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Simultaneous determination of cortisol and cortisone in human plasma by stable-isotope dilution mass spectrometry

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ABSTRACT

A method for the simultaneous determination of cortisol and cortisone in human plasma was developed using capillary gas chromatography–mass spectrometry–selected ion monitoring. [$^2H_5$]Cortisol and [$^2H_5$]cortisone were used as internal standards. Cortisol and cortisone in plasma were determined from the peak-height ratios of the [M − 31] fragment ions of the methoxime–trimethylsilyl derivatives of cortisol and [$^2H_5$]cortisol (*m/z* 605 and 610) and of cortisone and [$^2H_5$]cortisone (*m/z* 531 and 536). Sensitivity, specificity, precision, accuracy and reproducibility of the method were demonstrated to be satisfactory for measuring the circulating concentrations of cortisol and cortisone.

INTRODUCTION

The conversion of cortisol to cortisone by the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) is a major step in the metabolism of cortisol by many peripheral tissues [1]. 11β-HSD has been thought to regulate the local action of cortisol [2–4] and it has recently been reported that the defective oxidoreduction at C-11 is related to several diseases [5]. The introduction of highly sensitive radioimmunoassay (RIA) has greatly simplified the measurement of the circulating concentration of cortisone [6,7]. There are, however, serious restrictions to the use of RIA because of the difficulty encountered in achieving acceptable specificity owing to the cross-reactivity with other steroids.

We have previously described a sensitive and reliable gas chromatographic–mass spectrometric (GC–MS) method for determining plasma levels of cortisol by using [1,1,19,19,19-$^2H_5$]cortisol ([$^2H_5$]cortisol) as an internal standard [8]. The method has been applied to studies of detailed analysis of diurnal variations of plasma cortisol in paediatric patients with orthostatic dysregulation [9].

In this study, the same initial isolation and derivatization procedures employed for cortisol were successfully applied for the GC–MS determination of cortisone. This made possible the simultaneous determination of cortisol and cortisone in human plasma, in which the respective stable isotope-labelled analogues ([$^2H_5$]cortisol and [$^2H_5$]cortisone) served as ideal internal standards.

EXPERIMENTAL

Chemicals and reagents

[1,1,19,19,19-$^2H_5$]Cortisol ([$^2H_5$]cortisol) and [1,1,19,19,19-$^2H_5$]]cortisone ([$^2H_5$]cortisone) were synthesized in this laboratory as described

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elsewhere [8–10]. The isotopic composition of the stable isotope-labelled cortisol and cortisone was 97.94 atom% deuterium (d_5, 97.27; d_6, 1.14%; d_7, 1.59%). [4-^{14}C]Cortisol (2.0 GBq/mmol) was purchased from New England Nuclear Research Products (Boston, MA, USA). [4-^{14}]Cortisone was synthesized by oxidation with chromium trioxide after blocking [4-^{14}C]cortisol as the bis-methylenedioxy (BMD) derivative. Deprotection of [4-^{14}C]cortisone–BMD with 46% hydrofluoric acid followed by thin-layer chromatographic (TLC) purification [Kieselgel 60F_{254} (Merck, Darmstadt, Germany); chloroform–methanol (9:1, v/v)] furnished [4-^{14}C]cortisone with a specific activity of 1.2 MBq/mmol and a radiochemical purity of more than 83.6% as determined by radio-TLC (JTC-600, Aloka, Tokyo, Japan). Unlabelled cortisol and cortisone were purchased from Sigma (St. Louis, MO, USA). Methoxylamine hydrochloride and N,O-bis(trimethylsilyl)acetamide (BSA) were purchased from Eastman Kodak (Rochester, NY, USA) and Tokyo Kasei (Tokyo, Japan), respectively. All other chemicals and solvents were of analytical-reagent grade and were used as received.

Stock solutions

Stock solutions of cortisol (10.27 mg per 50 ml), cortisone (12.32 mg per 100 ml), [$^{2}H_5$]cortisol (811.50 μg per 50 ml) and [$^{2}H_5$]cortisone (174.35 μg per 50 ml) were prepared in methanol. All analyses were performed by diluting the stock solutions with methanol.

Gas chromatography–mass spectrometry–selected ion monitoring (GC–MS–SIM)

GC–MS–SIM measurements were made with a Shimadzu QP 1000 gas chromatograph–mass spectrometer equipped with a data processing system (Shimadzu, Kyoto, Japan). GC was performed on an SPB-1 fused-silica capillary column (7 m × 0.25 mm I.D.) with a 0.25-μm thin film (Supelco, Bellefonte, PA, USA). The mass spectrometer was operated in the electron-impact mode at an energy of 70 eV. Helium was used as the carrier gas at 29.4 kPa.

