Salivary Hormone Measurement Using LC/MS/MS: Specific and Patient-Friendly Tool for Assessment of Endocrine Function

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Saliva has recently been attracting attention as a patient-friendly available bio-fluid and an alternative to serum/plasma for hormone tests. LC coupled with atmospheric pressure ionization-MS/MS, especially electrospray ionization (ESI)-MS/MS, has been recently valued as a highly specific method in the analysis of salivary hormones. In this article, LC/ESI-MS/MS assays for salivary hormones are overviewed according to the papers that have been published during the last 5 years. Practical derivatization to enhance the detectabilities of hormones in ESI-MS/MS is also discussed, because a major disadvantage of using saliva is low hormone concentrations.

Key words saliva; hormone; LC/MS/MS; derivatization; endocrine function

1. INTRODUCTION

Specific and sensitive methods for the detection, characterization and quantification of hormones in biological fluids and tissues are essential for elucidation of the nature, diagnosis and treatment of endocrine disorders. Hormones 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, its specificity and accuracy are sometimes poor due to interference from other endogenous compounds. Furthermore, immunoassay does not have a simultaneous multi-analyte quantification capability. Among the alternative methods, LC coupled with atmospheric pressure ionization-MS/MS, especially electrospray ionization (ESI)-MS/MS, has been used for the analysis of hormones, such as steroids, catecholamines and their metabolites, and thyroid hormones (so-called low-molecular weight hormones), due to its specificity and versatility.1–3)

Although the blood serum or plasma specimen is conventionally used to assess the hormone status, saliva has recently been attracting attention as a new specimen for this purpose due to its easy and noninvasive nature of collection, and because various hormones can enter into saliva from blood circulation.4–6) A major disadvantage in the use of saliva is the low hormone concentrations, but the progress in LC/ESI-MS/MS, i.e., development of not only high-performance instruments (hardware) but also sensitivity enhancement procedures including derivatization (chemistry-based software) enables the analysis of the salivary hormones.

Based on this background information, in this review article the author presents an overview of LC/ESI-MS/MS assays for the salivary hormones and their applications according to the papers that have been published during the last 5 years (2007–2011).

2. ADVANTAGES AND DISADVANTAGES OF SALIVA AS A DIAGNOSTIC SPECIMEN

Serum or plasma is conventionally used for hormone testing; however, patients undergo pain whenever their blood is collected. In recent years, saliva has attracted much attention as an alternative to serum or plasma. The prime advantage of saliva is that it offers easy, noninvasive, stress-free and real-time repeated sampling where blood collection is either undesirable or difficult. No special training or equipment is needed for the saliva collection and patients can collect samples conveniently themselves, if required. Thus, it is expected that a saliva-based hormone test has a highly beneficial effect for both the patients and medical personnel. In addition, the levels of hormones in saliva generally reflect those of the serum/plasma free active hormones; the biological activity of hormones is a function of their free fraction.4–6) For the measurement of the serum/plasma free hormones, equilibrium dialysis or ultrafiltration is often required before assay, and therefore, the current serum/plasma free hormone test is not always the best method in terms of cost, simplicity and convenience. Salivary hormone measurement can be a simple and inexpensive alternative to serum/plasma free (active) hormone measurement.

Urine is also used in the evaluation of hormone status and can be collected without any special training and equipment. However, taking a steroid test as an example, most of the target steroids in urinalysis are inactive and hydrophilic metabolites, such as glucuronidated and sulfated conjugates; urinalysis can tell the change in hormone production/secretion but cannot tell the real-time active hormone status. Furthermore, urinary steroids are sometimes measured in the 24-h urine and this way of collection is often not easy.

A major disadvantage of using saliva is low hormone concentrations; the salivary hormone concentrations are generally
1–10% of the blood hormone concentrations. Furthermore, the collection of a large volume of saliva from a patient at one time is difficult, if not impossible, because the basal flow rate of saliva is approximately 0.5 mL/min. Therefore, a highly sensitive detection technique is required for a saliva-based test.

