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Determination of salivary dehydroepiandrosterone using liquid chromatography–tandem mass spectrometry combined with charged derivatization

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Abstract

A sensitive liquid chromatography–electrospray ionization-tandem mass spectrometric (LC–ESI-MS–MS) method for the quantification of dehydroepiandrosterone (DHEA) in human saliva has been developed and validated. The saliva was deproteinized with acetonitrile, purified using a Strata-X cartridge, derivatized with the permanently charged reagent, 2-hydrazino-1-methylpyridine (HMP), and subjected to LC–MS–MS. The derivatization with HMP was very effective for increasing the detectability of DHEA in the positive-ESI-MS. Quantification was based on the selected reaction monitoring and androsterone was used as an internal standard. This method allowed the reproducible and accurate quantification of the salivary DHEA using a 200-μl sample and the limit of quantitation for DHEA was 25 pg/ml. No significant matrix effect or change in the measured value by freeze/thaw repetition was observed. The developed method was applied to clinical studies, and produced satisfactory results.

Keywords: Dehydroepiandrosterone; Saliva; Liquid chromatography–electrospray ionization-tandem mass spectrometry; Derivatization; Clinical study

1. Introduction

Dehydroepiandrosterone (DHEA) 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, mainly the ovary, placenta and testis. Apart from serving as a precursor of these hormones, DHEA has been considered to have no obvious biological function. However, recent studies suggest that DHEA is involved in the prevention of diseases that frequently develop in the aged. The administration of large doses of DHEA to rodents has demonstrated a multitude of beneficial effects on the prevention of cancer, heart diseases, diabetes and obesity [1]. In vitro and animal studies have also shown that DHEA influences neuronal activity via interaction with neurotransmitter receptors and increases memory and learning performance [2,3]. Furthermore, in humans, the age-related decline in DHEA levels seems to be associated with depression, osteoporosis, autoimmune disease and the metabolic syndrome [4]. Although it is now 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.

The serum or plasma specimen is conventionally used to measure individual DHEA levels in humans [5–7]. On the contrary, 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 [8]. The use of saliva in steroid assay has another advantages; the levels of steroids in saliva generally reflect those of the unbound steroids with protein (*i.e.*, bioavailable steroids) in serum/plasma 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 [9].

Steroids have conventionally been measured in biological samples by immunoassay [10]. 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 poor due to interference from other endogenous steroids and lipids. Among the alternative methods, liquid chromatography

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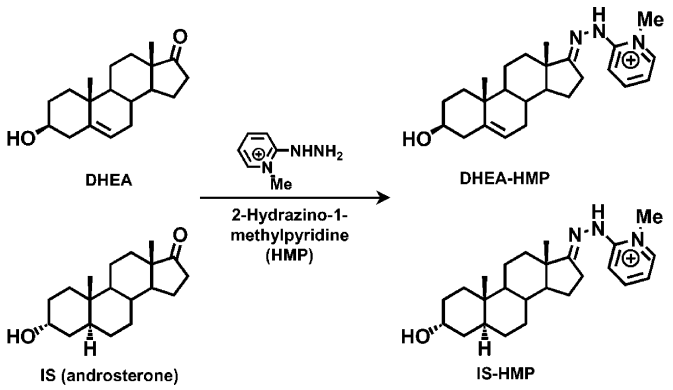

Fig. 1. Structures of DHEA, IS and their derivatives with HMP.

(LC) coupled with electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI)-mass spectrometry (MS) has been used for steroid analysis due to its specificity and versatility [11–13], but the ionization efficiencies of neutral steroids are generally low for either ESI or APCI; conventional LC–MS sometimes does not demonstrate the required sensitivity for the trace analysis of steroids. To overcome this, the chemical derivatization of steroids in LC–MS has recently been examined [14].

The best detectability with ESI-MS has been achieved in the analysis of compounds that are either ionic or can be readily ionized in solution. Based on this, we have developed a reagent having a permanently charged moiety, 2-hydrazino-1-methylpyridine (HMP), for oxosteroids and reported that the detectability of the HMP derivative of DHEA (DHEA-HMP, Fig. 1) was 1600 times superior to that of intact DHEA in the positive-ESI-MS [15]. In the present paper, we describe an LC–ESI-MS–MS method employing derivatization with HMP for the determination of trace amounts of DHEA in saliva using a small volume of sample. The application of the proposed method to clinical studies is also presented.