The splitless injector was used with a septum purge flow-rate of 5.0 ml/min and a split flow-rate of 30 ml/min. The purge activation time was 2 min. The initial column temperature was set at 150°C and 2 min after sample injection the temperature was increased at 20°C/min to 250°C, maintained there for 1 min, then increased at 10°C/min to 280°C. The temperature of the injector was 260°C and the ion source temperature was 280°C.

The multiple ion detector was focused on the characteristic fragment ions [M − 31] of the dimethoxime–tri(trimethylsilyl) derivatives of cortisol and of the dimethoxime–di(trimethylsilyl) derivatives of cortisone, *i.e.*, at m/z 605 for unlabelled cortisol, m/z 610 for the internal standard ([$^{2}H_5$]cortisol), m/z 531 for unlabelled cortisone and m/z 536 for the internal standard ([$^{2}H_5$]cortisone).

Sample preparation for GC–MS–SIM

Extraction. To 1.0 ml of human plasma were added 162.3 ng of [$^{2}H_5$]cortisol and 104.6 ng of [$^{2}H_5$]cortisone dissolved in 10 μl each of methanol and 2 ml of 0.5 *M* orthophosphoric acid solution. The plasma sample was applied to a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, USA). The cartridge was washed with 12 ml of distilled water and then eluted with 2 ml of methanol into a 2-ml V-vial. After evaporation of the eluate at 70°C under a stream of nitrogen, acetone (3 × 100 μl) was added to the residue and the sample was transferred into a 0.3-ml V-vial and then evaporated to dryness at room temperature under a stream of nitrogen.

Derivatization and purification. To the residue were added 100 μl of methoxyamine reagent (methoxyamine hydrochloride in pyridine, 5%, w/v) and the reaction mixture was heated at 100°C for 30 min. After cooling, the excess of reagent was removed under a stream of nitrogen. To the residue were added 50 μl of BSA and the vial was heated at 100°C for 2 h. After evaporation to dryness under a stream of nitrogen at room temperature, the residue was dissolved in *n*-hexane.

The *n*-hexane solution (3 × 100 μl) was applied to a Sep-Pak silica cartridge (Waters Assoc.), which was eluted with 7 ml of *n*-hexane.

The eluate was evaporated to dryness at 70°C under a stream of nitrogen and the residue was dissolved in 5 μl of carbon tetrachloride. A 0.7-μl portion of the solution was subjected to GC–MS.

Recovery

To 1.0 ml of human plasma were added 37 Bq of [4-^{14}C]cortisol or 30 Bq of [4-^{14}C]cortisone in ethanol and 2 ml of 0.5 *M* orthophosphoric acid solution. The plasma was applied to a Sep-Pak C_{18} cartridge as described above. The absolute recoveries for cortisol and cortisone were calculated by comparing the radioactivities before and after the extraction procedure.

Calibration

To each of five to seven standards containing known amounts of cortisol (10.27, 41.08, 102.7, 205.4 and 308.1 ng) and cortisone (1.232, 2.464, 6.160, 12.32, 49.28, 123.2 and 246.4 ng) dissolved in methanol, 162.3 ng of [$^{2}H_{5}$]cortisol and 104.6 ng of [$^{2}H_{5}$]cortisone were added. Each sample was prepared in triplicate. After evaporation of the solvent to dryness, the samples were derivatized and purified as described above. A 0.7-μl portion of a carbon tetrachloride solution (5 μl) was subjected to GC–MS. The peak-height ratios (*m/z* 605 to 610 and *m/z* 531 to 536) were determined in triplicate. The calibration graphs were obtained by an unweighted least-squares linear fitting of the peak-height ratios *versus* the amounts of cortisol or cortisone on each analysis of the standard mixtures.

Accuracy

Accuracy was determined by assaying five or six preparations of 1.0-ml portions of human plasma spiked with 10.27 or 30.81 ng of cortisol and 162.3 ng of [$^{2}H_{5}$]cortisol as the internal standard and with 10.80 or 21.08 ng of cortisone and 104.6 ng of [$^{2}H_{5}$]cortisone as the internal standard. After preparation of the sample for GC–MS–SIM as described above, the peak-height ratios (*m/z* 605 to 610 and *m/z* 531 to 536) were measured.

RESULTS AND DISCUSSION

The number and the stability of the label are fundamental aspects in stable isotope methodology coupled with GC–MS. To incorporate deuterium atoms into positions in the steroid skeleton, the incorporated deuterium is supposed not to be readily displaced by hydrogen under the conditions employed in the assay. The requirement for incorporating four or more deuterium atoms at predesignated positions of the cortisone molecule has not been easily achievable and the employment of deuterated analogues of cortisone as internal standards in quantification by GC–MS has not been established. We have recently synthesized cortisone labelled with five deuterium atoms at chemically stable sites ([1,1,19,19,19-$^{2}H_{5}$]cortisone) [10–12] and were therefore interested in developing GC–MS to measure simultaneously the small amounts of cortisol and cortisone in human plasma.

