Simultaneous Measurement of Serum Testosterone and Dihydrotestosterone by Liquid Chromatography–Tandem Mass Spectrometry

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BACKGROUND: Recent reports have described inherent problems with androgen immunoassays compared with mass spectrometry analyses.

METHODS: We developed a method for measuring serum testosterone (T) and 5α-dihydrotestosterone (DHT) simultaneously via liquid–liquid extraction followed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) with positive-mode electrospray ionization.

RESULTS: The DHT and T calibrators showed a linear response from 0.069 nmol/L to 34.4 nmol/L and 69.3 nmol/L, respectively. T interference in the DHT assay and vice versa were negligible. Within- and between-run imprecision values were <5% for both analytes. Percent recoveries of T and DHT spiked into samples at concentrations spanning the calibration curve were 100%–113% and 98%–107%, respectively. The lower limit of quantification was 0.069 nmol/L for both steroids. Serum T concentrations measured by LC-MS/MS were different from those obtained by RIA, especially at lower T concentrations. Serum DHT concentrations measured by LC-MS/MS were markedly lower than those generated by RIA because of the non-selectivity of the RIA without chromatography. The reference intervals (mean ± 2 SDs) determined for T and DHT were 9.2–33.7 nmol/L and 0.47–2.65 nmol/L, respectively, for 113 healthy adult men and 0.33–2.02 nmol/L and 0.09–0.91 nmol/L, respectively, for 133 healthy premenopausal women.

CONCLUSIONS: We have developed and validated a selective and precise method for simultaneous measurements of serum T and DHT that can be adopted for routine measurements of these androgens in health and disease in men and women.

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Testosterone (T) and 5α-dihydrotestosterone (DHT) are androgens crucial to the development of male reproductive organs and masculine characteristics in adults. The measurement of serum T is the cornerstone for the diagnosis and management of male hypogonadism and pubertal delay. Measurements of serum DHT and the DHT/T ratio are important for the measurement of androgen action during androgen replacement (1, 2). Use of transdermal scrotal patches and oral testosterone undecanoate treatment produce supraphysiological DHT concentrations (3–6). Because DHT is a more potent androgen than T, increased serum DHT concentrations in theory may cause greater growth of the prostate. The serum DHT concentration and the T/DHT ratio are clinically useful for monitoring 5α-reductase inhibitor treatment of benign prostate hyperplasia and for the prevention of prostate cancer (7, 8), as well as for assessing 5α-reductase deficiency. RIA has been used to measure serum T, yet comparisons of RIAs with liquid chromatography–tandem mass spectrometry (LC-MS/MS) and GC-MS have revealed systematic problems with RIA methods (9–13). Because of precision limitations and bias of the immunoassay methods at low serum T concentrations, these methods cannot be used to measure serum T concentrations in women and prepubertal children (10–12, 14). Methods for measuring serum T by derivatization followed by LC-MS/MS, including a reference method, have been described (15, 16). The

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Nonstandard abbreviations: T, testosterone; DHT, 5α-dihydrotestosterone; D2-T, 1,2-deuterated T; D3-DHT, 19,19,19-trideuterated DHT; LC-MS/MS, liquid chromatography–tandem mass spectrometry.
fact that the serum DHT concentration is only one tenth that of T makes serum DHT quantification more complex (3). RIA methods require extraction and chromatography (17–19) or oxidation of T by potassium permanganate (20, 21). Recently, methods with improved limits of quantification that use liquid- or solid-phase extraction, chromatography, derivatization, and GC-MS or LC-MS/MS have been applied to the measurement of T, DHT, and other androgens in rat tissues (22, 23), human adrenal cell culture medium (24), and human sera and testicular fluid (25, 26). None of the reported methods that measure T and DHT simultaneously have been validated for clinical samples. We report the development and validation of a method for simultaneous analysis of T and DHT in serum that uses simple organic-solvent extraction followed by LC-MS/MS analysis. We established reference ranges for T and DHT in healthy adult males and females, and in comparing RIA and LC-MS/MS methods for both T and DHT, we have revisited some issues regarding immunoassays.

