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Liquid chromatography–tandem mass spectrometric method for determination of salivary 17α-hydroxyprogesterone: A noninvasive tool for evaluating efficacy of hormone replacement therapy in congenital adrenal hyperplasia

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ABSTRACT

A sensitive liquid chromatography–electrospray ionization-tandem mass spectrometric (LC–ESI-MS–MS) method for the quantification of 17α-hydroxyprogesterone (17OHP) in human saliva has been developed and validated. The saliva was deproteinized with acetonitrile, purified using a Strata-X cartridge, derivatized with a highly proton-affinitive reagent, 2-hydrazinopyridine, and subjected to LC–MS–MS. Quantification was based on the selected reaction monitoring, and deuterated 17OHP was used as the internal standard. This method allowed the reproducible and accurate quantification of the salivary 17OHP using a 200-μl sample, and the limit of quantitation was 5.0 pg/ml. The developed method was applied to clinical studies. A linear relationship was found to be positive ($r^2 = 0.975$) between the blood 17OHP level and the salivary 17OHP level measured using the proposed method. The result from the salivary 17OHP measurement in patients with congenital adrenal hyperplasia demonstrated that the proposed method is very useful for monitoring of the therapeutic efficacy during hormone replacement therapy.

1. Introduction

Congenital adrenal hyperplasia (CAH), a disorder caused by an inherited deficiency of the enzyme, 21-hydroxylase, is the most common inborn error of the adrenal steroid pathways. The resulting hormone imbalances with decreased glucocorticoids and mineralocorticoids and increased 17α-hydroxyprogesterone (17OHP) and androgens can lead to life-threatening salt-wasting crises during the newborn period. Hormone replacement therapy (HRT) using a glucocorticoid, hydrocortisone (HC), and a synthetic mineralocorticoid, such as fludrocortisone, the basic therapeutic method for CAH, provides a substantial reduction in morbidity and mortality.

During the initial stage of HRT for CAH, the monitoring of the blood 17OHP is indispensable for determining the dose of HC. When the blood HC concentration increases by HRT, the 17OHP concentration decreases by the negative feedback mechanism of the hypothalamus–pituitary–adrenal axis. Furthermore, the blood 17OHP is a good index during follow-up of the CAH patients under HRT. However, the blood 17OHP monitoring is accompanied with significant hardships; a patient undergoes pain whenever their blood is collected, and a newborn patient and his/her parent sometimes require hospitalization for the repeated blood collections.

Saliva has recently been attracting attention as a new tool in clinical examinations and therapeutic drug monitoring due to its easy and noninvasive nature of collection [1,2]. The determinations of steroids, hormones and drugs in the saliva offer the advantage of repeated sampling, when blood collection is either undesirable or difficult. If the salivary 17OHP monitoring can be an alternative to the blood 17OHP monitoring, there is a highly beneficial effect for the patients and their parents. However, a major disadvantage in the use of saliva is the low 17OHP concentration, and only a few immunoassays, which were primarily developed for the serum 17OHP, have been applied to the measurement of the salivary 17OHP [3,4]. For these assays, care is required to address standardization issues as well as the differing matrices of serum and saliva [5]. On the other hand, liquid chromatography–mass spectrometry (LC–MS) has been recognized to be an alternative method for the determination of 17OHP; many successful applications of LC–MS to the determination of 17OHP in the serum/plasma [6–8] or dried blood spot [9,10] have been reported. However, to the best of our knowledge, only a report describes the development of LC–MS method for the salivary 17OHP quantification [6], but does

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not give the results of measurements for the saliva from the CAH patients.

Based on this background information, our objective was to develop an LC–tandem MS (MS–MS) method for the determination of the salivary 17OHP, which has a low invasiveness and less burden on patients. The method employs the derivatization with 2-hydrazinopyridine (HP) [11], which is effective for increasing the assay sensitivity. An application of the proposed method to a clinical study, i.e., the analysis of the change in the salivary 17OHP after the oral administration of HC, is also presented.

2. Experimental

2.1. Materials and chemicals

17OHP was purchased from Sigma (St. Louis, MO, USA). 11-Deoxycorticosterone and 16α-hydroxyprogesterone were obtained from Nacalai Tesque (Kyoto, Japan) and Steraloids (Nweport, RI, USA), respectively. A stock solution of 17OHP was prepared as a 100 μg/ml solution in ethanol. Subsequent dilutions were carried out with ethanol to prepare 0.1, 0.2, 0.5, 1, 2 and 5 ng/ml solutions. [2,2,6,6,21,21,21-$^2H_7$]-17OHP (internal standard, IS) was synthesized in our laboratories according to the known method [12,13], and dissolved in and diluted with ethanol to prepare a 5 ng/ml solution. HP was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). The 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-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 the flow rate of 0.2 ml/min at 40 °C. Acetonitrile–methanol-10 mM ammonium formate (5:3:1, v/v/v) was used as the mobile phase. The HP 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 50 eV and collision cell exit potential of 10 V. The precursor and product ions of the HP derivatives were as follows: 17OHP-HP, m/z 513.2 $[M+H]^+$ and 364.1, IS-HP, m/z 520.1 $[M+H]^+$ and 368.1. The LC–MS–MS conditions of the intact 17OHP were as follows: mobile phase: methanol-10 mM ammonium formate (7:3, v/v), declustering potential: 30 V, focusing potential: 380 V, collision energy: 35 eV, while the other parameters were the same as those of the HP derivative. The precursor and product ions of the intact 17OHP were m/z 331.3 $[M+H]^+$ and 109.0 (A-ring fragment ion), respectively.

