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Determination of salivary 17-ketosteroid sulfates using liquid chromatography–electrospray ionization-mass spectrometry

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Abstract

A simple and sensitive liquid chromatography–electrospray ionization-mass spectrometric (LC–ESI-MS) method for the simultaneous quantification of representative 17-ketosteroid sulfates, dehydroepiandrosterone sulfate (DHEAS), androsterone sulfate and epiandrosterone sulfate, in human saliva has been developed and validated. The saliva was deproteinized with acetonitrile, purified using a solid-phase extraction cartridge and subjected to LC–MS. Deuterium-labeled DHEAS was used as the internal standard and quantification was based on the selected ion monitoring (SIM) mode of each deprotonated molecule. This method allowed the reproducible and accurate quantification of the salivary sulfates using a 100-μl sample; the intra- and inter-assay coefficients of variation were below 6.8 and 6.3%, respectively, and the % accuracy values were quantitative for all the sulfates. The limits of quantitation for all the sulfates were 100 pg/ml. No significant matrix effect or change in the measured value by freeze/thaw repetitions was observed. The developed method was applied to clinical studies, and produced satisfactory results.

Keywords: 17-Ketosteroid sulfate; Saliva; LC–ESI-MS; Anti-aging; Clinical study

1. Introduction

Dehydroepiandrosterone (DHEA), a representative 17-ketosteroid, is synthesized by the steroidogenic enzyme P450c17 within the adrenal zona reticularis and then converted into active hormones, estrogens and androgens, in the peripheral tissues. Most (over 99%) of the DHEA is present as the sulfated conjugate (DHEAS) in the blood and DHEAS is second to cholesterol in abundance in circulating steroids in humans. Apart from serving as a precursor of estrogens and androgens, DHEA has been considered to have no obvious biological function. However, recent studies suggest that the age-related decline in DHEA levels seems to be associated with diabetes, depression, osteoporosis, the metabolic syndrome, and so on [1,2]. Although it is still unclear that these effects are due to DHEA itself or its downstream conversion products (estrogens and androgens), DHEA is now often designated as an anti-aging hormone and taken as a food supplement in the USA.

With the marked reduction in the production of DHEA during aging, the levels of androgens, such as testosterone (T), are of course decreased, which causes andropause [3]. T is activated to 5α-dihydrotestosterone in the target tissue for the androgen-dependent process, and then metabolized into inactive 17-ketosteroids, such as androsterone (A) and epiandrosterone (EA). The sulfated conjugates of these steroids (AS and EAS, respectively) are also present at relatively higher concentrations in humans and the measurement of their levels is useful for the elucidation of individual androgen status.

Saliva has recently been attracting attention as a new tool in clinical examinations and therapeutic drug monitoring due to its easy non-invasive nature of collection [4]. The use of saliva in steroid assays has other advantages; the levels of steroids in saliva generally reflect those of the free active steroids (i.e., non-protein-bound steroids) in the serum/plasma [5], and saliva assays are technically easier than serum/plasma assays due to the lower content of proteins and lipids. However, a major disadvantage in the use of saliva is the low analyte concentration; for example, the quantity of cortisol in saliva is less than one-tenth of the total cortisol in serum [6].

Steroids have conventionally been measured in biological samples by immunoassay [7]. Although this technique will doubtless continue to be the method of choice for routine use in the clinical field, its specificity and accuracy are sometimes

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poor due to interferences from other endogenous steroids and lipids. Among the alternative methods, liquid chromatography (LC) coupled with electrospray ionization (ESI) or atmospheric pressure chemical ionization-mass spectrometry (MS) has been used for steroid analysis due to its specificity, versatility and simultaneous multi-analyte quantification capability [8,9].

Based on this background information, in the present paper, we describe an LC–ESI-MS method for the simultaneous determination of 17-ketosteroid sulfates in saliva. The application of the proposed method to clinical studies is also presented.

