Validation of a High-Throughput Liquid Chromatography–Tandem Mass Spectrometry Method for Urinary Cortisol and Cortisone

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Background: Urinary free cortisol and cortisone measurements are useful in evaluation of Cushing syndrome, apparent mineralocorticoid excess, congenital adrenal hyperplasia, and adrenal insufficiency. To reduce analytical interference, improve accuracy, and shorten the analysis time, we developed a liquid chromatography–tandem mass spectrometry (LC-MS/MS) method for urinary cortisol and cortisone.

Methods: We added 190 pmol (70 ng) of stable isotope cortisol-9,11,12,12-d4 to 0.5 mL of urine as an internal standard before extraction. The urine was extracted with 4.5 mL of methylene chloride, washed, and dried, and 10 μL of the reconstituted extract was injected onto a reversed-phase C18 column and analyzed using a tandem mass spectrometer operating in the positive mode.

Results: Multiple calibration curves for urinary cortisol and cortisone exhibited consistent linearity and reproducibility in the range 7–828 nmol/L (0.25–30 μg/dL). Interassay CVs were 7.3–16% for mean concentrations of 6–726 nmol/L (0.2–26.3 μg/dL) for cortisol and cortisone. The detection limit was 6 nmol/L (0.2 μg/dL). Recovery of cortisol and cortisone added to urine was 97–123%. The regression equation for the LC-MS/MS (y) and HPLC (x) method for cortisol was: y = 1.11x + 0.03 μg cortisol/24 h (r² = 0.992; n = 99). The regression equation for the LC-MS/MS (y) and immunoassay (x) methods for cortisol was: y = 0.66x − 12.1 μg cortisol/24 h (r² = 0.67; n = 99).

Conclusion: The sensitivity and specificity of the LC-MS/MS method for urinary free cortisol and cortisone offer advantages over routine immunoassays or chromatographic methods because of elimination of drug interferences, high throughput, and short chromatographic run time.

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Analysis of urinary free cortisol (UFC) is a test of choice for the diagnosis of Cushing syndrome, along with plasma cortisol and corticotropin (adrenocorticotropic hormone) (1–3). UFC and cortisone measurements are also useful in evaluation of apparent mineralocorticoid excess, congenital adrenal hyperplasia, and adrenal insufficiency. Historically, various RIA methods were developed for UFC and have been replaced with more sensitive and specific luminescent immunoassay methods (4). Although all immunoassays for UFC use liquid–liquid extraction to eliminate interfering compounds, these methods are still susceptible to interferences from cortisone and/or other endogenous steroid metabolites and synthetic glucocorticoids, such as prednisolone (5). Another limitation of immunoassays is the lack of an internal standard to monitor variable recovery of cortisol in the extraction process. A recent review comparing immunoassay and chromatographic methods for UFC measurements clearly indicated that only chromatographic methods can accurately measure UFC (6, 7). These limitations of immunoassays for UFC have led to the development of more specific methods based on liquid chromatography with ultraviolet detection (LC-UV), liquid chromatography–mass spectrometry (LC-MS), and gas chromatography–mass spectrometry (GC-MS) (8, 9). The chromatographic methods not only have reduced interference for cortisol quantification, but also allow quantification of cortisone, an endogenous metabolite of UFC.
The mineralocorticoid properties of cortisol are regulated via its oxidation to cortisone by 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) at the mineralocorticoid receptors. Inactivation of cortisol to cortisone at the mineralocorticoid receptor prevents it from acting as a mineralocorticoid; otherwise, the very high concentration of cortisol in comparison with aldosterone would contribute to sodium and water retention. The absence of 11β-HSD2 activity leads to hypertension and is known as apparent mineralocorticoid excess (AME) syndrome (10). AME attributable to a lack of cortisol inactivation to cortisone arises from a mutation in the gene encoding 11β-HSD2 in the kidney. Recently, various publications have indicated that measurement of the direct ratio of cortisol to cortisone is a better indicator of 11β-HSD activity than the ratio of the urinary metabolites of cortisol and cortisone (11, 12). The LC-UV methods for UFC and cortisone require an analysis time longer than 20 min. This time is essential to obtain resolution for cortisol and cortisone and to assure that the commonly used synthetic corticosteroids and the more hydrophilic cortisol metabolites do not interfere with the cortisol and cortisone peaks.