Materials and Methods

SERUM SAMPLES
Serum samples were obtained from 113 healthy male volunteers (18–59 years; body mass index, 19.4–39.6 kg/m²) between 7 and 10 AM at screening sessions before participation in male contraceptive trials. These men had no relevant medical illness and had typical results in the physical examination, blood counts, routine serum chemistry tests, and luteinizing hormone, follicle-stimulating hormone, and T analyses (by RIA). We also recruited 133 healthy female participants (19–49 years; body mass index, 17.5–39.1 kg/m²) with no abnormal medical history, typical physical examination results, and regular menstrual cycles. Blood was collected into 30-mL red-top tubes (no additive) and centrifuged. The separated serum was removed and stored in small aliquots at -20 °C. Samples were obtained from women during the follicular phase of the menstrual cycle. In addition, we used both RIA and LC-MS/MS to analyze banked serum samples from hypogonadal men (19–83 years) that were obtained before treatment (baseline, 36 samples) and after treatment with transdermal T gels (60 samples) or transdermal DHT gels (78 samples) in previously reported studies (21, 27, 28). The study participants had given informed consent that the laboratory could use the collected samples for other research studies. The institutional review board approved the use of residual samples for this validation study. These serum samples were also stored at -20 °C until assayed.

REAGENTS
HPLC-grade ethyl acetate, hexane, methanol, water, and sodium hydroxide were purchased from Fisher Scientific. T and DHT (>99% pure) were purchased from Sigma–Aldrich. 1,2-Deuterated T (D2-T) (>98% pure; Cambridge Isotope Laboratories) was used as the internal standard for T measurements so that the isotopic peak of T due to the natural abundance of 13C would not interfere with the molecular ion of the recovery calibrator. 19,19,19-trideuterated DHT (D3-DHT) was synthesized by Barry Dent (>98% pure) and used as the internal standard for DHT measurements. Batches of steroid-free serum (MP Biomedicals) were treated with charcoal and were verified by LC-MS/MS to have undetectable T and DHT before use. Stock solutions of T (69.3 nmol/L) and DHT (68.8 nmol/L) were prepared in ethanol and diluted with methanol to final D2-T and D3-DHT concentrations of 10.4 nmol/L and 10.3 nmol/L, respectively. We prepared pools for imprecision studies by spiking steroid-free serum with T or DHT at concentrations spanning the low, middle, and high ranges of the calibration curve.

SAMPLE PREPARATION BEFORE LC-MS/MS ASSAY
We pipetted 25 μL of the internal standard solutions containing D2-T (0.26 pmol, 75 pg) or D3-DHT (0.25 pmol, 75 pg) into separate glass tubes (15 × 100 mm) and dried them under nitrogen. Aliquots (100 μL) of blank, calibrators, QC sera, and patient serum samples were then pipetted into the glass tubes, extracted for 2 min by vortex-mixing with 2 mL ethyl acetate/hexane solution (3 and 2 volumes, respectively), and allowed to stand for 10 min. The organic layer was then removed to another glass tube. The extraction procedure was repeated, and the organic phases were pooled. Acidic contaminants in the extract were removed by adding 350 μL of 0.1 mol/L sodium hydroxide to the pooled solvent extracts, vortex-mixing the samples for 2 min, allowing the tubes to stand for 15 min, transferring the organic layer to another glass tube, and drying under nitrogen. The dried sample extracts were reconstituted with 100 μL of an aqueous solution of 250 mL/L methanol and 26 mmol/L formic acid and transferred into 1.5-mL vials fitted with a 300-μL insert for LC-MS/MS analysis. We initially tested solid-phase extraction during the development of this method. This procedure was time-consuming, however, and required about 15 mL of solvent, and T and DHT recoveries were about 50% of those obtained with the simple liquid–liquid extraction method.