2. Experimental

2.1. Materials and chemicals

DHEAS, AS, EAS, etiocholanolone sulfate (ES) and epietiocholanolone sulfate (EES) were prepared from the corresponding unconjugated steroids (Steraloids, Newport, RI, USA) with the chlorosulfonic acid–pyridine complex in our laboratories [10]. Stock solutions of these steroid sulfates were prepared as 100 μg/ml solutions in ethanol. Subsequent dilutions were carried out with ethanol to prepare 0.5, 1, 2, 5, 10 and 20 ng/ml solutions. [7,7,16,16-$^2H_4$]-DHEAS (internal standard, IS), used in a previous study [11], was diluted with ethanol to prepare a 50 ng/ml solution. Strata-X cartridges (60 mg adsorbent; Phenomenex, Torrance, CA, USA) were successively washed with methanol (2 ml) and water (2 ml) prior to use. All other reagents and solvents were of analytical grade.

2.2. LC–MS(–MS)

LC–MS(–MS) was performed using an Applied Biosystems API 2000 triple stage quadrupole-mass spectrometer (Foster City, CA, USA) connected to a Shimadzu LC-10AT chromatograph (Kyoto, Japan). A J'sphere ODS H-80 column (4 μm, 150 mm × 2.0 mm i.d.; YMC, Kyoto) was used at a flow rate of 0.2 ml/min and 40 °C. Methanol-10 mM ammonium formate (4:3, v/v) was used as the mobile phase unless otherwise indicated. The sulfates were analyzed by ESI-MS in the negative-ion mode and the operating conditions were as follows: declustering potential, −70 V; focusing potential, −310 V; entrance potential, −10 V; ion spray voltage, −4 kV; curtain gas (nitrogen), 55 psi; ion source gas 1 (nitrogen), 40 psi; ion source gas 2 (nitrogen), 80 psi; turbo gas temperature, 500 °C and interface heater, on. The monitoring ions for the quantification of the respective sulfates were as follows [selected ion monitoring (SIM)]: DHEAS, *m/z* 367.0, AS and EAS, *m/z* 369.0 and IS, *m/z* 371.0. Nitrogen was used as the collision gas in the MS/MS experiment with the collision energy of 60 eV and collision cell exit potential of 10 V. The data were collected and quantified using Applied Biosystems Analyst software (Version 1.3.1).

2.3. Collection and pretreatment of saliva

Saliva was directly collected into a glass tube (without a collection device) from healthy male volunteers and immediately centrifuged at 1000 *g* (4 °C, 5 min). The supernatant was stored at −20 °C until use. The saliva (100 μl) was added to acetonitrile (200 μl) containing IS (500 pg), vortex-mixed for 30 s and centrifuged at 1000 *g* (4 °C, 5 min). The supernatant was diluted with water (1 ml), and the sample was passed through a Strata-X cartridge. After washing with water (2 ml) and ethyl acetate (1 ml), the sulfates were eluted with methanol (1.5 ml). After evaporation, the residue was dissolved in methanol-10 mM ammonium formate (1:1, v/v, 30 μl) and 10 μl of which was subjected to LC–MS.

2.4. Calibration curve and matrix effect

DHEAS, AS and EAS (10, 20, 50, 100, 200 or 500 pg each) and IS (500 pg) were dissolved in methanol-10 mM ammonium formate (1:1, v/v, 30 μl) and 10 μl of which was subjected LC–MS. The calibration curves were constructed by plotting the peak area ratios (DHEAS/IS, AS/IS or EAS/IS) versus the amounts of DHEAS, AS or EAS, respectively.

The matrix effect was examined by comparing the slope of the calibration curve constructed as described above and those of curves prepared by adding sulfates (10, 20, 50, 100, 200 and 500 pg each) to the saliva (100 μl) (matrix sample). The matrix samples were prepared using five different saliva.

