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Short communication

Quantitative analysis of human serum corticosterone by high-performance liquid chromatography coupled to electrospray ionization mass spectrometry

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Abstract

An original method based upon high-performance liquid chromatography coupled to electrospray ionization mass spectrometry has been developed for corticosterone (B) quantification in human serum. After extraction by diethyl ether using triamcinolone (T) as an internal standard, solutes are separated on a C_{18} microbore column (250×1.0 mm, I.D.), using acetonitrile–water–formic acid (40:59.9:0.1, v/v/v) as the mobile phase (flow-rate 40 μl/min). Detection is performed on an API 1 single quadrupole mass spectrometer equipped with a ESI interface and operated in positive ionization mode. Corticosterone quantifications were realized by computing peak area ratios (B/T) of the serum extracts analyzed in SIM mode (*m/z* 347 and *m/z* 395 for B and T, respectively), and comparing them with the calibration curve (r=0.998).

Keywords: Corticosterone

1. Introduction

Serum corticosterone (B compound) concentrations are of clinical significance in adrenal dysfunction. Its measurement may be of some help in apparent mineralocorticoid excess syndrome [1]. This steroid could also be a marker of malignancy in adrenal tumors [2,3].

Numerous methods have been described for the determination of corticosterone based on fluorimetry [4–7], radioimmunoassay [8,9], high-performance liquid chromatography (HPLC) [7–14]. Most of them, however have the disadvantage that their specificities are not absolute as certain amount of interference or cross-reactivity with other steroids may occur.

Normal human serum concentrations contain 21.7±8.1 (mean±SD) nmol/l of corticosterone [7], thus very sensitive and specific methods are required to measure the very low human serum concentrations of corticosterone.

HPLC has numerous advantages, e.g., very selective separations, comparatively short analysis times, and simple preliminary treatment of the sample.

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Electrospray ionization mass spectrometry (ESI-MS) has the advantage that it can easily be coupled to HPLC as the ion source is at atmospheric pressure and the detection is very sensitive and specific.

Here we describe a simple, rapid and highly specific and sensitive method by HPLC coupled to ESI-MS for quantitative determination of human serum corticosterone.

2. Experimental

2.1. Materials

Corticosterone (B, M_r=346.5) and the internal standard (I.S.) triamcinolone (T, M_r=394.4) were obtained from Sigma (St. Quentin Fallavier, France). Diethyl ether was obtained from Merck (Darmstadt, Germany). Acetonitrile was of HPLC grade from Scharlau (Barcelona, Spain). Formic acid was obtained from Merck.

The tuning and calibration solution for the mass spectrometer consisted of an equimolecular mixture of a 10^{-4} *M* solution of high-molecular-mass polypropylene glycols (PPGs) in water–methanol (50:50, v/v+2 m*M* ammonium formate+0.1% acetonitrile), provided by Perkin-Elmer Sciex (Foster City, CA, USA).

2.2. Chromatography

Chromatographic separations were carried out by HPLC using an Applied Biosystems 140B automated gradient controller pump model (Foster City, CA, USA) and its output was connected directly to ESI interface (splitless) by a silica capillary tube (75 μm I.D.). Samples were manually injected using a Hamilton Model 1702 (Reno, NV, USA) gastight syringe. The injector was a Rheodyne valve Model 2992 (USA) with a 25-μl loop (Cotati, CA, USA). Applications entailing continuous infusion of a definite analyte (e.g., MS tuning or spectrum determinations) were carried out using a precision, single-syringe low-pressure infusion pump (Havard Apparatus Model 11, South Natick, MA, USA) and a 1-ml gastight syringe (Hamilton Model 1001) for MS tuning and a 100-μl gastight syringe (Hamilton Model 1710) for spectrum determination of the standard.