LC-MS/MS
T and DHT analysis was conducted with a Shimadzu SCL-10Avp system controller, 2 Shimadzu LC-
10ADVP HPLC gradient pumps, and a Shimadzu DGU-14A online degasser attached to an Applied Biosystems API 5000 LC-MS/MS instrument. For the simultaneous detection of T and DHT, we used a Thermo Hypersil GOLD column (100 mm × 1 mm, 3 μm) with a gradient profile at a flow rate of 0.045 mL/min. Mobile phase A was methanol, and mobile phase B was an aqueous solution of 20 mL/L methanol and 26 mmol/L formic acid. The gradient profile was initially set to 35% A, ramped to 100% A over 12.5 min, held at 100% A for 5 min, and reequilibrated back to 35% A for 1 min. To analyze T and DHT, we used the turbo ion spray with electrospray ionization in the positive mode. The precursor/product ions for T and D2-T were monitored at 289.2/109.0 m/z, respectively; those for DHT and D3-DHT were monitored at 291.2/110.9 m/z, respectively.

PREPARATION OF CALIBRATORS
The concentrations of the calibrators in steroid-free serum were 0.035, 0.069, 0.173, 0.347, 0.867, 1.734, 10.40, 34.67, and 69.34 nmol/L for T assays and 0.034, 0.069, 0.172, 0.344, 0.861, 1.72, 3.44, 10.33, and 34.43 nmol/L for DHT assays. We used Analyst® software (Applied Biosystems) to construct the calibration curves from the known calibrator concentrations and the ratio of the analyte peak area to the area of the internal standard peak.

LC-MS/MS ASSAY VALIDATION
The LC-MS/MS method was validated according to the guidelines and best practices for chromatographic assays set forth by the US Food and Drug Administration (29,30). Ion-suppression studies were conducted with the postcolumn infusion method (31–33). A continuous infusion of T, D2-T, DHT, and D3-DHT at 1.734 nmol/L was introduced into the postcolumn effluent at a rate of 10 μL/min before it entered the mass spectrometer. We recorded the changes in the tandem mass spectrometry response of the respective multiple reaction monitoring transition to the injection of solvent (n = 3) and extract from steroid-free serum (n = 3) into the LC-MS/MS instrument.

RIA
T analysis by RIA was performed with a Coat-A-Count kit from Diagnostic Products Corporation (12,34). The DHT RIA was performed with a kit from Diagnostic Systems Laboratories as previously described (21). Results

T AND DHT CHROMATOGRAMS
Sample chromatograms of the T and DHT analyses of serum samples from a male volunteer and a female volunteer are shown in Fig. 1. The resolution of the peaks demonstrated that there were no interfering peaks. The overall run time was 14–16 min.

CALIBRATION CURVES, LINEARITY, AND PARALLELISM
The T and DHT calibration curves demonstrated linearity (r2 > 0.999 for both). Regression analyses of T calibration curves for 25 consecutive assays showed a mean (SD) slope of 0.0067 (0.0004), a mean y intercept of 0.0138 nmol/L (0.0014 nmol/L), and a negligible SD of residuals (2.08 × 10⁻¹⁶). The DHT calibration curves for 25 consecutive assays showed a mean (SD) slope of 0.0089 (0.0004), a mean y intercept of 0.0029 nmol/L (0.0020 nmol/L), and a negligible SD of residuals (1.02 × 10⁻¹⁶).

To test whether T and DHT in serum behaved in parallel with the calibrators, we used steroid-free serum to serially dilute serum samples with T and DHT concentrations spanning the low, medium, and high ranges of the calibration curve and then analyzed the diluted samples. The results showed that diluting 3 serum samples containing T concentrations of 0.94, 1.08, and 35.71 nmol/L with 1–7 volumes of steroid-free serum yielded mean T recoveries of 97%–100.4%. Similarly, 3 pooled samples with serum DHT concentrations of 0.90, 3.45, and 10.58 nmol/L diluted with 1–7 volumes of steroid-free serum yielded mean DHT recoveries of 94.5%–100.6%.