2.5. Recoveries of 17-ketosteroid sulfates and IS during pretreatment

The saliva (ca. 5 ml) was stirred with activated charcoal (0.6 g) overnight and then centrifuged at 1000 *g* (4 °C, 20 min). The supernatant was used as the steroid sulfate-free saliva, in which DHEAS, AS and EAS were not detected by the proposed method, to determine the recoveries of the sulfates and IS during pretreatment. The ethanolic solution of the mixed sulfates [DHEAS (200 pg), AS (100 pg) and EAS (20 pg) in 10 μl, spiked sample] or ethanol (10 μl, control sample) was added to the saliva (100 μl) and the resulting samples were pretreated. DHEAS (200 pg), AS (100 pg) and EAS (20 pg) were then added to only the control sample, and IS (500 pg) was added to both samples. The samples were subjected to LC–MS. The recoveries of the sulfates during pretreatment were calculated from the peak area ratios (DHEAS/IS, AS/IS or EAS/IS) of the spiked and control samples.

The ethanolic solution of IS (500 pg in 10 μl, spiked sample) or ethanol (10 μl, control sample) was added to the saliva (100 μl) and the resulting samples were pretreated. IS (500 pg) was then added to only the control sample, while DHEAS (500 pg) was added to both samples. The samples were subjected to LC–MS. The recovery of IS during pretreatment was calculated from the peak area ratios (IS/DHEAS) of the spiked and control samples.

2.6. Assay precision

The intra-assay precision was assessed by determining two saliva samples at different concentration levels ($n = 5$ for each sample) on 1 day. The inter-assay precision was assessed by

determining these samples over 5 days. The precision was determined as the coefficients of variation (CV, %).

2.7. Assay accuracy

Ethanol (10 μl, unspiked sample) or the ethanolic solution of the mixed sulfates [DHEAS (100 pg), AS (50 pg) and EAS (20 pg) in 10 μl, spiked sample] was added to the saliva (100 μl) (the spiked concentrations of DHEAS, AS and EAS were 1.0, 0.5 and 0.2 ng/ml, respectively, $n = 2$). After the addition of IS (500 pg), each of the resulting samples was pretreated and analyzed by LC–MS. The % accuracy values of the sulfates were defined as $F/(F_0 + A) \times 100$ (%), where F is the concentration of a sulfate in the spiked sample, F_0 is the concentration of a sulfate in the unspiked sample and A is the spiked concentration.

2.8. Limit of quantitation (LOQ)

The steroid sulfate-free saliva prepared by the charcoal-treatment was used to determine the LOQ. The LOQ was defined as the lowest concentration on the calibration curve of the analyte measured with an acceptable precision and accuracy (i.e., CV and relative error <15%) and with at least five times the response compared to the blank response.

2.9. Freeze/thaw stability

The freeze/thaw stabilities of the sulfates in the saliva were examined by determining two saliva samples before and after one and three freeze/thaw cycles.

2.10. Clinical studies

The saliva samples were obtained from healthy male volunteers known not to have received hormone supplementation, and used for the following studies (diurnal rhythm, age difference and DHEA administration study). The volunteers took no food and beverage within 30 min prior to the sample collection. The volunteers also did not brush their teeth within 2 h prior to sample collection to avoid blood contamination. Informed consent was obtained from all the volunteers. The statistical comparisons were performed using the Welch test in Microsoft Excel 2000 (Redmond, WA, USA).

