Radioimmunoassay and Liquid-Chromatographic Analysis for Free Cortisol in Urine Compared with Isotope Dilution–Mass Spectrometry

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I compared results by three different routine methods for analysis for urinary cortisol with those by a highly specific reference method based on isotope dilution–mass spectrometry (I). A “high-performance” liquid-chromatographic method (II) gave the most comparable results (regression coefficient 0.86, intercept 9 nmol/L). For some urines much lower values were obtained by I than by II. Two radioimmunoassay (III) methods, one involving direct assay and one involving extraction, gave less-accurate results (regression coefficients of 1.87 and 1.52 and intercepts of 86 and 12 nmol/L, respectively), although values obtained by III and by I correlated well (r = 0.95–0.99), indicating a relation between the free cortisol and the compounds interfering in III. The apparent accuracy for the extraction method was improved by using as calibration standards urine samples previously assayed by I (regression coefficient 0.90, intercept 6 nmol/L). All four methods investigated showed a statistically significant sex-related difference in 24-h urinary cortisol excretion; evidently such a finding should be a prerequisite in any such method proposed for routine use.

Additional Keyphrases: sex-related differences • reference methods • steroids

In 1959, Cope and Black (1) demonstrated the suitability of analysis for cortisol in urine in the diagnosis of adrenal hyperfunction. This has since been well confirmed. Different techniques for analysis have been used, and the normal values obtained with these different methods differ considerably, indicating different degrees of interference. In some previous studies (2–5), a statistically insignificant sex-related difference in the excretion of urinary free cortisol has been observed.

The aim of the present study was to set up a suitable reference method for assay of urinary cortisol and to use this method to evaluate the accuracy of some routine methods. In addition, I wanted to investigate possible sex-related differences in the 24-h urinary excretion of cortisol, as demonstrated by the different methods used.

Materials and Methods

Urine samples. Aliquots of 24-h urine specimens obtained from healthy volunteers (medical students and laboratory staff, ages 25–40 years) were centrifuged and stored frozen until analyzed.

Radioimmunoassay with extraction. Kits designed for analysis of serum and urine were obtained from Farmos Diagnostica, Turku, Finland, and the supplier's instructions were followed, with one modification. Urine samples were extracted with dichloromethane, and the organic phase was collected and washed once with 0.1 mol/L NaOH and then twice with redistilled water before radioimmunoassay. The standards included in the kit were used without the supplier-recommended extraction. In routine work, serum and urine samples were analyzed concurrently and the same standard curve was used for both. This modification decreased the values obtained for urine samples by 10–20%, as compared with those obtained with the procedure recommended by the manufacturer. The decrease of values is mainly from uncorrected losses in the extraction procedure of the samples, but the modification can be justified by the savings in time and kit components in routine use.

According to the manufacturer's specifications, the antibody used shows a cross reaction of 57% with prednisolone and 4% with 11-desoxycortisol (cortisol = 100%).

Radioimmunoassay without extraction (direct). Kits designed for analysis of both serum and urine were obtained from Amersham International Ltd. (formerly RCC), Amersham, U.K., and the instructions included in the kits were followed exactly. According to the specifications of the manufacturer, the antibody cross reacts with prednisolone, 11-desoxycortisol, and 17α-hydroxyprogesterone by 35, 1.8, and 1.7%, respectively (cortisol = 100%).

Radioimmunoassay with use of calibration standards with intact biological matrix. For calibration standards delivered by the kit manufacturers, I substituted urine samples that had been analyzed by isotope dilution–mass spectrometry (see below), with cortisol ranging between 8 and 419 nmol/L. The RIA procedures were then run as previously described, except that standards were treated the same as samples in the Farmos RIA procedure.

