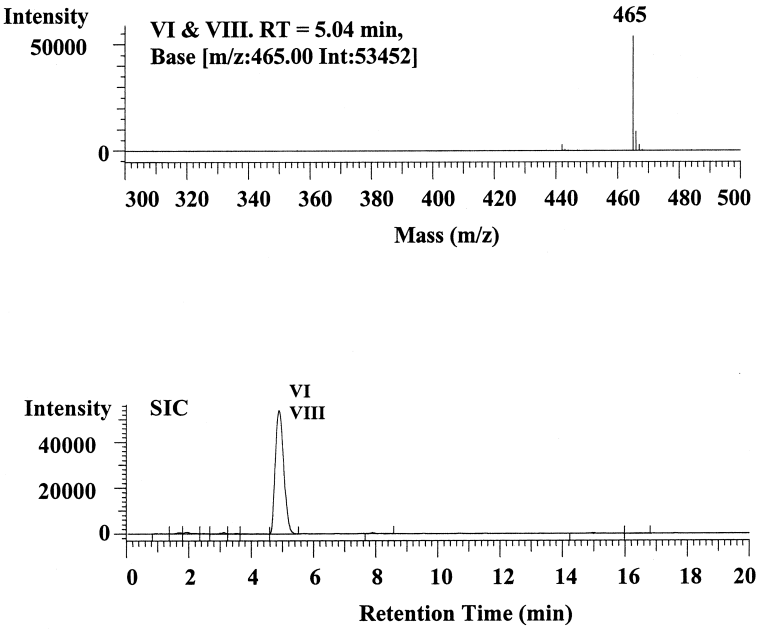


Fig. 4. Mass spectrum and selected ion chromatogram (SIC) for androsterone-3-β-glucuronide (VI) and etiocholanol-17-one-3-β-glucuronide (VIII) in urine.

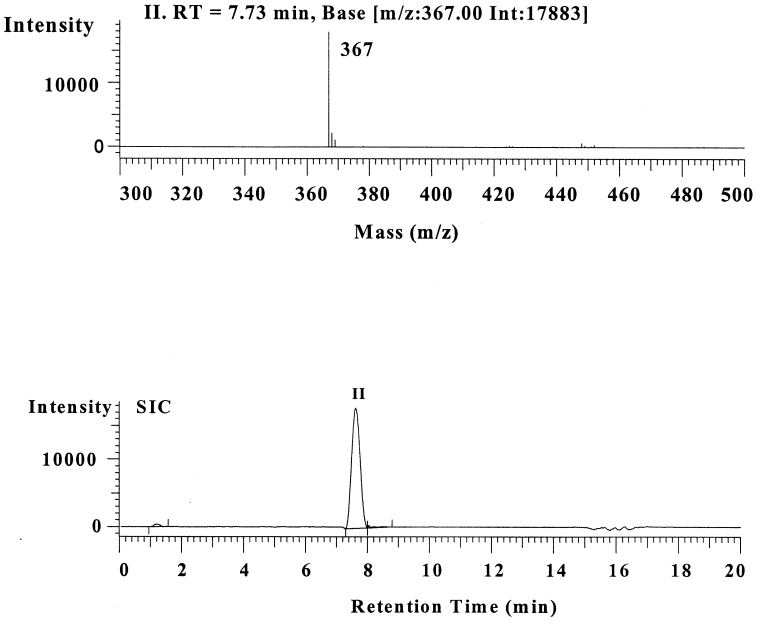


Fig. 5. Mass spectrum and selected ion chromatogram (SIC) of DHEA-S (II) in urine.

fication. Epiandrosterone-3-β-sulfate (IV), androsterone-3-β-sulfate (V) and etiocholanol-17-one-3-β-sulfate (VII) have the same $[M-1]^-$ ion at $m/z=369$ (Fig. 6). Fortunately, those three sulfates have different retention times that made the quantification of each conjugate possible.

