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# Determination of Serum Cortisol by Isotope-Dilution Liquid-Chromatography Electrospray Ionization Tandem Mass Spectrometry with On-line Extraction

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**A liquid chromatographic-mass spectrometric method for the determination of cortisol in serum using atmospheric pressure electrospray ionization and tandem mass spectrometry is described. During sample preparation, 150 µl of serum were deproteinized with methanol/zinc sulfate followed by on-line solid phase extraction employing column switching. Tri-deuterated cortisol was used as the internal standard. The following transitions were monitored: cortisol, 363>309 m/z; d3-cortisol, 366>312 m/z. The total runtime was 5 minutes. The method proved linear (0–500 µg/l; r=0.999), precise (total coefficient of variation between 5.0% and 3.2% at a mean cortisol concentration of 15.1 µg/l and 269 µg/l, respectively; n=16) and specific with regard to relevant endogenous and exogenous steroids.**

*Key words:* Cortisol; Liquid chromatography-tandem mass spectrometry (LC-tandem MS).

*Abbreviations:* LC-tandem MS, liquid chromatography-tandem mass spectrometry; MRM; multiple reaction monitoring.

## Introduction

Mass spectrometric methods for the determination of steroids in biological samples classically employ isotope dilution gas chromatography-mass spectrometry (1–5). These techniques, however, require laborious sample clean-up and long chromatographic run times; moreover, derivatization is necessary and completeness of derivatization and stability of derivatives are generally uncertain for individual samples.

The use of stable isotope internal standards is applicable to the emerging technique of liquid chromatography-mass spectrometry as well (6). The interfacing of HPLC to mass spectrometers by use of atmospheric pressure ionization is a major milestone for the application of chromatographic techniques in the clinical laboratory. The combination of two quadrupole mass spectrometers with interposed collision cell for the initiation of controlled molecule desintegrations compensates for the limited separation capacity of liquid chromatography. As a result, liquid chromatography-tandem mass spectrometry (LC-tandem MS) is a highly specific and versatile technology; most compounds (including macromolecules) can be ionized directly without derivatization, and the high specificity of detection reduces requirement for sample preparation. LC-tandem MS has been widely used in pharmacokinetic research; now, this technique enters specialized clinical laboratories.

Here, we describe a very rapid isotope-dilution LC-tandem MS method for the determination of cortisol in serum applicable to large series of specimens, especially in the context of cortisol immunoassay validation.

## Materials and Methods

### *Chemicals*

Cortisol was obtained from Sigma (Deisenhofen, Germany; >99%), tri-deuterated cortisol (cortisol-9,12,12-$d_3$; 98%) used as the internal standard was from Cambridge Isotope Laboratories (Andover, USA). Stock solutions were made in methanol (approximately 50 mg/l).

Zinc sulfate heptahydrate (Merck, Darmstadt, Germany) was dissolved in water to obtain a concentration of zinc sulfate of 50 g/l. A precipitation solution was made of methanol and this zinc sulfate solution by mixing 4/1 by volume.

Ammonium acetate was from Merck (Darmstadt, Germany); a 200 mM stock solution was made in water. Methanol and water were of HPLC-grade (Baker, Deventer, The Netherlands). Human albumin was from Dade Behring (Marburg, Germany).

### *Instruments*

A Waters Alliance 2690 HPLC module (Waters, Milford, USA) was used, coupled to a Micromass Quattro LC-tandem mass spectrometer (Micromass, Manchester, UK) with a split of approximately 1:10.

### *Standards*

A stock calibrator was prepared by spiking a solution of human albumin (70 g/l in isotone saline) with the cortisol stock solution to a concentration of 1000 µg/l; after careful mixing and overnight equlibration, working calibrators with the following concentrations were made by serial dilution: 500, 250, 125, 62.5, 31.3, 15.6, and 7.8 µg/l; additionally, a zero-calibrator was used.

