Performance Characteristics of a Novel Tandem Mass Spectrometry Assay For Serum Testosterone

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Background: Commercial immunoassays for testosterone (Te) may give inaccurate results for samples from women and children, leading to misdiagnosis and inappropriate treatment. We developed a sensitive and specific tandem mass spectrometric assay for measurement of Te at the concentrations encountered in women and children.

Methods: Te was extracted with methyl tert-butyl ether from 100 μL of serum or plasma, derivatized to form an oxime, and reextracted by solid-phase extraction. Instrumental analysis was performed on an API 4000 HPLC tandem mass spectrometer in the multiple-reaction monitoring (MRM) mode. The MRM transitions (m/z) were 304→124 and 304→112 for Te and 307→124 and 307→112 for d3-Te.

Results: Within- and between-run CVs were <12% and 7.9%, respectively. The limit of quantification was 0.0346 nmol/L (1 ng/dL). Reference intervals for sex hormone–binding globulin and total, free, and bioavailable Te were established for children of Tanner stages 1 through 5 and adult males and females.

Conclusions: The sensitivity and specificity of the method are adequate for analysis of Te in samples from women and children. The method requires small sample volumes, has adequate precision, and is not subject to interferences.

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Testosterone (Te) is the major androgen in males and is involved in development and maintenance of the male phenotype. In women, Te is also the predominant bioactive androgen, and circulating concentrations are 5%–10% of those in men. Te is important for many non–sex-specific functions, including growth, bone metabolism, and bone remodeling. A sensitive and specific assay for measurement of Te is needed in many clinical applications. In women, Te is frequently measured in cases of alopecia, acne, and hirsutism; for detection of androgen-secreting tumors of ovarian and adrenal origin; and to determine the minimum drug dose required to suppress androgen secretion in hyperandrogenic women (1–6). In children, the circulating Te concentration is determined for sex assignment of newborns and young infants with ambiguous genitalia, and in follow-up of children with precocious or delayed puberty (7–13). Te is also closely monitored in the follow-up of patients with congenital adrenal hyperplasia resulting from deficiency of 21-hydroxylase or other enzymes (14). Other clinical applications of Te measurement include evaluation of inborn errors of sex-steroid metabolism, disorders of puberty, and Te deficiency and therapeutic drug monitoring in cases of low-dose female hormone replacement therapy or antiandrogen treatment in some forms of cancer (5, 9, 11–15).

Immunoassays for Te have acceptable performance for concentrations characteristic of healthy men but lack specificity and precision at concentrations characteristic of women and children (16–18). Even high-sensitivity Te
immunoassays are inadequate for testing samples from women and children because physiologic serum concentrations are typically <1.73 nmol/L (<50 ng/dL) in adult women and <0.346 nmol/L (<10 ng/dL) in infants and children of both sexes. Taieb and coworkers (17, 18) reported that none of the commercially available automated assays for Te has adequate specificity for the analysis of Te in the serum of children and women. In addition, there is often very poor agreement among the results obtained by different immunoassays, even assays from the same manufacturer (17–26).

Numerous studies found acceptable concordance between immunoassay results for samples from men (27–29) but significant overestimation of the Te concentration in samples from women and children (19, 25, 30, 31). To eliminate interference from binding globulins and structurally related molecules, RIAs have been combined with extraction and chromatographic separation, but these assays do not compare well with isotope-dilution mass spectrometry (MS) (18, 32). Because interference is less likely in gas chromatographic (GC)-MS than in nontargeted methods, GC-MS is often used as a reference method for steroid analysis (35–38). GC-MS methods typically require at least 1 mL of sample, however, and their throughput is limited.

We developed a sensitive and specific liquid chromatography–tandem MS (LC-MS/MS) assay to measure Te at the concentrations encountered in women and children and used isotope-dilution MS to establish reference intervals for males and females of different Tanner stages and ages.