The HPLC separations were performed on a 5 μm C_{18} Microbore (Vydac) column (250×1.0 mm I.D.), operated at ambient temperature. The elution was achieved isocratically (flow-rate 40 μl/min) with a mobile phase of acetonitrile–water–formic acid (40:59.9:0.1, v/v/v). Before use this mobile phase was degassed. Due to the microbore column, an equilibration time of at least 5 h was necessary before performing a series of analyses. Aliquots (25 μl) of supernatant samples were analysed by HPLC coupled with ESI-MS. At the end of each series, the column was thoroughly rinsed with a mixture of acetonitrile–deionized water (30:70, v/v) at a flow-rate of 40 μl/min for 2 h, and stored.

2.3. Mass spectrometry

ESI-MS was performed on an API 1 single quadrupole mass spectrometer (Perkin-Elmer Sciex), equipped with an ion-spray (nebulizer-assisted electrospray) source (Sciex, Toronto, Canada). The system was monitored by an Apple Macintosh computer equipped with the softwares Tune v.2.4.1, MacSpec v.3.3 and MacQuan v.1.4 (Perkin-Elmer Sciex) for instrument control and data acquisition, processing and solute quantification, respectively.

Air (purity grade N_{50}, i.e., 99.999%, L'Air Liquide, Paris, France) was employed as the nebulizing gas at a pressure of 50 p.s.i. (flow-rate 1 l/min) (1 p.s.i.=6894.76 Pa). The instrument was operated in the positive ionization mode with a voltage of 5 kV applied to the sprayer during all experiments. Spectra were recorded at an orifice voltage of +90 V. To prevent solvent vapours and contaminants from entering the vacuum chamber, the area in front of the orifice was continuously flushed with a ''curtain gas'' ($N_{60,}$ purity 99.9999, from L'Air Liquide, operating pressure 30 p.s.i.) at a flow-rate of 1 l/min during all experiments. The temperature of the curtain gas was 50°C. The system was tuned by PPGs, monitoring the ions at *m*/*z* 59, 326, 520, 906, 1254, 1545, 1836 and 2010 for mass calibration, lens optimization and peak width adjustments.

For direct analysis, corticosterone standard was dissolved in 20% acetonitrile in water, 0.1% formic acid (2.88 pmol/μl). The solutions were continuous-

ly infused with a medical infusion pump (Model 11, Harvard Apparatus) at a flow-rate of 5 μl/min. Ion spray mass spectra were acquired at unit resolution by scanning from m/z 100 to 600 with a step size of 0.1 and a dwell time of 2 ms. MacSpec was used for calculation of the molecular masses of corticosterone during direct analysis.

For quantitative determinations of corticosterone, ESI-MS data for corticosterone and triamcinolone (I.S.) were collected in the selected ion monitoring (SIM) mode (dwell time 100 ms) at m/z 347 and 395 respectively. Quantitative determinations were performed by MacQuan v.1.4.

2.4. Extraction procedure

Serum samples were either used at once or stored at −20°C until analysis. A 10-ml volume of diethyl ether was added to 1 ml of serum sample; this was vortex mixed for 3 min to extract the steroid in to the solvent and then centrifuged at 2500 *g* for 10 min.

After freezing the mixture for 15 min at −25°C, the lower frozen aqueous phase was separated from the upper organic layer. The organic layer was transfered to a 15-ml test tube, and evaporated to dryness in a water bath at 45°C under a nitrogen stream (99.99% pure, Euregaz, Fampeux, France). Before injection, 50 μl of the solvent methanol–water (60:40, v/v) was added to the dried human serum extract, this was vortex mixed for 60 s and 25 μl portions were injected into the chromatograph.

2.5. Biological samples

Human serum samples were collected from normal subjects in the morning (T_0) and 60 min after adrenal stimulation by an injection of tetracosactide (ACTH) (synacthene 0.25 mg i.v. Ciba-Giegy Rueil-Malmaison, France) (T_{60}).

3. Results and discussion

By using HPLC–ESI-MS, we have developed a quantitative assay for corticosterone in human serum. By employing SIM, a higher degree of selectivity was obtained compared to the potential cross-reactivity prevalent in immunoassay methods.

