Simultaneous determination of salivary testosterone and dehydroepiandrosterone using LC–MS/MS: Method development and evaluation of applicability for diagnosis and medication for late-onset hypogonadism☆

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ABSTRACT

Late-onset hypogonadism (LOH) is a male-specific disorder caused by the age-related decline in androgens, such as testosterone (T). A sensitive liquid chromatography–electrospray ionization-tandem mass spectrometric (LC–ESI-MS/MS) method for the simultaneous quantification of T and its precursor, dehydroepiandrosterone (DHEA), in human saliva has been developed and validated. The saliva was deprotenized with acetonitrile, purified using a Strata-X cartridge, derivatized with 2-hydrazino-1-methylpyridine, and subjected to LC–MS/MS. The recovery rates of the steroids during the pretreatment were about 90%. Quantification was based on selected reaction monitoring using characteristic transitions, and deuterated T and DHEA were used as internal standards. This method allowed the reproducible (inter- and intra-assay precisions, <2.9%) and accurate (accuracy, 98.5–101.8%) quantification of the salivary androgens using a 500-μl sample and the limits of quantification for both androgens were 10 pg/ml. As preliminary steps in the practical application of the developed method in diagnosis and medication for LOH, the diurnal rhythms, inter-day alternations and age differences in the salivary T and DHEA were examined; the method found that the salivary T and DHEA show specific diurnal rhythms, significant alternations in early morning and pronouncedly decline with age. The method also enabled the determination of the changes in the individual T and DHEA levels after the DHEA supplementation, which is expected to be a new and easy medication for LOH. Thus, the developed method has satisfactory applicability in the diagnosis and medication for LOH.

1. Introduction

Interest has arisen during the last 10 years as to the possible connection between the age-related decline in circulating androgens and symptoms of aging in men. A new aging-related syndrome, late-onset hypogonadism [LOH, also known as partial androgen deficiency in aging male (PADAM)], has been identified. LOH is defined as a clinical and biochemical syndrome associated with advancing age and is characterized by typical symptoms (i.e., decrease in intellectual activity and cognitive functions, depressed mood and irritability, sleep disturbances, decrease in muscle volume and strength, increase in visceral fat, decrease in body hair and skin alterations, decreased bone mineral density as well as decreased libido) and a deficiency in serum testosterone (T) levels [1].

In healthy adult men, 98% of circulating T is bound to serum proteins, primarily sex hormone binding globulin (SHBG) and albumin, while only 1–2% of serum T is free of bound protein [2]. Free T is available to target tissue for androgenic action, and age-related decline in T level has been seen more frequently in free T than in total T [3–5]. For these reasons, free T is currently being used to diagnose LOH. In Japan, the DPC·Free Testosterone kit (Mitsubishi Kagaku Iatron, Tokyo, Japan) is exclusively used for measuring

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serum free T; this kit is a solid phase radioimmunoassay (RIA) using an ^{125}I-labelled T analogue for the direct quantitative estimation of free T in serum and does not require equilibrium dialysis or ultrafiltration before assaying.

Alternatively, salivary T has recently been attracting attention for the diagnosis of LOH [6–8]. Saliva collection is easy, noninvasive and repeatable, and may be performed by the patient with no need for the involvement of medical personnel, if so desired. The levels of steroids in saliva generally reflect those of the free active steroids, not the protein binding forms, in serum; indeed, a significant correlation between salivary and serum free T concentrations was also described in healthy men [6–8], suggesting that the measurement of salivary T may be a useful index of serum free T.

Dehydroepiandrosterone (DHEA), the precursor of T and estrogens, has been heretofore considered to have no obvious biological function. However, recent studies suggest that the age-related decline in serum DHEA level seems to be associated with depression, osteoporosis and the metabolic syndrome [9], which are common with symptoms observed in LOH. Although it is now unclear that these symptoms are caused by the decrease in DHEA itself or its downstream conversion products (T and estrogens), DHEA is now often designated as an anti-aging hormone and taken as a food supplement in the United States. DHEA can serve as a senility indicator and might be useful in diagnosing LOH. Furthermore, the studies using RIA proved that the salivary DHEA positively correlates with serum DHEA [10,11]. Based on this information, it is expected that the simultaneous measurement of T and DHEA in saliva proves more helpful in the assessment of androgen status in men and consequently in the diagnosis and medication for LOH than the measurement of T alone.

Steroids are conventionally measured in biological samples by immunoassay. Although this technique will doubtless continue to be the method of choice for routine use in the clinical field, especially in a large population-based test, it does not have a simultaneous multi-analyte quantification capability. Several immunoassays, which were primarily developed for the serum/plasma steroids, have been applied to the measurement of the salivary steroids, but for these assays, care is required to address standardization issues as well as the differing matrices of serum/plasma and saliva [12]. For example, Rey et al. [13] failed to reveal a detectable T concentration in saliva using direct RIA, which had been primarily developed for the serum free T. Among the alternative methods, liquid chromatography (LC) coupled with electrospray ionization (ESI)-tandem mass spectrometry (MS/MS) has been used for steroid analysis due to its high specificity and versatility. Recently, an LC–MS/MS method for the determination of salivary T was reported [8]. Because T has the ESI-active structure (3-oxo-4-ene-steroid) [14], it can be sensitively analyzed in LC–ESI-MS/MS. However, it is challenging to develop an LC–MS/MS assay that enables the quantification of the salivary DHEA (3β-hydroxy-5-ene-steroid) together with salivary T, because DHEA has an extremely low response for ESI-MS. As a procedure to overcome this problem, we have reported a derivatization using 2-hydrazino-1-methylpyridine (HMP) [15], by which the detection response of DHEA in ESI-MS can be enhanced sufficiently for the analysis of salivary DHEA [16].

Based on this background information, the primary objective of this study is to develop and validate an LC–ESI-MS/MS method for the simultaneous determination of T and DHEA in saliva. The secondary objective is to prove the applicability of the method in the assessment of androgen status and consequently in the diagnosis and medication of LOH.