**Materials and Methods**

**CALIBRATORS AND REAGENTS**

Te and d5-Te (both 98% purity) were purchased from Sigma. Stock solutions were prepared in methanol at concentrations of 3.46 mmol/L (1 g/L) and 0.346 mmol/L (0.1 g/L) for Te and d5-Te, respectively. Working calibrators and internal standards were prepared at concentrations of 8.65 and 17.3 nmol/L (2.5 and 5 μg/L), respectively. With every set of samples, Te calibrators were prepared in 10 g/L bovine serum albumin at concentrations of 0.346, 1.73, 3.46, and 6.92 nmol/L (10, 50, 100, and 200 ng/dL). Controls analyzed with every batch of samples were human plasma samples containing 0.415 and 7.23 nmol/L (12 and 209 ng/dL) Te, respectively. HPLC-grade water, methanol, methyl tert-butyl ether (MTBE), and acetonitrile were obtained from Fisher Scientific. Hydroxylamine hydrochloride, sodium hydroxide, trifluoroacetic acid, and formic acid were obtained from Sigma. Strata X solid-phase extraction (SPE) columns were purchased from Phenomenex.

**SAMPLE PREPARATION**

We transferred 0.1-mL portions of calibrators, controls, and patient serum to microcentrifuge tubes and added 20 μL of a working internal standard, d5-Te, to each tube. We then extracted the samples with 1.5 mL of MTBE and transferred the upper organic layer to clean glass tubes. After evaporating the solvent under nitrogen in a 35 °C water bath, we redissolved the dried residue for derivatization in 300 μL of an aqueous hydroxylamine solution (1.5 mol/L, pH 10), vortex-mixed the tubes, incubated them in a heating block at 60 °C for 5 min, and added 2 mL of water to each tube. The derivatized Te was extracted with SPE as follows. The SPE columns were conditioned with methanol followed by water, and the samples were applied to the columns. After the columns were washed with 200 mL/L acetonitrile in water and dried for 10 min, Te was eluted with MTBE. The solvent was evaporated and the residue reconstituted with 75 μL of mobile phase; the samples were then transferred to autosampler vials, sealed, and assayed.

**LC-MS/MS**

We validated the method on an API 4000™ triple-quadrupole mass spectrometer (Applied Biosystems/MD Sciex) equipped with a V-spray ionization source operated at 700 °C. The system included an Agilent HPLC pump series 1100 and a Perkin-Elmer series 200 autosampler. The chromatographic separation was performed on a 50 × 2.0 (i.d.) mm Phenomenex Luna C18 column with 5-μm particles. The oven temperature was 50 °C. The mobile phase consisted of methanol–0.22 mol/L aqueous formic acid (70:30 by volume) and was delivered at a flow rate of 0.25 mL/min. The injection volume was 15 μL, and the total instrumental analysis time was 3 min. Between injections, the autosampler injection syringe was washed 12 times with methanol–water (4:1 by volume) containing 1 mL/L trifluoroacetic acid. The quadrupoles Q1 and Q3 were tuned for unit resolution, and the MS conditions were optimized for maximum signal intensity. The instrument was operated in positive-ion mode with the following optimized voltages: ion spray, 4200 V; declustering potential, 85 V; entrance potential, 10 V; collision energy, 40 V; collision cell exit potential, 5 V. Nitrogen was the collision gas. The data were acquired and processed with Analyst 1.4™ software (Applied Biosystems/MD Sciex). We performed a quantitative calibration with every batch of samples and used these results in conjunction with the intensities of the transitions of internal standards to calculate Te concentrations in unknown samples. We used an API 3000™ (Applied Biosystems/MD Sciex) triple-quadrupole mass spectrometer to study relative sensitivity among different ion sources and for initial evaluation of the method performance.