3. COLLECTION OF SALIVA

Most of the saliva in man is produced by three pairs of major salivary glands (parotid, submandibular and sublingual) but a small contribution is made by the numerous small buccal glands which line the mouth. Each type of salivary gland secretes a characteristic type of saliva. Furthermore, in healthy subjects, gingival crevicular fluid (from the tooth-gum margin) may constitute up to 0.5% of the volume of mixed saliva. It should be noticed that the composition of the gingival crevicular fluid is similar to that of plasma. Saliva produced by a single salivary gland can be collected by cannulation of a single salivary duct or by other devices such as a micropipette, but the test based on this type of sampling is of little practical use because a high degree of training is required for the collection of the specimen. Therefore, for the collection of a sample on a patient basis, the mixed whole saliva, which contains gingival crevicular fluid, is the only practical alternative in the clinical field, and in this article, the mixed whole saliva is simply described as saliva.

Saliva collection methods may impact analytical results. Today several methods and devices are available to more easily collect saliva. The most commonly used devices are cotton rolls, such as Salivette (Sarstedt, Nümbrecht, Germany). However, when a cotton-based 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 and contaminants from the device sometimes interfere with the analysis.

Alternatively, the stimulated saliva collection by chewing gum or other devices are often used to collect an adequate sample volume of saliva within a shorter time. Stimulation by these methods usually increases saliva flow by 2–5 times (basal flow rate, 0.5 mL/min versus increased flow rate, 1–3 mL/min). However, some reports show that the stimulated saliva collection decreases the concentrations of some steroids, such as dehydroepiandrosterone sulfate (DHEAS), in the saliva (see Section 4 for details).

Taken together, the collection of unstimulated saliva into a collection tube without a collection device can avoid the above-mentioned pitfalls. In the many research studies which claim to have utilized unstimulated saliva, the subjects have usually been asked to spit or drool their saliva directly into a collection tube. Because active spitting is considered sufficient stimulus to increase saliva flow rate, passive drooling is preferred to collect unstimulated saliva.

Although 99.5% of saliva is water, saliva contains mucins; the mucins make pipetting difficult due to their viscosity and sometimes interfere with the analysis. Most of the mucins may be denatured by freezing and thawing the sample; the denatured mucins also tend to adsorb much of the debris from the sample and the freezing eliminates most of the froth associated with a fresh saliva sample. Consequently, most researchers have advocated freezing and thawing, then centrifuging samples before analysis.

4. TRANSPORT OF HORMONES TO SALIVA

Some hormones commonly measured in serum/plasma, such as steroids, amine-derivatives, peptide and protein hormones, can also be detected in saliva. Hormones can enter into saliva from blood circulation by a variety of mechanisms. The speed at which hormones can be transferred from blood into saliva is controlled by passage through the lipophilic layers of the capillaries and glandular epithelial cells. Figure 1 schematically shows two modes of entry of hormones into saliva. For lipophilic compounds, such as unconjugated steroids, which are highly membrane-permeable, the major route for entry into saliva is rapid diffusion through the acinar cells (the transcellular route) and the rate of diffusion across the cells is so fast that their concentrations are independent of the rate of saliva flow. In contrast, conjugated steroids, such as DHEAS, have limited lipid solubility and are therefore membrane-impermeant. Very small amounts of such compounds may enter the saliva via the tight junctions of the acinar cells (the paracellular route) at a low and constant rate, and their concentrations are significantly influenced by saliva flow rate (the concentration decreases with an increased saliva flow rate). Therefore, the salivary level of a conjugated
The mechanism of hydrophilic compounds for entry into saliva is via the paracellular route.\textsuperscript{4,5} This mechanism is restricted to compounds with a molecular weight of <1900 and therefore, peptide and protein hormones cannot enter via the paracellular route.\textsuperscript{5} Although some peptide hormones such as insulin are actively transported into saliva from their tissue of origin,\textsuperscript{17} the presence of peptide and protein hormones in saliva is almost certainly due to the contamination of the saliva by gingival crevicular fluid, whose composition is similar to that of plasma.\textsuperscript{4,5} For catecholamine metabolites, melatonin and thyroxine, their modes of entry into saliva have not been completely elucidated at the present time.