2. Experimental

2.1. Materials and chemicals

T and DHEA were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Stock solutions of T and DHEA 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. [19,19,19-^{2}H$_3$]-T (D$_3$-T) [17] and [2,2,4,6-^{2}H$_4$]-DHEA (D$_4$-DHEA) [18] were donated from Teikoku Hormone Medical Research Center (Kawasaki, Japan) and used as internal standards (ISs). ISs were dissolved in and diluted with ethanol to prepare 5.0 ng/ml solutions. Other steroids were purchased from Steraloids (Newport, RI, USA). HMP was synthesized in our laboratories as previously reported [15]. Strata-X cartridges (60 mg adsorbent; Phenomenex, Torrance, CA, USA) were successively preconditioned 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-20AD chromatograph (Kyoto, Japan). A YMC-Pack Pro C18 RS column (5 μm, 150 mm × 2.0 mm i.d.; YMC, Kyoto) was used at a flow rate of 0.2 ml/min at 40 °C. Methanol–10 mM ammonium formate (14:5, v/v) was used as the mobile phase. 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 a collision energy of 60 eV and a collision cell exit potential of 10 V. The precursor and product ions of the HMP derivatives for SRM were as follows: T-HMP, m/z 394.1 and 108.2; DHEA-HMP, m/z 394.1 and 109.2; D$_3$-T-HMP, m/z 397.3 and 108.2; and D$_4$-DHEA, m/z 398.1 and 109.0. The transitions of m/z 394.1 → 109.2 and m/z 398.1 → 109.0 were monitored for 0–5.5 min after injection, and the transitions of m/z 394.1 → 108.2 and m/z 397.3 → 108.2 were monitored for 5.5–7.5 min.

2.3. Collection and pretreatment of saliva

Saliva (*ca.* 1 ml) was directly collected into a collecting tube (without a collection device) from male volunteers who did not take hormone supplements or drugs that influence androgen biosynthesis and metabolism, and stored below −15 °C (in household refrigerator or laboratory freezer) until use. The volunteers took no food and beverage within 30 min prior to the sample collection. The volunteers also did not brush their teeth within 1 h prior to sample collection to avoid blood contamination. Written informed consent was obtained from all the volunteers. The experimental procedures were conducted in accordance with the ethical standards of the Helsinki Declaration and approved by the Ethics Committee of Kanazawa University.

After thawing, the saliva sample was centrifuged at 1000 × *g* (4 °C, 5 min) and the supernatant (500 μl) was added to acetonitrile (1 ml) containing ISs (50 pg each), vortex-mixed for 30 s and centrifuged at 1000 × *g* (4 °C, 5 min). The supernatant was diluted with water (1.5 ml), and the sample was passed through a Strata-X cartridge. 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 dissolved in ethanol (30 μl) and added

with a freshly prepared solution of HMP (10 μg) in ethanol (50 μl) containing 25 μg of trifluoroacetic acid. The mixture was kept at 60 °C for 1 h. After removal of the solvent, the products were dissolved in the methanol–10 mM ammonium formate (1:1, v/v, 30 μl), 10 μl of which was subjected to LC–MS/MS.

2.4. Calibration curves

The saliva (5 ml) obtained from a healthy volunteer was stirred overnight with activated charcoal (0.5 g, Norit EXW, Nacalai Tesque) and then centrifuged at $1000 \times g$ (4 °C, 20 min). The supernatant, in which T and DHEA were not detected by the proposed method, was used as the steroid-free saliva to construct the calibration curves. The steroid-free saliva (500 μl) was spiked with T and DHEA (5.0, 10, 20, 50, 100 and 200 pg each; corresponding to 10, 20, 40, 100, 200 and 400 pg/ml each) and the ISs (50 pg each), which was then pretreated, derivatized and subjected to LC–MS/MS. The calibration curves were constructed by plotting the peak area ratio of T to D_3-T or DHEA to D_4-DHEA (y) versus the concentration of T or DHEA (x, pg/ml).

2.5. Method validation

2.5.1. Recoveries of T, DHEA and ISs during pretreatment

The recoveries of T and DHEA were calculated from the peak area ratios of T/D_3-T and $DHEA/D_4$-DHEA, respectively, in samples A and C as described below. The recoveries of ISs were calculated from the peak area ratios of D_3-T/T and D_4-DHEA/DHEA in samples B and C as described below.

Sample A: The charcoal-treated (steroid-free) saliva (500 μl) spiked with T and DHEA (20 pg each) was pretreated. After the addition of ISs (50 pg each) to this pretreated saliva, the resulting sample was derivatized and subjected to LC–MS/MS.

Sample B: The charcoal-treated saliva (500 μl) spiked with ISs (50 pg each) was pretreated. After the addition of T and DHEA (20 pg each) to this pretreated saliva, the resulting sample was derivatized and subjected to LC–MS/MS.

Sample C: The charcoal-treated saliva (500 μl) was pretreated. After the addition of T, DHEA (20 pg each) and ISs (50 pg each) to this pretreated saliva, the resulting sample was derivatized and subjected to LC–MS/MS.

2.5.2. Matrix effect

The matrix effect (ion suppression or ion enhancement of analytes by saliva components) was examined by comparison of peak areas of the derivatized T and DHEA in matrix sample with those in standard sample described below.

Standard sample: T and DHEA (20 pg each) was derivatized and subjected to LC–MS/MS.

Matrix sample: The charcoal-treated (steroid-free) saliva (500 μl) obtained from five different volunteers was pretreated. After the addition of T and DHEA (20 pg each) to this pretreated saliva, the resulting sample was derivatized and subjected to LC–MS/MS.

2.5.3. 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 relative standard deviation (R.S.D., %).