3.3. Linearity and quantification limit

The standard solution at various concentrations (60, 30, 18, 12, 3, 1, 0.6, 0.2 ng/μl of total 17-KS) was analyzed as described with 10 ng/μl of total 17-KS solution as external standard. Linearity was expressed as a regression line forced through zero as shown in Table 1. Correlation coefficients for the seven 17-ketosteroids were between 0.9725 to 0.9976. The limit of quantification (LOQ) for each 17-ketosteroid was determined by calculating the coefficient of variation (C.V.) of the peak areas with less than 20% out of five injections. The limit of quantification (LOQ) for 17-ketosteroids were from 3 to 30 pg/μl. The upper limit of linearity was determined by >0.97 correlation coefficients based on a broader range of measurements. Over the upper calibration limit, the linearity gradually disappeared, because of overflow of the ion trap in MS. The linearity range demonstrated from this analysis covered the normal concentration of total 17-ketosteroids (3.04±2.54 ng/μl) in urine.

3.4. Standard recovery and stability

At the concentrations of 1.4 and 4.7 ng/μl of total spiked standards, recovery for 17-ketosteroids was in the range of 64.8% to 98.5% (Table 2). The recovery yields for DHEA-G (64.8%–68.0%) and DHEA-S (70.9%–72.4%) at two different concentrations were consistent with published reports [24]. Furthermore, total 17-ketosteroid sulfates composed of the four major sulfates, DHEA-S (II), epiandrosterone-3-β-sulfate (IV), androsterone-3-β-sulfate (V), and etiocholanol-17-one-3-β-sulfate (VII), showed consistent recovery from the test concentrations, of 85.9% and 85.7%, respectively. 17-ketosteroid sulfates and their glucuronides were stable for 2 months at −10±0.5°C with recovery of 96.1%–107.2%, without significant deviation (C.V.≤4.9%).

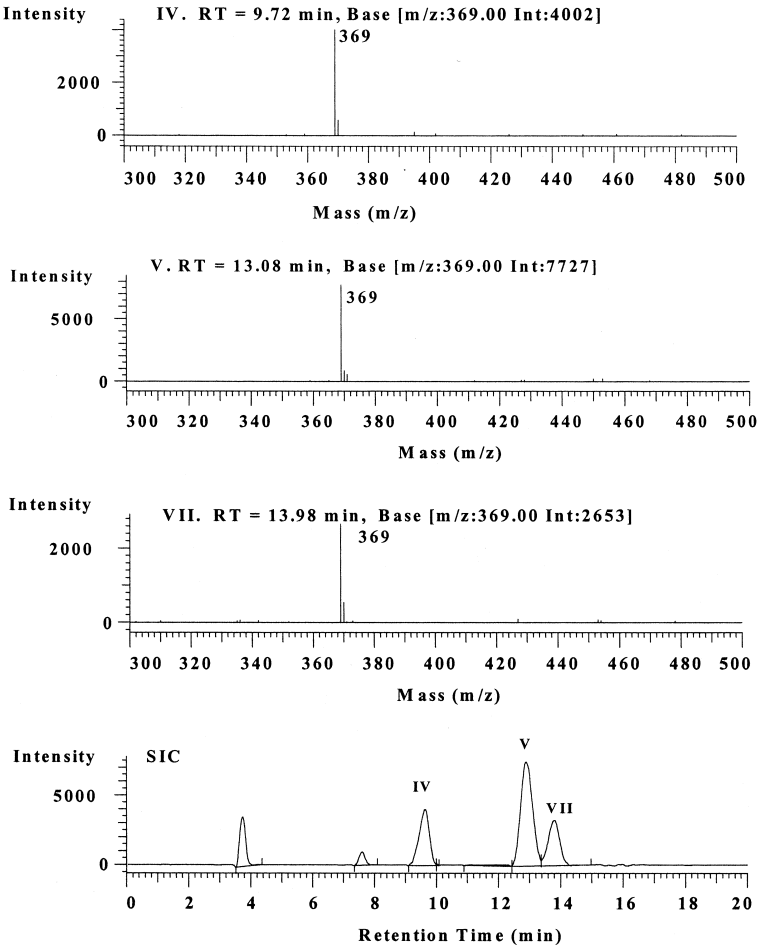


Fig. 6. Mass spectrum and selected ion chromatogram (SIC) of epiandrosterone-3-β-sulfate (IV), androsterone-3-β-sulfate (V) and etiocholanol-17-one-3-β-sulfate (VII) in urine.