2. Experimental

2.1. Materials and chemicals

DHEA, androsterone [internal standard (IS)], epiandrosterone and testosterone were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). A stock solution of DHEA was 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. IS was dissolved in and diluted with ethanol to prepare a 50 ng/ml solution. HMP was synthesized in our laboratories as previously reported [15]. Strata-X cartridges (60 mg adsorbent; Phenomenex, Torrance, CA, USA) were successively washed with ethyl acetate (2 ml), 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 at 40 °C. Acetonitrile-10 mM ammonium formate (1:1, v/v) was used as the mobile phase unless otherwise indicated. The HMP derivatives of the steroids were analyzed by ESI-MS in the positive-ion mode and the conditions were as follows: declustering potential: 80 V, focusing potential: 200 V, entrance potential: 10 V, ion spray voltage: 5 kV, curtain gas (nitrogen): 45 psi, ion source gas 1 (nitrogen): 80 psi, ion source gas 2 (nitrogen): 80 psi, turbo gas temperature: 500 °C and interface heater: on. Nitrogen was used as the collision gas in the selected reaction monitoring (SRM) mode with the collision energy of 60 eV and collision cell exit potential of 10 V. The precursor and monitoring ions of the HMP derivatives were as follows: DHEA-HMP, *m/z* 394.3 and 109.2, IS-HMP, *m/z* 396.3 and 109.2.

2.3. Collection and pretreatment of saliva

Saliva was directly collected into a glass tube (without a collection device) from healthy volunteers and immediately centrifuged at 1000 × *g* (4 °C, 5 min). The supernatant was stored at −20 °C until use. The saliva (200 μl) was added to acetonitrile (500 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, which has a reversed-phase polymeric sorbent. After washing with water (2 ml) and methanol–water (7:3, v/v) (2 ml), the steroids were eluted with ethyl acetate (2 ml). After evaporation, the residue was subjected to derivatization with HMP as described below.

2.4. Derivatization reaction

To the standard or pretreated saliva sample in ethanol (30 μl), a freshly prepared solution of HMP (10 μg) in ethanol (50 μl) containing 25 μg of trifluoroacetic acid was added, and the mixture was kept at 60 °C for 1 h. After removal of the solvents, the product was dissolved in the methanol–10 mM ammonium formate (1:1, v/v, 30 μl), 10 μl of which was subjected to LC–MS–MS.

2.5. Calibration curve

Each tube containing DHEA (5, 10, 20, 50, 100 or 200 pg) and IS (500 pg) was derivatized as described above. The calibration curves were constructed by plotting the peak area ratios (DHEA/IS) versus the amounts of DHEA.

2.6. Method validation

2.6.1. Recoveries of DHEA and IS during pretreatment

The ethanolic solution of DHEA (50 pg in 10 μl; spiked sample) or ethanol (10 μl; control sample) was added to the saliva (200 μl) and the resulting samples were pretreated. DHEA (50 pg) was then added to only the control sample, and IS

(500 pg) was add to both samples. After derivatization, the samples were subjected to LC–MS–MS. The recovery of DHEA during pretreatment was calculated from the peak area ratios (DHEA/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 (200 μl) and the resulting samples were pretreated. IS (500 pg) was then added to only the control sample, and epiandrosterone (500 pg) was add to both samples; epiandrosterone was used as the external standard, because its HMP derivative (t_R 6.9 min) was completely separated from IS-HMP and behaved in the same manner as IS-HMP in ESI-MS–MS (*m/z* 396.3 → 109.2). After derivatization, the samples were subjected to LC–MS–MS. The recovery of IS during pretreatment was calculated from the peak area ratios (IS/epiandrosterone) of the spiked and control samples.

2.6.2. Matrix effect

The matrix effect was examined by comparing the slope of the calibration curve constructed as described above and those of curves prepared by adding DHEA (5, 10, 20, 50, 100 and 200 pg) to saliva (200 μl) (matrix sample). The matrix samples were prepared using five different saliva.