Despite their advantages over immunoassays, LC-UV methods are still susceptible to some interferences, the most notable being carbamazepine and its hydroxy metabolites (13). To resolve the carbamazepine interferences, LC-MS or GC-MS analysis is required (14). Although chromatographic methods with MS detection provide specific quantification of cortisol, they have not been widely implemented because of low throughput and the higher cost of instrumentation. The introduction of liquid chromatography–tandem mass spectrometry (LC-MS/MS) to the clinical laboratory has overcome the limitations of single quadrupole MS methods for the analysis of various analytes in the clinical laboratory (15–17). The advantages of sample processing and analysis time seem to make these methods more cost-effective than conventional methods, such as HPLC with UV detection (HPLC-UV), LC-MS, and GC-MS. We have previously presented preliminary validation data for a LC-MS/MS method using electrospray ionization (ESI) for UFC and cortisone (15). A similar method was also published by Nassar et al. (17), who used a different mode of ionization, i.e., atmospheric chemical ionization (APCI). Both approaches are robust and have shorter run times. We have addressed additional issues related to UFC analysis by LC-MS/MS, including the following: (a) validation for the cortisone metabolite as part of the UFC method; (b) comparison with methodologies used at present in clinical laboratories; and (c) determination of the reference intervals for cortisol and cortisone. In this study we describe in detail a high-throughput LC-MS/MS method for the simultaneous analysis of UFC and cortisone with no known interferences.

Materials and Methods

Cortisol, cortisone, and estriol were purchased from Sigma. Cortisol-9,11,12,12-d4 was purchased from Cambridge Isotope Laboratories with an isotopic enrichment of 98%. We prepared 2.8 mmol/L (1 g/L) stock solutions of cortisol, cortisone, and cortisol-9,11,12,12-d4 in HPLC-grade methanol. Working solutions were prepared by diluting stock solutions with methanol–water (70:30 by volume) containing 4 μmol/L (1 μg/mL) estriol. Methanol and methylene chloride were HPLC grade (EM Science). All other chemicals were of the highest purity available from commercial sources.

To determine reference intervals, we obtained 24-h urine collections, using 10 g of boric acid as preservative. The reference interval study was approved by the Institutional Review Board of the Mayo Foundation. Reference interval data for urinary cortisol and cortisone were collected by LC-MS/MS and analyzed using a statistical method suggested by O’Brien and Dyck (18). Briefly, linear regression analysis was used to adjust for differences in significant trend or variability across age or gender groups. Significance was defined as P < 0.05. Z-Scores were then created to adjust for the significant age or gender effects. The 2.5 and 97.5 percentiles were then selected and transformed back to the measured units, creating a central 95% reference interval. The SAS statistical software system was used to perform all of these analyses.

For stability studies, four samples were collected and split into two aliquots, with one aliquot preserved with boric acid (0.5 g/100-mL specimen) and the other preserved with acetic acid (2.5 mL of 500 g/L acetic acid per 200-mL specimen). A 1.0-mL sample was taken from each aliquot and frozen at −70 °C. Each aliquot was again split into two aliquots, with one aliquot preserved at ambient temperature and one aliquot at 4 °C. A 1.0-mL sample was removed from each of the four aliquots on days 1, 3, and 7 and frozen at −70 °C. All samples were thawed and assayed for cortisol and cortisone. For the freeze/thaw study, four 1.0-mL aliquots were immediately taken from each of the original two aliquots for each sample collected. The first aliquot was stored at 4 °C, the second aliquot was frozen at −70 °C and thawed once, the third aliquot was frozen and thawed twice, and the fourth aliquot was frozen and thawed three times. All four aliquots were then assayed for cortisol and cortisone.