### *Sample preparation*

A semi-automated sample preparation with a manual deproteinization step and on-line solid phase extraction using automatic column-switching was applied.

In 1.5 ml polypropylene cups, 150 µl of calibrator or serum samples, respectively, were precipitated with methanol/zinc

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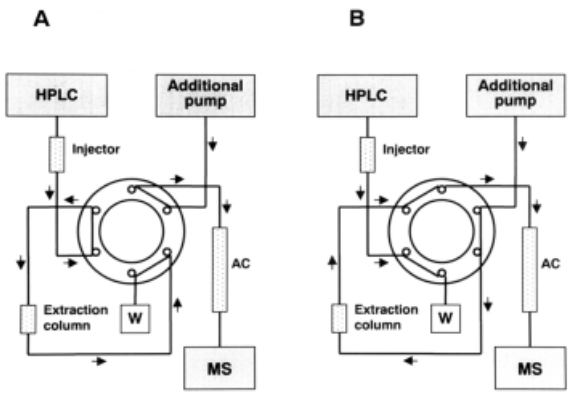


**Fig. 1** Column-switching scheme of the on-line extraction procedure (W, waste; AC, analytical column). A: Loading the sample onto the extraction column; B: elution from the extraction column onto the analytical column and analytical chromatography.

sulfate (50 g/l) 4/1 v/v containing 200 µg/l tri-deuterated cortisol as the internal standard. After vigorous vortex mixing, the samples were centrifuged with a benchtop centrifuge at 15000 *g* for 10 min; 150 µl of the clear supernatant were transferred into HPLC vials and placed into the autosampler.

For on-line extration a Waters Oasis HLB column (HLB 1.0 × 50 mm; Waters, Milford, USA) was used together with a Rheodyne six-port high-pressure switching valve (Rheodyne, Rohnert Park, CA, USA) installed into the Waters 2690 separation module and controlled by the Micromass Masslynx 3.4 software.

The extraction procedure consisted of three steps: first 50 µl of deproteinized sample were injected and loaded onto the extraction column in valve position A (Figure 1); the mobile phase was water/methanol, 95/5 v/v delivered at a flow rate of 2 ml/min; potentially interfering compounds were washed into the waste. In parallel, the analytical column (Reprosil pur C18-AQ, 125 × 2 mm; 5 µm, Maisch, Ammerbuch, Germany) was equilibrated with methanol/2 mM ammonium acetate, 75/25 v/v at a flow rate of 0.4 ml/min. After 1 min, the switching valve changed to position B; the extraction column was then eluted in the back-flush mode onto the analytical column. After 2 min, the valve switched back to position A. During analytical chromatography onto the mass spectrometer in postion A the extraction column was washed with methanol at a flow rate of 2 ml/min for 1 min and subsequently re-equilibrated with water-methanol 95/5 v/v. Both extraction and analytical column were kept at 40 °C in a column oven. The retention time of cortisol and d3-cortisol was approximately 2.9 min. After injection onto the extraction column the total run-time was 5 min.

*Mass spectrometric conditions*

Electrospray atmospheric pressure ionization in the positive mode was used; the source parameters were tuned to obtain the $[M+H]^+$ molecular ions of cortisol and d3-cortisol, respectively (363 and 366 m/z); the following settings resulted in optimal ion yield: capillary voltage, 3.0 kV; cone voltage, 30V; source temperature 85 °C; desolvation temperature 300 °C at a nitrogen flow of approximately 630 ml/min and a nebulizer gas flow of approximately 75 ml/min.

The collision energy with argon as the collision gas was 17 V. Under these conditions, an intense daughter ion of 309 m/z was obtained from cortisol, and of 312 m/z from tri-deuterated cortisol used as the internal standard. Thus, the following ion transitions were used for multiple reaction monitoring (MRM) from minute 1 to 5 after sample injection: cortisol 363>309 m/z and d3-cortisol 366>312 m/z. The dwell time in both MRM traces was 0.5 s, interchannel delay and interscan delay were both 0.15 s.