2.5.4. Analytical recovery (assay accuracy)

Ethanol (20 μl; unspiked sample) or the ethanolic solution of T and DHEA (5.0 or 15 pg each in 20 μl; spiked sample) was added to the saliva (500 μl) (the spiked concentrations of steroids were 0, 10 and 30 pg/ml, respectively, $n = 2$). After the addition of ISs (50 pg each), each of the resulting samples was pretreated, derivatized and analyzed by LC–MS/MS. The analytical recoveries of steroids were defined as $F/(F_0 + A) \times 100$ (%), where F is the concentration of T or DHEA in the spiked sample, F_0 is the concentration of T or DHEA in the unspiked sample and A is the spiked concentration.

2.5.5. Limits of quantification (LOQs)

The steroid-free saliva was used to determine the LOQs. 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 5 times the response compared to the blank response.

2.5.6. Stabilities of T and DHEA in saliva

The stabilities of T and DHEA in the saliva at room temperature (*ca.* 20 °C) and in ice up to 3 h after collection were examined. The freeze/thaw stabilities of T and DHEA in the saliva were also examined before and after 1 and 3 freeze/thaw cycles. The saliva samples were collected from six volunteers, portions of them were left to stand at room temperature and the rest were iced or frozen and then stored at −20 °C. The measured values of the samples analyzed immediately after the collection were taken as 100%.

2.6. Clinical studies

2.6.1. Diurnal rhythms of salivary T and DHEA

Four volunteers (24–39 years old) got up no later than 6:45 and their saliva was collected at 7:00, 8:00, 9:00, 11:00, 13:00, 15:00, 17:00 and 19:00. The salivary T and DHEA were determined using the proposed method.

2.6.2. Inter-day alternations of salivary T and DHEA

Four volunteers (22–39 years old) got up no later than 6:45 and their saliva was collected at 7:00 and 11:00 for 3 consecutive days, although their waking times (5:30–6:45) varied from day to day. The salivary T and DHEA were determined using the proposed method, and their inter-day alternations were evaluated as the R.S.D.

2.6.3. Age differences in salivary T and DHEA

The saliva samples were collected from 114 volunteers (21–89 years old) at 9:00–14:00 and divided into 4 groups according to their ages; Group 1 [20s (mean ± standard deviation (S.D.), 23.8 ± 2.8 years), $n = 24$], Group 2 [30s and 40s (39.1 ± 5.2 years), $n = 28$], Group 3 [50s and 60s (59.4 ± 5.7 years), $n = 32$] and Group 4 [70s and 80s (77.3 ± 4.7 years), $n = 30$]. The salivary T and DHEA were determined using the proposed method. The statistical analysis was performed using Pearson's correlation coefficient test and the Kruskal–Wallis test followed by the Steel–Dwass test. A P value of <0.05 was considered statistically significant.

2.6.4. DHEA administration study

Four volunteers (24–39 years old) got up no later than 6:45 and took a DHEA tablet (25 mg, Schiff, Salt Lake, UT, USA) at 9:00. Their saliva was collected immediately before the administration, at 9:30, 10:00, 11:00, 12:00, 13:00, 15:00, 17:00, 19:00, 21:00, 7:00 on the following morning (the waking times; no later than 6:45) and 9:00 on the following morning. The salivary T and DHEA were determined using the proposed method.

3. Results and discussion

3.1. Optimization of LC–ESI-MS/MS conditions

For the ESI-MS operating in the positive-ion mode, the HMP derivatives of T, DHEA and ISs provided intense molecular cations, $[M]^+$, as the base peak ions. During the collisional activation with a 60 eV collision energy, the N–N bond of the hydrazone was cleaved, and characteristic and intense product ions at m/z 108 (T and D_3-T, [N-methylpyridine + NH]$^+$) or m/z 109 (DHEA and D_4-DHEA, [N-methylpyridine + NH$_2$]$^+$) were produced from the respective $[M]^+$ (Fig. 1). We could not provide a definite reason why the HMP derivatives of T and DHEA produced different product ions. By the collisional activation, the HMP derivatives of 3- and 20-oxosteroids produce the [N-methylpyridine + MH]$^+$ (m/z 108), while those of 17-oxosteroids always produce the [N-methylpyridine + NH$_2$]$^+$ (m/z 109). Based on these results, the transitions described in Section 2.2 were monitored for the determination of the salivary T and DHEA.

Due to the formation of the E- and Z-isomers during the derivatization of the oxosteroids with HMP [15], the derivatives sometimes produce twin peaks on their chromatograms. In principle, higher sensitivity could be realized with the convergence of twin peaks into a single peak. We examined various LC conditions, but the twin peaks of the derivatized T could not be converged into a single peak. On the contrary, when a YMC-Pack Pro C18 RS column with the mobile phase of methanol–10 mM ammonium acetate (14:5, v/v) was used, the derivatized DHEA gave a single peak. Under these LC conditions, the peaks of T-HMP [twin peaks, retention times (t_Rs)

Fig. 1. Chemical structures and product ion mass spectra of (a) T-HMP and (b) DHEA-HMP. The ESI-MS/MS conditions are described in Section 2.2.

5.2 and 6.6 min] and DHEA-HMP (t_R 3.8 min) were completely separated with a relatively short chromatographic run time (8 min per one analysis). Based on the above results, the LC–MS/MS conditions described in Section 2.2 was used in the following studies. For the quantification of T, the slower-eluting isomer of T-HMP (t_R 6.6 min) was used, because it was the major isomer and chromatographically well separated from endogenous interfering substances.

3.2. Collection and pretreatment of saliva

The stimulated saliva collection (i.e., collection after chewing gum and other commercially available devices for a few minutes or citric acid stimulation) sometimes influences, mostly decreases, the analyte concentrations in the saliva [19]. Although the salivary T concentration was reported to be independent of saliva flow rate [7], the unstimulated saliva collection, which has less effect on analytical results than the stimulated saliva collection, was employed in this study. 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 [20]. 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 a false DHEA concentration. Based on these issues, the saliva was directly collected into a glass tube without the use of any collection devices (this procedure is sometimes called passive drool). About 1 ml of saliva could be collected from all the volunteers within 5 min.