Table 1
Linearity of 17-KS standards

17-Ketosteroid	Retention time (min)	Correlation coefficient	Linearity range (ng/μl)	LOQ (pg/μl)
DHEA-G (III)	2.64	0.9976	0.01–3.08	3
Androsterone-3-β-glucuronide (VI) and etiocholanolone-3-β-glucuronide (VIII)	5.04	0.9927	0.08–20.23	30
DHEA-S (II)	7.73	0.9724	0.03–3.51	10
Epiandrosterone-3-β-sulfate (IV)	9.72	0.9845	0.01– 1.35	4
Androsterone-3-β-sulfate (V)	13.08	0.9725	0.05–3.92	20
Etiocholanolone-3-β-sulfate (VII)	13.98	0.9802	0.05–3.66	20

Table 2
17-KS standard recovery

17-Ketosteroid	Baseline concentration (ng/μl)	Spiked standard		Spiked standard	
		Concentration (ng/μl)	Recovery (%)	Concentration (ng/μl)	Recovery (%)
DHEA-G (III)	0.108	0.074	64.8	0.246	68.0
Androsterone-3-β-glucuronide (VI) and etiocholanolone-3-β- glucuronide (VIII)	6.067	0.486	89.2	1.618	83.2
DHEA-S (II)	1.510	0.169	70.9	0.562	72.4
Epiandrosterone-3-β-sulfate (IV)	0.376	0.065	86.7	0.215	90.2
Androsterone-3-β-sulfate (V)	1.309	0.314	87.6	1.044	85.3
Etiocholanolone-3-β-sulfate (VII)	0.594	0.293	98.5	0.976	94.8

3.5. Urine specimen analysis

A total of 66 normal human volunteers participated in this study, which included 49 females and 17 males, aged 40 to 65 with average age of 50.3. Three urine samples were disqualified due to incomplete collection. Urine specimens collected in a period of 24-h were thawed at room temperature and processed as described on the same day of receipt. Individual 17-ketosteroid was quantified and summarized in Table 3. Subjects were grouped based on their age. The mean 24-h secretion amount of individual 17-KS±two times standard error was listed in the each age group. The secretion amount of all 17-KS in 24-h urine showed negative correlation with age based on Pearson coefficient with statistical significance ($P<0.05$) except DHEA-G. Urine DHEA-S secretion had a mean value of 342.7 μg/24-h out of 63 urine samples in the range of 0 to 3922.1 μg/24-h. The Pearson correlation coefficient of DHEA-S 24-h secretion against age is −0.2940 with statistical significance ($P=0.0193$). This result is consistent with previous studies that demonstrated high serum and urinary DHEA-S (II) levels in young adults and decreasing with age [2,3].

Urinary total 17-ketosteroid sulfates that include compound II, IV, V and VII had been used to represent an index of overall adrenal androgen production, at least before the onset of puberty [13]. It also has been reported as declining with the beginning of disease until reaching very low levels during severe disease or old age [9]. Based on our observation, both 24-h secretion amount and concentration of 17-KS-S in 24-h urine specimen showed very significant ($P<0.002$) negative correlation against age (Table 4). Their similar Pearson correlation coefficients indicated an equal correlation with age. Gender has been reported as a factor that affects secretion of DHEA, DHEA-S, and total 17-KS-S [4]. Our analysis also confirmed that males have significantly higher 24-h secretion amount of DHEA-S and total 17-KS-S in urine than in the same

Table 3
Means±two times standard errors for 24-h urinary secretion (μg/24-h) 17-KS conjugates by age