IMPRECISION
We evaluated intraassay (within-run) imprecision by analyzing 30 replicate sets of samples with T and DHT concentrations at the low, medium, and high ranges of the calibration curve, and we assessed interassay (total) imprecision by analyzing replicate sets of samples with T and DHT concentrations at the low, medium, and high ranges of the calibration curve over 25 separate runs. The calculated within-run CV for all pools was approximately 3%, and the total CV over the 25 assays was <5% (Table 1).

RECOVERY
In 5 different experiments, the mean concentrations of T and DHT in assays of steroid-free serum with no added steroid were 0.016 nmol/L and 0.0024 nmol/L, respectively. Recovery was defined as the amount measured in the assay expressed as a percentage of the added amount. Mean recoveries in T assays for samples containing 0.069–69.3 nmol/L T were 100%–110% (CVs, 1.2%–5.7%); see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol54/issue11. A 21% CV value was associated with the lowest spiked T concentration (0.035 nmol/L); the mean recovery at this concentration was 113%. Mean recoveries in DHT
assays for samples containing 0.034–34.4 nmol/L DHT were 98.5%–106.9% (CVs, 1.6%–12%); see Table 1 in the online Data Supplement.

LOWER LIMIT OF QUANTIFICATION
The lower limit of quantification, defined as the smallest quantity that can be reproducibly measured with a CV of <20% and a signal-to-noise ratio of >5 in the chromatogram, was 0.069 nmol/L for both T and DHT (see Table 1 in the online Data Supplement).

INTERFERENCE, ION-SUPPRESSION, AND STABILITY STUDIES
We performed selectivity (specificity) studies by running dilutions of potential cross-reacting steroids (androgens, progestins, and estrogens) at concentrations up to 100-fold greater than the concentration of the highest calibrator (0.35–3500 nmol/L). In the T assay, the interference was 0.5% for androstenedione, 0.009% for 17α-hydroxyprogesterone, and 0.004% for DHT; interference was not detectable for progesterone, estradiol, dehydroepiandrosterone, cortisol, and cholesterol. In the DHT assay, the interference was 0.056% for 3α-androstenediol, 0.03% for T, and not detectable for all of the other steroids tested.

Table 2 in the online Data Supplement shows the interference caused by adding T to 34.7 nmol/L in serum samples with various DHT concentrations and by adding 34.4 nmol/L DHT to serum with various T concentrations before the T assay. The data showed that the interference was negligible and not clinically significant. The only exception was at a DHT concentration of 0.0067 nmol/L, for which the addition of 34.7
nmol/L T (i.e., 5000-fold more T than DHT) caused a 33.4% increase in the measured DHT concentration, which could be due to the imprecision of measurement at such a low DHT concentration or to interference by the very high relative T concentration.

We evaluated the potential for chemical interference by over-the-counter drugs and by additives used in blood-collection tubes. There was no interference by ascorbic acid, salicylic acid, or acetaminophen. Blood samples collected into red-top tubes (no additive) and into tubes containing EDTA, citrate, or heparin anticoagulant exhibited no significant differences in T and DHT values (data not shown). Tubes containing fluoride lowered measured serum T and DHT concentrations by a mean of 18%–21% (n = 11005) in LC-MS/MS assays.

Ion-suppression studies were conducted to test for potential matrix effects. We compared and measured the changes in the baseline response of the respective multiple reaction monitoring transition for T, D2-T, DHT, and D3-DHT to the injection of solvent and extract from steroid-free serum into the LC-MS/MS. Ion suppression was not present for T, DHT, and their respective isotopes at the respective retention times of T and DHT (see Fig. 1 in the online Data Supplement).

We evaluated sample stability by studying the effects of 10 freeze and thaw cycles on serum samples stored at −20 °C. Serum T and DHT concentrations measured over multiple freezing and thawing cycles showed CVs of <5% (2.2%–4.9% for T, 3.3%–4.7% for DHT).