Sample Preparation

A 2-mL portion of a 24-h urine collection was centrifuged for 5 min at 600g to remove all sediment and particulate matter. A 0.5-mL fraction of the centrifuged urine was transferred to a 13 × 100 mm borosilicate glass culture tube and mixed with 35 μL of 6 nmol/L (2.0 μg/mL) cortisol-9,11,12,12-d4. A dispensette (Brinkmann Instruments Inc.) was used to add 4.5 mL of methylene chloride to each urine sample. The urine extraction tubes were
mixed for 30 s on a multitube vortex-mixer. The urines were then centrifuged for 5 min at 600g, and the upper aqueous layer was aspirated and discarded.

The methylene chloride fraction was washed with 1.0 mL of 0.1 mol/L sodium hydroxide, 0.1 mol/L hydrochloric acid, and 1.0 mL of distilled water, with each aqueous upper layer being aspirated and discarded. The washed methylene chloride was evaporated under nitrogen at 45 °C in an N-Vap. The dried extract was reconstituted with 125 μL of methanol–water (70:30 by volume) containing 4 μmol/L (1 μg/mL) estradiol and thoroughly mixed by vortex-mixing to ensure complete reconstitution. The estradiol in the reconstitution solvent was added to prevent the loss of extracted cortisol and cortisone from the sample by nonspecific binding with the glass surface. The sample tubes were centrifuged for 5 min at 600g, and the solution was gently transferred to autosampler vials containing 300-μL glass inserts. Assay calibrators were prepared in methanol–water (70:30 by volume) containing estradiol with corresponding cortisol and cortisone concentrations of 7, 28, 138, 413, and 825 nmol/L (0.25, 1.0, 5.0, 15.0, and 30 μg/dL).

METHODS

A triple-quadruple mass spectrometer, API 2000™ (Applied Biosystems), operating with an ESI source was used. A Perkin-Elmer Series 200 pump and autosampler were used for sample introduction. Cortisol, cortisol-d4, and cortisone were chromatographically resolved from the more abundant hydrophilic urine components by use of a reversed-phase column (LC-18; 33 × 4.6 mm; Supelco) combined with a precolumn filter (C18: 4 × 2 mm; Phenomenex®). The chromatographic mobile phase was composed of methanol and water (56:44 by volume) and delivered at a flow rate of 1.0 mL/min. The flow delivered to the Turbolonspray® was reduced by splitting, with a net flow rate of 200 μL/min to the ionization probe of the mass spectrometer. Cortisone and cortisol were chromatographically resolved, whereas cortisol and cortisol-d4 co-eluted with a retention time of ~1.7 min. The instrument analysis time per sample was 3.0 min. All results were generated in the positive-ion mode. Product ion scans of the three steroids were generated using 13.8 μmol/L (5 μg/mL) solutions prepared in methanol–water (50:50 by volume) containing 0.2 mmol/L ammonium acetate and delivered at a continuous flow via infusion pump at 10 μL/min. In the selected-reaction monitoring mode, the instrument monitored the m/z 363.0 to m/z 121.1, m/z 361.0 to m/z 163.0, and m/z 366.9 to m/z 121.2 transitions for cortisol, cortisone, and cortisol-d4, respectively. The mass spectrometer operating conditions consisted of a source heater probe temperature of 300 °C, with a Turbolonspray voltage of 5000 V, curtain gas at 1.8 L/min, and collision gas at 1.1 L/min. The nebulizing and heater gas settings were 6.5 and 8.3 L/min, respectively. Data acquisition and quantitative processing were accomplished using Analyst™ software, Ver. 1.1 (Applied Biosystems).

Method validation, including studies of precision, recovery, interference, reference values, linearity, preservatives, and stability, was completed using the LC-MS/MS method. We compared UFC results obtained for 98 patient samples by the LC-MS/MS method with results obtained by an immunoassay (ACS:180) and a LC-UV method. All methods used a preanalysis sample preparation that required solvent extraction with methylene chloride. Both of the comparison methods had been used in our laboratory and have recently been replaced by the LC-MS/MS method.