The saliva was deproteinized in acetonitrile and purified using a Strata-X cartridge. The steroid fraction was then treated with excess HMP. Thus, our method employed only a one-step solid-phase extraction for the purification of the saliva samples, and the recovery rates [mean ± S.D. from five different saliva samples] of T, DHEA, D_3-T and D_4-DHEA during the pretreatment were 89.8 ± 1.7, 89.9 ± 2.3, 90.0 ± 1.2 and 90.7 ± 1.5%, respectively. The reproducibility of the recovery rates was satisfactory, and there was no significant difference between the analytes and ISs.

3.3. Effect of derivatization on assay sensitivity

The effect of the derivatization on the assay sensitivity was examined as follows. Into the steroid-free saliva (500 µl), T and DHEA (5.0 pg each) were spiked (concentrations, 10 pg/ml each). This sample was pretreated and then derivatized with HMP. As shown in Fig. 2b, the peaks of the derivatized T and DHEA were observed with signal-to-noise ratios (S/N) of over 5. On the other hand, when the sample was analyzed without derivatization, equal S/N values were obtained at the concentrations of 40 pg/ml of T and 20 ng/ml of DHEA [these samples were prepared by spiking T (20 pg) and DHEA (10 ng) into the same saliva (500 µl)] (chromatograms not shown). This result proves that the HMP-derivatization increases the assay sensitivity by 4 times for T and 2000 times for DHEA. This sensitivity enhancement significantly contributed to reducing the sample volume, which was an important point in developing a method applicable in the clinical field.

3.4. Method validation

3.4.1. Assay specificity

The chromatograms shown in Fig. 2a were obtained from the charcoal-treated saliva (steroid-free saliva). Although some endogenous low molecular weight compounds may be removed
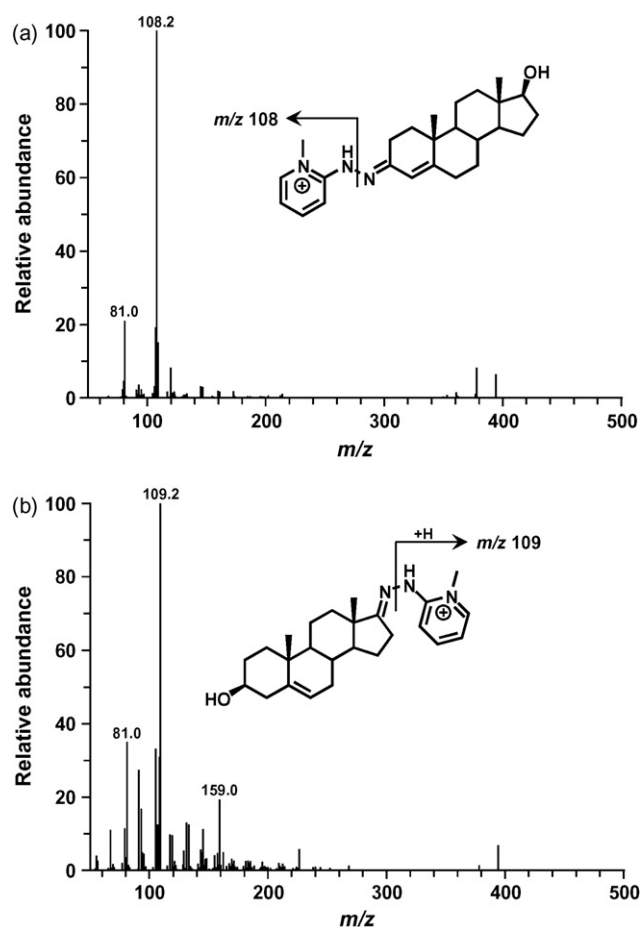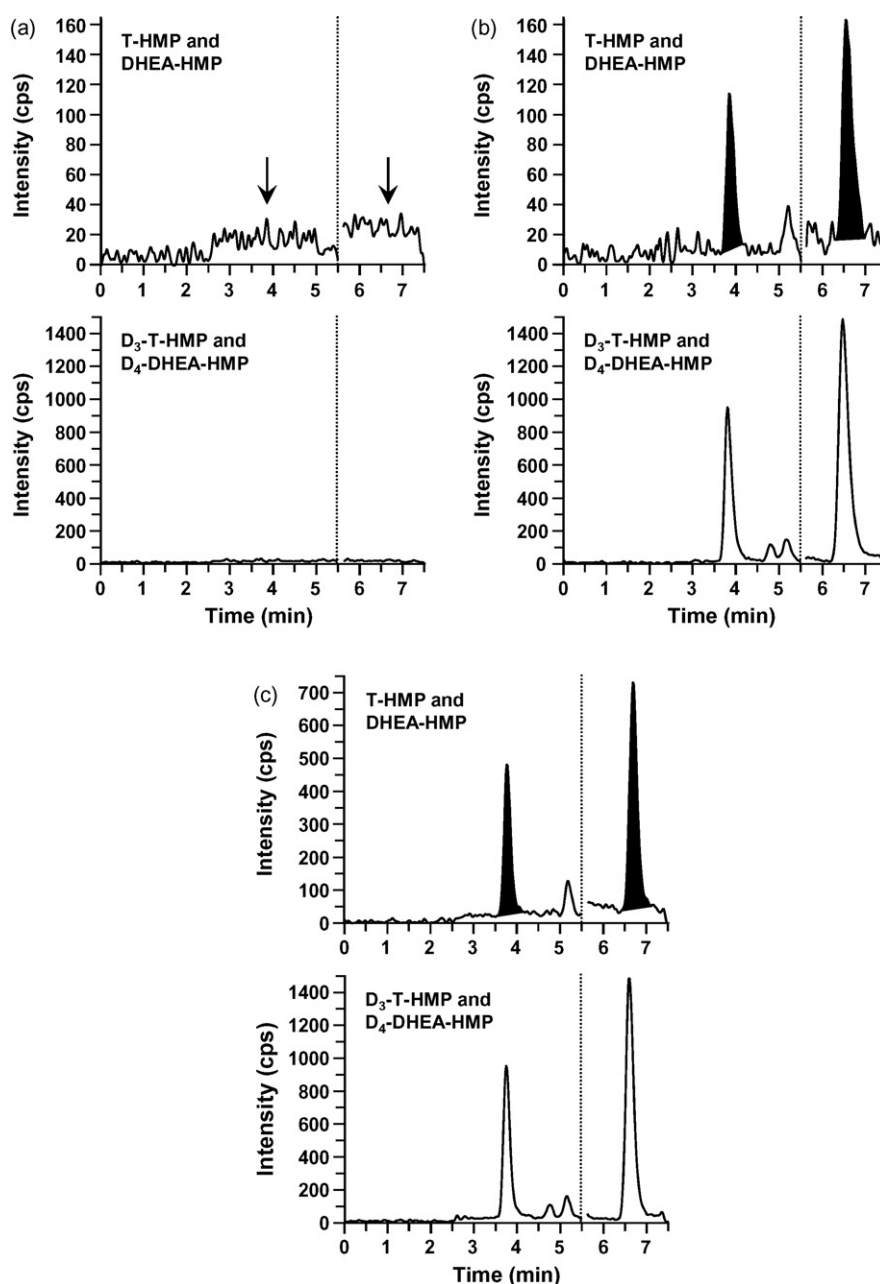
Fig. 2. Chromatograms of derivatized T, DHEA and ISs in (a) charcoal-treated saliva (without spiking IS), (b) charcoal-treated saliva spiked with T, DHEA (10 pg/ml each) and ISs (100 pg/ml each) and (c) saliva obtained from a subject (29 years old) with ISs (100 pg/ml each). The arrow indicates the elution position of the T (t_R 6.6 min) and DHEA (t_R 3.8 min) derivatives. The measured concentrations of T and DHEA in the sample of the chromatograms (c) were 44.7 and 51.3 pg/ml, respectively. The LC–MS/MS conditions are described in Section 2.2.