2.3. Collection and pretreatment of saliva

The saliva was directly collected into a glass tube (without a collection device) unless otherwise indicated. The healthy volunteers and CAH patients except for a newborn took no food and beverage within 30 min prior to the sample collection. They also did not brush their teeth within 1 h prior to the sample collection to avoid any blood contamination. Saliva from the newborn patient was collected using a disposable plastic pipet. The saliva was centrifuged at 1000 × g (4 °C, 5 min) and the supernatant was stored at −20 °C until use. Informed consent was obtained from all the subjects or their parents.

The saliva (200 μl) was added to acetonitrile (500 μl) containing IS (50 pg), vortex-mixed for 30 s and then 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 methanol–water (1:1, v/v) (2 ml), the steroids were eluted with ethyl acetate (2 ml). After evaporation, the residue was subjected to derivatization with HP as described in Section 2.5. When the salivary 17OHP concentration was over 250 pg/ml, 50 or 100 μl of saliva was diluted with water (150 or 100 μl) and then pretreated as already described.

2.4. Calibration curve

The saliva (15 ml) was stirred overnight with activated charcoal (1.5 g, Norit EXW, Nacalai Tesque) and then centrifuged at 1000 × g (4 °C, 20 min). The supernatant, in which 17OHP was not detected by the proposed method, was used as the 17OHP-free saliva to construct the calibration curve. The 17OHP-free saliva (200 μl) was spiked with 17OHP (1.0, 2.0, 5.0, 10, 20 and 50 pg; corresponding to 5.0, 10, 25, 50, 100 and 250 pg/ml) and the IS (50 pg), which was then pretreated, derivatized and subjected to LC–MS–MS. The calibration curve was constructed by plotting the peak area ratio of 17OHP to IS (y) versus the concentration of 17OHP (x, pg/ml).

2.5. Derivatization reaction

To the samples (standard steroid, the calibration sample or test sample) in ethanol (30 μl), a freshly prepared solution of HP (10 μg) in ethanol (50 μl) containing 25 μg of trifluoroacetic acid was added, and this mixture was then subjected to an ultrasonic treatment in a CS-20 water bath (Shibata Scientific Technology, Tokyo; oscillation frequency, 46 kHz) at ambient temperature (ca. 20 °C) for 15 min. After removal of the solvents, the product was dissolved in methanol-10 mM ammonium formate (1:1, v/v, 30 μl), 10 μl of which was subjected to LC–MS–MS.

2.6. Method validation

2.6.1. Recoveries of 17OHP and IS during pretreatment

The 17OHP-free saliva was used to determine the recoveries of 17OHP and IS during the pretreatment. The recovery of 17OHP was calculated from the peak area ratio (17OHP/IS) in samples A and B as described below. The recovery of IS was calculated from the peak area ratio (IS/17OHP) in samples C and D as described below.

Sample A: The ethanolic solution of 17OHP (5 pg in 10 μl) was added to the 17OHP-free saliva (200 μl) and the resulting sample was pretreated. After the addition of IS (50 pg), the sample was derivatized and subjected to LC–MS–MS.

Sample B: 17OHP (5 pg) and IS (50 pg) were added to the pretreated 17OHP-free saliva (200 μl), derivatized and subjected to LC–MS–MS.

Sample C: The ethanolic solution of IS (50 pg in 10 μl) was added to the 17OHP-free saliva (200 μl) and the resulting sample was pretreated. After the addition of 17OHP (50 pg), the sample was derivatized and subjected to LC–MS–MS.

Sample D: 17OHP (50 pg) and IS (50 pg) were added to the 17OHP-free saliva (200 μl) that had been pretreated, derivatized and subjected to LC–MS–MS.

2.6.2. Assay precision and accuracy

Ethanol (10 μl; unspiked sample) or the ethanolic solution of 17OHP (2 or 20 pg in 10 μl; spiked sample) was added to the saliva (200 μl) obtained from a healthy adult male volunteer. Each of

the resulting samples was pretreated, derivatized and analyzed by LC–MS–MS. The intra-assay precision was assessed by determining these samples ($n = 5$ 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., %). The percent accuracy (analytical recovery) was defined as $F/(F_0 + A) \times 100$ (%), where F is the concentration of 17OHP in the spiked sample, F_0 is the concentration of 17OHP in the unspiked sample and A is the spiked concentration.

2.6.3. Limit of quantitation (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 5 times the response compared to the blank response.

2.6.4. Effect of sample volume on measured value

The salivary 17OHP concentrations obtained from the analysis using 200 μl of the samples were compared with those from the analysis using 50 or 100 μl of the samples.