RESULTS

LC-MS/MS CHARACTERISTICS OF CORTISOL AND CORTISONE

The mass spectrum for an infusion of 13.8 μmol/L (5 μg/mL) cortisol is shown in Fig. 1. The (Q1) scans were done using ESI in the positive-ion mode. The expected [M + 1] protonated molecular ions are m/z 363, 367, and 361 for cortisol, cortisol-d4, and cortisone, respectively. The transmission of these protonated molecular ions into the collision cell and the subsequent scanning of the second resolving quadrupole (Q3) for fragments yielded the product ion scan for cortisol shown in Fig. 1B. These two experiments determined the molecular and fragmentation ions on which all future analysis were based. The fragments for cortisol and cortisol-d4 correspond to the cleavage of ring B at the bonds common with ring C of the steroid molecule. Because of the C=O group at position 11 for cortisone, the ionization pattern was different for this molecule, and the fragment probably resulted from the cleavage of ring C instead of ring B in cortisol. We selected the m/z 121, 121, and 163 ions as product ions for cortisol, cortisol-d4, and cortisone, respectively.

The extracted ion chromatogram shown in Fig. 2 represents a 10-μL injection of a cortisol and cortisone calibrator at 828 nmol/L (30 μg/dL) containing 70 ng of cortisol-d4. The retention times were 1.3 and 1.7 min for cortisone and cortisol, respectively, with a total run time of 3 min. The chromatographic separation produced baseline resolution even for the highest concentration calibrator (30 μg/dL). The signal-to-noise ratios at the lowest calibration dose, 7 nmol/L (0.25 μg/dL), were 48:1 and 60:1 for cortisol and cortisone, respectively.

PRECISION

The inter- and intraassay precision for urine free cortisol and cortisone is summarized in Table 1. The pools were divided into sufficient aliquots and stored at −20 °C. Interassay CVs ranged from 7.3% to 12% and 9.2% to 16% for cortisol and cortisone, respectively, at concentrations ranging from 6 to 726 nmol/L (0.2–26.3 μg/dL; n = 20). Intraassay CVs ranged from 2.0% to 21% at concentrations ranging from 6 to 419.5 nmol/L (0.2–15.2 μg/dL; n = 20). These determinations were done using pooled urine from
Fig. 1. ESI mass spectrum (Q1 scan) of cortisol in positive mode, showing the [M + 1]$^+$ molecular ion (A), and product ion scan of the protonated cortisol ion (m/z 363), showing the major transition fragment at m/z 121 (B).

Cortisol [13.8 μmol/L (5 μg/mL)] prepared in methanol–H$_2$O containing 0.2 mmol/L ammonium acetate was infused at 10 μL/min. amu, atomic mass units.
Fig. 2. Extracted ion chromatogram of the 828 nmol/L (30 μg/dL) calibrator containing cortisol, cortisone, and cortisol-d₄.

healthy individuals to which appropriate doses of each analyte were added. The functional sensitivity, or limit of quantification, of the assay for cortisol and cortisone was 6 nmol/L (0.20 μg/dL), based on an interassay CV of ~20% at that concentration. This method is at least 2.5 times more sensitive than the method published by Nasar et al. (17), which used LC-MS/MS and APCI.

RECOVERY
Cortisol and cortisone were added at three concentrations ranging from 55 to 668 nmol/L (2–25 μg/dL) to four patient samples with endogenous concentrations and assayed in single determinations. For individual samples, calculated recoveries ranged from 97% to 106% (mean, 101%) and from 109% to 123% (mean, 114%) for cortisol and cortisone, respectively, by the LC-MS/MS method with cortisol-d₄ as an internal standard. The absolute recovery was 85% for cortisol and cortisol-d₄ and 102% for cortisone. The increased calculated recovery of 114% for cortisone probably was attributable to the lower absolute recovery of cortisol-d₄, the internal standard used for the analysis of both cortisol and cortisone. This could be corrected by the use of deuterium-labeled cortisone as an internal standard, which was not commercially available at the time of this study.