For quantification, the response of calibrator and serum samples was calculated as the peak area ratio of the MRM trace of cortisol and the internal standard d3-cortisol, respectively.

The methanolic stock solution of tri-deuterated cortisol used as the internal standard was analyzed using the assay to exclude possible hydrogen/deuterium exchange; as specified by the manufacturer, a ratio of 365 Da to 362 Da cortisol of 98:2 was found.

*Assay validation*

To study the linearity ot the method, regression analysis of the six calibrator samples run in four batches was carried out. To study the accuracy of the method, external quality control materials were analyzed in triplicate (Lyphochek® Immunoassay Plus Controls, Bio-Rad Laboratories, Munich, Germany; Lot 40090); the target cortisol concentrations in these materials at three levels were determined applying an isotope dilution gas chromatography-mass spectrometry reference method (1) by INSTAND e.V. (Düsseldorf, Germany).

To investigate the imprecision of the method, three human serum pools were prepared from residual clinical samples, at a low, medium and high concentration range. Analyses were performed in four batches over a 1-year period, each in quadruplicate to calculate the total coefficient of variation.

The specificity of the assay was verified towards relevant endogenous steroids (corticosterone, 17α-OH-progesterone, tetrahydrocortisone, tetrahydrocortisol, 6β-hydroxycortisol, DHEA, and DHEA-S) as well as for relevant synthetic steroids (dexamethasone, prednisolone, methylprednisolone, prednisone) using standard solutions of the compounds as samples without adding the internal standard (each containing 1000 µg/l, DHEA-S 10 mg/l).

To test the applicability of the method to human serum, 44 clinical samples taken before and 60 min after *i.v.* dose of 250 µg synthetic ACTH 1–24 (Synacthen®, Novartis Pharma, Nürnberg, Germany) were analyzed after routine determination of cortisol by an automated immunoassay (Elecsys 2010 Cortisol™, Roche, Mannheim, Germany).

## Results

The assay proved linear over the full calibration range (0–500 µg/l) with r>0.999 in the four batches. The regression equation for the calibration curve (mean slope and intercept of the four batches) was: cortisol [µg/l]=197 × response + 1.2 µg/l.

The analysis of external quality control materials revealed a close agreement with the respective target concentrations determined by a reference method (mean of three determinations): for Lyphochek® Immunoassay Plus Controls level 1, the observed value was 31 µg/l with the target 33 µg/l; for level 2, the observed value was 150 µg/l with the target 164 µg/l; and for level 3, the observed value was 269 µg/l with the target 263.

The total coefficient of variation (n=16) was 5.0% for the low pool (15.1 µg/l), 3.7% for the normal pool

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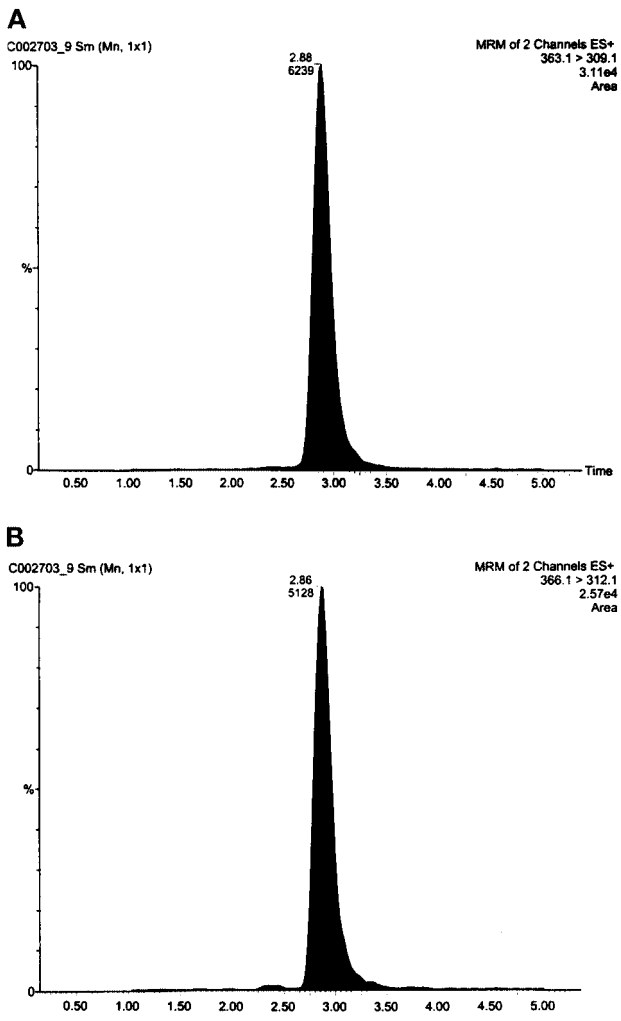