5. STEROID HORMONES

5.1. Relationship of Structure and Detection Response in ESI-MS/MS, and Basic Idea of Derivatization

Steroids with the 3-oxo-4-ene structure, such as cortisol, progesterone and testosterone, show relatively high responses in the positive ESI-MS/MS, because they are more proton-affinitive than most other neutral steroids (5-ene-steroids, 5αβ-reduced steroids and estrogens) and can provide some characteristic product ions in MS/MS.\textsuperscript{50} Moreover, the salivary levels of cortisol (in a ng/mL range) and testosterone (in tens of pg/mL range) are relatively high. Therefore, their salivary concentrations can be readily determined by using a current instrument. The salivary progesterone can be also determined for nonpregnant women at the luteal phase (in tens of pg/mL range) and pregnant women (in hundreds of pg/mL range) by LC/positive-ESI-MS/MS.\textsuperscript{59} On the other hand, the ionization efficiencies of 5-ene-steroids, 5αβ-reduced steroids and estrogens are low for ESI; to improve their detectabilities, some derivatization procedures have been proposed.\textsuperscript{7,20}

Because the ESI process occurs in the liquid phase, the best detectability with ESI-MS has been achieved for the analysis of compounds that are either ionic or can be readily ionized in solution. Therefore, the basic idea for enhancing the detection sensitivity in ESI-MS is the introduction of a proton-affinitive moiety or a permanently charged moiety to the target steroid. Successful examples of derivatization for the analysis of salivary steroids are shown in Table 1 and are discussed in detail later. LC/MS/MS methods for the salivary aldosterone (a major mineralocorticoid) and estradiol (the most active estrogen) have not yet been reported but will be developed and validated in the near future, because derivatization methods which enable the detection of attomole levels of these steroids were recently reported.\textsuperscript{21,22}

Table 1. Effect of Derivatization in Salivary Steroid Measurement Using LC/ESI-MS/MS

<table>
<thead>
<tr>
<th>Derivatization reagent\textsuperscript{a}</th>
<th>Sample volume (mL)</th>
<th>Limit of quantification (pg/mL)\textsuperscript{b}</th>
<th>Increasing detectability\textsuperscript{c}</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Intact steroid</td>
<td>Derivative</td>
<td></td>
</tr>
<tr>
<td>17α-Hydroxyprogesterone [17(0H)P]</td>
<td>HP</td>
<td>0.2</td>
<td>25</td>
<td>5.0</td>
</tr>
<tr>
<td>Testosterone</td>
<td>HMP</td>
<td>0.5</td>
<td>40</td>
<td>10</td>
</tr>
<tr>
<td>Dehydroepiandrosterone (DHEA)</td>
<td>HMP</td>
<td>0.5</td>
<td>20000</td>
<td>10</td>
</tr>
<tr>
<td>25-Hydroxyvitamin D\textsubscript{3}</td>
<td>PTAD</td>
<td>1.0</td>
<td>400</td>
<td>4.0</td>
</tr>
</tbody>
</table>

\textsuperscript{a} HP, 2-hydrazinopyridine; HMP, 2-hydrazino-1-methylpyridine; PTAD, 4-phenyl-1,2,4-triazoline-3,5-dione. \textsuperscript{b} Limits of quantification were determined using an Applied Biosystems API 2000 triple quadrupole mass spectrometer with the selected reaction monitoring mode. \textsuperscript{c} The detection responses of intact steroids are taken as 1.
5.2. Cortisol and Cortisone  By far the best established and accepted application for hormone analysis in saliva is the use of salivary cortisol in the diagnosis of Cushing’s syndrome. The level of cortisol circulation is also considered to be a marker of stress, and the noninvasive and stress-free collection of saliva has become particularly popular for use in psychological investigations.