LINEARITY
The interassay variability for the five-point calibration curve for cortisol and cortisone, constructed with calibrator concentrations of 7–828 nmol/L (0.25–30 μg/dL), was assessed. The set of calibrators, which was run with each analysis, was monitored for 6 consecutive days. Multiple calibration curves were linear and reproducible with the following linear regression equations: y = 0.99x + 0.08 μg/dL for cortisol and y = 0.99x + 0.1 μg/dL for cortisone (r² = 0.999). The linearity of the assay was assessed by extracting three patient samples at sample volumes of 500, 400, 300, 200, and 100 μL. The expected value for each sample volume was calculated based on the result obtained for the 500-μL sample. The linearity was evaluated by dividing the observed value by the expected value for each sample. The percentages of the expected results for cortisol and cortisone were 95–109% (mean, 101%) and 97–106% (mean, 102%), respectively, for urine specimens containing 22–1024 nmol/L (0.8–37.1 μg/dL) cortisol or cortisone.

STABILITY STUDIES FOR UFC IN DIFFERENT PRESERVATIVES
Cortisol was stable for 7 days at ambient temperature and 4 °C in both acetic acid and boric acid, with the day 7 values differing from the day 0 values by an average of 2.5% (range, −11.4% to 9.8%). Cortisone was stable for 3 days at ambient temperature in both acetic acid and boric acid, with the day 3 values differing from the day 0 values by an average of −5.6% (range, −13.3% to 2.9%), and was stable for 7 days at 4 °C in both acetic acid and boric acid, with the day 7 values differing from the day 0 values by an average of −6.6% (range, −14.5% to 0.4%). Cortisol and cortisone were stable for three freeze/thaw cycles in both acetic acid and boric acid, with the results obtained after three freeze/thaw cycles differing from the baselines values by an average of −3.7% (range, −21.4% to 4.5%).

METHOD COMPARISON
Unused portions of 98 urine specimens were analyzed by an in-house LC-UV method for cortisol and cortisone and were reanalyzed by the LC-MS/MS method. Unused portions of 99 urine specimens analyzed by an automated chemiluminescence immunoassay for cortisol were reanalyzed by the LC-MS/MS method. All three methods include preanalysis sample preparation that requires extraction with methylene chloride. The HPLC-UV method resolves cortisol and cortisone from other synthetic steroids.

Linear regression analysis between the LC-MS/MS (y) method and the chemiluminescence immunoassay (x) for cortisol gave a slope of 0.655 (95% confidence interval, 0.56–0.74), a y-intercept = −12.1 μg/dL (95% confidence interval, −24 to 0.33 μg/dL), and a correlation coefficient

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**Table 1. Precision characteristics of LC-MS/MS method for urinary cortisol and cortisone.**

<table>
<thead>
<tr>
<th></th>
<th>Cortisol</th>
<th>Cortisone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean concentration, nmol/L (μg/dL)</td>
<td>CV, %</td>
<td>Mean concentration, nmol/L (μg/dL)</td>
</tr>
<tr>
<td>Interassay (n = 20)</td>
<td>6 (0.2)</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>55 (2.0)</td>
<td>7.3</td>
</tr>
<tr>
<td></td>
<td>163 (5.9)</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>654 (23.7)</td>
<td>7.5</td>
</tr>
<tr>
<td>Intraassay (n = 20)</td>
<td>6 (0.2)</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>61 (2.2)</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>420 (15.2)</td>
<td>2.0</td>
</tr>
</tbody>
</table>
The slope of \( r^2 \) of 0.67. The slope of \(<1\) for the difference between these methods is not attributable to differences in the values assigned to the calibrators. We assayed the low and high calibrators for the chemiluminescence immunoassay (ACS:180) method by the LC-MS/MS method and found that they had the assigned values of 93 and 1045 nmol/L (3.4 and 38 μg/dL), respectively. The Bland–Altman plot comparing the LC-MS/MS method with the immunoassay method showed a concentration-dependent negative bias (Fig. 3). We calculated the regression line for the variation of mean difference as a function of the average values of the two methods, as recommended by Bland and Altman in a recent publication (19). The concentration-dependent bias was related to cross-reactivity of cortisone, prednisone, and prednisolone in the immunoassay. In contrast, the Bland–Altman plot comparing the LC-MS/MS method with the LC-UV method (Fig. 4) showed no bias. Cross-reactivity attributable to cortisone and drug interferences (prednisone and prednisolone) in the immunoassay accounted for individual sample bias as these compounds do not interfere or cross-react in the LC-MS/MS or the LC-UV methods. As the cortisol concentration increases, the concentration of its metabolite, cortisone, also increases so that a considerable portion of the immunoassay result is cortisone cross-reactivity, which is not observed in the LC-MS/MS and LC-UV methods.