**ASSAY PERFORMANCE CHARACTERISTICS**

Evaluation of the method performance included imprecision, limit of detection (LOD), limit of quantification (LOQ), upper limit of linearity, and recovery. Method imprecision was determined from analysis of 2 human serum samples analyzed as controls [concentrations of 0.415 and 7.23 nmol/L (12 and 209 ng/dL)]. The samples...
were analyzed in duplicate on 20 consecutive days. In addition, the imprecision of the method was determined by analyzing 3 replicates per run of 10 g/L bovine serum albumin samples containing Te at concentrations of 0.139, 0.866, 2.43, 10.38, and 20.18 nmol/L (4, 25, 70, 300, and 700 ng/dL) over a 3-day period. The instrument imprecision was determined by repetitive injections of an extracted patient sample containing 11.0 381 nmol/L (11 ng/dL) Te from the same vial. Method linearity was evaluated by analyzing calibrators prepared at 0.35, 28, 55.6, 83.2, 110.8, and 138.4 nmol/L (10, 800, 1600, 2400, 3200, and 4000 ng/dL). Method sensitivity was determined by analyzing calibrators containing progressively lower concentrations of Te. According to accuracy criteria, we maintained accuracy within 15% and imprecision (CV) at <20% and used a branching ratio of the mass transitions within 30% of the value set by the calibration to determine the upper limit of linearity of the assay. The branching ratio is the ratio of peak intensities of 2 transitions of Te (304→112/304→124) and $d_3$-Te (307→112/307→124), used to assure specificity of the detection (39). The branching ratios were compared with the ratios of the same transitions in the calibrator containing 1.73 nmol/L (50 ng/dL) Te. The LOQ was the lowest concentration at which Te peaks were present in both transitions at the expected retention time with a branching ratio within established limits and the observed concentration was within ±20% of the expected concentration. The LOD was the lowest concentration at which Te peaks were present in both transitions at the expected retention time. Each calibrator was analyzed in duplicate over a 2-day period. Recovery of the sample preparation was determined by analyzing a patient sample containing 1.71 nmol/L (35 ng/dL) endogenous Te enriched with 1.73 nmol/L (50 ng/dL) Te. The observed difference in the concentrations was compared with the expected concentration.

To determine cross-reactivity, we analyzed steroids with structures similar to that of Te, derivatized and extracted according to the method. We also assayed more than 3000 random patient samples to detect potential drug- or disease-related interferences. A branching ratio of the transitions outside of established limits, broadening of the Te peak, split peaks, or a significant increase in background were interpreted as potential interferences.

We evaluated ion suppression by analyzing extracted samples from females, injected in the flow of a solution of derivatized Te prepared at a concentration of 14 nmol/L (400 ng/dL) and infused with a syringe at flow rate of 1.2 mL/h. The patient samples were prepared by the method outlined above. A decrease in the intensity of the baseline in the mass transitions of Te was considered a criterion for evaluation of ion suppression.

### RESULTS

The LC-MS/MS method was compared with a high-sensitivity automated immunoassay for Te (Vitros ECI; Ortho-Clinical Diagnostics), LC-MS/MS at a commercial reference laboratory (Esoterix Inc., Austin, TX), and GC-MS (27). For method comparison with the immunoassay, we analyzed all samples collected for the adult reference interval studies. For the comparison with the LC-MS/MS and GC-MS methods, we analyzed subsets of 20 and 15 serum samples, respectively. To account for imprecision in both the reference and the evaluated methods, we evaluated the results with Deming regression (41).

### EVALUATION OF THE METHOD

We studied the ionization efficiency of nonderivatized Te in positive-ion mode with mobile phase containing various solvents and buffers with electrospray ionization (ESI), heated nebulizer [atmospheric pressure chemical ionization (APCI)], and atmospheric pressure photoionization (APPI) ion sources on an API 3000 tandem mass spectrometer. Samples containing 34.7 nmol/L (1000 ng/dL) Te were injected in mobile phase (flow rate, 250 μL/min). The [M+H]$^+$ molecular ion of underivatized Te was observed with each ionization method. The absolute signal intensity did not change significantly in the experiments with different ion sources, but the signal-to-noise ratio of the [M+H]$^+$ molecular ion of Te was highest with the APPI, followed by the APCI and ESI ion sources (Fig. 1). In experiments with the APPI ion source, we used toluene as a dopant in an amount that was 10% of that of the other mobile phase components.