2.6.5. Stability of 17OHP in saliva

The stability of 17OHP in the saliva at room temperature (ca. 25 °C) up to 3 h after collection was examined. The freeze/thaw stability of 17OHP in the saliva was also examined before and after 1 and 3 freeze/thaw cycles. The saliva samples were collected from 2 volunteers and portions of them were left to stand at room temperature and the rest were frozen and stored at −20 °C. The measured values of the samples analyzed immediately after the collection were taken as 100%.

2.7. Clinical studies

2.7.1. Correlation between salivary and blood 17OHP concentrations

The saliva samples collected from a CAH patient were analyzed using the proposed method. The blood was also collected at the same time as the saliva collection and the serum was then separated. The serum 17OHP concentration was determined by radioimmunoassay (DPC·17α-OH-progesterone kit, Mitsubishi Kagaku Iatron, Tokyo) at Mitsubishi Chemical Medience (Tokyo). Ten saliva and blood samples were collected from a male patient (9 years old) during his hospital treatment with sufficient time between them.

The statistical analysis was performed using Peason's correlation coefficient test.

2.7.2. Monitoring of salivary 17OHP at the dose-setting of HC

The saliva samples collected from a newborn patient, who is in hospital for determining the dose of HC, were analyzed using the proposed method. The date and time of the HC administration and saliva collection are summarized in Table 1. The HC dose is also described in Table 1. The blood was collected from his heel at the same time when saliva was collected and filter-paper forms (No. 545 filter-paper, Advantec Toyo, Tokyo) were prepared in the usual way. The blood 17OHP level was determined by enzyme-linked immunosorbent assay (D-ELISA 17OHP kit, Eiken Chemical, Tokyo) after the extraction of 17OHP from the blood spot with diethyl ether at the Tokyo Health Service Association (Tokyo).

2.7.3. Monitoring of salivary 17OHP in CAH patients under HRT

The saliva samples collected from the CAH patients under HRT were analyzed using the proposed method. The saliva was collected immediately before and 2 h after the administration of HC. All the patients also took a synthetic mineralocorticoid, fludrocortisone acetate (Florinef®), (0.04–0.1 mg/day) as a substitution for aldosterone.

Table 1
Date and hour of the HC administration and saliva/blood collection for a newborn patient

Date	Time	Dose of HC	Saliva/blood collection
November 11	08:00	8 mg	No
	16:00	8 mg	Yes
	18:00	–	Yes
November 12	00:00	8 mg	Yes
	02:00	–	Yes
	08:00	8 mg	Yes
	10:00	–	Yes
November 20	08:00	6 mg	No
	10:00	–	Yes
	16:00	6 mg	Yes
	18:00	–	Yes
November 21	00:00	6 mg	Yes
	02:00	–	Yes
	08:00	6 mg	Yes
November 27	08:00	4 mg	No
	10:30	–	Yes
	16:00	4 mg	Yes
	18:00	–	Yes
	22:00	4 mg	Yes
November 28	00:00	–	Yes
	08:00	4 mg	Yes

2.7.4. Determination of 17OHP in serum or dried blood spot using immunoassay

The DPC·17α-OH-progesterone kit and D-ELISA 17OHP kit were used for the measurement of 17OHP in serum and dried blood spot, respectively. These kits were well validated and widely used in Japan for the samples from children and newborns. The antibodies used in these kits were prepared using the haptenic derivatives conjugated with carrier proteins at the C7 position of 17OHP. The cross-reactivities of these antibodies for 17α-hydroxypregnenolone (17OHPreg) and its sulfate (17OHPreg-S), which are considered to be the major causes for the false elevated 17OHP value in the immunoassay-based newborn screening for CAH [14], are little as to be neglected; cross-reactivity, 17OHPreg, 1.5% and 17OHPreg-S, 0.4% for DPC·17α-OH-progesterone kit and 17OHPreg, 1.0% and 17OHPreg-S, 0.9% for D-ELISA 17OHP kit.

3. Results and discussion

3.1. LC–ESI-MS–MS of HP derivatives

For the ESI-MS operating in the positive-ion mode, the HP derivatives of 17OHP and IS provided intense protonated molecules $[M+H]^+$, as the base peak ions (Fig. 1a and b). The product ion mass spectra of the derivatives employing their protonated molecules as the precursor ions and a 50 eV collision energy are shown in Fig. 1c and d, respectively, in which the intense product ions were observed at m/z 364.1 ($[M+H-149]^+$) and 368.1 ($[M+H-153]^+$), respectively. These product ions could not be fully assigned, but it was inferred that they were probably formed by elimination of the side chain fragment (the HP moiety with C20 and C21) and CH_3 based on the difference in the m/z values between 17OHP-HP and IS-HP. The product ions were specific ions of the HP derivatives and had satisfactory intensities. Based on these results, the transitions of m/z 513.4 → 364.1 (17OHP-HP) and m/z 520.4 → 368.1 (IS-HP) were monitored for the determination of the salivary 17OHP.