### Table 1. Intraassay (within-run) and interassay (total) imprecision for T and DHT assays.a

<table>
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<th>T, nmol/L</th>
<th>CV, %</th>
<th>DHT, nmol/L</th>
<th>CV, %</th>
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<tr>
<td>Within-run imprecision (30 replicates)</td>
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<tr>
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<td>9.50 (0.30)</td>
<td>3.1</td>
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</table>

*Data are presented as the mean (SD). Concentrations are based on a molecular mass of 288.43 for T and 290.44 for DHT. When samples from female individuals were assayed, a QC serum with extra-low T and DHT concentrations (approximately 0.86 nmol/L) was included in each run (total imprecision <5%).

### Fig. 2. Comparison of serum T concentrations measured by LC-MS/MS and RIA.

(A), Deming regression. (B), Bland–Altman plot. y axis represents the difference in serum T concentrations obtained with the 2 assays (LC-MS/MS - RIA), and the x axis represents the mean of the serum T concentrations measured by LC-MS/MS and RIA [i.e., (LC-MS/MS + RIA)/2].

### COMPARISON OF LC-MS/MS AND RIA METHODS

For comparison studies of T methods, we analyzed 145 patient samples by both LC-MS/MS and RIA. Deming regression analysis showed the following relationship: 

\[
\text{LC-MS/MS} = 1.06(RIA) - 20.6 \text{ nmol/L} (r^2 = 0.9785; \text{Fig. } 2A)\]

Bland–Altman plots (35–37) showed that the mean (SD) difference (i.e., LC-MS/MS – RIA) between the 2 methods was −0.15 nmol/L (0.11 nmol/L) (Fig. 2B). The mean ratio of T concentrations measured by RIA to those obtained by LC-MS/MS was 1.06, with large variation in the ratio (from 0.5–2.5) occurring at low T concentrations (data not shown).

We similarly compared the 2 methods for DHT analysis. We measured DHT in 187 patient samples by...
LC-MS/MS and by RIA with potassium permanganate oxidation of T. Serum DHT concentrations measured by LC-MS/MS and RIA demonstrated a lower correlation than the T assays \( r^2 = 0.8649; \) \( LC-MS/MS = 0.590(\text{RIA}) + 0.275 \text{nmol/L} \) (Fig. 3A). Bland–Altman plots showed serum DHT concentrations measured by LC-MS/MS were consistently lower than those obtained with the RIA [mean (SD), 2.4 nmol/L (3.5 nmol/L)]. The difference in DHT concentration between the 2 methods increased with the concentration of DHT in the sample (Fig. 3B). The mean ratio of the DHT concentration measured by RIA to that measured by LC-MS/MS was 2.01; the ratio varied greatly (1–4.8) at low serum DHT concentrations (data not shown).

REFERENCE INTERVALS FOR SERUM T AND DHT IN HEALTHY MEN AND WOMEN
To incorporate the skewness of the T and DHT distributions, we evaluated the limits of the reference intervals as antilogarithms of the mean ± 2 SDs of the log-transformed serum T and DHT concentrations in samples from healthy adult volunteers. For adult males, the reference intervals established from 113 healthy volunteers were 9.2–33.7 nmol/L for T and 0.47–2.65 nmol/L for DHT. The reference intervals for serum T and DHT concentrations in 133 adult females (follicular phase) with typical menstrual histories were 0.33–2.02 nmol/L for T and 0.09–0.91 nmol/L for DHT. The distributions of serum T and DHT concentrations in healthy individuals are shown in Fig. 4.
Discussion

We developed and validated an LC-MS/MS method for the simultaneous measurement of serum T and DHT that is suitable for use in a clinical laboratory. We showed that the interference of T in the DHT assay and vice versa were small for concentrations within the anticipated clinical ranges and were not clinically relevant. Furthermore, our validation studies demonstrated both total and within-run CVs were <5% and yielded T and DHT recoveries of 105% and 103%, respectively, with CVs of <15% throughout the calibration curve.