A modified method of extraction by diethyl ether was chosen as already described by Wei et al. [20]. This method has numerous advantages. It is simple, rapid and gives very high and reproducible extraction efficiencies.

Microbore HPLC coupled to ESI-MS has a huge advantage in terms of sensitivity and instrument ease of operation. Indeed, the microbore-type column and the absence of post-column splitting allows 40 μl to be analyzed by MS. Isocratic elution was chosen for low noise and short equilibrium time. In our experience the major ion peak for corticosterone and the I.S. was always found to be m/z 347 $(M+H)^+$ and m/z 395 $(M+H)^+$, respectively at different orifice voltages. Whereas the highest abundance of these ions was found at +90 V which was used for corticosterone quantitation. SIM was employed to obtain a high degree of selectivity and sensitivity required for corticosterone quantitation in human serum, as the morning human corticosterone levels are very low. A dwell time of 100 ms was chosen for sharpness and symmetry of corticosterone and the I.S. peaks. The major advantages of this technique are its simplicity and sensitivity.

A full scan background subtracted positive ion mass spectrum of a pure standard of corticosterone in 20% acetonitrile in water, 0.1% formic acid is shown in Fig. 1 (orifice voltage +90 V). This was recorded from a continuous, 5 μl/min syringe infusion of a 28.8 pmol/μl solution of the analyte. The m/z 347 was represented by $(M+H)^+$, as already shown by Wong et al. [15].

Fig. 2 presents SIM (m/z 347) chromatogram of corticosterone standard 0.45 pmol/μl obtained by decimal dilution of a 2.88 nmol/μl methanolic solution of corticosterone in methanol–water (60:40, v/v). The chromatogram was recorded in SIM mode at m/z 347, an orifice voltage +90 V and dwell time 100 ms. The additional peak observed in the chromatogram is 18-hydroxy-desoxycorticosterone, added in the standard as we had doubt that it could interfere while corticosterone quantification in serum. The HPLC separation was performed on a microbore-type column, as such columns are well adapted to the low-flow-rates imposed by the ESI technique, thus require no post-column splitting. The elution was achieved isocratically (flow-rate 40 μl/min) with a mobile phase of acetonitrile–water–

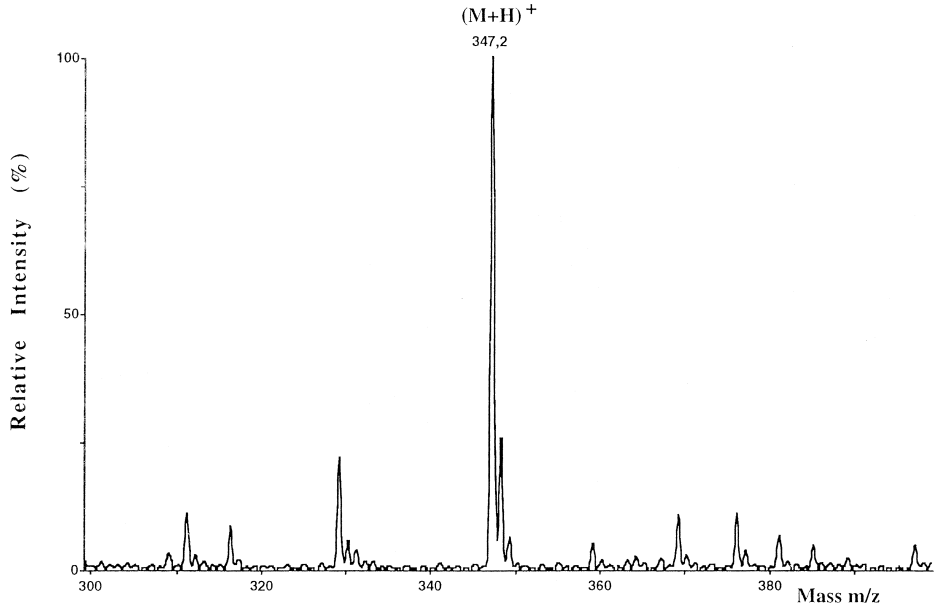

Fig. 1. 28.8 pmol/μl methanolic solution of corticosterone standard. Direct ESI-MS analysis (orifice voltage +90 V).