Due to the formation of the *E*- and *Z*-isomers during the derivatization of the 3-oxosteroids with HP [11], the derivatives

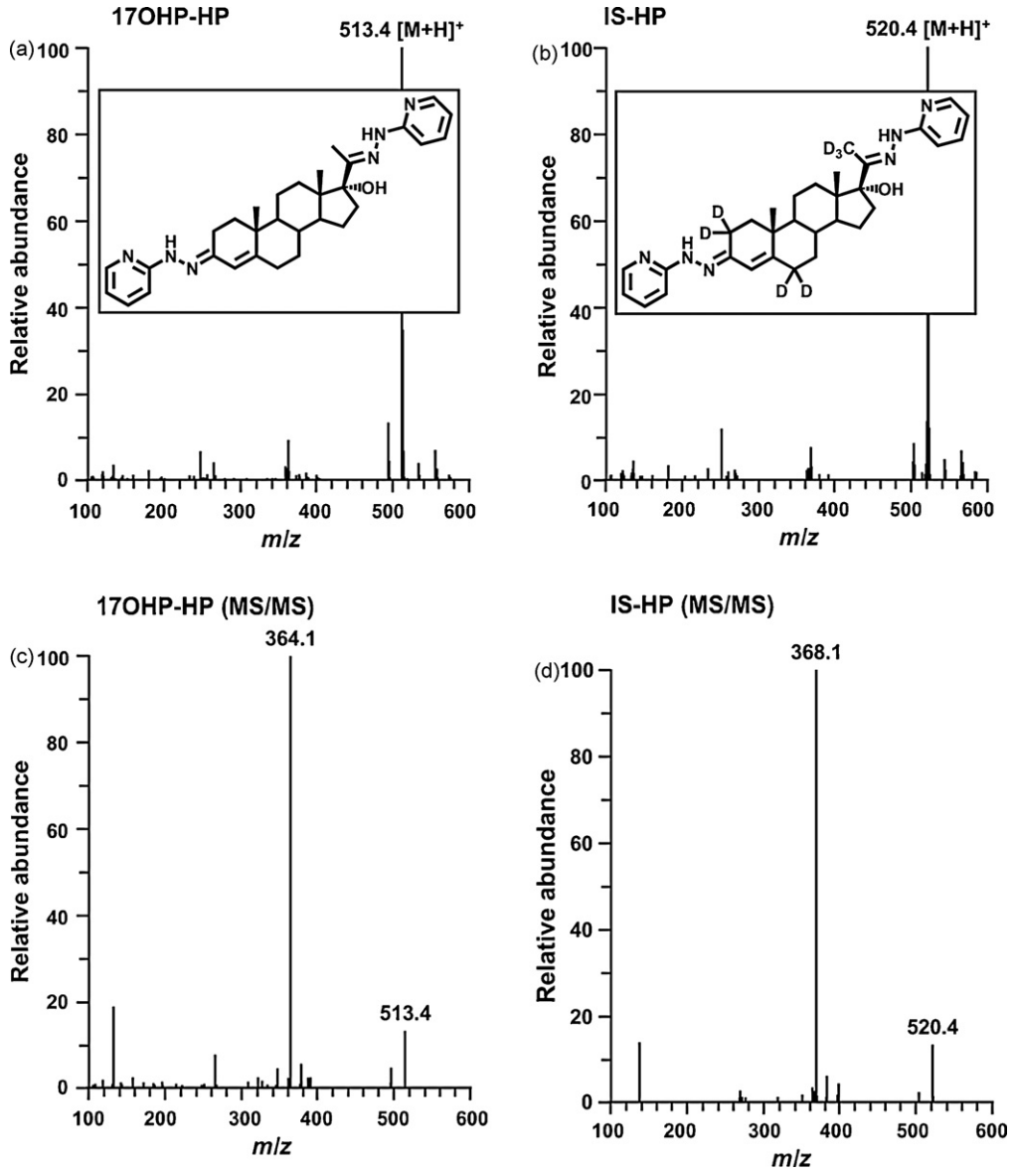

Fig. 1. ESI mass spectra of (a) 17OHP-HP and (b) IS-HP; product ion mass spectra of (c) 17OHP-HP and (d) IS-HP.

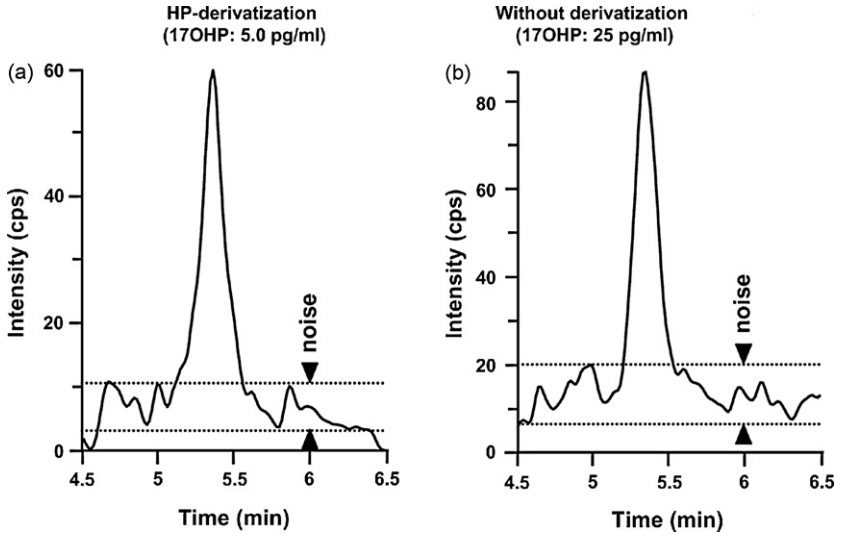

Fig. 2. Chromatograms of 17OHP in charcoal-treated saliva: (a) the sample spiked with 17OHP (5.0 pg/ml) was analyzed after the HP-derivatization and (b) the sample spiked with 17OHP (25 pg/ml) was analyzed without derivatization.