**Fig. 2** Representative multiple reaction monitoring chromatogram of a serum sample. m/z 363>309 represents the transition of cortisol (A), m/z 366>312 the transition of trideuterated cortisol used as the internal standard (B).

(58.1 µg/l) and 3.2% for the high pool (269 µg/l). At a cortisol concentration of 1 µg/l (in the matrix used for preparation of the calibrators) the signal-to-noise ratio was 4:1; at a concentration of 50 µg/l, representing the lower limit of the reference range (sampling in the morning), the signal-to-noise ratio was 36:1.

The compounds investigated for analytical specificity did not generate responses in any of the two used multiple reaction monitoring traces. Pure multiple reaction monitoring chromatograms for cortisol and the internal standard were obtained in all clinical samples (Figure 2); no additional peaks were found, and the peak shape was identical to that found in calibrators with an equally low degree of back-ground signal; the mean peak area of the internal standard in patients' samples was equal to that found in calibrator samples, ruling out differential matrix effects.

Good agreement was found between serum cortisol results obtained with the Roche Elecsys 2010 Cortisol® assay and the method described here (r=0.97, Elecsys cortisol = 0.9 × TMS cortisol – 1.0 µg/l; range 51–480 µg/l).

## Discussion

We described an isotope dilution mass spectrometric method for the determination of cortisol in serum that applies electrospray atmospheric pressure ionization after on-line solid phase extraction and liquid chromatographic separation of samples.

The method proved accurate, linear, precise, and specific towards relevant endogenous and exogenous compounds. The use of a stable isotope internal standard (that has identical chemical properties compared to native cortisol) and the highly specific technology of tandem mass-spectrometry (with quantification based on a specific transition reaction) suggests the potential to further develop this method as a reference method.

In the fragmentation spectra of both cortisol and d3-cortisol, a 120 m/z fragment was predominant; this relatively nonspecific fragment represents the ring-A moiety common to many steroids with a similar chemical structure. Therefore, the slightly less abundant fragments 309 m/z and 312 m/z for the internal standard were preferred for quantification. The use of 2 mM ammonium acetate instead of water resulted in approximately tenfold higher ionization yield.

Compared to the published GC-MS methods for the quantification of cortisol (1–5), the LC-tandem MS assay described here is by far less laborious, since a semi-automated sample extraction is used and derivatization is not necessary. Both the short hands-on time for sample preparation and the reduced analytical runtime make this method applicable to large series of samples. In a routine clinical setting the method offers the possibility to monitor the adrenal function during therapy with synthetic steroids, since the method proved highly specific toward these drugs. This is an important advantage compared to the available cortisol immunoassays and makes the method especially useful to help avoid hypocortisolism when administration of exogenous steroids is reduced.

In the future, LC-tandem MS systems will probably be more widely used clinically for therapeutic drug monitoring and metabolic profiling (6). Our results demonstrate that this technology – if available – may also be useful for highly specific serum cortisol measurement.

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