together with steroids by the charcoal-treatment, these chromatograms prove that no interfering peak was derived from the endogenous components and the derivatization reagent at the elution positions of the derivatized T, DHEA and ISs. The chromatograms obtained from a volunteer are shown in Fig. 2c, in which the peaks corresponding to the derivatized T (t_R 6.6 min) and DHEA (t_R 3.8 min) were clearly detected with satisfactory shapes.

In order to examine the assay specificity, the ESI-MS and -MS/MS spectra of the HMP derivatives of endogenous androgens were recorded under the LC–MS(/MS) conditions described in Section 2.2. The base peaks, most abundant product ions and t_Rs of the respective steroids were as follows; androstenedione [m/z 497.4 $[M-1]^+$, m/z 108.0 and t_R 5.4 and 6.5 min (twin peaks)], 5α-dihydrotestosterone [m/z 396.2 $[M]^+$, m/z 108.0 and t_R 6.5

and 6.8 min (twin peaks)], androsterone (m/z 396.2 $[M]^+$, m/z 109.0 and t_R 5.7 min), epiandrosterone (m/z 396.2 $[M]^+$, m/z 108.9 and t_R 4.1 min), etiocholanolone (m/z 396.2 $[M]^+$, m/z 109.0 and t_R 5.7 min) and epietiocholanolone (m/z 396.2 $[M]^+$, m/z 109.0 and t_R 4.4 min). All the derivatized androgens were not at all detected by the selected transitions for T-HMP and DHEA-HMP, when 200 pg of them was injected. However, the HMP derivatives of 5α-dihydrotestosterone and epiandrosterone were closely eluted with T-HMP and DHEA-HMP, respectively, and isotropic peaks of their $[M]^+$ overlapped the $[M]^+$ of D_3-T-HMP and D_4-DHEA-HMP. Therefore, we carefully examined the occurrence of 5α-dihydrotestosterone and epiandrosterone in the saliva. In five saliva samples obtained from different subjects, these two steroids were not detected at all. Furthermore, even if 25 mg of DHEA was

Table 1
Precision in determination of salivary T and DHEA.

Variation	Saliva A		Saliva B	
	T	DHEA	T	DHEA
Intra-assay				
Mean ± S.D. (pg/ml, $n=5$)	60.6 ± 0.84	64.2 ± 0.76	31.1 ± 0.89	35.4 ± 0.88
R.S.D. (%)	1.4	1.2	2.9	2.5
Inter-assay				
Mean ± S.D. (pg/ml, $n=5$)	60.3 ± 0.94	64.6 ± 0.88	30.8 ± 0.90	35.2 ± 0.82
R.S.D. (%)	1.6	1.4	2.9	2.3

Table 3
Stabilities of T and DHEA in saliva.

Treatment	Percentage of initial concentration[a]	
	T	DHEA
Room temperature, 1 h	96.9 ± 1.9	98.5 ± 0.8
Room temperature, 3 h	90.3 ± 3.7	95.7 ± 1.0
In ice, 1 h	99.3 ± 0.5	98.8 ± 1.1
In ice, 3 h	97.3 ± 0.8	98.1 ± 1.1
Freeze/thaw, 1 cycle	99.7 ± 1.1	99.7 ± 0.7
Freeze/thaw, 3 cycles	97.8 ± 0.8	98.1 ± 0.4

[a] The measured values immediately after the collection were taken as 100%. The results represent mean ± S.D. of six different saliva.

administered to the volunteers (see Sections 2.6.4 and 3.5.4), 5α-dihydrotestosterone and epiandrosterone were also not detected in their saliva at all ($n=4$). These results show that the salivary concentrations of 5α-dihydrotestosterone and epiandrosterone were below our detection limits (5 pg/ml for both the steroids), even if the saliva contains these steroids. Based on these data, we concluded that the endogenous androgens do not interfere with the quantifications of T and DHEA.

Matrix effect (ion suppression or enhancement of the analytes by saliva components which co-elute with the analytes from LC column) may impair assay accuracy and precision. For this reason, matrix effect was evaluated by the method described in Section 2.5.2. The peak areas of derivatized T and DHEA in the matrix sample were 84.8 ± 3.9 and 85.5 ± 4.0% ($n=5$) of those in the standard sample, respectively. Thus, saliva components slightly suppressed the ionization of the analytes, but this method uses deuterated forms of the analytes as ISs and therefore, the matrix effect on assay accuracy and precision would be negligible.