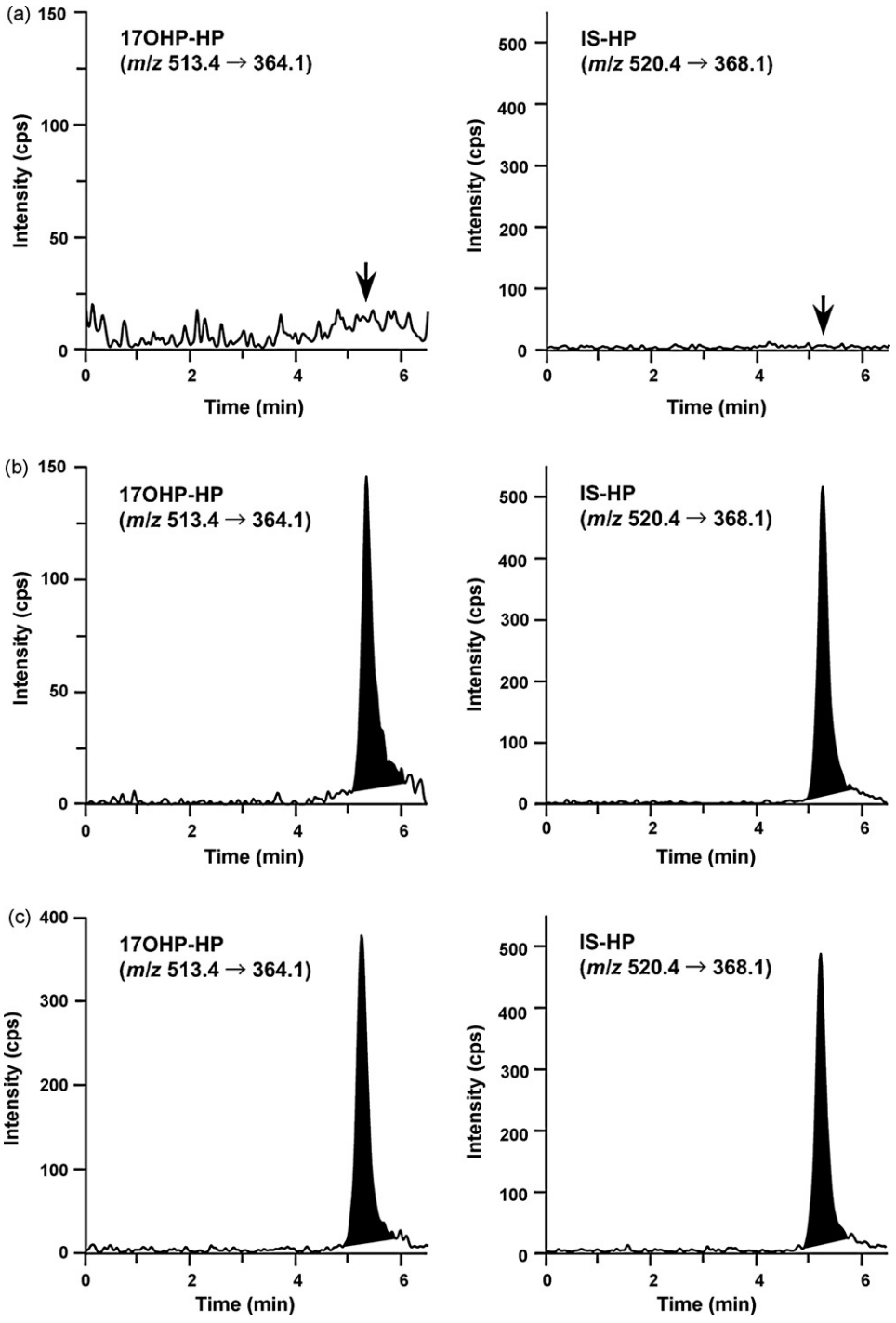

Fig. 3. Chromatograms of derivatized 17OHP and IS in saliva: (a) the charcoal-treated saliva without IS (the arrows indicate the elution positions of the derivatized 17OHP and IS), (b) the saliva obtained from a healthy male subject with IS (the measured concentration of 17OHP was 22.9 pg/ml) and (c) the saliva obtained from a CAH patient with IS (the measured concentration of 17OHP was 58.2 pg/ml).

sometimes produce twin peaks on their chromatograms. However, when a YMC-Pack Pro C18 RS column with the mobile phase of acetonitrile–methanol-10 mM ammonium acetate (5:3:1, v/v/v) was used, the derivatized 17OHP and IS gave single peaks at 5.4 and 5.3 min, respectively.

