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Liquid chromatography–tandem mass spectrometry assay for human serum testosterone and trideuterated testosterone

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Abstract

A liquid chromatography tandem mass spectrometry assay for serum testosterone (T) and trideuterated testosterone (d_3T) was developed in order to support clinical research studies that determine the pharmacokinetics, production rate, and clearance of testosterone by administration of trideuterated testosterone. After adding 19-nortestosterone as the internal standard (I.S.), sodium acetate buffer, and ether, to a serum aliquot, the mixture was shaken and centrifuged, and the ether was dried. The extract was reconstituted in methanol and 15 μl was injected into a liquid chromatograph equipped with an autosampler and Applied Biosystems-Sciex API 300 triple quadrupole mass spectrometer operated in the positive ion mode. T, d_3T, and I.S. were monitored with transitions m/z 289 to m/z 97, m/z 292 to m/z 97, and m/z 275 to m/z 109, respectively. The two calibration curves were linear over the entire measurement range of 0–20 ng/ml for T and 0–2.0 ng/ml for d_3T. The LOQs for T and d_3T were 0.5 ng/ml and 0.05 ng/ml. The recoveries for T and d_3T were 91.5 and 96.4%. For T at 1.25 ng/ml and 4.0 ng/ml, the intra-day precision (RSD, %) was 3.9 and 4.3% and intra-day accuracy 0.01 and 4.5%, respectively. The inter-day precision at these levels was 5.3 and 5.4% and inter-day accuracy was 1.9 and 0.3%. For d_3T at 0.125 ng/ml and 0.4 ng/ml, the intra-day precision (RSD, %) was 2.8 and 8.3% and intra-day accuracy was 1.8 and 5.6%. The inter-day precision at these levels was 10.0 and 7.6% and inter-day accuracy was 5.7 and 3.4%. The concentrations of T in the 38 healthy subjects ranged from 2.5 to 14.0 ng/ml (mean 6.2 ng/ml).

Keywords: Testosterone

1. Introduction

Testosterone (T) is one of the most important and most commonly measured serum hormones. Clinicians use serum T measurements to diagnose and monitor various disorders such as hypogonadism, testicular dysfunction, hirsutism, virilization, alopecia, prostate disease, adrenal hyperplasia, and ageing. Most of these measurements are performed with immunoassays [1] because of their simplicity, rapidity, and relatively low cost, and despite occasional concerns about their reliability and validity [2].

Testosterone circulates in plasma non-specifically

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bound to albumin, specifically bound to sex hormone binding globulin, and unbound (free). Serum total T has been quantitated by immunoassay [3], gas chromatography–mass spectrometry (GC–MS) [4,5], liquid chromatography–mass spectrometry (LC–MS) [6,7], and liquid chromatography–tandem mass spectrometry (LC–MS–MS) [8,9]. MS-based methods are often used in research studies or to confirm immunoassay results [10]. The advantage of chromatography coupled with MS is the high specificity not available with immunoassays.

Studies designed to determine the production rate and clearance of T typically utilize the isotope-dilution method wherein the dilution of a tracer amount of radioactively labeled hormone is monitored [11]. These measurements have contributed to the understanding of many conditions and diseases [12–15], however, they require radioactivity counting methods and complex separations.

More recently, clearance studies have utilized stable, deuterium-labeled tracers in place of ^{3}H- or ^{14}C-labeled homologs. The principal advantages of deuterated steroids are higher recoveries, the ability to measure deuterated and endogenous species simultaneously, the unambiguous nature of MS data, and safety and ethical considerations. These considerations led investigators to develop GC–MS methods for quantitating endogenous and trideuterated T in man [5,16]. The advantages of LC–MS–MS include simplified sample preparation (underivatized steroids can be analyzed directly), high recovery, improved signal-to-noise ratio, and less difficulty with interferences due to MS–MS principles.