3.4.2. Calibration curves and LOQs

The calibration curves were constructed using the steroid-free saliva prepared from five different volunteers' saliva. The regression lines obtained from the combination of five calibration curves were $y = 0.01013x + 0.00502$ with a correlation coefficient (r) of 0.999 within the range of 10–400 pg/ml for T and $y = 0.01023x + 0.00469$ with an r of 0.999 within the range of 10–400 pg/ml for DHEA. The R.S.D.s of the slope and intercept ($n=5$) were 0.9 and 5.5%, respectively, for T and 1.5 and 8.1%, respectively, for DHEA; these results demonstrate that the individual difference in the saliva matrix does not affect the detection responses of the derivatized steroids. The R.S.D.s and relative errors of the back-calculated concentration at 10 pg/ml for T were 3.4 and 2.8%, respectively. Those for DHEA were 2.0 and 4.9%, respectively. The peaks corresponding to the respective steroids at 10 pg/ml were clearly observed with S/N values of more than 5 as shown Fig. 2b. Based on these results, the LOQs of both T and DHEA were determined to be 10 pg/ml, when 500 μl of saliva was used.

3.4.3. Assay precision and analytical recovery (assay accuracy)

The assay precision was examined using two healthy volunteers' saliva, which contained different concentrations of T and DHEA

(Table 1). The intra-assay R.S.D.s for both the steroids were less than 2.9%, and good inter-assay R.S.D.s (less than 2.9%) were also obtained. The assay accuracy was evaluated as the analytical recovery. As shown in Table 2, satisfactory recovery rates ranging from 98.5 to 101.8% were obtained. These data indicate that the present method is highly reproducible and accurate.

3.4.4. Stabilities of T and DHEA in saliva

Table 3 shows that salivary steroids gradually decrease when the sample is allowed to stand at room temperature; about 10% of T was lost after 3 h. However, the decrease was significantly prevented by cooling the saliva in ice, and the losses of the steroids in the sample left in ice for 3 h were less than 3%. These data demonstrate that it is necessary to refrigerate or freeze saliva in transit in order to obtain correct androgen levels, if subjects collect saliva at their home and carry the samples to the hospital or clinical laboratory; in this study, all the samples were kept freezing in transit. The salivary T and DHEA were stable up to 3 freeze/thaw cycles (Table 3).

3.5. Clinical studies

As preliminary steps in the practical application of the developed method in diagnosis and medication for LOH, some clinical studies were performed to obtain fundamental data.

3.5.1. Diurnal rhythms and inter-day alternations of salivary T and DHEA

In order to utilize the measured values of salivary androgens for the diagnosis and medication for LOH, attention should be paid not only to the saliva collection method but also to the collection time. For this reason, the diurnal rhythms and inter-day alternations of salivary T and DHEA were examined.

The diurnal rhythms of the salivary T and DHEA are presented in Fig. 3. Four volunteers showed that their T and DHEA levels were higher after awakening (7:00) and then sharply decreased to lower values at 9:00 with a small change thereafter. Thus, the salivary T and DHEA show clear diurnal rhythms just as the serum T and DHEA do. Individual differences in the T and DHEA levels were also larger in the early morning.
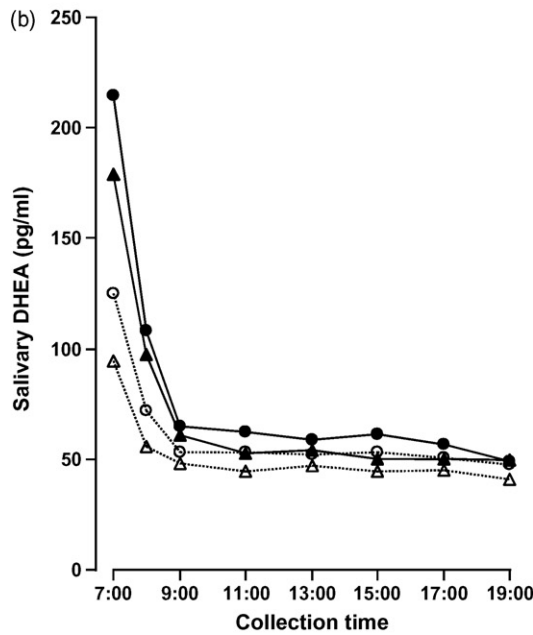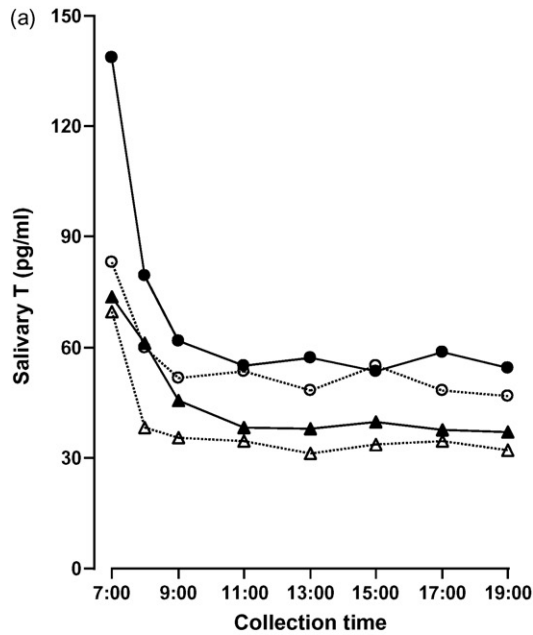
Table 2
Analytical recoveries (assay accuracy) in determination of salivary T and DHEA.