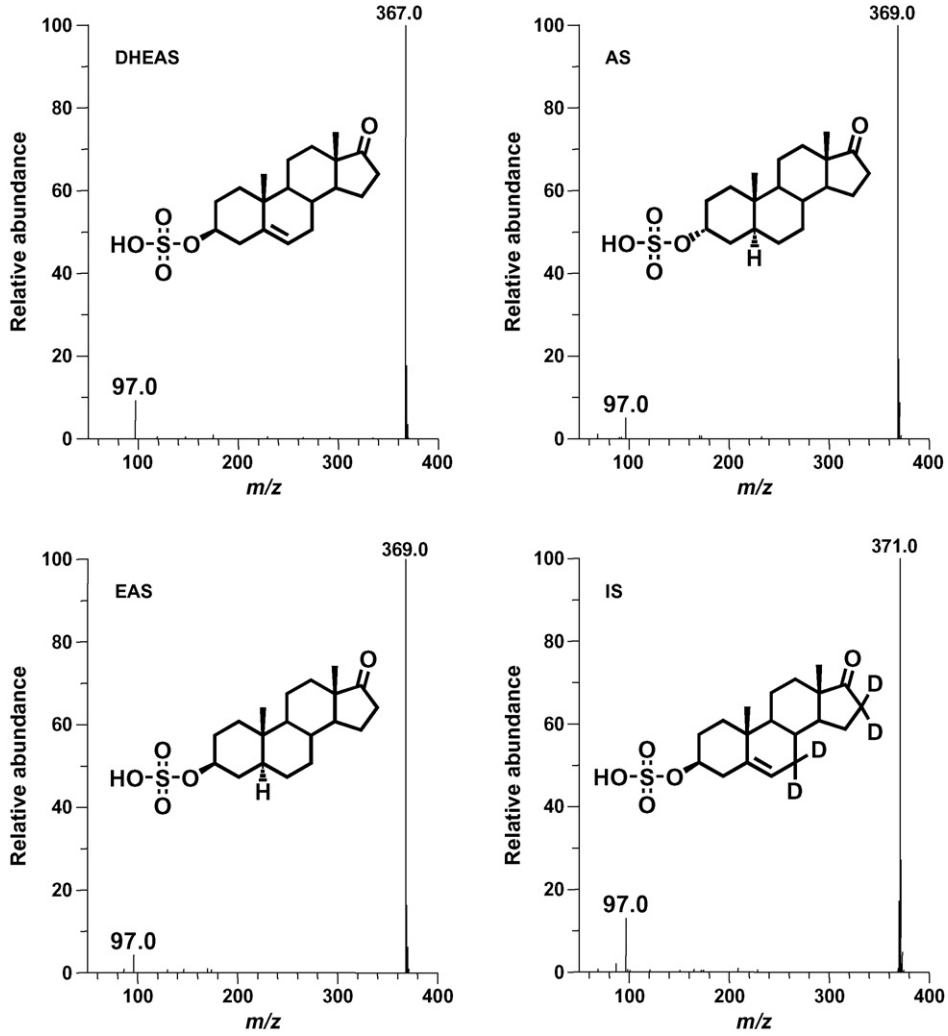

Fig. 1. ESI mass spectra of 17-ketosteroid sulfates and IS. MS conditions are described in Section 2.2.

2.10.1. Diurnal rhythm

The saliva samples collected from seven male (22–27 years old) volunteers at 9:00, 11:00, 13:00 and 15:00 h were analyzed using the proposed method.

2.10.2. Age difference

The saliva samples collected from male volunteers in their twenties ($n = 5$) and sixties ($n = 5$) at 15:00 h were analyzed using the proposed method.

2.10.3. DHEA administration study

DHEA (25 mg per tablet) (Schiff, Salt Lake, UT, USA) was orally administered to five healthy male volunteers (24–37 years old) at 9:00 h once daily for 3 days and their saliva was collected at 13:00 h on each day. The saliva was also collected at 13:00 h on 1, 2 and 3 days before the administration and on 1 and 3 days after the last administration.

3. Results and discussion

3.1. Chromatographic and mass spectrometric behavior of 17-ketosteroid sulfates

For the ESI-MS operating in the negative-ion mode, the sulfates and IS provided their deprotonated molecules, $[M–H]^-$, as the base ion peaks together with $[SO_4H]^-$ at *m/z* 97 (Fig. 1). The use of the selected reaction monitoring (SRM) mode may allow for the discrimination and quantification of the sulfates from a saliva matrix without the need for a long chromatographic separation due to its high specificity. However, the characteristic product ion was not detected except for the $[SO_4H]^-$, but the intensity of the ion was not high enough to use as the monitoring ion. For example, the signal to noise ratio (S/N) of the salivary DHEAS of a male volunteer was 31 when it was measured by the SIM mode (*m/z* 367.0), while it fell to 3.6 in the SRM mode (*m/z* 367.0 → 97.0) (chromatograms are not shown). Therefore, the determination of the sulfates was done with SIM and each deprotonated molecule was chosen as the monitoring ion.