Evaluation of the method sensitivity with the APPI ion source showed insufficient sensitivity for the intended
clinical application of measuring Te in samples from women and children. We therefore evaluated various derivatizing reagents to improve the ionization efficiency and decrease the LOD. The oximation reaction with hydroxylamine greatly increased sensitivity and was adopted for the method. Hydroxylamine reacts with keto groups to form oxime derivatives. Comparison of the detection sensitivity for the derivatized Te in positive-ion mode showed the highest sensitivity with the ESI, followed by the APPI and APCI ion sources (Fig. 1). Evaluation of the method performance on API 3000 and API 4000 tandem mass spectrometers (using conditions optimal for each instrument and oxime derivative of Te) showed a sensitivity ~8-fold higher than that of the API 4000. Ionization was more efficient when the ammonium formate buffer was replaced with formic acid (Fig. 1).

Because the sample preparation is specific to neutral molecules containing keto groups, LC separation plays a lesser role in the method. We evaluated the potential for ion suppression by injecting extracted samples with Te concentrations <0.346 nmol/L (10 ng/dL) in the flow of Te oxime derivative prepared at a concentration of 14 nmol/L (400 ng/dL) infused with a syringe pump at constant flow rate. We observed a decrease in the baseline in the samples at a retention time of 0.5 min; the baseline recovered by 0.8 min, and no ion suppression was observed at the retention time of Te (1.8 min). The chromatograms of 2 multiple-reaction monitoring transitions of Te for calibrators and a patient sample are presented in Fig. 2. We used the calibrator containing 1.73 nmol/L (50 ng/dL) Te to set a threshold for the branching ratio of the Te mass transitions. The acceptance limit for the branching ratio was established in every batch of samples as ±30% of the value observed in the 1.73 nmol/L (50 ng/dL) calibrator.

Within-run, between-run, and total imprecision data are shown in Table 1. The CV at the lowest concentration evaluated, 0.139 nmol/L (4 ng/dL), was 13% and was <8% at concentrations >0.347 nmol/L (10 ng/dL). The total CV for the controls analyzed with routine batches of samples (n = 20) was 9.9% at a concentration of 0.415 nmol/L (12 ng/dL) and 5.1% at 7.23 nmol/L (209 ng/dL). Method imprecision was comparable to methods for other steroids present at similar concentrations (36, 37, 42). For a sample containing 0.346 nmol/L (10 ng/dL) Te, 1.5 pg was injected on the column, and the corresponding mean (SD) signal-to-noise ratio (n = 8) was 32 (5). The overall method recovery was 98%. The assay was linear up to 138 nmol/L (4000 ng/dL) with inaccuracy <5% at the highest
concentration \((r = 0.998)\). The LOQ of the method was 0.0345 nmol/L (1 ng/dL), and the LOD was 0.0173 nmol/L (0.5 ng/dL).

A set of 20 serum samples from female volunteers was analyzed by our method and by an LC-MS/MS method of Esoterix Inc., and a set of 15 samples was analyzed by a GC-MS method (27) (Fig. 3). Deming regression analysis gave a regression line with the equation:

\[ y = 1.01x - 0.09 \text{ ng/dL} \quad (r = 0.953; S_{yx} = 2.86 \text{ ng/dL}). \]

We also compared the method performance with that of the Vitros ECi immunoassay. A total of 216 split patient samples (150 samples from females and 66 samples from males) were analyzed by the methods (in groups of 10 to 35 samples per batch), and the results were compared. When all of the results (samples from men and women) were included in the method comparison with the Vitros ECi method, the Deming regression equation, correlation coefficient, and standard error of the residuals were as follows: Vitros ECi = 1.15 × LC-MS/MS + 11.2 ng/dL \((r = 0.976; S_{yx} = 5.0 \text{ ng/dL})\). The results (Fig. 4) showed substantial disagreement between the methods at concentrations <1.73 nmol/L (50 ng/dL). Agreement between the methods was better for results with higher Te concentrations. The branching ratio of the 2 mass transitions for Te and \(d_3\)-Te in >98% of the patient samples was within the acceptance limits established by the calibrators analyzed with each batch of samples.