Initial efforts to develop a GC–MS method proved difficult due to the length and complexity of the extraction procedures and to a low and non-uniform recovery. Previous workers have reported on T quantitation by LC–MS and LC–MS–MS. Tiller et al. [8] report on the analysis of testosterone, hydrocortisone, and SR 27417 by LC–MS–MS. Their assay includes simultaneous quantitation of the above analytes, but not of deuterated testosterone, and they do not explicitly report LOD, LOQ, precision, or accuracy. Draisci et al. [9] measured T, 19-nortestosterone, progesterone and metabolites in bovine serum, but not in human serum. Ma and Kim [7] developed an LC–MS method but only applied it to standards. Yergey et al. [6] report on isotope dilution work using LC–MS, however, the high sample size volume (50 ml) and use of a thermospray interface make it unlikely that such a method would be selected today. The present assay was designed to develop an LC–MS–MS method for simultaneous measurement of endogenous and trideuterated T in human serum.

2. Experimental

2.1. Chemicals and reagents

Methanol, diethyl ether, sodium acetate and formic acid were HPLC grade (Fisher, Pittsburgh, PA, USA). Water was double distilled, then purified by ion-exchange resin and filtered through a 0.22-μm filter. Trideuterated T [16,16,17-$^{2}H_3$ testosterone, d_3T] and dideuterated T [1,2-$^{2}H_2$ testosterone] were obtained from MSD Isotopes, Montreal, Canada (now CDN Isotopes, Point-Claire, Canada), while T, 19-nortestosterone, and methyl-testosterone were obtained from Sigma–Aldrich (St. Louis, MO, USA). The charcoal filtered human serum was obtained from American Biology Inc. (San Antonio, TX, USA).

2.2. Samples

The blood samples were obtained from 38 healthy male subjects participating in studies concerned with estimating production rate and plasma clearance of testosterone by infusing trideuterated T. The study was approved by the Human Subjects Committee and all subjects gave written informed consent. The d_3T dose was calculated for each subject based on a 24-h T production rate of 7 mg/1.7 m^2 of body surface area [5]. Ten percent of this dose was administered as a bolus at time zero, and the remainder was infused at constant rate for 24 h. At time zero a baseline blood sample was drawn before the bolus dose was administered. Subsequent blood samples were drawn at times 3, 4, 5 and 12 h. The total number of blood samples was 190 (38 subjects×5 samples each). The serum tubes were allowed to stand for about 15–30 min and then centrifuged at 1000 *g* for 15 min at 4 °C and stored at −20 °C until assayed. The T production rates of

the subjects will be presented in a separate publication.

2.3. Sample preparation procedure

Fifty microliters of 0.2 ng/μl 19-nortestosterone in methanol–water (70:30, v/v) solution was added to 2 ml of serum, followed by 2 ml of saturated sodium acetate buffer (0.5 *M*, pH 5.5) and 5 ml of diethyl ether. The mixture was vortexed, shaken for 10 min, and centrifuged for 10 min. The ether layer was transferred to a clean tube and evaporated to dryness under nitrogen. The extract was reconstituted in 100 μl of methanol, vortexed, centrifuged for 10 min, and the supernatant transferred to 2-ml vials with inserts. Fifteen microliters was injected into the LC–MS–MS system.

2.4. Liquid chromatography–mass spectrometry

An LC-10A binary pump LC (Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with a Series 200 autosampler (Applied Biosytems, Foster City, CA, USA), was used for the analysis. The LC was controlled by Applied Biosystems-Sciex software. The column was a Hypersil BDS C_{18} (Keystone, Bellefonte, PA, USA) (150×2.1 mm I.D., 3 μm). Gradient elution was used at room temperature. Solvent A was 0.1% formic acid and solvent B was methanol. The gradient program began with 50% B for 0.5 min, ramped to 90% B at 9 min returned to 50% of B in 1 min, and was held for 6 min. The flow-rate was 0.2 ml/min.