To the best of the author’s knowledge, the first validated LC/MS/MS assay of salivary cortisol was reported by Jönsen et al. in 2003.\textsuperscript{25} Since then, a number of LC/MS/MS methods have also been reported for salivary cortisol measurement.\textsuperscript{24–28} In some of these methods, the simultaneous determination of cortisol and its inactive metabolite, cortisone, was done.\textsuperscript{25,27,28} The salivary glands contain enzymes (11β-hydroxysteroid dehydrogenase) capable of converting cortisol to cortisone,\textsuperscript{29} and it appears that the salivary cortisol is largely derived from enzymatic conversion of plasma cortisol by the salivary glands; Perogamvros et al.\textsuperscript{30} describes that salivary cortisol has potential as a useful surrogate for serum free cortisol.\textsuperscript{30} The measurements of salivary cortisol (and cortisone) have usually been done by the selected reaction monitoring (SRM) mode after online/off-line solid phase extraction (SPE) of saliva (100–250 µL) without derivatization.\textsuperscript{31} The biggest problem in the study was the lack of sensitivity for the DHEA measurement. Testosterone (3-oxo-4-ene-steroid) was much lower, and its LOQ was as high as 20 ng/mL. Therefore, the method employed derivatization with 2-hydrazino-1-methylpyridine (HMP) for increasing the sensitivity of DHEA (Fig. 3a). HMP has the methylpyridyl group and also provided specificity for the DHEA analysis. The saliva was deproteinized with acetonitrile, purified using a reversed-phase SPE cartridge, derivatized with a highly proton-affinitive reagent, 2-hydrazinopyridine (HP),\textsuperscript{32} and subjected to LC/MS/MS. Although 17(OH)P has the 3-oxo-4-ene structure, derivatization was employed due to its low salivary level; the HP-derivatization increased the assay sensitivity by 5 times [limit of quantification (LOQ), 5.0 pg/mL] and significantly contributed to reducing the sample volume, which was an important point in developing a method applicable for newborn and child patients. Deuterated 17(OH)P was used as the internal standard (IS). The salivary 17(OH)P concentration determined using the developed method was well correlated with the blood 17(OH)P concentration, which has been conventionally used as a means of monitoring the therapeutic efficacy of HRT for CAH. Figure 2 shows the results of the measurements of 17(OH)P in the saliva and blood sample in the dose-setting of cortisol for HRT; the changes in the two levels showed reasonable agreement. Thus, the saliva-based test could be an alternative to the blood test for the dose-setting of cortisol.

5.4. Testosterone and Dehydroepiandrosterone  In the salivary androgen assays using LC/MS/MS, studies are concerned with late onset hypogonadism (LOH).\textsuperscript{34,35} 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 levels.\textsuperscript{37} Currently, serum free testosterone is being used for the diagnosis of LOH. However, to measure serum free testosterone, equilibrium dialysis or ultrafiltration, which is complex, time-consuming and costly, is often required before assay. Alternatively, salivary testosterone has recently been attracting attention for the diagnosis of LOH, because the salivary steroid concentrations generally reflect the serum free steroid concentrations.\textsuperscript{4–6}