3.2. Collection and pretreatment of saliva

Some devices, such as Salivette (Sarstedt, Nümbrecht, Germany), are often used to collect the saliva. However, no collection device was used in this study for the following reasons. (1) 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 [15]. (2) Contaminants from the device sometimes interfere with the analysis [16]; indeed, when Salivette was used for the collection of the saliva, the noise peaks increased in the SRM chromatograms (data not shown). (3) No collection device is currently available for the collection of saliva from a newborn.

The saliva was deproteinized in acetonitrile and purified using a Strata-X cartridge. The steroid fraction was then treated with an

excess of HP. 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 5 different saliva samples] of 17OHP and IS during the pretreatment were 89.9 ± 1.7 and 88.1 ± 1.8%, respectively. The reproducibility of the recovery rates was satisfactory and there was no significant difference between the analyte and IS.

3.3. Effect of derivatization on assay sensitivity

The effect of the derivatization on the assay sensitivity was examined by the following two tests. (1) When 17OHP in 200 μl of the saliva obtained from a healthy volunteer was analyzed as its HP derivative, a peak was clearly observed with a signal to noise ratio (S/N) of more than 8 (the 17OHP concentration of this sample was 8.5 pg/ml). On the contrary, without derivatization, 800 μl of the same saliva was required to obtain the equal S/N value. (2) To the 17OHP-free saliva (200 μl), 17OHP (1.0 pg) was spiked (concentration, 5.0 pg/ml). This sample was pretreated and then derivatized with HP. As shown in Fig. 2a, the peak of the derivatized 17OHP was observed with an S/N of 6. On the other hand, when the sample was analyzed without derivatization, an equal S/N value was at least obtained at the concentration of 25 pg/ml [this sample was prepared by spiking 17OHP (5.0 pg) to the same saliva (200 μl)] (Fig. 2b). The results obtained from the above two tests prove that the HP-derivatization increases the assay sensitivity by over 4 times. This sensitivity enhancement significantly contributed to reducing the sample volume, which was an important point in developing a method applicable for newborn and child patients.

3.4. Method validation

3.4.1. Specificity

The chromatograms shown in Fig. 3a were obtained from the charcoal-treated saliva in which 17OHP was not detected. Although some endogenous components may be removed together with 17OHP 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 17OHP and IS (5.4 and 5.3 min, respectively, indicated by arrows). The HP derivatives of 16α-hydroxyprogesterone (t_R 3.5 min) and 11-deoxycorticosterone (t_R 8.4 min) were chromatographically well separated from 17OHP-HP, though the m/z values of their protonated molecules are identical. The HP derivatives of other endogenous steroids [17α-hydroxypregnenolone, 16α-hydroxypregnenolone, pregnenolone, progesterone, 11-deoxycortisol, 21-deoxycortisol, cortisol, cortisone, androstenedione, testosterone and dehydroepiandrosterone] do not have a molecular mass that would de detected by the selected transition.

Typical chromatograms of the saliva sample obtained from a healthy volunteer and a CAH patient are shown in Fig. 3b and c, respectively. The peaks corresponding to the derivatized 17OHP and IS were clearly observed at 5.4 and 5.3 min, respectively, and their shapes were satisfactory.

3.4.2. Calibration curve and LOQ

The regression line obtained from the combination of 5 standard curves was $y = 0.0302x + 0.00231$ with a correlation coefficient (r) of 0.999 within the range of 5.0–250 pg/ml. The R.S.D. values of the slope and intercept were 1.8 and 5.7%, respectively. The R.S.D. value and relative error (RE) of the back-calculated concentration at the minimum point (5.0 pg/ml) were 3.0% and 1.2%, respectively. The peak of the derivatized 17OHP at this concentration was observed with an S/N of more than 6 as described in Section 3.3 (Fig. 2a).

Table 2
Precision and accuracy

	Measured[a] (pg/ml)	Expected[b] (pg/ml)	Precision (R.S.D., %)	Accuracy[c] (%)
Intra-assay				
Intact	11.8 ± 0.4	–	3.4	–
Spiked (10 pg/ml)	22.1 ± 0.8	21.8	3.6	101.4
Spiked (100 pg/ml)	111.1 ± 2.5	111.8	2.3	99.4
Inter-assay				
Intact	11.7 ± 0.3	–	2.6	–
Spiked (10 pg/ml)	22.2 ± 0.7	21.7	3.2	102.3
Spiked (100 pg/ml)	111.5 ± 1.3	111.7	1.2	99.8

[a] Mean ± S.D. ($n = 5$).
[b] Expected values were calculated on the basis of the average values of the intact samples.
[c] Measured value / expected value × 100.

Based on these results, the LOD was determined to be 5.0 pg/ml when 200 μl of saliva was used.

3.4.3. Assay precision and accuracy

The intra-assay ($n = 5$) R.S.D. values were less than 3.6% and good inter-assay ($n = 5$) R.S.D. values (less than 3.2%) were also obtained, as shown in Table 2.