Structurally related compounds [methyltestosterone, nandralone, dihydrotestosterone, trans-dehydrotestosterone, trans-androsterone, dehydroepiandrosterone (DHEA), and androstenedione] were evaluated for potential interference with the method. The steroids were enriched to a concentration of 500 000 ng/dL in human serum containing 0.345 nmol/L (10 ng/dL) Te. Nandrolone produced a peak that eluted earlier than Te but did not affect the Te result. Androstenedione has 2 keto groups and produced single \((m/z 302)\) and double \((m/z 317)\) oxime derivatives. DHEA is an isomer of Te that is different from Te by the positions of the keto and hydroxyl groups. DHEA produced peaks at both mass transitions of Te at a retention time of 0.88 relative to Te and did not interfere with the method. The branching ratio of the transitions of DHEA was 8 (0.5) vs 1 (0.1) for Te. Androstenedione has 2 keto groups and produced single \((m/z 302)\) and double \((m/z 317)\) oxime derivatives. A second isotopic peak \((A + 2)\) in the molecular ion of androstenedione inoxime is an isobar of Te that has the same product ions as Te and increased the apparent concentration of Te by \(\sim 0.4\%\). Hemolyzed and lipemic samples were analyzed by the method and did not interfere with the method performance. No difference in Te concentrations or recovery was observed for different collection tubes (serum separator tubes, EDTA, heparin). Stored Te showed no degradation after 4 weeks at refrigerator temperatures and after 12 months at \(-20^\circ \text{C}\). In evaluating carryover potential, we did not detect Te in the negative control analyzed after a sample containing 173 nmol/L (5000 ng/dL) Te.

### Table 1. Method imprecision.

<table>
<thead>
<tr>
<th>Concentration, nmol/L (ng/dL)</th>
<th>CV, %</th>
<th>Within-run</th>
<th>Between-run/day</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.139 (4)a</td>
<td></td>
<td>12</td>
<td>5.9</td>
<td>13</td>
</tr>
<tr>
<td>0.866 (25)a</td>
<td></td>
<td>5.3</td>
<td>5.3</td>
<td>7.5</td>
</tr>
<tr>
<td>2.43 (70)a</td>
<td></td>
<td>2.4</td>
<td>4.7</td>
<td>5.3</td>
</tr>
<tr>
<td>10.38 (300)a</td>
<td></td>
<td>3.3</td>
<td>3.9</td>
<td>5.1</td>
</tr>
<tr>
<td>20.18 (700)a</td>
<td></td>
<td>1.9</td>
<td>2.5</td>
<td>3.1</td>
</tr>
<tr>
<td>0.415 (12)b</td>
<td></td>
<td>6.2</td>
<td>7.9</td>
<td>9.9</td>
</tr>
<tr>
<td>7.23 (209)b</td>
<td></td>
<td>2.3</td>
<td>4.5</td>
<td>5.1</td>
</tr>
</tbody>
</table>

\(a\) Three replicates per run over 3 days.

\(b\) Two replicates per run over 20 days.

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**Fig. 3. Bland–Altman plot of differences between the LC-MS/MS method and the reference LC-MS/MS and GC-MS methods with samples from females.**

The mean difference was 0.2 ng/dL (0.007 nmol/L). The hatched lines represent 2 SE.

**Fig. 4. Bland–Altman plot of differences between the LC-MS/MS method and the Vitros ECi method for samples with concentration <50 ng/dL (1.73 nmol/L; \(n = 150\)).**

The mean difference was 4.1 ng/dL (0.142 nmol/L).
The LC-MS/MS method was used to establish reference intervals for total, free, and bioavailable Te in volunteers of different age and Tanner stage groups (Tables 2 and 3). Nonparametric reference intervals were determined based on the central 95% interval.

**Discussion**

Published LC-MS/MS methods for measurement of Te have sufficient sensitivity and accuracy for analyzing samples from healthy adult men, but are not sufficiently sensitive for measurement of the low concentrations characteristic of females and children (43). Recently, Cawood et al. (32) published an LC-MS/MS method for the routine analysis of Te that had an LOD of 0.3 nmol/L (8.6 ng/dL), which is adequate for the analysis of majority of female specimens but not sufficiently sensitive for measurement of Te in children. We improved the sensitivity of the assay through optimization of the sample preparation, analysis of derivatized Te vs nonderivatized TE [as in Ref. (32)], finding conditions for efficient ionization, and elimination of ion suppression. Because high sensitivity is required, a large number of coextracted compounds could potentially produce background noise and affect method performance. One class of compounds present in biological samples that may cause ion suppression is phospholipids. An efficient way of purifying biological samples, which does not coextract phospholipids, is liquid–liquid extraction with MTBE (44, 45). A combination of liquid–liquid extraction, formation of the Te oxime derivative, and SPE on polymer adsorbent allowed high-sensitivity, selective analysis of Te. Our sample preparation procedure thus removed the majority