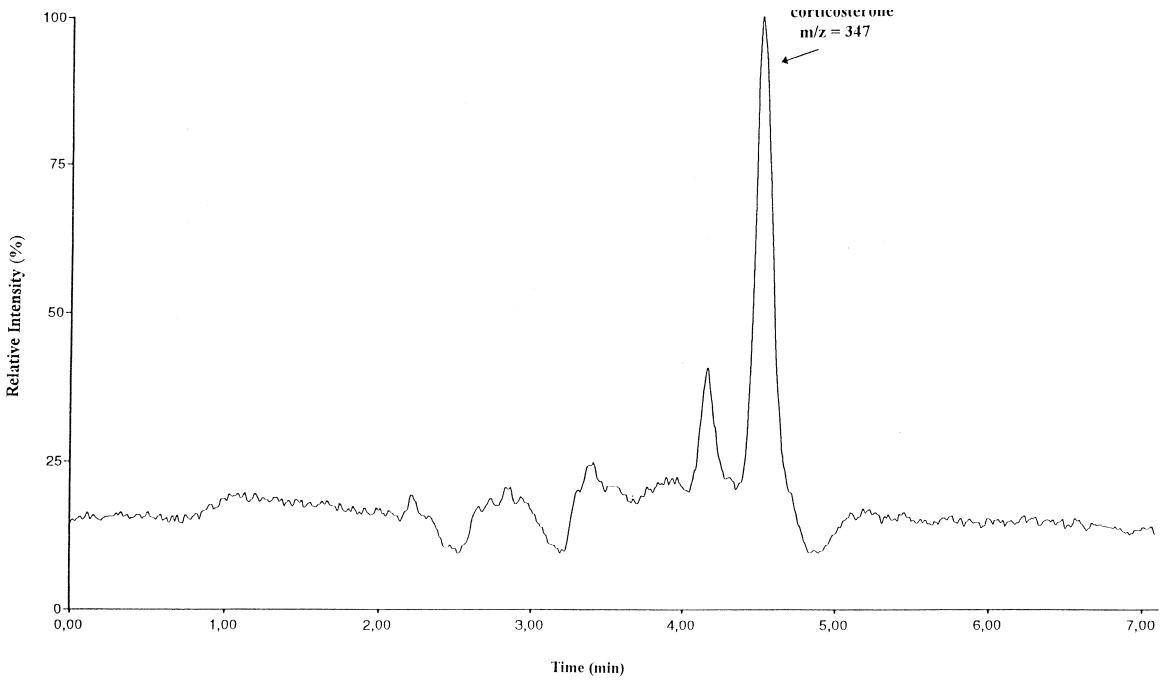

Fig. 2. Chromatogram of corticosterone standard (0.45 pmol/μl) by HPLC coupled to ESI-MS. Data recorded in SIM mode at m/z 347. Column: 5 μm C_{18} Microbore (Vydac), 250×1.0 mm I.D. Eluent: acetonitrile–water–formic acid (40:59.9:0.1, v/v/v); flow-rate 40 μl/min.