An Applied Biosystems-Sciex API 300 triple quadrupole mass spectrometer (Applied Biosystems-Sciex, Thornhill, Ontario, Canada) coupled to a liquid chromatograph and equipped with an APCI interface was used to perform the analysis. MS–MS spectra were recorded in the positive mode using the Turbo-Ion spray interface. The ion-spray voltage was set to 5000 V, the orifice and ring voltages were 25 and 180 V, the collision energy was 28 eV and the collision gas was nitrogen. The spectra were recorded from *m*/*z* 50 to *m*/*z* 300 with a dwell time of 1 ms.

The LC–MS–MS was operated in the positive ion mode using a corona charge current of 2 mA. The collision gas and energy were nitrogen and 28 eV, respectively. The temperature of the heated nebulizer was 350 °C and the protonated molecular ions [M–H^+] were used as parent ions.

Testosterone (T) was monitored by using transition *m*/*z* 289 to *m*/*z* 97, trideuterated T, transitions *m*/*z* 292 to *m*/*z* 97 and *m*/*z* 292 to *m*/*z* 109, and 19-nortestosterone, transition *m*/*z* 275 to *m*/*z* 109. The dwell time was 400 ms. The MS–MS experiments were run with a resolution of 0.8 u for both the first and third quadrupole. Integration of the peaks of analytes and internal standard, calibration curves, and unknown sample concentration computations were performed by MacQuan version 1.6 software from Applied Biosystems-Sciex. The statistical computations were performed with SAS system release 8.0 (SAS Institute Inc. Cary, NC, USA).

2.5. Calibration and quantitation

The calibration curves were prepared daily by spiking charcoal filtered human serum with the d_3T, T, and I.S. solutions (70% methanol–water mixture) to obtain concentrations in the range of 0.05–2.0 ng/ml and 0.5–20 ng/ml, respectively. They were analyzed on four different days to obtain LOQ data. Three replicates of blank serum were spiked at 0.125 and 0.4 ng/ml of d_3T and 1.25 and 4 ng/ml of T and analyzed on each of 3 days to obtain accuracy and precision data for the method. For the sample analysis, calibration curve samples were prepared each day.

3. Results and discussion

3.1. Chromatography and mass spectrometry

The main goal of the method development was to obtain good sensitivity for d_3T because the expected concentration range was up to 10 times lower then the serum T concentrations. The ideal internal standards for LC–MS or GC–MS analysis are deuterated analogues of analytes. They elute at practically the same retention times as the analytes and undergo the same changes in ion suppression or instrument instability. Unfortunately in our case the most desirable I.S., the trideuterated analogue of testosterone,

was our analyte. The next most desirable I.S., methyl-testosterone, had a response rate that was too low in the conditions that were optimal for T and d_3T. Therefore, we used 19-nortestosterone as I.S. The retention times of the I.S. and analytes were not identical but they were not too far apart (0.5 min), thus we expected ion suppression or instrument instability to affect the I.S. to the same extent as the analytes.

The response factor for 19-nortestosterone was lower than for T and d_3T, therefore the amount added was larger. The MS–MS spectra of testosterone (T), d_3T, and 19-nortestosterone are presented in Fig. 1. The fragmentation of T and d_3T is limited to two prominent fragments, m/z 97 and m/z 109.

The exact fragmentation mechanism is not known but it is safe to conclude that fragments m/z 97 and m/z 109 originated from the A ring. This is because the MS–MS spectrum of $[^2H]_2$ testosterone, in which the deuterium atoms are on the A ring in positions 1 and 2, shows (Fig. 2) similar fragmentation with a shift of 2 m/z units (m/z 99 and m/z 111).

Fig. 3 shows a representative chromatogram from the analysis of one sample from a subject that received d_3T. The transitions monitored were m/z 289→m/z 97 for testosterone, m/z 292→m/z 97 for d3-testosterone, and m/z 275→m/z 109 for 19-nortestosterone. The transition m/z 292→m/z 109 was not used for d_3T because many samples had an interfering peak in the m/z 292→m/z 109 window. The retention times of T, d_3T and 19-nortestosterone were 9.2 (RRT 1.06), 9.2 (RRT 1.06) and 8.7 min, respectively. The shapes of the peaks were satisfactory, the internal standard peak was well resolved from those of the analytes and there were no interferences in the region. Fig. 4 shows a repre-