Linear regression analysis for correlation between the MS/MS method and LC-UV method for cortisol gave a slope of 1.11 (95% confidence interval, 1.08–1.14), a \( y \)-intercept of 0.03 μg cortisol/24 h (95% confidence interval, −0.16 to 0.1 μg cortisol/24 h), and a correlation coefficient \( r^2 \) = 0.992. Linear regression analysis for cortisone between these methods gave a slope of 0.96 (95% confidence interval, 0.9–1.0), a \( y \)-intercept of 0.32 μg cortisol/24 h (95% confidence interval, −0.1 to 0.7 μg cortisol/24 h), and a correlation coefficient \( r^2 \) of 0.974. In the Bland–Altman plot, the variation in the mean difference increased with increasing average values of these methods. To normalize the effect of the increasing concentration, the percentage of difference rather than the net difference between the two methods was plotted against the average of these methods, as shown in Fig. 4 (19). The difference in the cross-reactivity associated with immunoassays or chromatographic methods is also reflected in the reference intervals for adult UFC values, e.g., <299 nmol/24 h (<108 μg/24 h) for the immunoassay, 14–152 nmol/24 h (5–55 μg/24 h) for LC-UV, and 8–166 nmol/L (3–60 μg/24 h) for LC-MS/MS (Table 2).

### Interferences

Interfering and coeluting peaks attributable to drugs in LC-UV methods for UFC can make peak identification and quantification difficult. Interferences from some of the commonly encountered synthetic corticosteroids, in-

#### Table 2. Reference intervals for UFC and cortisone by age and sex, determined by LC-MS/MS method.

<table>
<thead>
<tr>
<th>Age, years</th>
<th>n</th>
<th>Cortisol, μg/24 h</th>
<th>Cortisone, μg/24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>3–8</td>
<td>23</td>
<td>1.4–20</td>
<td>6–41</td>
</tr>
<tr>
<td>9–12</td>
<td>26</td>
<td>2.6–37</td>
<td>10–73</td>
</tr>
<tr>
<td>13–17</td>
<td>25</td>
<td>4.0–56</td>
<td>15–108</td>
</tr>
<tr>
<td>18–70+</td>
<td>83</td>
<td>4.2–60</td>
<td>17–141</td>
</tr>
<tr>
<td>Males</td>
<td>104</td>
<td>3.0–43</td>
<td>15–122</td>
</tr>
<tr>
<td>Females</td>
<td></td>
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</table>
cluding prednisone, prednisolone, and dexamethasone, were studied by adding these compounds to urine specimens and comparing the retention times and measured concentrations of cortisol and cortisone with the previously determined values. Addition of the three synthetic corticosteroids at 550 nmol/L (20 μg/dL) produced no deviations in retention time or changes in concentration from the previously determined cortisol or cortisone values. The drug carbamazepine and its metabolites have been reported to cause peak interference with LC-UV methods, and results may be falsely increased (13). The analysis of UFC by a LC-UV method for a patient on such a drug is shown in Fig. 5A. LC-MS/MS analysis of the same urine specimen produced precise calculation of UFC with no interference attributable to carbamazepine or its metabolites (Fig. 5B).

