Quantitative measurement of salivary testosterone in Korean adults by stable isotope-dilution liquid chromatography-electrospray-tandem mass spectrometry

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Salivary testosterone levels in Korean adults were quantitatively measured for the first time by liquid chromatography-electrospray-tandem mass spectrometry (LC ESI MS/MS). Salivary testosterone was separated on a multiple reaction monitoring (MRM) chromatogram within 7 min. The LC ESI MS/MS assay was validated over the linearity range of 0.01-2.00 ng/ml (r = 0.99987) using testosterone-d3 as an internal standard. The lower limit of quantification (LOQ) was 0.01 ng/ml. The intra- and inter-assay precisions were 1.54% to 4.09% and 0.96% to 4.29%, respectively. The mean recovery was 93.32% (range 88.43-98.05%). The validated assay was then applied to measure the salivary testosterone levels of Korean adults. In men, the salivary testosterone level collected between 9:00-11:00 am was approximately 2.8 times higher than that in women (P < 0.0001). Salivary testosterone levels in both sexes negatively correlated with age. The present assay would also be useful in measuring salivary testosterone levels in clinical laboratories. [BMB reports 2010; 43(11): 761-765]

INTRODUCTION

Testosterone is produced by testicular Leydig cells in males and by the adrenal glands, ovaries, and peripheral conversion of circulating androstenedione in females (1). In men, testosterone is known to be necessary for the maintenance of spermatogenesis, secondary sexual characteristics, bone density, muscle mass, and libido (2). Serum testosterone concentration in women is known to be approximately 5-10% of that in men and is considered to be important in the maintenance of bone mineral density, mood, and libido (1, 3).

Measurement of testosterone has many clinical applications and is essential in the evaluation of androgenic status and monitoring of stimulatory, suppressive, or replacement therapy in children and adults. Especially, testosterone in men is largely measured to evaluate late-onset hypogonadism (LOH) (4-7), whereas the steroid in women is measured to evaluate hyperandrogenism such as hirsutism, acne, alopecia, and oligo-amenorrhea (8, 9).

In both men and women, the majority of circulating testosterone is bound to proteins. In healthy adult men, about 98% of circulating testosterone is bound to serum proteins, primarily sex hormone-binding globulin (SHBG) and albumin, whereas only 1-2% of serum testosterone is free of bound protein (10). Albumin-bound testosterone and free testosterone are referred to as bioavailable testosterone, which is thought to be a good index of androgen activity (11). A significant correlation between salivary and serum free testosterone has been demonstrated in healthy subjects, indicating that salivary testosterone can be a good index of serum free testosterone (6, 12-14). Due to these reasons, salivary testosterone has recently attracted attention for use in the evaluation of physiological and pathological conditions based on steroid assays.

Among the steroid assays, immunoassay-based methods for salivary testosterone evaluation are known to be unsatisfactory due to cross-reactivity between a variety of endogenous materials, and the measured values often overestimate the true concentrations, especially at the low levels of testosterone typically found in women, children, men with androgen deficiencies, and patients undergoing anti-androgenic therapies (15-17). To circumvent these problems, LC MS/MS assay has been used as a highly specific and sensitive tool for evaluating salivary testosterone in healthy adults in Japan or USA, using protocols specified by the Federal Drug Administration (FDA) (4, 5, 18). However, to the best of our knowledge, no study reported the evaluation of salivary testosterone levels in Korean adults of both sexes by LC MS/MS assay.

The aims of the present study were to validate the LC ESI MS/MS method for measuring testosterone concentrations in saliva samples from both sexes and to quantify the salivary testosterone levels in Korean adults.
RESULTS AND DISCUSSION

Chromatographic separation
Salivary testosterone was eluted within 7 min by the gradient elution method without any interfering substances (Fig. 1B, C, D). No ion suppression was observed. The testosterone was clearly separated in the MRM chromatogram of an extracted saliva sample. The retention times of testosterone and testosterone-d3 were 6.25 min and 6.22 min, respectively (Fig. 1C, D). This result indicates that the rapid analytical time would permit high-throughput measurement of salivary testosterone.

Validation performance
LC ESI MS/MS assay for measuring salivary testosterone levels was validated. The assay was linear from 0.01 to 2.00 ng/ml (Fig. 1A). The regression coefficient (r) of the calibration curve (y = 0.62189x + 0.00469) was 0.99987, indicating excellent linearity. The LOQ was 0.01 ng/ml (Table 1). In our study, the LOQ was low enough to quantitatively measure salivary testosterone (Fig. 1B-D). Intra- and inter-day (n=5) accuracies ranged from 92.40% to 97.72% and from 96.28% to 96.90%, respectively, whereas intra- and inter-day precisions (n=5) ranged from 1.54% to 4.09% and from 0.96% to 4.29%. The average recoveries of salivary testosterone in triplicate were 88.43% for 0.05 ng/ml spike, 93.48% for 0.50 ng/ml spike, and 98.05% for 2.00 ng/ml spike (Table 1). These results indicate that the present analytical method was within internationally accepted criteria and was fast, highly reproducible, accurate, specific, and sensitive.

Salivary testosterone measurements from healthy adults in Korea
The average concentrations of salivary testosterone were 69.36 ± 17.95 pg/ml (range: 38.50-99.50 pg/ml) for men and 25.52 ± 9.80 pg/ml (10.10-46.00 pg/ml) for women between 9:00-11:00 a.m. At the collection time, the salivary testosterone level in men was approximately 2.8 times higher than that in women (P < 0.0001) (Fig. 2A and Table 2). It is well known that salivary testosterone in human is subject to significant diurnal rhythms and individual differences. Further, inter-day alterations in steroid levels in the early morning (6:00-7:00 a.m.) are larger than in the late morning (9:00-11:00 a.m.), as evidenced by LC ESI MS/MS assay (4, 5). To avoid the individual differences and inter-day alterations in salivary testosterone level in the early morning, we analyzed saliva samples collected between 9:00-11:00. As pre-

Table 1. Intra- and inter-day accuracy, precision, recovery, and LOQ of the LC ESI MS/MS assay for measuring salivary testosterone

<table>
<thead>
<tr>
<th>Concentration (ng/ml)</th>
<th>Intra-assay (n = 5)</th>
<th>Inter-assay (n = 5)</th>
<th>Recovery (%)</th>
<th>LOQ (ng/ml)</th>
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<td>Accuracy (%)</td>
<td>Precision (%)</td>
<td>Accuracy (%)</td>
<td>Precision (%)</td>
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</tbody>
</table>

Fig. 1. (A) Calibration curve at spiked concentrations of 0.01 to 2.00 ng/ml for testosterone from steroid-free saliva. (B) MRM chromatogram of an extracted saliva sample spiked with testosterone at LOQ concentration of 0.01 ng/ml. (C) MRM chromatogram of an extracted saliva sample (1 ml) containing 0.01 ng of testosterone and a 100 μl aliquot of testosterone-d3 solution with a concentration of 100 ng/ml. (D) MRM chromatogram of an extracted saliva sample (1 ml) containing 1.00 ng of testosterone and a 100 μl aliquot of testosterone-d3 solution with a concentration of 100 ng/ml.