Added[a]	Saliva A				Saliva B			
	T		DHEA		T		DHEA	
	Measured[a,b]	Recovery (%)	Measured[a,b]	Recovery (%)	Measured[a,b]	Recovery (%)	Measured[a,b]	Recovery (%)
0	60.3	–	64.6	–	30.8	–	35.2	–
10	70.0	99.6	74.0	99.2	40.8	100.0	46.0	101.8
30	89.7	99.3	94.6	100.0	59.9	98.5	65.4	100.3

[a] Unit: pg/ml.
[b] Mean of duplicate assays.

Fig. 3. Diurnal rhythms of (a) salivary T and (b) DHEA in four volunteers.
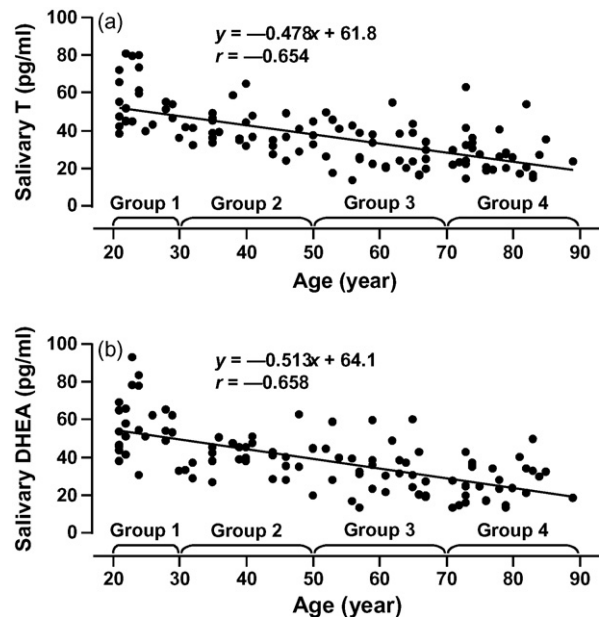

Fig. 4. Age-related changes in (a) salivary T and (b) DHEA ($n = 114$).

greatly influenced by the waking time; all the volunteers got up no later than 6:45, but their waking times considerably varied from day to day. The control of the waking times of subjects may minimize the inter-day alternations in the early morning androgen levels, but it is not practicable to control the waking times for all the subjects who undergo the salivary androgen test. Based on the above results, the saliva should be collected after 9:00 when the salivary T and DHEA are used in the diagnosis for androgen-dependent diseases; basal steroid levels would provide more accurate information.

3.5.2. Age differences in salivary T and DHEA

The age differences in the salivary T and DHEA were examined. The saliva was collected at 9:00–14:00, because individual differences and inter-day alternations in the steroid levels were large in the early morning as mentioned above. Fig. 4a and b shows the relationship of salivary T and DHEA, respectively, with age. The T levels decreased substantially in Group 2 (mean ± S.D., 39.2 ± 9.0 pg/ml, $P < 0.05$), when compared with Group 1 (55.0 ± 13.6 pg/ml), and decreased further in Group 3 (30.8 ± 10.9 pg/ml, $P < 0.05$, when compared with Group 2). There was no statistical difference between Groups 3 and 4 (27.6 ± 10.9 pg/ml) in the T level. The mean T level in Group 4 was almost half of that in Group 1, and 19 of 30 subjects in Group 4 had lower levels than the mean − 2S.D. (27.8 pg/ml) of the T level of Group 1. The result of linear regression analysis also showed that the salivary T level significantly declined with age (Fig. 4a, $P < 0.001$ in Pearson's correlation coefficient test). A further large-scale study (over 1000 subjects) will give the standard diagnostic criteria for LOH (i.e., standard value for indication of hormone replacement therapy) in the saliva-based test.
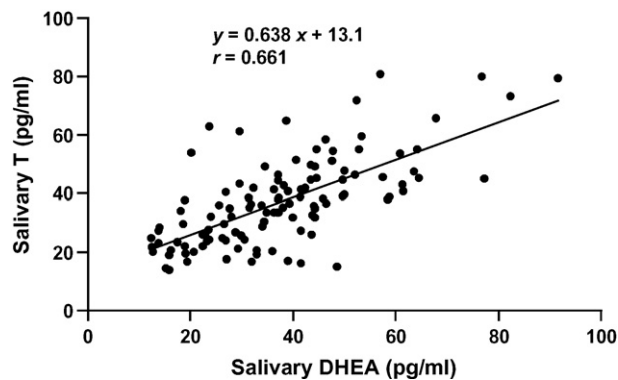
Significant inter-day alternations were observed in the early morning (7:00) salivary T (R.S.D., 6.5–20.5%) and DHEA levels (R.S.D., 7.1–36.6%), while the alternations of the levels at 11:00 were negligible (R.S.D.s were less than 3.9 and 3.7% for T and DHEA, respectively) (Table 4). The levels in the early morning seem to be

Table 4
Inter-day alternations in salivary T and DHEA.

	Concentration (pg/ml)[a] and R.S.D. (%) at 7:00		Concentration (pg/ml)[a] and R.S.D. (%) at 11:00	
	T	DHEA	T	DHEA
Subject C	59.9 ± 12.3 and 20.5	83.8 ± 30.7 and 36.6	33.5 ± 1.3 and 3.9	46.1 ± 1.7 and 3.7
Subject D	109.1 ± 7.1 and 6.5	93.8 ± 6.7 and 7.1	50.5 ± 1.2 and 2.4	49.0 ± 1.3 and 2.7
Subject E	130.9 ± 22.4 and 17.1	110.6 ± 13.0 and 11.8	78.2 ± 1.3 and 1.7	55.6 ± 1.1 and 2.0
Subject F	77.0 ± 14.1 and 18.3	80.4 ± 14.3 and 17.8	53.2 ± 1.0 and 1.9	53.0 ± 1.0 and 1.9

[a] Mean ± S.D. ($n = 3$).

Fig. 5. Correlation between salivary T and DHEA levels ($n = 114$).