Our method is novel because we achieved equivalent limits of quantification for DHT and T by means of a simple liquid–liquid extraction method, without derivatization. Previous reported mass spectrometry methods for serum T and DHT require larger sample volumes (750 μL), extraction, and column chromatography followed by derivatization before analysis (22, 38). Such methods may not be suitable for routine analysis of large numbers of samples. In another study, LC-MS/MS coupled with in-line C18 solid-phase extraction was used to measure DHT and T in cell culture medium. The limit of quantification for this method was 0.17 nmol/L for T and 3.34 nmol/L for DHT (24). The lower limit of quantification of the present study was 2-fold lower for T and 50-fold lower for DHT. Another LC-MS/MS method, which was designed to simultaneously measure the testicular interstitial fluid concentrations of T, DHT, estradiol, and 3α-androstanediol, used 20 μL of testicular fluid, which had high concentrations of androgens. The lowest calibrator concentration was 0.35 nmol/L for T and 0.068 for DHT (25). Increased sensitivity in the picomole (15) or femtomole (26) range with the LC-MS/MS method for both T and DHT required derivatization of T and DHT to oximes before chromatography. Introduction of a derivatization step in the method could increase measurement error and the time required to complete the assay. The use of the API 5000 LC-MS/MS system in our method provided the required ability to detect clinically relevant low T and DHT concentrations in men without the derivatization or purification step required of a solid-phase system. The lower limit of quantification of 0.069 nmol/L for both T and DHT in our method was sufficiently low to quantify T and DHT concentrations in serum samples from female individuals, allowing us to generate a reference interval for T and DHT from 133 healthy female volunteers. We recognize that the sample size may not be adequate to fully define the reference intervals for men and women at various ages, as in a prior report (15), and to determine whether the female reference intervals vary with the phase of the menstrual cycle (39–43). The ability to measure serum DHT at such low concentrations will allow future studies to define the role of DHT in females.

The results of our comparison of the LC-MS/MS and solid-phase RIA methods concurred with those of previous studies (10–12). Despite an excellent correlation between LC-MS/MS and RIA methods, the serum T values obtained with the 2 methods were different at relatively low T values, as has previously been reported (10–12, 14). There have been very few comparison studies of DHT methods. In a recent report, Hsing et al. (38) compared RIA and mass spectrometry methods for assessing assays of 12 sex steroids in human sera. T and DHT were measured with good imprecision by RIA after Celite chromatography and by GC-MS after thin-layer chromatography and derivatization. RIA measurements of T and DHT, as well as other steroids, were consistently higher than those obtained by mass spectrometry. Mean serum T and DHT concentrations obtained by GC-MS were about 87% and 75%, respectively, of the mean concentrations measured by RIA. In our study, analyses of 187 pedigreed samples from eugonadal and hypogonadal men before and after DHT treatment yielded a lower correlation between the RIA and LC-MS/MS methods for serum DHT than for serum T. Serum DHT concentrations measured by LC-MS/MS were on average only 58.6% of those obtained with RIA. The difference between the 2 methods in serum DHT concentration increased with the absolute DHT concentration. The large differences between the 2 methods shown in our study may have been exaggerated by the very high supraphysiological DHT concentrations observed in samples obtained from men receiving treatment with exogenous DHT (21). The discrepancy between the DHT values measured by LC-MS/MS and RIA might be due to the lack of specificity of the DHT antibody in the RIA method and the potassium permanganate treatment possibly not having removed interfering substances other than T.

The novelty of our approach lies in the ability to simultaneously measure T and DHT, even at very low serum concentrations of these steroids. In addition, the assay’s characteristics of a sample-preparation procedure that does not use solid-phase extraction and derivatization while still achieving the desired specificity and detection limits may allow this method to be easily adopted for routine use by clinical chemistry laboratories.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design,
acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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Consultant or Advisory Role: None declared.

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