REFERENCE INTERVALS FOR UFC AND CORTISONE

We used the LC-MS/MS method to more comprehensively study UFC and cortisone reference intervals for pediatric as well as adult population, using 24-h urine collections. The nonparametric reference intervals (central 95th percentiles) for cortisol and cortisone were determined as a function of age and sex, as shown in Table 2. These values were determined using 24-h urine specimens from 125 males and 140 females between the ages of 3 and 82 years. UFC and cortisone increased up to 17 years of age, and no significant differences were observed in individuals ≥70 years of age. No significant difference in males and females was seen until puberty. After puberty, males were found to have 1.4-fold higher UFC, but no such difference was observed for cortisone. Previously, cortisol concentrations in adults were reported to be 14–152 nmol/24 h (5–55 μg/24 h) by HPLC-UV methods with no segregation based on age or sex. However, the lower cortisol concentrations measured in adult females by the LC-MS/MS method in the present study confirmed the male-to-female ratio of 1.4:1 reported in a recent publication (6).

**Discussion**

The measurement of UFC is considered the best screening test for hypercortisolism (1). The unbound plasma fraction of cortisol is excreted by glomerular ultrafiltration. Analysis of UFC and cortisone in a complex matrix such as urine is a challenge for the laboratory because urine also contains many compounds, including cortisol metabolites, that have polarities and chemical structures similar to those of cortisol and cortisone. The falsely increased cortisol values attributable to interference by endogenous steroids or synthetic steroids in immunoassays have been documented. In alternative HPLC-UV methods, interfering compounds still require separation and resolution from UFC, which prolongs chromatographic run times and decreases throughput. Clinicians also must base decisions on method-specific reference intervals, which are very different for immunoassays than for chromatographic methods (6). Compared with the HPLC-UV method for urine cortisol, the present LC-MS/MS method is not only more sensitive [e.g., lower limit of quantification, 6 vs 27.6 nmol/L (0.2 vs 1 μg/dL)], but is also very specific.

The unique design of the tandem MS contributes to the enhanced specificity and sensitivity of the proposed method for UFC. The nebulizer vaporizes and ionizes the eluting compounds and eliminates the need for derivatization as required for the GC-MS method. The Q0 region focuses the ionized vapor into the first quadrupole, which selects the protonated parent cortisol and cortisone molecular [M + H]⁺ ions and sends them to the collision cell, where collision-induced dissociation of cortisol and cortisone occurs. The second quadrupole selects fragments m/z 121 and 163 (from the collision-induced dissociation), called daughter ions, of cortisol and cortisone, respectively. By combining the parent and daughter ions to produce a signal, MS/MS provides a sensitivity and specificity greater than those of conventional LC-UV, LC-MS, and GC-MS methods. The increased sensitivity...
and specificity for UFC provide cleaner chromatograms by eliminating interferences, thus shortening run times, which helps in increasing throughput for a high-volume laboratory. The LC-MS/MS method for UFC also uses a deuterated internal standard (cortisol-d4) to provide the most accurate recovery for extractions. A suitable deuterated internal standard could not be found for cortisone, so cortisol-d4 was used as the internal standard for cortisol and cortisone. The washes in the sample preparation not only remove interferents, but also enhance calculated recovery for cortisone with cortisol-d4 as the internal standard. Analysis of extracts without these washes showed a calculated recovery of only 70% for cortisone. Cortisol calculated recovery was 100% with or without the washes.