2.6.3. Analytical recovery

Ethanol (10 μl; unspiked sample) or the ethanolic solution of DHEA (10 or 20 pg in 10 μl; spiked sample) was added to the saliva (200 μl) (the spiked concentration of DHEA was 0, 50 and 100 pg/ml, respectively, $n = 2$). After the addition of IS (500 pg), each of the resulting samples was pretreated, derivatized and analyzed by LC–MS–MS. The analytical recovery of DHEA was defined as $F/(F_0 + A) \times 100(\%)$, where F is the concentration of DHEA in the spiked sample, F_0 is the concentration DHEA in the unspiked sample and A is the spiked concentration.

2.6.4. Assay precision

The intra-assay precision was assessed by determining two saliva samples at different concentration levels ($n = 5$ for each sample) on a day. The inter-assay precision was assessed by determining these samples over 5 days. The precision was determined as the relative standard deviation (R.S.D., %).

2.6.5. Limit of quantitation (LOQ)

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 DHEA-free saliva, in which DHEA was not detected by the proposed method, 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.*, R.S.D. and relative error <15%) and with at least five times the response compared to the blank response.

2.6.6. Freeze/thaw stability

The freeze/thaw stability of DHEA in saliva was examined by determining two saliva samples before and after 1 and 3 freeze/thaw cycles.

2.7. Clinical studies

The saliva samples were obtained from healthy male and female volunteers known not to have received hormone supplementation. 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.

2.7.1. Diurnal rhythm

The saliva samples collected from three male (22–23 years old) and three female (22–24 years old) volunteers at 9:00, 11:00, 13:00 and 15:00 h were analyzed using the proposed method.

2.7.2. Age and sex differences in salivary DHEA

The saliva samples collected from 10 male volunteers in their 20s, 10 male volunteers in their 60s and 10 female volunteers in their 20s at 15:00 h were analyzed using the proposed method. The statistical comparisons were performed using Welch test in Microsoft Excel 2000 (Redmond, WA, USA).

2.7.3. DHEA administration study

DHEA (25 mg per tablet) (Schiff, Salt Lake, UT, USA) was orally administered to two healthy male volunteers (37 and 26 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 administration and 1 and 3 days after the last administration.

3. Results and discussion

3.1. LC–ESI-MS–MS of HMP derivative

For the ESI-MS operating in the positive-ion mode, the HMP derivatives of DHEA and IS provided only their molecular cations, $[M]^+$ (Fig. 2a and b). The high selective SRM analysis coupled with a suitable pretreatment procedure may allow for the discrimination and quantification of DHEA from a biological matrix without the need for a long chromatographic separation. The product ion mass spectrum of DHEA-HMP employing $[M]^+$ as the precursor ions and a 60 eV collision energy is shown in Fig. 2c, in which the base product ion was observed at *m/z* 109.2. This product ion was assigned as $[N\text{-methylpyridine} + NH_2]^+$ formed by the cleavage of the N–N bond of the hydrazone. The IS derivative showed the same fragmentation pattern (Fig. 2d). Based on these results, the SRM mode using the $[M]^+$ (DHEA; *m/z* 394.3 and IS; *m/z* 396.3) and $[N\text{-methylpyridine} + NH_2]^+$ (DHEA and IS; *m/z* 109.2) as the precursor and monitoring ions, respectively, was employed in the following quantitative analysis.

Because the difference in the *m/z* of the precursor ions of DHEA-HMP and IS-HMP is only 2 units and the isotopic molecular ions of DHEA-HMP have some influence on the peak area of IS-HMP, the chromatographic separation of these derivatives is necessary for the accurate quantification. When a J'sphere ODS H-80 column with the mobile phase of acetonitrile-10 mM