**Table 2. Summary of reference interval data for SHBG and total, free, and bioavailable Te by Tanner stage.**

<table>
<thead>
<tr>
<th>Testosterone</th>
<th>Total, nmol/L (ng/dL)</th>
<th>Free, pmol/L (pg/mL)</th>
<th>Bioavailable, nmol/L (ng/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TS 1</td>
<td>147</td>
<td>25–144</td>
<td>0.588 (17) 7.63 (2.2) 0.010–0.190 (0.3–5.5)</td>
</tr>
<tr>
<td>TS 2</td>
<td>66</td>
<td>13–106</td>
<td>0.138–1.35 (4–39) 1.39–15.61 (0.4–4.5) 0.042–0.519 (1.2–15)</td>
</tr>
<tr>
<td>TS 3</td>
<td>85</td>
<td>10–82</td>
<td>0.346–2.07 (10–60) 4.51–26.02 (1.3–7.5) 0.131–0.969 (3.8–28)</td>
</tr>
<tr>
<td>TS 4</td>
<td>77</td>
<td>12–147</td>
<td>0.277–2.18 (8–63) 3.82–53.78 (1.1–15.5) 0.097–1.35 (2.8–39)</td>
</tr>
<tr>
<td>TS 5</td>
<td>123</td>
<td>15–150</td>
<td>0.346–2.07 (10–60) 2.77–31.92 (0.8–9.2) 0.076–0.796 (2.2–23)</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>16</td>
<td>17–100</td>
<td>0.138–0.865 (4–25) 1.39–13.19 (0.4–3.8) 0.038–0.329 (1.1–9.5)</td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TS 1</td>
<td>143</td>
<td>21–144</td>
<td>0.657 (19) 12.84 (3.7) 0.010–0.450 (0.3–13)</td>
</tr>
<tr>
<td>TS 2</td>
<td>66</td>
<td>18–141</td>
<td>0.069–5.16 (2–149) 1.04–72.87 (0.3–21) 0.010–2.04 (0.3–59)</td>
</tr>
<tr>
<td>TS 3</td>
<td>65</td>
<td>11–87</td>
<td>0.242–26.37 (7–762) 3.47–340 (1–98) 0.066–1.24 (1.9–296)</td>
</tr>
</tbody>
</table>