Age	*N*	DHEA-G (III)	Androsterone-G (VI) and etiocholanolone-G (VII)	DHEA-S (II)	Epiandrosterone-S (IV)	Androsterone-S (V)	Etiocholanolone-S (VII)
40–45	19	32.8±17.1	3227.4±974.7	478.1±242.3	169.2±86.0	772.1±320.3	351.6±181.7
46–50	20	49.5±46.0	3406.3±1901.4	518.3±402.1	92.3±47.6	526.1±252.2	200.0±91.3
51–55	10	29.4±10.0	2658.4±947.1	96.0±58.3	27.4±22.5	266.4±122.1	76.67±68.46
56–65	14	17.5±7.6	1171.7±569.6	84.5±80.7	30.9±25.9	121.3±92.8	96.2±60.8
Pearson correlation coefficient		−0.1058	−0.2750	−0.2940	−0.4073	−0.4508	−0.3619
P value		0.4094	0.0292	0.0193	0.0009	0.0002	0.0036

Table 4
Means±two times standard errors for 24-h urinary total 17-KS-S, creatinine and ACI by age

Age	*N*	Average age	Total 17-KS-S secretion in 24-h (μg/24 h)	Total 17-KS-S concentration (ng/μl)	Creatinine concentration (μg/μl)	Creatinine secretion in 24-h (mg/24 h)	ACI
40–45	19	42.68	1771.12±786.85	1.40±0.66	0.93±0.21	1134.08±256.68	1.50±0.68
46–50	20	48.75	1336.40±750.74	1.27±0.60	0.99±0.23	1513.44±1277.88	1.45±0.53
51–55	10	52.90	466.29±228.08	0.38±0.21	0.64±0.15	890.94±201.8	0.56±0.32
56–65	14	61.14	187.90±93.95	0.27±0.10	0.61±0.14	601.40±187.1	0.53±0.26
Pearson correlation coefficient			−0.4026	−0.3976	−0.2600	−0.1312	−0.3571
P value			0.0011	0.0013	0.0396	0.3053	0.0041

age of females, *P* values equal to 0.0046 and 0.0012, respectively, after analysis of covariance.

Urinary secretion amount of creatinine in 24-h showed a decrease against age without statistical significance ($P=0.3053$). Urinary creatinine concentration has been utilized as a normalization factor to eliminate the cumbersome 24-h urine collection. Total 17-KS-S concentration normalized with creatinine concentration (ACI) has been reported decreasing with aging and falling health [9,10]. As shown in Table 4, this study confirmed that ACI decreased with age for both genders. The Pearson correlation analysis demonstrated a negative slope with statistical significance ($P=0.0041$). However, gender was not significant after analysis of covariance with ACI as an outcome variable.

Current methods used to determine biological age include measurements of skin thickness, strength, stamina, body composition, reaction time, hearing, visual acuity [25], and whole blood lymphocyte analysis [26]. Unfortunately, most of these tests do not directly measure aging as an active, kinetic process but rather as clinical endpoints. However, a ratio of urinary total 17-ketosteroid sulfate concentrations (ng/μl) to creatinine concentration (μg/μl) expressed as the ACI (Anabolic/Catabolic Index) has been reported that it is sensitive enough to reflect the full range of repair/rebuild activity in muscles, organs, connective tissue, immune and nervous system [4,9,10]. This LC–MS method will supply a sensitive and accurate tool to quantify individual 17-KS conjugate in human urine, which could be used in establishment of relation between the steroids and aging/disease, and in recommendation of proper dosages for DHEA/DHEA-S replacement therapy.

4. Conclusions

A high-performance liquid chromatography and ion trap mass spectroscopic method for the quantification of individual urinary 17-ketosteroid sulfates and glucuronides was developed as a simple, accurate and high-throughput research and diagnostic technique. A normal range of 24-h urinary secretion of individual 17-KS and creatinine from 63 samples has been reported. The statistically significant negative correlation between age and 24-h secretion of all 17-KS conjugates but DHEA-G has been observed. The total 17-ketosteroid sulfate concentration normalized by creatinine (ACI) showed negative correlations with age similar to that of total 17-KS-S concentration and 24-h secretion amount. It could be utilized as a convenient and sensitive biomarker of aging-related DHEA/DHEA-S deficiency.

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