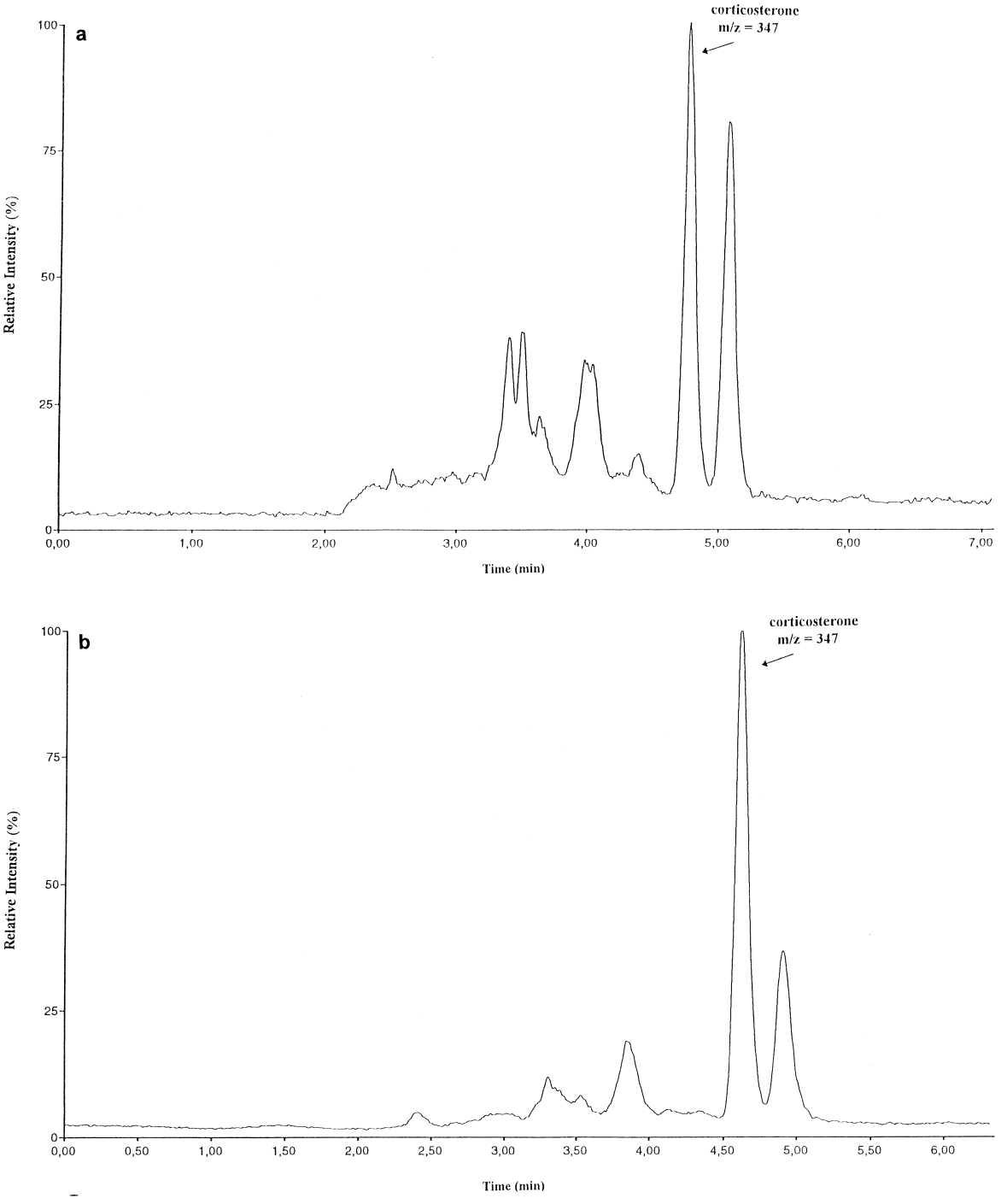

Fig. 3. (a) Chromatogram of human serum pool corticosterone determined at 0.52 pmol/μl (before adrenal administration: T_0) by HPLC coupled to ESI-MS. Data recorded in SIM mode at m/z 347. Column: 5 μm C_{18} Microbore (Vydac), 250×1.0 mm I.D. Eluent: acetonitrile–water–formic acid (40:59.9:0.1, v/v/v); flow-rate 40 μl/min. (b) Chromatogram of human serum pool corticosterone determined at 1.89 pmol/μl (60 min after adrenal administration: T_{60}) by HPLC coupled to ESI-MS. Data recorded in SIM mode at m/z 347. Conditions as in (a).

formic acid (40:59.9:0.1 v/v/v). Under the chromatographic conditions described above, average retention times for corticosterone and the internal standard triamcinolone were 4.6 and 3.4 min, respectively.