"High-performance" liquid chromatography (HPLC).1 The method used was a synthesis of those described by Rose and Jusko (6) and van den Berg et al. (7). To a 2-mL urine sample, about 100 ng of prednisolone and a tracer amount of [3H]prednisolone (about 60 000 cpm; Amersham International) were added, as internal standard and radioactive marker, respectively. In the preparation of the standard curve, I substituted urine for water, with NBS cortisol (National Bureau of Standards, Washington, DC) 0–400 nmol/L final concentration, being added. The samples were then extracted with 8 mL of dichloromethane and the organic phase was removed and evaporated. The residue was subjected to thin-layer chromatography (TLC) in benzene/absolute ethanol (4/1 by vol). With this solvent system, cortisol and prednisolone did not separate. The radioactive zone was detected with a radio scanner (Berthold Dünnschicht Scanner II; Berthold, Wildbad, F.R.G.). The appropriate zone was scraped off and the silica gel was extracted with 5 mL of ethyl acetate. After evaporation of the separated solvent, the residue was dissolved in 100 μL of dichloromethane/absolute ethanol (97/3 by vol) (mobile phase in the HPLC-analysis), and about 70 μL of this solution was injected into a Model 6000A liquid chromatograph (Waters Associates, Milford, MA 01757) equipped with an H.C. Pellosil precolumn (Reeve Angel, Clifton, NJ) and a column consisting of Nucleosil-NO2 (5 μm particle size; Supelco Inc., Bellefonte, PA 16823). The column pressure was

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1 Nonstandard abbreviations: HPLC, “high-performance” liquid chromatography; TLC, thin-layer chromatography; and ID-MS, isotope dilution–mass spectrometry.
Fig. 1. Regression analysis between the ID-MS method (x-axis) and the different routine methods
The solid lines are the regression lines and the dotted lines are the unit slope lines. © Outlier (cf. Results). RCC = Amersham. Note differences in scale for results by each method.
5500 kPa, the flow rate 1.0 mL/min. The detector wavelength was 254 nm and the amplification 0.005 A full scale.

Isotope dilution--mass spectrometry (ID--MS). ID--MS analysis was performed essentially as described previously (8) except that a purification step (TLC) was introduced before gas chromatography--mass spectrometry. To a 2-mL urine sample, about 100 ng of [4-14C]cortisol (30 000 cpm; Amer- sham International) was added as internal standard. In the preparation of the standard curve, I substituted urine for water, with NBS cortisol, 0--400 nmol/L final concentration, added. The sample work-up procedure was the same as for the HPLC method. After evaporation of the ethyl acetate extract, the methoxime-trimethylsilyl derivative was formed and dissolved in 30 μL of hexane. About 3--5 μL was injected into an LKB-2091 gas chromatograph--mass spectrometer (LKB Instruments, Bromma, Sweden). The column was 1.5% SE-30 on Chromosorb W, 80--100 mesh, 2 mm × 1.5 m; the flow rate of the carrier gas, helium, was 20 mL/min. The column temperature was 250 °C and the temperatures of the ion source and the flash heater were 275 and 290 °C, respectively. The electron energy was 20 eV and the trap current 60 μA. The electron multiplier sensitivity was set to 650. The first channel of the ion detector was focused on m/z 605 and the second on m/z 607. The amplification of both channels was 100--200-fold.

Statistics. The regression lines were calculated by the least-squares method, with results by the ID--MS method as the independent variable. The statistical significance of the slopes and the intercepts was calculated by Student's t-test (9). The ranges for male and female subjects were calculated after logarithmic transformation. The statistical analysis of differences between male and female urinary cortisol excretion was by unpaired t-test. The within-assay variation (CV) was calculated from duplicate determinations (10).

Results

The ID--MS method used as a reference method in the present study is a modification of the method previously described (8). Introduction of the purification step was found to be necessary for accurate determinations. To test the specificity of the assay, I divided three urine samples of different concentrations into two aliquots, and analyzed one-half by RIA and the other half by ID--MS as previously described (cf. Materials and Methods), but further purified the other half by means of HPLC before gas chromatography--mass spectrometry. The results obtained for the duplicate samples were identical, thus confirming the specificity of the assay.

The CV of the method as calculated from duplicate determinations on 40 samples was 5.8%.

Figure 1 and Table 1 show the results of a comparison between the ID--MS method and the different routine methods. Both RIA methods gave significantly higher values than ID--MS, whereas the HPLC method gave values more comparable with those by ID--MS. Three urine samples, however, gave much higher values by HPLC than by ID--MS. These three outliers (indicated in Figure 1) were excluded from the statistical evaluation. All the slopes in the different regression analyses differed significantly from 1 (Table 1). The intercept in the comparison between ID--MS and the Amersham (RCC) method differed significantly from zero, but did not in the other two regression analyses.