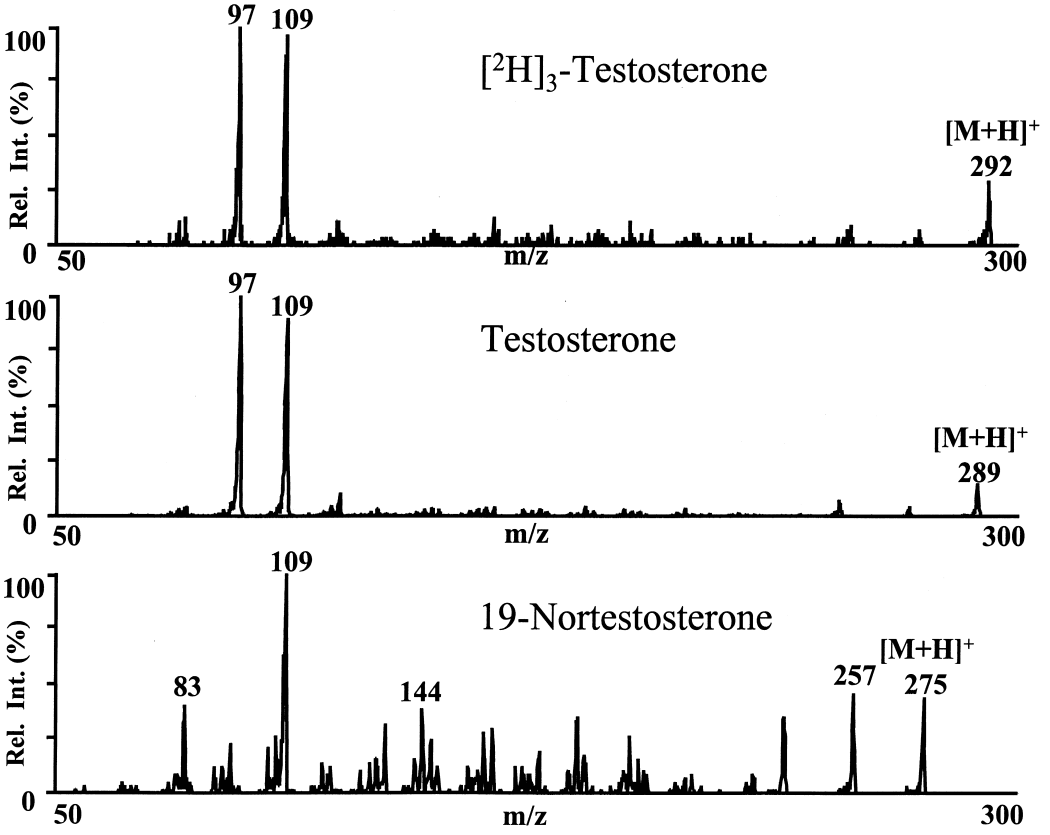

Fig. 1. MS–MS spectra of d_3-testosterone, testosterone and 19-nortestosterone.