A satisfactory separation of DHEAS, AS and EAS was achieved within 9 min using a J'sphere ODS H-80 column with the mobile phase of methanol-10 mM ammonium formate (4:3, v/v); DHEAS, retention time (t_R) 4.7 min; AS, 7.6 min; EAS, 5.5 min and IS, 4.7 min.

3.2. Collection and pretreatment of saliva

The stimulated saliva collections (i.e., collection after chewing Parafilm, gum and other commercially available devices for a few minutes or citric acid stimulation) may influence, mostly decrease, the analyte concentrations in the saliva [4]. In this study, the unstimulated saliva collection, which has less effect on analytical results than the stimulated saliva collections, was employed. Some devices, such as Salivette® (Sarstedt, Nümbrecht, Germany), are often used in the unstimulated saliva collections. However, when a collection device is used, it is difficult to precisely determine the analyte concentration, because the recovery of the analyte from the device is not always quantitative [4]. Furthermore, contaminants from the device sometimes interfere with the analysis [12]. For these reasons, the saliva was directly collected into a glass tube without the use of any collection devices in the present study.

The saliva was deproteinized in acetonitrile and purified using a Strata-X cartridge. Thus, our method employed only a one-step solid-phase extraction for the purification of the saliva samples and the recovery rates [mean ± standard deviation (S.D.) from five different saliva samples] of DHEAS, AS, EAS and IS during the pretreatment were 65.9 ± 1.9, 69.7 ± 1.5, 67.0 ± 1.1 and 66.3 ± 2.2%, respectively. The reproducibility of the recovery rates was satisfactory and there was no significant difference between the analytes and IS.

3.3. Assay specificity

A typical chromatogram of the saliva sample obtained from a healthy volunteer with IS is shown in Fig. 2. The peaks corresponding to DHEAS, AS and EAS were clearly observed at 4.7, 7.6 and 5.5 min, respectively, in the chromatogram. ES and EES, the 5β-isomers of AS and EAS, respectively, showed their deprotonated molecules at *m/z* 369 in the negative-ESI-MS, which was very similar to those of AS and EAS. Moreover, ES (t_R 7.1 min) and EES (t_R 5.5 min) were closely eluted with AS and EAS, respectively. Therefore, the peak purities of the saliva sample were confirmed by the different LC system [column; YMC-Pack C8 (5 μm, 150 mm × 2.0 mm i.d.) and mobile phase; acetonitrile-10 mM ammonium formate (1:3, v/v)]; the t_R values of AS (29.3 min) and EAS (23.9 min) in the saliva

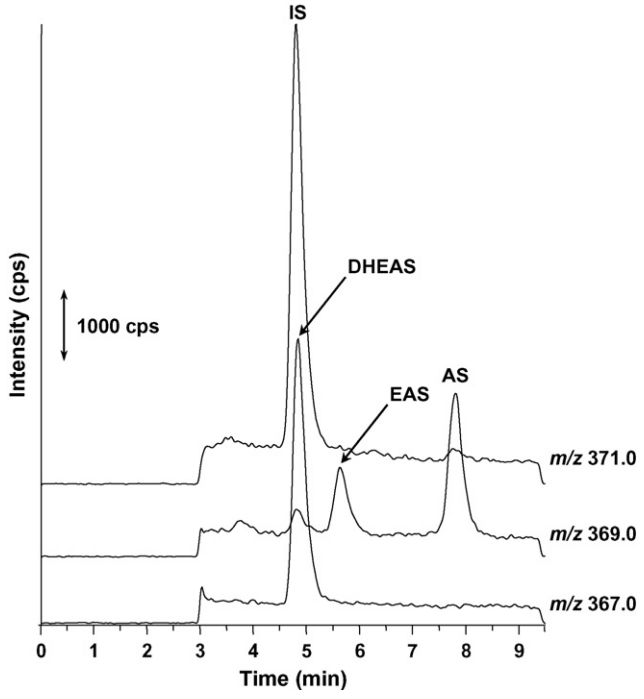

Fig. 2. Chromatograms of 17-ketosteroid sulfates and IS in saliva obtained from a healthy male subject. LC–MS conditions are described in Section 2.2. The measured concentrations of DHEAS, AS and EAS were 2.71, 1.34 and 0.49 ng/ml, respectively.