The saliva to which known amounts of 17OHP had been added were pretreated and analyzed in order to examine the assay accuracy. Satisfactory values ranging from 99.4 to 102.3% were obtained (Table 2). We are well aware that a calibration curve should be preferentially constructed with a sample of the same matrix, but it is difficult to obtain a saliva sample that does not contain 17OHP. Therefore, the calibration curve constructed with the charcoal-treated saliva spiked with 17OHP was employed in the present study. However, the above results demonstrate that the salivary 17OHP can be accurately determined using the curve. In addition, significant ion suppression or ion enhancement for the derivatized 17OHP due to the saliva matrix was not observed. These data indicate that the present method is highly reproducible and accurate.

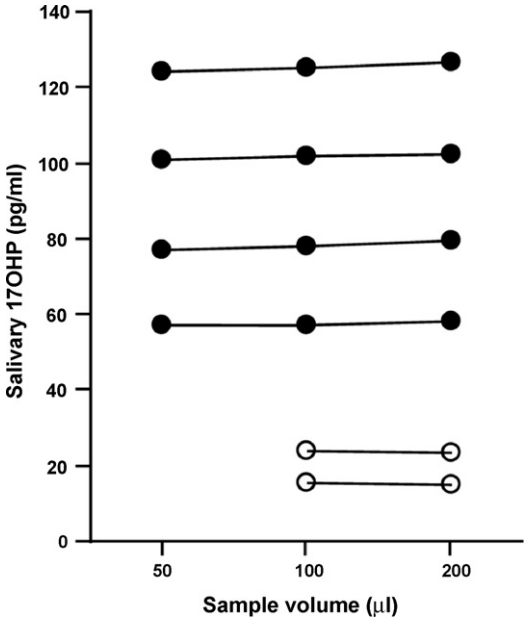

Fig. 4. Effect of sample volume on measured value. (○) Healthy volunteers and (●) CAH patients.

Table 3
Stability of 17OHP in saliva

Treatment	Percentage of initial concentration[a]	
	Saliva A	Saliva B
Room temperature 1 h	96.1	97.3
Room temperature 3 h	95.0	95.1
Freeze/thaw 1 cycle	105.8	102.4
Freeze/thaw 3 cycles	102.8	99.6

[a] The measured values immediately after the collection were taken as 100%.

3.4.4. Effect of sample volume on measured value

As shown in Fig. 4, the 17OHP concentrations obtained from the analysis using 200 μl of the samples and those using 50 or 100 μl of the samples were quite similar. This result indicates that 50 or 100 μl of saliva should be used when the 17OHP concentration is over 250 pg/ml (the highest concentration on the calibration curve for a 200-μl saliva aliquot). The result of this test also demonstrates that the measured value is not affected very much, when the salivary components (matrix) increase and decrease.

3.4.5. Stability of 17OHP in saliva

Table 3 shows that 17OHP is stable in saliva at room temperature for up to 3 h. This indicates that patients (or their parents) can easily carry the saliva samples, which they (or their parents) collected at their home, to the hospital/clinical laboratory; it is not necessary to keep the samples frozen or to refrigerate them within the first 3 h. The salivary 17OHP was also stable up to 3 freeze/thaw cycles (Table 3). Furthermore, it was possible to store the saliva at −20 °C without loss of the 17OHP for at least 6 months.

3.5. Clinical studies

3.5.1. Correlation between the salivary and blood 17OHP concentrations

As mentioned in the introductory section, the blood 17OHP concentration is conventionally used as the index of therapeutic efficacy during the HRT of CAH. In order to put this new method to practical use in the clinical field, it is important to examine the correlation between the blood 17OHP concentration and the salivary 17OHP concentration measured by the method. Some papers describe that immunoassay generally overestimates the blood 17OHP concentration [6,10], while other papers demonstrate that immunoassay and LC–MS–MS yield results in reasonable agreement [7,17]. The major cause for this difference is the specificities of antibodies used. As described in Section 2.7.4, this study used DPC·17α-OH-progesterone kit for the measurement of the serum 17OHP, which is highly specific for 17OHP. As is obvious from Fig. 5, a good correlation ($r^2 = 0.975$, Peason's correlation coefficient test, $P < 0.01$) was observed between the serum and salivary levels. This result reveals that there is a reliable ratio between the blood and saliva concentrations and that the salivary 17OHP assay can be a noninvasive tool for the monitoring of the therapeutic efficacy during the HRT of CAH.

3.5.2. Monitoring of salivary 17OHP at the dose-setting of HC

During the initial stage of the HRT for CAH, the monitoring of the blood 17OHP is carried out for determining the dose of HC. If the salivary 17OHP monitoring can be an alternative to the blood 17OHP monitoring, there is a significantly beneficial effect for the patients and their parents.