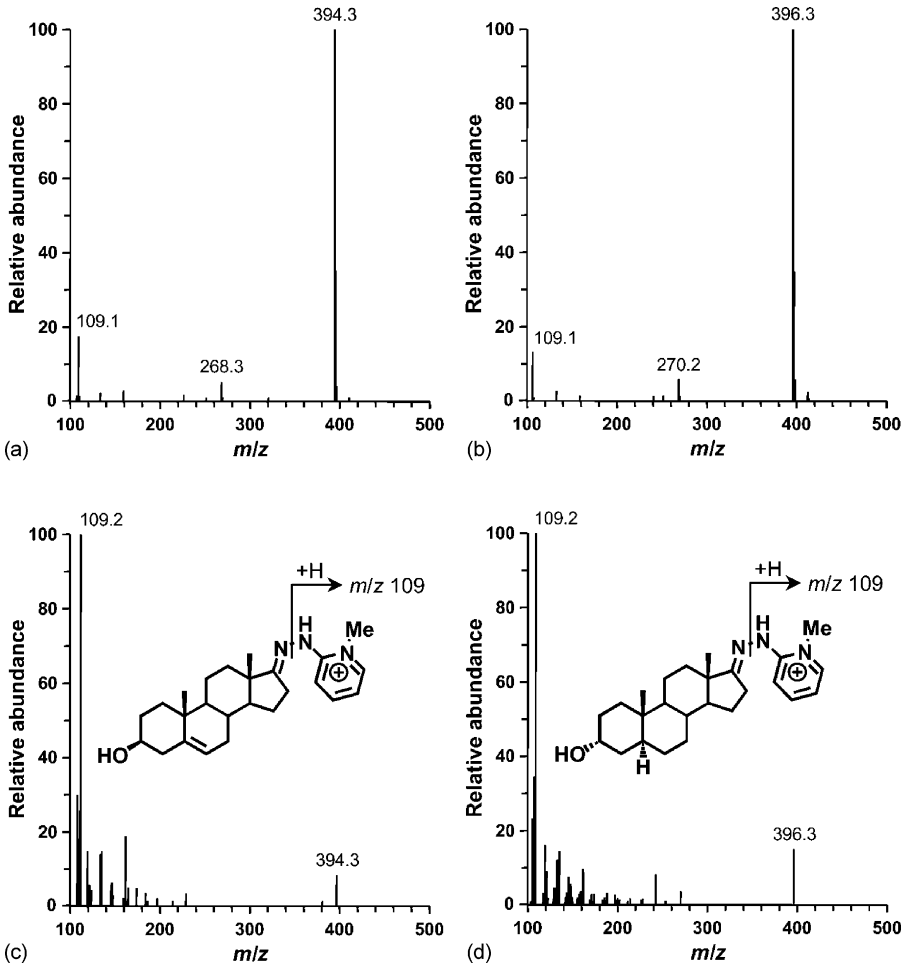

Fig. 2. ESI mass spectra of (a) DHEA-HMP and (b) IS-HMP; product ion mass spectra of (c) DHEA-HMP and (d) IS-HMP.

ammonium formate (1:1, v/v) was used, the complete chromatographic separation of the derivatives was achieved; DHEA-HMP; retention time (t_R) 5.7 min and IS-HMP: 9.5 min.

3.2. Collection and pretreatment of saliva

Some devices, such as Salivette (Sarstedt, Nümbrecht, Germany), are often used to collect the saliva. 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. Furthermore, contaminants from the device sometimes interfere with the analysis. Indeed, when Salivette was used for the collection of the saliva, a large peak, which was derived from its cotton, was observed at the elution position of the DHEA derivative in the SRM chromatogram, which caused false (overestimated) DHEA concentration. Based on this result, the saliva was directly collected into a glass tube without the use of any collection devices.

The saliva was deproteinized in acetonitrile and purified using a Strata-X cartridge. The steroid fraction was then treated with an excess of HMP. 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 DHEA and IS during the pretreatment were 85.4 ± 4.2 and 86.6 ± 0.7%, respectively. The reproducibility of the recovery rates was satisfactory and there was no significant difference between the analyte and IS.

3.3. Method validation

3.3.1. Specificity and limit of detection (LOD)

The chromatograms shown in Fig. 3a was obtained from the charcoal-treated saliva spiked with IS, in which DHEA was not detected. The charcoal-treated specimens have been often used in the validation studies of steroid analysis [16,17]. Although some endogenous components may be removed together with DHEA by the charcoal-treatment, this chromatogram is evidence that there was no interfering peak derived from the endogenous components and the derivatization reagent at the elution positions of the derivatized DHEA (5.7 min, indicated by an arrow). The HMP derivative of testosterone (t_R 6.8 and 9.5 min; *E*- and *Z*-isomers) was chromatographically well separated from DHEA-HMP, though the *m*/*z* values of their molecular cations are identical.