In spite of the significant inaccuracy of the two RIA methods, very high coefficients of correlation were obtained (r = 0.95--0.99).

The values obtained with the HPLC method were based on single determinations, whereas the values obtained with the two RIA methods were based on duplicate determinations.

The within-assay CV of the RIA/Farmos and the RIA/Amersham method, as calculated from duplicate determinations, was 5.3 and 4.7%, respectively. The within-assay CV of the HPLC method, as calculated from duplicate determinations of serum samples by the same method as the determinations of cortisol in urine samples in the present investigation, was 2.7%. All analytical variations were within the expected range.

To improve the accuracy of the RIA methods, I used standards intact in the biological matrix that had been assayed by ID--MS (Table 2). This improved the apparent accuracy of the kit from Farmos, but did not work out at all with the kit from Amersham.

The range for 24-h urinary cortisol excretion obtained with the ID--MS method for 20 men was 42--352 nmol/24 h (mean 122 nmol/24 h) and for 25 women 13--199 nmol/24 h (mean 51 nmol/24 h). The difference between the ranges was statistically significant (p <0.001). This sex-related difference in the 24-h urinary cortisol excretion was found to be significant also with the three routine methods used (p <0.05--<0.001).

Discussion

According to current concepts, ID--MS has a higher potential for accuracy than most other techniques. However, when such a method for assay of serum cortisol (8) is applied to the assay of free urinary cortisol, interferences were sometimes observed. Introduction of a purification step (TLC) before gas chromatography--mass spectrometry was necessary. Still further purification did not change the results obtained; therefore, the accuracy of the present modification is probably sufficient for its purpose. The imprecision of the method was relatively high, but should be of little importance as compared with the high inaccuracy of the different routine methods.

In our hands, the HPLC method turned out to be the most nearly accurate of the different routine methods tested. In some urines, however, interferences produced falsely high values (Figure 1). Considerably higher values were obtained with the two RIA

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<th>Table 1. Statistical Evaluation of the Comparison between the Routine Methods (y) and the ID--MS Method (x)</th>
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a Significantly different from unity (p <0.001). b Significantly different from unity (p <0.05). c Significantly different from zero (p <0.001). d Not significant.

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<th>Table 2. Statistical Evaluation of the Comparison between ID--MS (x) and the RIA Methods (y) with Use of Calibration Standards in Intact Biological Matrix</th>
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Footnotes same as for Table 1.
methods than with the comparison method. The method from Farmos gave more nearly accurate results than did the direct method from Amersham. Extracting the samples before analysis in the Amersham method did not improve the accuracy, thus excluding cortisol conjugates as being responsible for the higher degree of inaccuracy.

Despite the falsely high values obtained by the two RIA methods, their correlation with ID-MS was very high, which may explain the suitability of the RIA methods in the diagnosis of adrenal hypo- and hyperfunction. Evidently, the ratio between free cortisol and the compounds interfering in the RIA is relatively constant over a broad concentration range. Whether this is the case also in pathological states remains to be established.

In a previous work on RIA of plasma cortisol (11), it was possible to increase the accuracy of the determination by using calibration standards with an intact biological matrix that had been assayed by ID-MS. For successful use of such a calibration procedure the antibodies must be assumed to cross react only with compounds that occur in the same concentration in all urine samples or covariate with the specific compound assayed. The present study indicates that the antibodies from Farmos are more suitable for such a calibration procedure with respect to urine cortisol than are the antibodies from Amersham. Pending a more extensive investigation involving samples from patients with different diseases, general use of such a calibration procedure can not be recommended, however.

The concentrations of free cortisol in urine determined with the present reference method were similar to those found by Schönhöfer et al. (12) when these authors combined HPLC and RIA. That method also can be used as a comparison method. Theoretically, however, ID-MS should give more nearly accurate results.

In contrast to some previous reports (2–5), the present study showed a significant sex-related difference in the excretion of urinary free cortisol. I conclude that one should be able to demonstrate such a sex-related difference with any reliable routine method.

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References