Table 1
Summary of calibration curves for 17-ketosteroid sulfates

	Slope[a] (CV)	Intercept[a] (CV)	Correlation coefficient (r)	Measurable range (pg/tube)
DHEAS	$(2.306 \pm 0.026) \times 10^{-3}$ (1.1%)	$(7.301 \pm 0.372) \times 10^{-3}$ (5.1%)	0.999	10–500
AS	$(2.551 \pm 0.038) \times 10^{-3}$ (1.5%)	$(5.847 \pm 0.290) \times 10^{-3}$ (5.0%)	0.999	10–500
EAS	$(3.060 \pm 0.063) \times 10^{-3}$ (2.1%)	$(3.831 \pm 0.177) \times 10^{-3}$ (4.6%)	0.999	10–500

[a] Mean ± S.D. ($n = 5$).

completely agreed with those of the standard samples under these LC conditions, in which ES (t_R 31.3 min) and EES (t_R 22.9 min) showed different t_R values. We further examined the occurrence of ES and EES in the saliva obtained from five different volunteers, and they were not detected at all. These data demonstrate that the other endogenous steroids do not interfere with the present assays.

3.4. Calibration curves and LOQ

The regression lines obtained from the combination of five standard curves are summarized in Table 1. All the CV values of the slopes and intercepts were below 2.1 and 5.1%, respectively.

To determine the extent to which the saliva matrix affects the quantification, the slopes of the above calibration curves were compared to those of the curves prepared with the matrix sample [11,13]. As a result, the slopes of the latter were $(2.245 \pm 0.095) \times 10^{-3}$ (mean ± S.D., $n = 5$, CV 4.2%) for DHEAS, $(2.627 \pm 0.085) \times 10^{-3}$ (CV 3.2%) for AS and $(3.162 \pm 0.065) \times 10^{-3}$ (CV 2.1%) for EAS, which were practically identical to the slopes of the curves constructed using the standard solutions. This result clearly revealed that the saliva matrix did not affect the calibration curves. Based on this result and the fact that it is not always easy to prepare the steroid sulfate-free saliva, the calibration curves were constructed using the standard solutions in the following studies. The applicability of these curves to the salivary 17-ketosteroid sulfate assays was also examined in the accuracy test that will be discussed later.

Although some endogenous components may be removed together with the steroid sulfates by the charcoal-treatment, the charcoal-treated saliva was used to determine the LOQs. Incidentally, the charcoal-treated specimens have been often used in the endogenous steroid assays as the blank matrices [11,14]. To the charcoal-treated saliva (100 μl), the respective sulfates (10 pg each) were spiked (concentration, 100 pg/ml each) and the sample was then pretreated and subjected to LC–MS. The peaks corresponding to the respective sulfates were clearly observed with an S/N of about 6. The measured values (mean ± S.D., $n = 5$) of DHEAS, AS and EAS were 100.1 ± 5.8 pg/ml (CV 5.8%, relative error 0.1%), 99.7 ± 9.8 pg/ml (CV 9.8%, relative error −0.3%) and 106.6 ± 4.4 pg/ml (CV 4.1%, relative error 6.6%), respectively. A sample below 100 pg/ml of the sulfates did not meet the criterion for the LOQ described in the experimental section. Therefore, the LOQs for the respective sulfates were determined to be 100 pg/ml when 100 μl of saliva was used.

3.5. Assay precision and accuracy

The intra-assay ($n = 5$) CV values for all the sulfates were less than 6.8%, and good inter-assay ($n = 5$) CV values (less than 6.3%) were also obtained, as shown in Table 2.