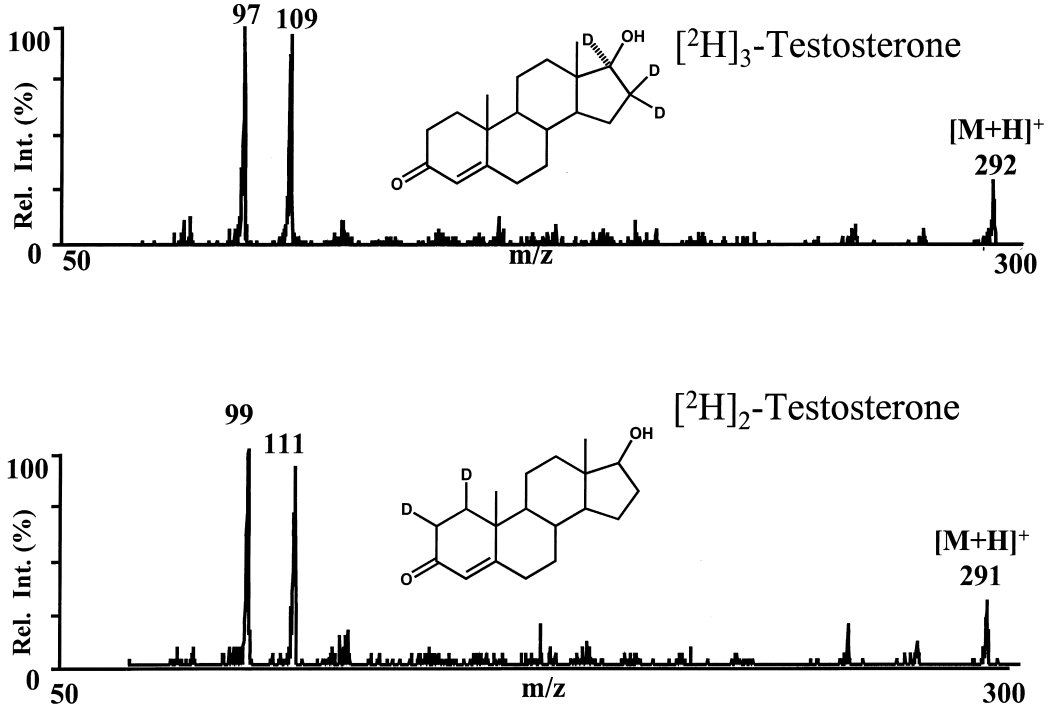

Fig. 2. MS–MS spectra of d_3-testosterone, and d_2-testosterone.

sentative chromatogram from one baseline serum sample obtained from a subject before d_3T was administered. The expected peaks are observed for T and 19- nortestosterone, and the d_3T window contains no peaks.

3.2. Calibration curves

The calibration curves for T and d_3T ware calculated using weighted least-squares linear regression (model: weight=1/conc^2) and they were linear during the validation and sample analysis period. The average slope, intercept, and r^2 for d_3T were 0.7808, 0.0726 and 0.995 respectively, and for T, the average slope, intercept and r^2 were 0.1971, 0.0398 and 0.996.

The LOQs were determined by the Lang and Bolton procedure [17]. The 0.5 ng/ml calibrator was analyzed twice on 4 different days and the mean daily T/I.S. ratio was calculated. The overall mean of the daily means was 0.0638 (SD=0.0197). The overall mean exceeded 3 SDs (0.0638>3×0.0197). In addition, the overall mean was greater than 0 (P=0.0075). This procedure establishes that the LOQ for T is at least 0.5 ng/ml. The 0.05 ng/ml calibrator was analyzed twice on 4 different days and the mean daily d_3T/I.S. ratio was calculated. The overall mean of the daily means was 0.112 (SD= 0.0134). The overall mean exceeded 3 SDs (0.112> 3×0.0134). In addition, the overall mean was greater than 0 (P=0.0075). This procedure establishes that the LOQ for d_3T is at least 0.05 ng/ml. Draisci et al. [9] had an LOQ (0.1 ng/ml) lower than ours (0.5 ng/ml).

3.3. Recovery, accuracy, and precision

The assay recovery was assessed by extracting spiked serum and comparing the result with non-extracted calibrators. The recoveries were 91.5% for T and 96.4% for d_3T. Draisci et al. [9] had a slightly lower extraction efficiency for T (85.3%). The intra-day and inter-day precision and accuracy data for T and d_3T are presented in Tables 1 and 2. Precision was determined by calculating the relative standard deviation (RSD, %) for the repeated measurements