Shibayama et al.\textsuperscript{36} developed a stable-isotope dilution LC/positive ESI-MS/MS method that enables the simultaneous determination of testosterone and its precursor, dehydroepiandrosterone (DHEA), in saliva,\textsuperscript{37} because the age-related decline in DHEA production also seems to be associated with LOH.\textsuperscript{35} The biggest problem in the study was the lack of sensitivity for the DHEA measurement. Testosterone (3-oxo-4-ene-steroid) showed a relatively high response in ESI-MS/MS, but the detection response of intact DHEA (5-ene-steroid) was much lower, and its LOQ was as high as 20 ng/mL. Therefore, the method employed derivatization with 2-hydrizin-1-methylpyridine (HMP) for increasing the sensitivity of DHEA (Fig. 3a). HMP has the methylpyridyl group as the charged moiety and quantitatively reacted with the androgens to produce the permanently-charged derivatives.\textsuperscript{39} The derivatives gave intense [M]+ ions in the positive ESI-MS and also provided specific product ions during MS/MS. As a consequence, a 2000-times sensitivity improvement was achieved for the DHEA analysis. The saliva was deproteinized and then purified using a reversed-phase SPE cartridge. The androgen fraction was then treated with HMP. 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 (Fig. 3b) and the LOQs for both androgens were 10 pg/mL. The method could noninvasively detect the age-related decline in the testosterone \( (r=0.66) \) and DHEA \( (r=0.65) \) productions. When the mean – 2SD of the androgen levels of the healthy subjects in their 20s were used as the provisional cutoff values (i.e., diagnostic criteria for LOH), more than half of the subjects in their 70s and 80s were suspected of LOH (Fig. 4). The method also enabled the determination of the changes in the individual testosterone and DHEA levels after the DHEA supplementation, which is expected to be a new and easy medication for LOH. Thus, the developed method had satisfactory applicability in the diagnosis and medication for LOH.

An LC/positive ESI-MS/MS assay developed by Matsui et al. enabled the simultaneous determination of testosterone and cortisol without derivatization.\(^ {36} \) As described above, LOH causes a variety of symptoms and the differential diagnosis is relatively difficult, including psychological disorders, stress, and mood disturbances. Because the salivary cortisol level is a good marker of stress, the simultaneous determination of testosterone and cortisol is expected to be useful for the differential diagnosis of LOH and stress-induced disorders. The LOQs of the method were 5 and 10 pg/mL for testosterone and cortisol, respectively, when a 1-mL saliva was used. The study also confirmed that the salivary testosterone levels negatively correlate with age \( (r=0.64) \), but there is no relationship between salivary cortisol and age \( (r=0.03) \).

5.5. 25-Hydroxyvitamin D\(_3\) The measurements of the serum/plasma concentration of the vitamin D metabolites are widely used for the diagnostic assessment and the follow-up of several diseases (osteoporosis, renal osteodystrophy, parathyroid gland disorders and sarcoidosis).\(^ {40} \) Among vitamin D metabolites, 25-hydroxyvitamin D\(_3\) [25(OH)D\(_3\), a pro-hormone of calcitriol] is the best-established indicator of the vitamin D status.

Higashi et al. developed a sensitive LC/positive ESI-MS/MS method for the determination of 25(OH)D\(_3\) in human saliva.\(^ {41} \) The saliva (1 mL) was deproteinized with acetonitrile, purified using a reversed-phase SPE cartridge, derivatized with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD), and subjected to LC/MS/MS. The PTAD derivative was much more easily ionized in positive ESI-MS and efficiently produced a characteristic product ion during MS/MS, compared to the intact 25(OH)D\(_3\), which resulted in a 100-times enhancement of sensitivity in the SRM mode. Methylamine was also used as the mobile phase additive to enhance the assay sensitivity (2 times); an intense adduct ion \([M+CH_3NH_3]^+\) was formed by the addition of methylamine. The LOQ was 2.0 pg/mL and the method was used for clinical studies. A linear relationship was found to be positive \( (r^2=0.830) \) between the serum 25(OH)D\(_3\) level, which is conventionally used as the means of assessing the vitamin D status, and the salivary 25(OH)D\(_3\) level measured using the developed method. The method also enabled the detection of the increase in the salivary 25(OH)D\(_3\) level after the supplementation of vitamin D\(_3\).