Typical chromatograms of the saliva samples obtained from a healthy volunteer with IS and without IS are shown in Fig. 4a and b, respectively. The peaks corresponding to the derivatized DHEA and IS were clearly observed at 5.7 and 9.5 min, respec-

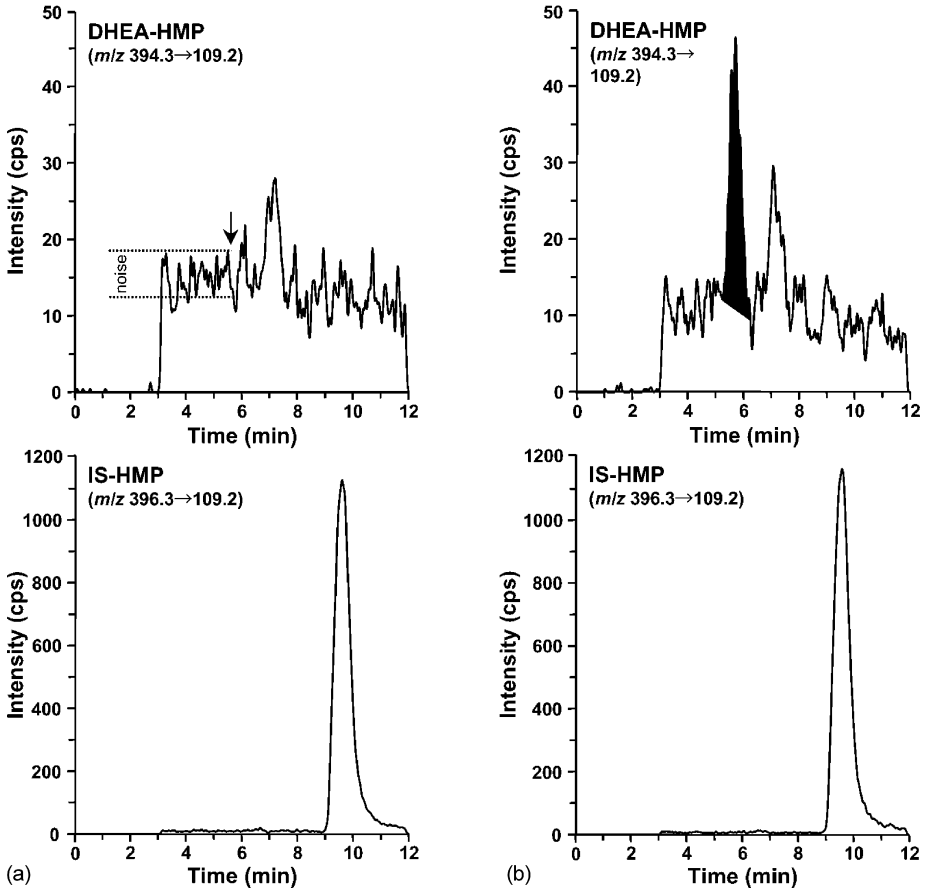

Fig. 3. Chromatograms of derivatized DHEA and IS in (a) charcoal-treated saliva spiked with IS (2.5 ng/ml) and (b) charcoal-treated saliva spiked with DHEA (25 pg/ml) and IS (2.5 ng/ml). The arrow indicates the elution position of the DHEA derivative.

tively, in Fig. 4a. As can be seen in Fig. 4b, there is no interfering peak at the elution position of the IS derivative (indicated by an arrow); androsterone was not detected in the saliva. Even if 25 mg of DHEA was administered to the volunteer, androsterone was not detected in the saliva at all. Furthermore, there is no possibility of dosing androsterone as a drug or a supplement. These data prove androsterone to be the suitable IS in the salivary DHEA assay.

When the LOD is defined as the signal equivalent to three times the noise, that of DHEA in saliva was 13 pg/ml for the 200 μl sample aliquot (chromatogram not shown).

3.3.2. Calibration curves and LOQ

The calibration curve was constructed by plotting the peak area ratios (DHEA/IS, y) versus the amounts (pg) of DHEA per tube (x) using the standard solutions. The regression line obtained from the combination of five standard curves was $y = 0.00269x + 0.00847$ with a correlation coefficient (r^2) of 0.999 within the range of 5–200 pg per tube. The R.S.D. values of the slope and intercept were 0.9 and 4.7%, respectively.