Although no statistical differences were observed between Groups 2 (39.5 ± 8.0 pg/ml) and 3 (33.0 ± 12.7 pg/ml) and between Groups 3 and 4 (25.3 ± 9.5 pg/ml), the salivary DHEA also significantly decreased with age from the level of Group 1 (57.0 ± 14.9 pg/ml) (Fig. 4b); $P < 0.05$ in Group 1 versus Groups 2, 3 and 4, and Group 2 versus Group 4. Thus, our saliva assay demonstrated that both the T and DHEA levels significantly decreased with age. These observations were in accordance with previous studies for salivary T [6] and DHEA [11].

3.5.3. Correlation between salivary T and DHEA

A Pearson's correlation (r, P) was calculated for the comparison of the salivary T and DHEA levels ($n = 114$). Although the T level was correlated with the DHEA level (Fig. 5, $r = 0.681$, $P < 0.001$), this was not sufficient to calculate one steroid level from another steroid level. Because T is produced from DHEA via androstene-dione or androst-5-ene-3β,17β-diol, the T levels in tissues and fluids are generally influenced by the amount of DHEA secreted from the adrenals. However, the two-step metabolism by the action of 3β-hydroxysteroid dehydrogenase (HSD) and 17β-HSD is required for the conversion of DHEA into T. Therefore, the individual difference in the activities of these enzymes causes a significant alternation in the T/DHEA ratio. For these reasons, the correlation between the salivary T and DHEA is not extremely high.

3.5.4. DHEA administration study

Biweekly-to-monthly intramuscular injections of testosterone enanthate are widely used for androgen replacement therapy in Japan [21], because it is economical and generally well tolerated. On the contrary, DHEA is also taken orally as a convenient androgen supplement in the United States. In this study, four healthy volunteers took DHEA (25 mg) at 9:00, and the time course of the salivary T and DHEA concentrations were monitored (Fig. 6).

The DHEA concentration started to increase at 2 h after the administration and reached a maximum concentration at 3 or 4 h after the administration (Fig. 6b). The concentration returned to the basal level within 8 h and elevated again the next morning due to the diurnal rhythm. The T concentration also reached a maximum concentration at 3 (one volunteer) or 4 h (three volunteers) after the administration and then returned to the basal level within 8 h (Fig. 6a). Thus, the time courses of the salivary T and DHEA concentrations were very similar. If the increase in the T level by DHEA supplementation is also observed in LOH patients, the oral DHEA supplementation would be a novel method in the medication for LOH, because it is much easier than the intramuscular injections of testosterone enanthate. At this time, there is insufficient evidence for the use of DHEA supplementation in the medication for LOH, and
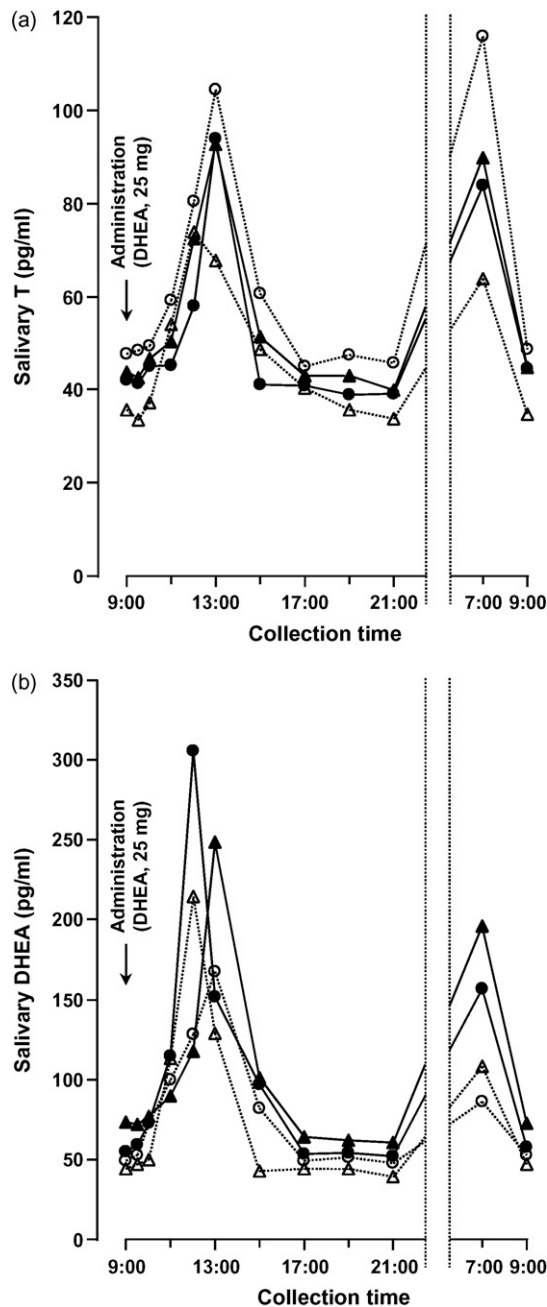

Fig. 6. Changes in (a) salivary T and (b) DHEA levels in four volunteers after the oral administration of DHEA (25 mg).

an effort should be made to accumulate data for the development of the new medication. Our LC–MS/MS method enables the simultaneous determination of T and DHEA and therefore is expected to be useful in the development of the LOH medication using DHEA.

4. Conclusion

We have demonstrated the LC–ESI-MS/MS method for the simultaneous determination of T and DHEA in human saliva. The method was specific, accurate and reproducible and successfully applied to clinical studies. This method was able to detect the diurnal rhythms and the age-related declines in the salivary T and DHEA and was also applicable to the determination of the changes in the

individual T and DHEA levels after DHEA supplementation. This well-characterized method will prove helpful in the diagnosis and medication for LOH, because salivary T and DHEA are noninvasive and reliable substitutes for serum androgens. The method is also expected to be a useful tool in the development of a new medication for LOH and other androgen deficiency using DHEA supplementation. However, at this time, the present method is not applicable to the saliva from patients with gingivitis, because such a sample is contaminated by considerable blood and the salivary steroid levels are artificially elevated in the patients. To expand the clinical utility of the present method, a method quantifying blood leakage into the saliva should be validated. Such studies are now in progress in our laboratories.

Acknowledgments

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