The saliva to which known amounts of the sulfates had been added were pretreated and analyzed in order to examine the assay accuracy. Satisfactory % accuracy values ranging from 95.8 to 103.5% were obtained (Table 2). Although the calibration curves were constructed using the standard sulfate solutions in the present study as already mentioned, this result demonstrates that the salivary 17-ketosteroid sulfates can be accurately determined using the curves. These data indicate that the present method is highly reproducible and accurate.

Table 2
Assay precision and accuracy

	Saliva A			Saliva B		
	DHEAS	AS	EAS	DHEAS	AS	EAS
Intact sample						
Intra-assay						
Mean ± S.D. (ng/ml, $n = 5$)	2.60 ± 0.042	0.63 ± 0.017	0.14 ± 0.010	2.77 ± 0.070	1.49 ± 0.049	0.24 ± 0.012
CV (%)	1.6	2.7	6.8	2.5	3.3	5.0
Intra-assay						
Mean ± S.D. (ng/ml, $n = 5$)	2.62 ± 0.018	0.64 ± 0.013	0.16 ± 0.010	2.80 ± 0.031	1.54 ± 0.044	0.24 ± 0.013
CV (%)	0.7	2.0	6.3	1.1	2.9	5.4
Spiked sample[a]						
Mean (ng/ml, $n = 2$)	3.56	1.18	0.34	3.84	2.09	0.45
Accuracy (%)[b]	98.3	103.5	95.8	101.1	102.5	100.9

[a] Spiked concentration: DHEAS, 1.0 ng/ml; AS, 0.5 ng/ml and EAS, 0.2 ng/ml.
[b] % Accuracy values were calculated based on the values obtained in the inter-assay variation test.

Table 3
Freeze/thaw stabilities of 17-ketosteroid sulfates in saliva

	DHEAS, Freeze/thaw cycle			AS, Freeze/thaw cycle			EAS, Freeze/thaw cycle		
	0	1	3	0	1	3	0	1	3
Saliva C									
Concentration (ng/ml)	3.81	3.71	3.85	1.38	1.42	1.41	0.28	0.27	0.28
Percentage of initial concentration	100.0	97.6	101.3	100.0	103.0	102.0	100.0	98.8	101.4
Saliva D									
Concentration (ng/ml)	3.52	3.52	3.57	0.99	0.99	0.97	0.17	0.18	0.17
Percentage of initial concentration	100.0	99.9	101.3	100.0	99.5	98.1	100.0	102.7	97.6

3.6. Freeze/thaw stability

The freeze/thaw stabilities of the sulfates in the saliva were examined, which indicated that the sulfates were stable up to three freeze/thaw cycles (Table 3). Furthermore, it was possible to store the saliva at −20 °C without any loss of the sulfates for at least 6 months.

3.7. Diurnal rhythms of salivary 17-ketostroid sulfates

No remarkable diurnal rhythms were observed in any of the sulfates (Fig. 3). The diurnal level changes in the salivary sulfate are in contrast to that of the salivary DHEA, which has a higher concentration in the morning [12].

3.8. Age difference in salivary 17-ketostroid sulfates

Age differences in the salivary 17-ketostroid sulfates were observed ($P < 0.05$) in the DHEAS (twenties, 2.19 ± 0.47 ng/ml and sixties, 1.11 ± 0.58 ng/ml) and AS (twenties, 1.05 ± 0.34 ng/ml and sixties, 0.56 ± 0.20 ng/ml) levels as expected, but there is no significant difference in the EAS level (twenties, 0.25 ± 0.11 ng/ml and sixties, 0.20 ± 0.10 ng/ml).

3.9. DHEA administration study

The changes in the salivary sulfate levels after the oral administration of DHEA (25 mg), which is often taken as an anti-aging food supplement in the USA, are shown in Fig. 4. The results are expressed as a percentage of the measured concentrations of respective sulfates at day 1 and are means ± S.D. of values from five male volunteers. Before the administration (days 1–3), there was no significant change in all the sulfate levels. The DHEAS level immediately increased after the administration and reached more than twice the concentration of the basal level on day 6. The concentration significantly decreased within 1 day after the last administration. The AS and EAS levels were also elevated following the increase in the DHEAS level by the DHEA supplementation.