To determine the extent to which the saliva matrix affects the quantification, the slope of the above calibration curve was compared to that of the curve prepared with the matrix sample. As a result, the slope of the latter was 0.00280 ± 0.00017 (mean ± S.D., $n = 5$, R.S.D. 5.9%), which was practically identical to the slope of the curve constructed with standard solutions. This result clearly revealed that the saliva matrix did not affect the calibration curve. Based on this result and the fact that it is not always easy to prepare the DHEA-free saliva, the calibration curve was constructed using the standard solutions in the following studies. The applicability of this curve to the salivary DHEA assay was also examined in the analytical recovery test that will be discussed later.

To the charcoal-treated saliva (200 μl), DHEA (5 pg) was spiked (concentration; 25 pg/ml) and the sample was then pretreated, derivatized and subjected to LC–MS–MS. As shown in Fig. 3b, the peak was clearly observed with the signal to noise ratio (S/N) of about 6. The measured value (mean ± S.D., $n = 5$) of DHEA was 25.9 ± 2.3 pg/ml (R.S.D. 8.8%, relative error 3.6%). A sample below 25 pg/ml did not meet the criterion for LOQ described in the experimental section. Therefore, the LOQ was determined to be 25 pg/ml when 200 μl of saliva was used.

3.3.3. Assay precision and analytical recovery

The intra-assay ($n = 5$) R.S.D. values were less than 5.3% and good inter-assay ($n = 5$) R.S.D. values (less than 3.2%) were also obtained, as shown in Table 1.

The saliva to which known amounts of DHEA had been added were pretreated and analyzed in order to examine the analyti-

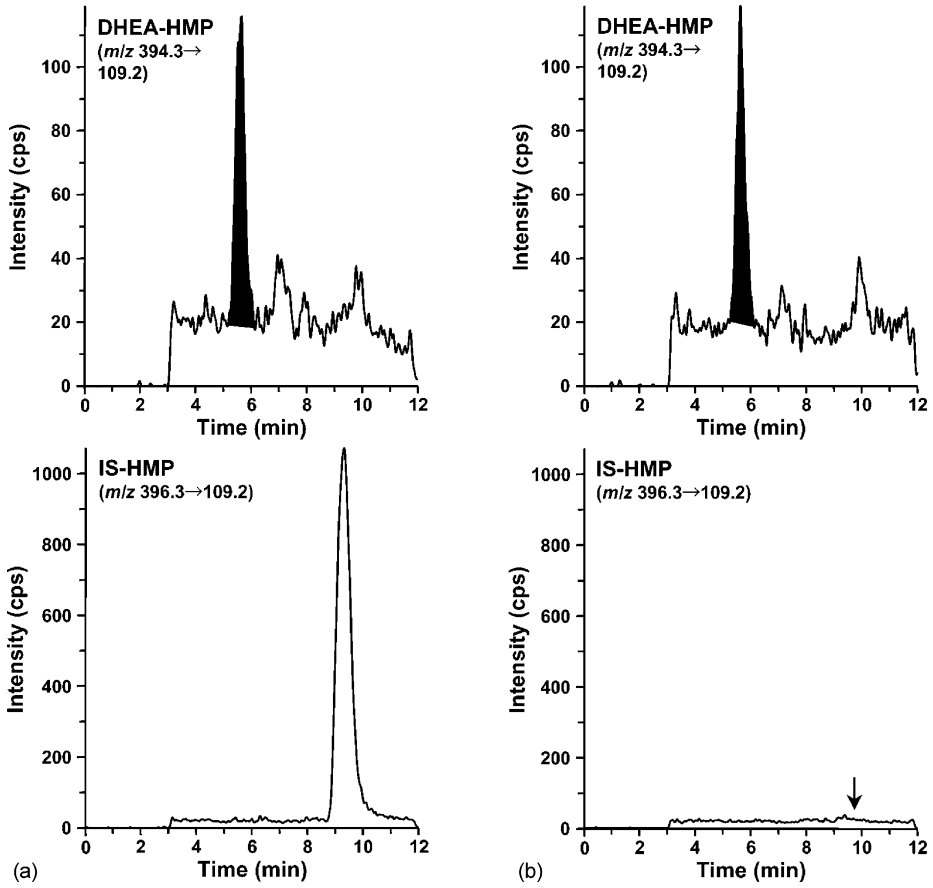

Fig. 4. Chromatograms of derivatized DHEA and IS in saliva obtained from a healthy male subject (a) with IS (2.5 ng/ml) and (b) without IS. The arrow indicates the elution position of the IS derivative. The measured concentration of DHEA was 85.1 pg/ml.

cal recovery. A satisfactory recovery rate ranging from 98.0 to 102.7% was obtained (Table 1). Although the calibration curve was constructed using the standard solutions of DHEA in the present study as mentioned above, this result demonstrates that the salivary DHEA can be accurately determined using the curve. These data indicate that the present method is highly reproducible and accurate.