The LC-MS/MS instrument is very specific and sensitive, but injection of unextracted urine samples can cause significant loss in sensitivity as a result of suppression of the ions by matrix components in the urine. It may be difficult to maintain the high sensitivity of the instrument when unextracted urine samples are analyzed 24 h a day and 7 days a week. Under these conditions, the instrument may require frequent maintenance, as opposed to the low maintenance required for running cleaner samples in a high-throughput laboratory. Our experience has shown that, compared with the dilute-and-shoot approach, liquid–liquid or solid-phase extraction followed by concentrating the sample provides consistent sensitivity and specificity for endocrine analytes by LC-MS/MS methods. The 3-min run time for analysis of UFC and cortisone has allowed us to transfer our entire workload from immunoassays and HPLC-UV to the LC-MS/MS method without any impact on turnaround time. This LC-MS/MS method has improved accuracy, reduced analytical interference, and increased sample throughput for our clinical laboratory. A similar method that measures only cortisol has also been published, which uses APCI and a tandem quadrupole spectrometer (17). In our preliminary experiments with an APCI source, we observed an exchange between the deuterium atoms of the internal standard and the hydrogen atoms of water. This led to the formation of unlabeled steroids, which caused overestimation of steroids, especially at low concentrations. We have not observed this phenomenon with ESI. The functional sensitivity that we obtained using the ESI mode of ionization and liquid–liquid extraction was <6 nmol/L (<0.2 μg/dL) vs the 14 nmol/L (0.5 μg/dL) reported by Nassar et al. (17), using APCI.

URINE FREE CORTISOL/CORTISONE RATIO AND AME

The AME syndrome is an inherited form of hypertension (11). This disorder results from an inability of the enzyme 11-β-HSD2 to inactivate cortisol to cortisone (Scheme I). In the absence of active 11-β-HSD2, cortisol manifests mineralocorticoid properties. The diagnosis of AME is usually based on an increased ratio of cortisol to cortisone-reduced metabolites in the urine (tetrahydrocortisol plus allotetrahydrocortisol to tetrahydrocortisone). Recently the ratio of UFC to cortisone, as measured by a GC-MS method, was used to study two variants of AME in 24 patients (20). Urinary free cortisone concentrations are usually 2–3 times higher than UFC concentrations, but higher UFC values with low cortisone are observed in patients with hypertension attributable to AME. The LC-MS/MS-ESI method has been validated for both cortisol and cortisone, and the ratio can aid in the diagnosis of AME syndrome. The role of 11-β-HSD2 inactivation in Cushing syndrome has not yet been fully studied. 11-β-HSD2 is also inhibited by licorice, carbenoxolone, and other commonly used drugs, which leads to increased UFC concentrations (13). For timely diagnosis of AME, the use of LC methods to obtain the cortisol/cortisone ratio and molecular tests for 11-β-HSD2 mutations may be beneficial. A LC-MS/MS method for UFC and cortisone will permit future studies to correlate the enzymatic activity of 11-β-HSD2 in diseases associated with novel mutations of 11-β-HSD2. In vivo, cortisone is converted back to bioactive cortisol by cortisone reductase (11-β-HSD1). Decreased 11-β-HSD1 activity in some individuals can reduce the efficacy of cortisone treatment, so hydrocortisone (cortisol) is used for patients with congenital adrenal hyperplasia or inflammatory conditions. We consider the inclusion of cortisone in the method to be clinically useful. We have also determined for the first time age- and sex-specific reference intervals for UFC and cortisone by a LC-MS/MS method, which was lacking in previous publications.

Because of advantages of the LC-MS/MS methodology, many clinical laboratories have implemented this technology. Currently this technology is being used in laboratories for assays such as homocysteine, 17-hydroxyprogesterone, urine catecholamines, vanillylmandelic acid, homovanillic acid, and urine metanephrines (21–25). The proposed LC-MS/MS method for UFC compared very well with the HPLC-UV method and was not susceptible to the interferences attributable to cross-reactivity with endogenous and exogenous steroids as observed in the immunoassays.

In summary, the sensitivity and specificity of the LC-MS/MS method for UFC and cortisone offer advantages...
over immunoassays and HPLC methods; the LC-MS/MS method also eliminates drug interferences and has high throughput and short chromatographic run time. The age- and sex-specific reference intervals for UFC and cortisone obtained by the LC-MS/MS method will help improve the diagnostic efficacy of UFC and cortisone tests.

References