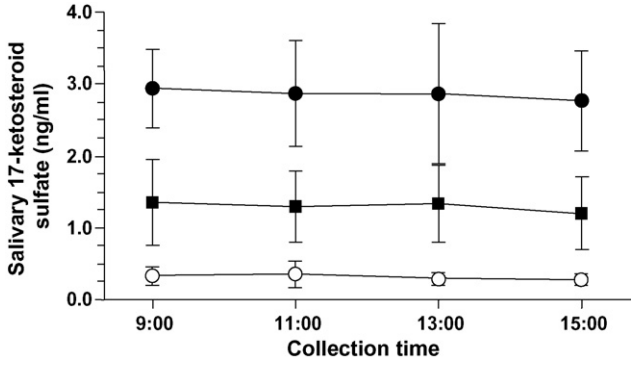

Fig. 3. Diurnal rhythm of salivary 17-ketosteroid sulfates. Closed circle: DHEAS, closed square: AS and open circle: EAS. Data are mean ± S.D. of seven male volunteers.

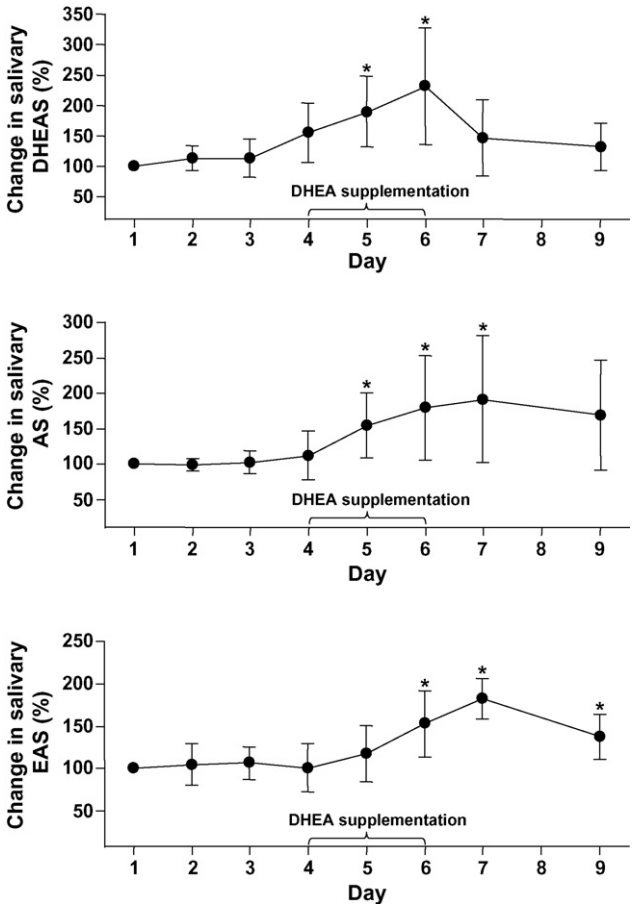

Fig. 4. Changes in salivary 17-ketosteroid sulfate levels in five male volunteers after the oral administration of DHEA (25 mg). Data are expressed as a percentage of the measured concentrations of respective sulfates at day 1 and are means ± S.D. of values from five volunteers. $^{*}P < 0.05$ vs. the measured concentrations of respective sulfates at day 3 (the day before the administration).

4. Conclusion

We have demonstrated the LC–ESI-MS method for the determination of 17-ketosteroid sulfates in human saliva. The method was specific, accurate and reproducible, and successfully applied to clinical studies. This method was able to detect the age-related decline in the salivary DHEAS and AS, and also applicable for the determination of the change in the individual sulfate levels after the DHEA supplementation. This well-characterized method will prove helpful in the elucidation of the physiological functions of DHEAS and in the diagnosis of androgen-related diseases, because the collection of saliva compared to blood and urine is a non-invasive technique that can be easily applied many times to the same person.

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