3.3.4. Freeze/thaw stability

The freeze/thaw stability of DHEA in saliva was examined, which indicated that DHEA was stable up to 3 freeze/thaw cycles (Table 2). Furthermore, it was possible to store the saliva at −20 °C without loss of DHEA for at least 6 months.

3.4. Clinical studies

3.4.1. Diurnal rhythm

The diurnal rhythm of the salivary DHEA is presented in Fig. 5. Three volunteers showed that their DHEA levels were higher at 9:00 h then sharply decreased to lower values at 11:00 h with no significant change thereafter. This rhythm was similar to that reported by Hucklebridge et al. [18]. In another three volun-

Table 1
Precision and analytical recovery

	Saliva A				Saliva B			
	Measured[a] (pg/ml)	Expected[b] (pg/ml)	Precision/AR[c]		Measured (pg/ml)	Expected (pg/ml)	Precision/AR	
Intact (intra-assay)	119.9 ± 2.2	–	R.S.D.	1.9%	54.7 ± 2.9	–	R.S.D.	5.3%
Intact (inter-assay)	121.8 ± 1.4	–	R.S.D.	1.2%	54.3 ± 1.7	–	R.S.D.	3.2%
Spiked (50 pg/ml)	170.3	171.8	AR	99.1%	107.0	104.3	AR	102.6%
Spiked (100 pg/ml)	217.4	221.8	AR	98.0%	158.4	154.3	AR	102.7%

[a] Mean ± S.D. ($n = 5$) in the intra- and inter-assay variation tests and mean ($n = 2$) in the analytical recovery test.
[b] Expected values were calculated based on the values obtained in the inter-assay variation test.
[c] AR: analytical recovery (measured value/expected value × 100).

Table 2
Freeze/thaw stability of DHEA in saliva

		Freeze/thaw cycle		
		0	1	3
Saliva C	Concentration (pg/ml)	80.4	82.0	78.5
	Percentage of initial concentration	100.0	102.1	97.6
Saliva D	Concentration (pg/ml)	29.7	30.4	31.1
	Percentage of initial concentration	100.0	102.3	104.7

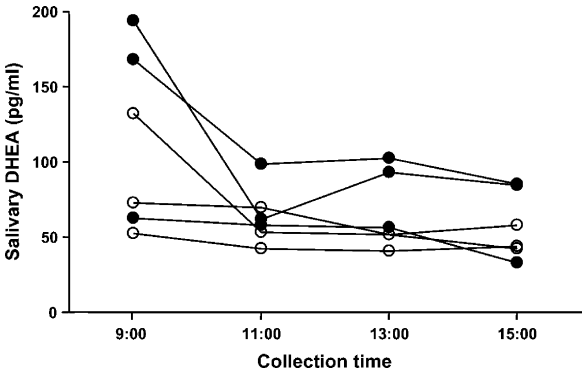

Fig. 5. Diurnal rhythm of salivary DHEA. Open symbols; male volunteers and closed symbols; female volunteers.

teers, their salivary DHEA levels in the morning were relatively low and gradually decreased. For all volunteers, the morning level was higher than the evening level. The diurnal rhythm of DHEA is caused by its primary secretagogue, the adrenocorticotropic hormone, the secretion of which increases in the morning.

3.4.2. Age and sex differences in salivary DHEA

The age change and sex difference in the salivary DHEA were examined. The saliva was collected at 15:00 h, because individual differences in the DHEA level were large in the morning (Fig. 5). The measured values were 56.6 ± 14.6 pg/ml (mean $\pm$ S.D., $n = 10$) in the male volunteers in their 20s, 32.5 ± 7.0 pg/ml in the male volunteers in their 60s and 60.1 ± 18.0 pg/ml in the female volunteers in their 20s. An age difference was observed ($P < 0.05$) as expected, but there is no significant difference between the sexes.