**Table 3. Summary of reference interval data for SHBG and total, free, and bioavailable Te by age.**

<table>
<thead>
<tr>
<th>Testosterone</th>
<th>Total, nmol/L (ng/dL)</th>
<th>Free, pmol/L (pg/mL)</th>
<th>Bioavailable, nmol/L (ng/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7–9</td>
<td>103</td>
<td>31–143</td>
<td>0.519 (15) 6.25 (1.8) 0.010–0.173 (0.3–5.0)</td>
</tr>
<tr>
<td>10–11</td>
<td>69</td>
<td>15–145</td>
<td>0.069–1.45 (2–42) 0.347–12.14 (0.1–3.5) 0.014–0.332 (0.4–9.6)</td>
</tr>
<tr>
<td>12–13</td>
<td>69</td>
<td>10–108</td>
<td>0.208–2.21 (96–64) 3.12–23.60 (0.9–6.8) 0.059–0.650 (1.7–18.8)</td>
</tr>
<tr>
<td>14–15</td>
<td>70</td>
<td>11–106</td>
<td>0.311–1.70 (9–49) 4.16–26.02 (1.2–7.5) 0.104–0.782 (3.0–22.6)</td>
</tr>
<tr>
<td>16–17</td>
<td>70</td>
<td>16–160</td>
<td>0.277–2.18 (8–63) 4.16–34.35 (1.2–9.9) 0.114–0.990 (3.3–28.6)</td>
</tr>
<tr>
<td>18–30</td>
<td>55</td>
<td>18–203</td>
<td>0.381–2.04 (11–59) 2.78–25.68 (0.8–7.4) 0.076–0.713 (2.2–20.6)</td>
</tr>
<tr>
<td>31–40</td>
<td>43</td>
<td>20–126</td>
<td>0.381–1.94 (11–56) 4.51–31.92 (1.3–9.2) 0.142–0.882 (4.1–25.5)</td>
</tr>
<tr>
<td>41–51</td>
<td>18</td>
<td>26–110</td>
<td>0.311–1.90 (9–55) 3.82–20.13 (1.1–5.8) 0.097–0.571 (2.8–16.5)</td>
</tr>
<tr>
<td>50–62 (menopause)</td>
<td>16</td>
<td>18–97</td>
<td>0.208–0.865 (6–25) 2.08–13.19 (0.6–3.8) 0.052–0.325 (1.5–9.4)</td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7–9</td>
<td>105</td>
<td>24–170</td>
<td>0.311 (9) 3.12 (0.9) 0.010–0.097 (0.3–2.8)</td>
</tr>
<tr>
<td>10–11</td>
<td>68</td>
<td>20–133</td>
<td>0.069–0.197 (2–57) 0.35–21.86 (0.1–6.3) 0.003–0.619 (0.1–17.9)</td>
</tr>
<tr>
<td>12–13</td>
<td>69</td>
<td>15–144</td>
<td>0.242–25.85 (7–747) 1.73–340 (0.5–98) 0.048–9.96 (1.4–288)</td>
</tr>
</tbody>
</table>
of compounds that may potentially cause ion suppression and interference.

Preliminary experiments for evaluation of the method with nonderivatized Te (mass transitions of \(m/z\) 289\(\rightarrow\)109 and 289\(\rightarrow\)97) using an ESI ion source demonstrated a LOQ similar to that described by Cawood et al. [0.346 nmol/L (10 ng/dL)] (32). Because sensitivity was insufficient for our intended clinical applications, we evaluated derivatization as a means to improve the ionization efficiency and sensitivity. Derivatization with hydroxylamine was highly effective; the reaction is fast, quantitative, and goes to completion, and the derivative is stable. Data for the comparison of the intensity of the molecular ion with different ionization techniques for nonderivatized Te and the Te oxime are shown in Fig. 1. Derivatization with formation of Te oxime improved the ionization efficiency considerably; this improvement is likely related to the tertiary amine moiety present within Te oxime. The derivative was efficiently ionized at relatively low voltage. Evaluation of different mobile phase additives showed that the most efficient ionization was observed with an ESI ion source and a mobile phase consisting of methanol and 0.022 mol/L aqueous formic acid. The LOQ and precision of the assay incorporating oxime derivatization was adequate for Te measurement in clinical samples from women and children.

Chromatographic separation on a short reversed-phase LC column provides sufficient specificity, prevents ion suppression, and allows selective detection of Te. The method has high specificity, which is assured through monitoring of 2 unique mass transitions each for Te and \(d_3\)-Te and no known interference. Use of 2 transitions and monitoring of 2 unique mass transitions each for Te and \(d_3\)-Te has high specificity, which is assured through strict adherence to quality control, evaluation of system performance, and batch acceptance criteria.

RESULTS

Levels of SHBG and Te were consistent with observations by Fitzgerald and Herold (27) and Taieb et al. (18): Immunoassay results agreed with an MS-based method for samples from healthy men, but in samples from women, the methods correlated poorly with each other. Possible reasons for the lack of agreement between the results obtained with the immunoassays are matrix effects and cross-reactivity of the antibody with structurally related steroids (17–27). Low physiologic concentrations of Te in women and children and limited sample size for pediatric samples challenge method sensitivity. The results obtained by most immunoassays for samples from women and children are inaccurate because of insufficient method specificity at low Te concentrations (16–18, 27). Because interfering substances and the degree of interference vary among assays from different manufacturers, results obtained with different immunoassays often do not agree with each other and cannot be used interchangeably. To address this issue, we recommend using the isotope-dilution LC-MS/MS method for measurement of Te in women and children.