6. NON-STEROID HORMONES

6.1. Catecholamine Metabolites Epinephrine and norepinephrine are detectable in human saliva, but their source is still uncertain.\(^ {42} \) They seem to originate by diffusion from blood, but there is also an amount of salivary catecholamines derived by direct release from sympathetic nervous terminals, so their concentrations are poorly correlated with those of serum/plasma. Salivary catecholamine is therefore not considered a useful index of general sympathetic tone. On the contrary, the studies using HPLC demonstrated that the concentrations of methylated catecholine metabolites, such as homovanillic acid (HVA) and 3-methoxy-4-hydroxyphenylethylene glycol (MHPG), in saliva correlated with their plasma levels.\(^ {43,44} \)

A method using the derivatization with 2-picolylamine (PA) followed by LC/positive ESI-MS/MS enabled the detection of HVA with a 0.1-mL saliva.\(^ {45} \) The detection response of
the derivative was increased by 86-fold over the intact HVA. When an analogue of HVA, 4-hydroxy-3-methoxyphenylpropionic acid, was used as the IS, the intra-assay precision (n=5) was 3.6%. Although no LC/MS/MS method for salivary MHPG has been reported, the author found that it can be readily analyzed with a 20-µL sample by LC/positive ESI-MS/MS after conversion into the triacetate (Fig. 5, the details will be reported elsewhere).

6.2. Melatonin Circadian disruption, in relation to night work, can have several possible health consequences, and melatonin, which functions as a circadian pacemaker, is often determined in several bio-fluids. An LC/positive ESI-MS/MS method published recently is able to quantify melatonin in saliva with the limit of detection of 4 pmol/L.46) Using this method, salivary testosterone and cortisol, which also show significant diurnal rhythms, were determined simultaneously. The pilot study among four healthy volunteers proved that the method is useful for monitoring the circadian rhythms for the three hormones.

6.3. Thyroxine Thyroid hormones are essential for regulation of biological processes such as growth, metabolism, neurodevelopment, and protein synthesis. Measurements of the circulating thyroxine (T4) concentrations in blood are important to assess thyroid hormone status, diagnose thyroid disorders and monitor treatment. Most circulating T4 is bound to serum proteins, primarily thyroxine binding globulin (TBG), while only less than 0.1% of serum T4 is free of bound protein.47) Total circulating T4 concentration is susceptible to the effect of the TBG concentration, and therefore, serum free T4 is currently used to diagnose thyroid disorders. Salivary T4 measurement may be a simple and cost-effective alternative to serum/plasma free T4 measurement. However, there is little information about the possibility of salivary T4 determination. Based on this background information, Higashi et al. developed and validated a stable isotope-dilution LC/positive ESI-MS/MS method for the determination of T4 in saliva.48) The saliva (400 µL) was deproteinized with methanol, purified using a reversed-phase SPE cartridge, and subjected to LC/MS/MS. Quantification was based on the SRM and the LOQ was 25.0 pg/mL. A pilot study using the developed method found that there is a diagnosable difference in the salivary T4 concentration between the euthyroid subjects [n=16, 69.9±29.0 pg/mL (mean±S.D.)] and the patients with Graves disease (n=2, 675 and 195 pg/mL). Based on this result, the salivary T4 measurement seems to be useful at least in the diagnosis of Graves disease.

7. CONCLUSION

This review highlights recent advances in the determination of salivary hormones using LC/ESI-MS/MS. Although saliva has not yet become a mainstream specimen for hormone tests, the saliva-based test is an upcoming tool for the patient-friendly assessment of endocrine functions. LC/MS/MS is now the best methodology for the saliva-based hormone test and plays an important role especially in salivary steroid measurements. With the further progress in LC/ESI-MS/MS (both in instruments and chemistry-based software, such as derivatization), the author believes that the saliva-based hormone test will be integrated into clinical practice in the near future.

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REFERENCES

7) Higashi T, Shimada K. Derivatization of neutral steroids to enhance the mass sensitivity, Professor Toshimasa Toyo'oka (University of Shizuoka) and colleagues. The author's studies cited in this article were supported in part by the Japan Society for the Promotion of Science (JSPS) and the Japan Science and Technology Agency (JST).

REFERENCES


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