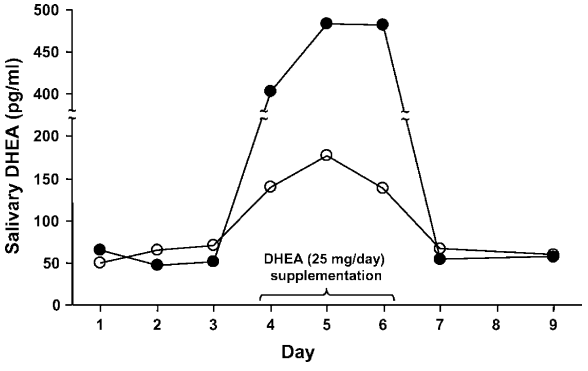

Fig. 6. Changes in salivary DHEA levels in two male volunteers after the oral administration of DHEA (25 mg).

3.4.3. DHEA administration study

The change in the salivary DHEA level after the oral administration of DHEA (25 mg) is shown in Fig. 6. The DHEA concentration immediately increased after the administration and was almost constant at the higher levels during the administration period. The concentration returned to the basal level within 1 day after the last administration.

4. Conclusion

We have demonstrated the LC–ESI-MS–MS method combined with derivatization for the determination of DHEA in human saliva. The derivatization with HMP increased the detectability of DHEA in the positive-ESI-MS, and the LOQ of the salivary DHEA was 25 pg/ml for a 200-μl sample aliquot. The method was specific, accurate and reproducible and successfully applied to clinical studies. This method was able to detect the diurnal rhythm and the age-related decline in the salivary DHEA, and also applicable for the determination of the change in the individual DHEA levels after the DHEA supplementation. This well-characterized method will prove helpful in the elucidation of the physiological functions of DHEA, because saliva can be non-invasively collected.

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References

[1] F. Svec, J.R. Porter, Proc. Soc. Exp. Biol. Med. 218 (1998) 174.
[2] M. Vallée, W. Mayo, M. Le Moal, Brain Res. Rev. 37 (2001) 301.
[3] S.H. Mellon, L.D. Griffin, Trends Endocrinol. Metab. 13 (2002) 35.
[4] W. Arlt, Best Pract. Res. Clin. Endocrinol. Metab. 18 (2004) 363.
[5] S. Boschi, R. De Iasio, P. Mesini, G.F. Bolelli, R. Sciajno, R. Pasquali, M. Capelli, Clin. Chim. Acta 231 (1994) 107.
[6] M.A. Zemaitis, P.D. Kroboth, J. Chromatogr. B 716 (1998) 19.
[7] Y. Matsuzaki, S. Yoshida, A. Honda, T. Miyazaki, N. Tanaka, A. Takizawa, Y. Fujimoto, H. Miyazaki, Steroids 69 (2004) 817.
[8] R.E. Choo, M.A. Huestis, Clin. Chem. Lab. Med. 42 (2004) 1273.
[9] P.T. Kefalides, Ann. Intern. Med. 131 (1999) 991.
[10] H.L.J. Makin, D.B. Gower, D.N. Kirk (Eds.), Steroid Analysis, Blackie, London, 1995.
[11] K. Shimada, K. Mitamura, T. Higashi, J. Chromatogr. A 935 (2001) 141.
[12] A. Marwah, P. Marwah, H. Lardy, J. Chromatogr. A 935 (2001) 279.
[13] Y.-C. Ma, H.-Y. Kim, J. Am. Soc. Mass Spectrom. 8 (1997) 1010.
[14] T. Higashi, K. Shimada, Anal. Bioanal. Chem. 378 (2004) 875.
[15] T. Higashi, A. Yamauchi, K. Shimada, J. Chromatogr. B 825 (2005) 214.
[16] M.L. Cawood, H.P. Field, C.G. Ford, S. Gillingwater, A. Kicman, D. Cowan, J.H. Barth, Clin. Chem. 51 (2005) 1472.
[17] K. Mitamura, Y. Nagaoka, K. Shimada, S. Honma, M. Namiki, E. Koh, A. Mizokami, J. Chromatogr. B 796 (2003) 121.
[18] F. Hucklebridge, T. Hussain, P. Evans, A. Clow, Phychoneuroendocrinology 30 (2005) 51.