Absolute recovery was determined by extracting and assaying human serum pool loaded with corticosterone at a concentration of 0.29 pmol/μl. The recoveries were 93.6±8% (mean±SD) in six samples analyzed.

Accuracy and precision for the assay were determined by extracting and assaying human serum pool with corticosterone at 0.52 pmol/μl (ten replicates). The measured concentrations (mean±2SD) were 0.52±0.04 pmol/μl (relative standard deviation, RSD 3.8%). The day-to-day precision, estimated by daily analysis of an aliquot of human serum pool at 0.52 pmol/μl over a period of three days was <5%. The lower limit of detection defined as a signal-to-noise ratio of 3 was reached with a 0.18 pmol/μl, i.e., 4.5 pmol (25 μl) corticosterone injected.

Fig. 3a shows a SIM (m/z 347) chromatogram of human serum pool before adrenal stimulation (T_0), corticosterone 0.52 pmol/μl (solvent methanol–water, 60:40, v/v). Where as Fig. 3b shows the SIM (m/z 347) chromatogram of human serum pool corticosterone after adrenal stimulation by synacthen (T_{60}), 1.89 pmol/μl (solvent methanol–water, 60:40, v/v).

Corticosterone quantifications were realized by computing peak area ratios (corticosterone/triamcinolone) of the serum extracts analyzed in SIM mode, and comparing them with calibration curve at concentrations of 0.45, 0.90, 1.8, 3.6 and 7.2 pmol/μl (each analysis performed in duplicate). The samples were extracted by diethyl ether, containing triamcinolone as internal standard. The data were collected in the SIM mode (dwell time 100 ms) at m/z 347 for corticosterone and m/z 395 for triamcinolone (I.S.). Results for the calibration curve showed good linearity (r=0.998) over the concentration range tested, with an equation of y=1.072x+0.102 (y=B concentration in pmol/μl; x=B area/T area). MacQuan v.1.4 was used for corticosterone quantification.

As it is well known that corticosterone levels are increased after adrenal stimulation by ACTH [2,3,17], we confirmed it by our method by performing a series of analyses in seventeen subjects before adrenal stimulation (T_0) and in ten subjects after adrenal stimulation by ACTH. Human serum concentration with this method before adrenal stimulation (T_0) was 0.49±0.10 pmol/μl, i.e., 24.7±5.4 (mean±SD) nmol/l (1 ml serum extracted, evaporated to dryness, and after addition of 50 μl of solvent, 25 μl injected), which is quiet similar to concentration already reported in literature [3,7]. Where as human serum concentration after adrenal stimulation (T_{60}) increased 2–6-fold.

4. Conclusions

The present method is the first described for quantitative analysis of corticosterone in human serum by means of HPLC–ESI-MS. Most of the methods already described for corticosterone determinations have some disadvantages. Radioimmunoassay (RIA) has traditionally been used for corticosterone analysis due to its sensitivity [9]. The commonly employed RIA techniques use antiserum which significantly crossreacts with precursors and metabolites of B, and with other endogenous steroids and their metabolites [18,19]. By employing MS, a higher degree of selectivity was obtained compared to the potential cross-reactivity prevalent in RIA methods. A further advantage of our method is the elimination of the handling of radioactive-labeled materials. HPLC coupled to UV detection is less-sensitive [15,16]. Methods utilizing gas chromatography require time consuming and labor-intensive derivatization, necessary for the analyte to be in a volatile state for analysis. The method we developed requires no derivatizing of the sample, is simple, rapid, highly specific and sensitive owing to the simple extraction procedure and mass detection.

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