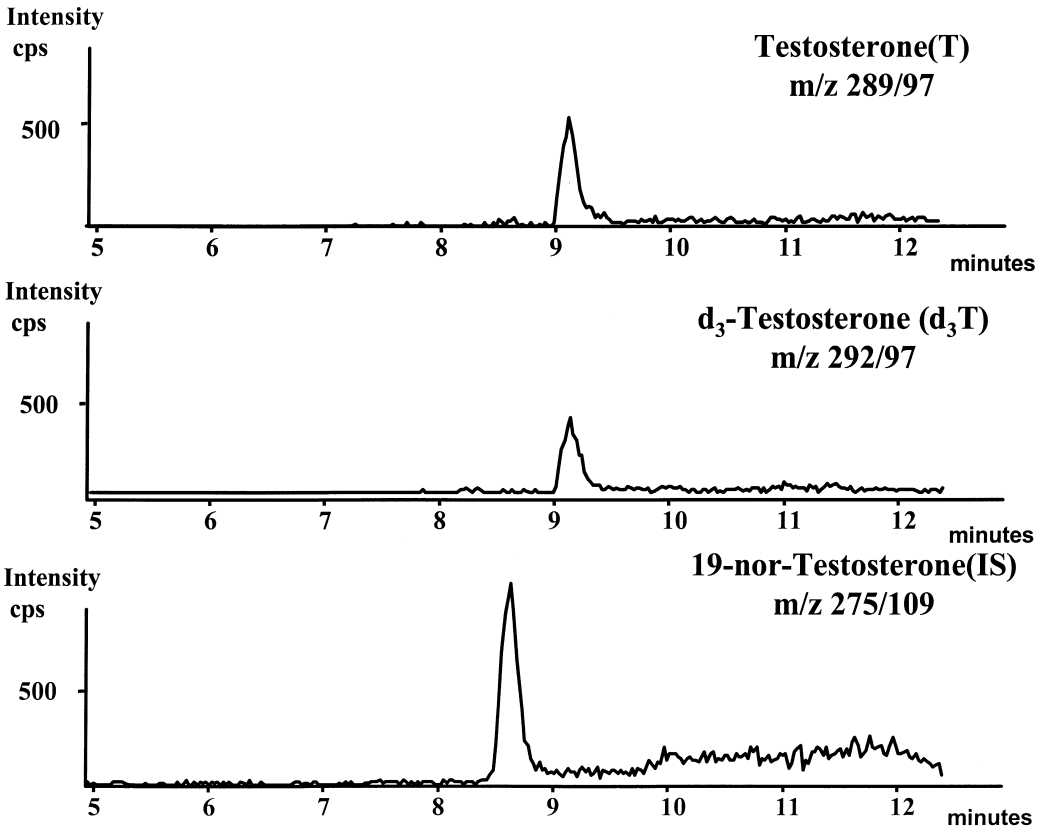

Fig. 3. SRM LC–MS–MS chromatogram of subject serum after administration of d_3T.

and accuracy by calculating the difference between nominal values and measured values for the spiked samples (RE, %). The intra-day precision and accuracy as well as inter-day precision and accuracy were adequate for the purpose of establishing production rates of testosterone.

3.4. Serum concentrations of T and d_3T in the study samples

The mean serum T concentration for all 190 samples was 6.2 ng/ml (range 2.5–14.0 ng/ml). These values are typical for normal healthy males [3]. The mean d_3T serum concentration for the samples collected 3, 4, 5, and 12 h after initiating the infusion was 0.23 ng/ml (range<LOD=0.65 ng/ml, N=80 samples, 20 subjects). Table 3 shows that d_3T is absent in the baseline sample collected at time zero. The 3-, 4-, 5- and 12-h samples show that the steady state is achieved. The T concentration declines slightly during the infusion as expected from prior studies [5].