Some potential pitfalls of the method are cross-contamination between samples during sample preparation and carryover during instrumental analysis. The large variation in Te concentrations attributable to sex and age can contribute to this potential problem. This is why successful implementation of the LC-MS/MS method requires strict adherence to quality control, evaluation of system performance, and batch acceptance criteria.

CHANGES IN SHBG AND TE CONCENTRATIONS IN MALES AND FEMALES BY TANNER STAGE AND AGE

Results of the analysis of samples from volunteers of different Tanner stages are summarized in Table 2. Boys and girls at Tanner stage (TS) 1 had comparable SHBG reference intervals. After TS 1, the SHBG concentrations decreased. In females, the lowest reference limits were observed at TS 3, followed by an increase through TS 4 and TS 5 back to the values characteristic of TS 1. SHBG concentrations in males gradually decreased between TS 1 and TS 4 to adult reference limits. The reference limits for total Te in females increased starting from TS 1, reached concentrations characteristic of adult females by TS 3, and then decreased to prepubertal concentrations at menopause. In males, the greatest increase in total Te reference limits was observed between TS 2 and TS 3 (∼5-fold), followed by a 15% increase between TS 3 and TS 4 and a 15% decrease between TS 4 and TS 5. The highest reference limits for free Te in females were observed at TS 4, followed by a steady decrease of ∼50% by TS 5 and an additional decrease to prepubertal concentrations at menopause. The highest increase in free Te concentrations in males (∼3.5 fold) was observed between TS 2 and TS 3, followed by an ∼70% increase between TS 3 and TS 4 and an additional ∼40% increase between TS 4 and TS 5. The highest reference limits for bioavailable Te in females were observed at TS 4, followed by decreases of ∼50% by TS 5 and ∼60% at menopause. The greatest increase of the bioavailable Te reference limits in males (∼5-fold) was between TS 2 and TS 3, followed by steady increase of an additional ∼80% by TS 5.

The results of analysis of samples from volunteers in different age groups are summarized in Table 3. In
females, SHBG reference limits decreased by ~30% between ages 10 and 13, followed by an increase in reference limits to the peak values observed in the 18- to 30-year age group and an ~30% decrease in the 31- to 40-year age group back to the concentrations observed in teenagers. Total Te concentrations in females started to increase at age 10–11 years, reached reference limits characteristic of adult women by age 12–13 years, and remained at these concentrations until menopause, when they decreased, on average, by ~60%. The concentration of bioavailable Te in females increased starting from age 10–11 years and reached adult concentrations by age 16–17 years, with a parallel increase in free Te concentrations in females from age 7 through 17 years. The concentrations of bioavailable and free Te in women peaked between ages 17 and 40 years, followed by a decrease to prepubertal concentrations at menopause.

In males 7–13 years of age, SHBG concentrations were stable and then decreased by ~50% to concentrations characteristic of adult males. The total Te reference limits increased at age 10–11 years, then increased to the concentrations characteristic of adult men by age 16–17 years. The total Te concentrations did not differ significantly among the 16–17, 18–30, and 31–52 age groups. Concentrations of free and bioavailable Te in males started to increase at age 10, reached maximum values at age 16–17 years, and decreased, on average, by ~30% at age 31–52 years.

In conclusion, we have developed a highly sensitive and specific LC-MS/MS method suitable for analysis of Te in children and preand postmenopausal women and established reference intervals for total, free, and bioavailable Te and SHBG for males and females of different Tanner stages and age groups. Measurement of Te by LC-MS/MS was precise and accurate down to 0.0347 nmol/L (1 ng/dL). With a 3-min run time, the assay is a good alternative to immunoassays in clinical reference laboratories because it is free of interference and cross-reactivity with structurally related compounds. Compared with GC-MS methods for Te analysis, the LC-MS/MS method offers better sensitivity along with higher throughput. The small sample volume required for this test may reduce the volume of blood drawn from patients, a useful feature for pediatric testing.

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References
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