The change in the salivary 17OHP concentrations after the oral administration of HC was measured by our method and compared to that in the blood 17OHP concentrations. Fig. 6 shows the results of the measurements of 17OHP in the saliva and dried blood filter-paper sample. Generally, the changes in the two levels showed

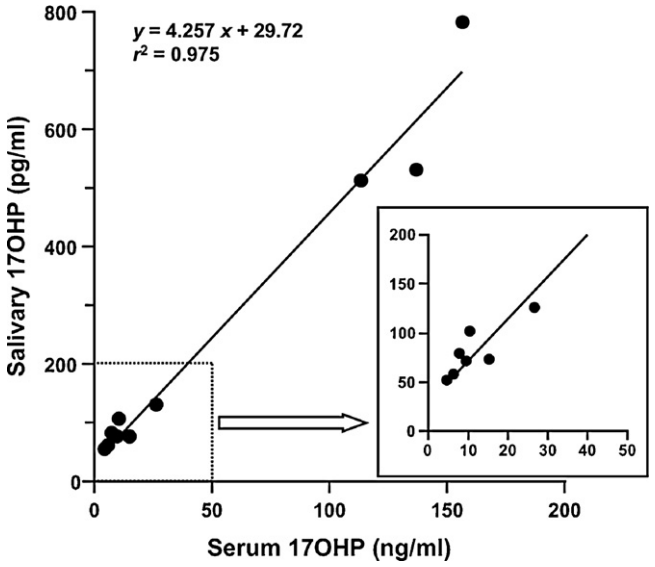

Fig. 5. Correlation between the salivary and serum 17OHP concentrations.

reasonable agreement. Continuous decreases in the salivary and blood 17OHP concentrations attributable to the administration of 8 mg of HC (3 times a day at intervals of 8 h) were recognized. Based on these results, HC at a dose of 8 mg was administered to this newborn patient at 8 h intervals. Thus, the saliva assay can also be an alternative to the blood assay for the dose-setting of HC. However, at the 2 points asterisked in Fig. 6, the salivary concentrations were quite different from those expected from the blood concentrations. Although we cannot provide a certain cause for the disagreement at these points, the salivary level may be possibly overestimated, if the saliva is contaminated with blood or mother's milk. In order to put the proposed saliva assay to practical use in the clinical field, it is necessary to continue examining the saliva collection method for a newborn.

3.5.3. Monitoring of salivary 17OHP in CAH patients under HRT

The salivary 17OHP levels of the CAH patients (9.3 ± 2.9 years old, 6 males and 4 females) under HRT were measured using the proposed method. The saliva was collected immediately before and 2 h after the administration of HC and fludrocortisone. The measured results and the patient characteristics are shown in Table 4. In all the patients, the salivary 17OHP level significantly decreased after the administration; still, the decreased level of the patients was much higher than the level (13.1 ± 2.8 pg/ml) of the healthy child volunteers (8.9 ± 1.7 years old, 5 males and 9 females).

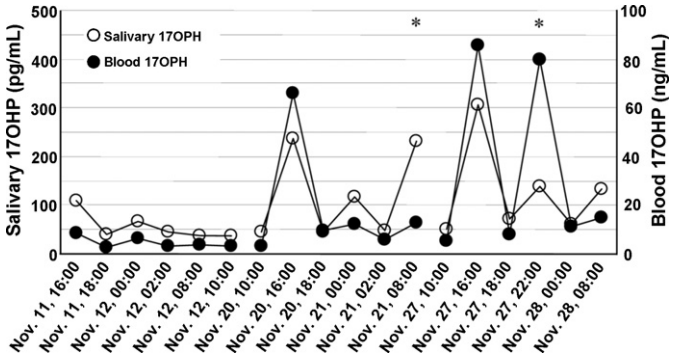

Fig. 6. Monitoring of salivary and blood 17OHP concentrations in a newborn CAH patient at the dose-setting of HC. The date and time of the administration of HC and the HC dose are summarized in Table 1.

Table 4
Salivary 17OHP concentration in CAH patients under HRT

CAH patient	Sex	Age (year)	Daily dose of HC[a] (mg)	17OHP concentration (pg/ml)[b]	
				Before administration	After administration
1	Male	12	25	455	176
2	Male	11	24	327	112
3	Male	4	13	32.4	22.7
4	Male	8	15	46.5	27.6
5	Male	9	35	528	102
6	Male	9	35	508	73.2
7	Female	5	9	41.0	24.8
8	Female	11	15	260	66.7
9	Female	12	24	263	58.3
10	Female	12	22	378	106

[a] HC is daily administrated in 3 doses at intervals of about 8 h.
[b] When the concentration was over 250 pg/ml, 50 or 100 μl of saliva was used.

4. Conclusion

We have demonstrated the LC–ESI-MS–MS method for the determination of 17OHP in human saliva. The method was specific, accurate and reproducible, and able to quantify 5 pg/ml of salivary 17OHP using a 200-μl sample. The salivary 17OHP concentration determined using the developed method was well correlated with the blood 17OHP concentration, which has been conventionally used as a means of monitoring the therapeutic efficacy of HRT for CAH. This method was also able to detect the decrease in the salivary 17OHP level after the HC administration. This well-characterized method can be an alternative to the blood 17OHP monitoring during HRT, because saliva collection is noninvasive, easy, inexpensive and may be performed by the patient with no need for the involvement of medical personnel, if so desired.

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