4. Conclusions

This report described the quantitation of natural serum testosterone and trideuterated testosterone in the same samples by LC–MS–MS. The assay is suitable for supporting clinical studies designed to determine the clearance and production rate of T by infusing d_3T. The advantage of this LC–MS–MS method over GC–MS is a much simpler sample preparation. Immunoassays cannot be used for this application because they do not distinguish between trideuterated and natural testosterone. The assay is a simple and rapid method for simultaneous quantita-

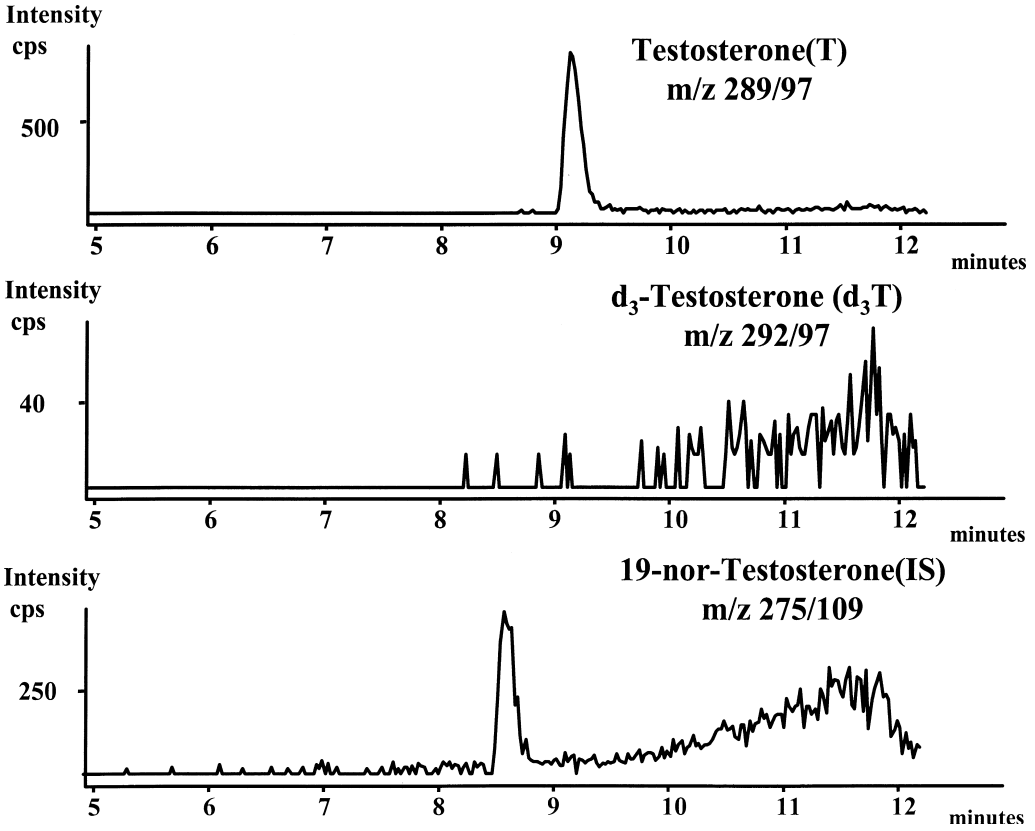

Fig. 4. SRM LC–MS–MS chromatogram of subject serum before administration of d_3T.

Table 1
Intra-day accuracy and precision for T and d_3T in human serum

Analyte	Level (ng/ml)	*n*	Mean (ng/ml)	Precision (RSD, %)	Accuracy (RE, %)
d_3T	0.125	3	0.127	2.8	1.8
	0.400	3	0.422	8.3	5.6
T	1.25	3	1.25	3.9	0.01
	4.00	3	4.18	4.3	4.5

Table 2
Inter-day accuracy and precision for T and d_3T in human serum

Analyte	Level (ng/ml)	*n*	Mean (ng/ml)	Precision (RSD, %)	Accuracy (RE, %)
d_3T	0.125	9	0.131	10.0	5.7
	0.400	9	0.413	7.6	3.4
T	1.25	9	1.27	5.3	1.9
	4.00	9	4.01	5.4	0.3

Table 3
Concentrations of T and d_3T in serum during d_3T infusion of one subject

Time (h)	T (ng/ml)	d_3T (ng/ml)
0	4.2	0.00
3	4.7	0.51
4	4.4	0.55
5	4.3	0.52
12	3.3	0.51

tion of T and d_3T in human serum provided that the LOD for T need not be lower than 0.5 ng/ml.

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