The conditions employed for the formation of the methoxime–trimethylsilyl (MO–TMS) derivative of cortisol [8] were found to produce a thermally stable MO–TMS derivative of cortisone with good GC behaviour. Fig. 1 shows the electron-impact mass spectra of the MO–TMS derivatives of unlabelled cortisone and [$^{2}H_{5}$]cortisone. These mass spectra were similar to those of cortisol and [$^{2}H_{5}$]cortisol [8] in that the relative intensities of the fragment ions [M − 31] at *m/z* 531 (unlabelled) and 536 (labelled) were prominent compared with those of the molecular ions at *m/z* 562 and 567. Methoxime formation at the C-11 carbonyl group of cortisone did not occur [13]. The [M − 31] fragment ions at *m/z* 531 and 536 were selected as the ions to be monitored for the GC–MS analysis of cortisone.

The absolute recovery of cortisone from human plasma examined by using [^{14}C]cortisone was found to be 87.8–99.5% during the initial extraction procedure using Sep-Pak C_{18} cartridges. The high extraction efficiency obtained for cortisone was comparable to that obtained for cortisol (Table I). Fig. 2 shows the selected ion recordings of the MO–TMS derivatives of cortisol (*m/z* 605) and [$^{2}H_{5}$]cortisol (*m/z* 610) and

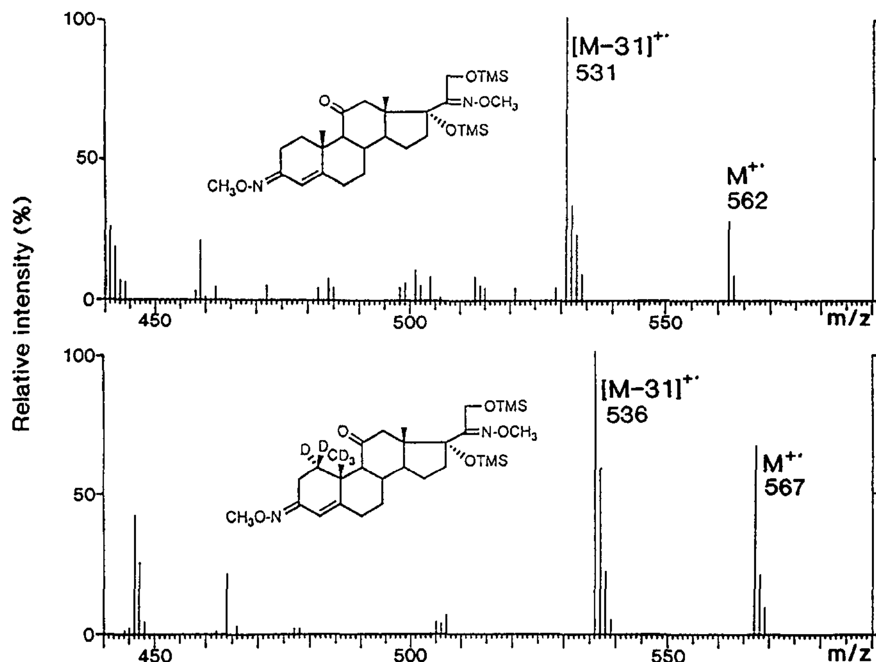

Fig. 1. Mass spectra of MO–TMS derivatives of cortisone (top) and [$^2H_5$]cortisone (bottom).

of cortisone (*m/z* 531) and [$^2H_5$]cortisone (*m/z* 536) for a processed plasma sample spiked with 162.3 ng of [$^2H_5$]cortisol and 104.6 ng of [$^2H_5$]cortisone. Sharp and single peaks of the respective MO–TMS derivatives of cortisol and [$^2H_5$]cortisol and of cortisone and [$^2H_5$]cortisone appeared without interfering peaks with retention times close to those of these derivatives. The purification procedure using the Sep-Pak silica cartridge after the MO–TMS derivatization removed interfering peaks from the plasma sample on the selected ion recordings for the simultaneous measurement of cortisol and cortisone.

TABLE I

RECOVERY OF CORTISONE AND CORTISOL FROM HUMAN PLASMA

Compound	Recovery (%)	
	Individual values	Mean ± S.D.
Cortisone	99.5, 96.2, 95.6, 87.8, 90.9	94.0 ± 4.63
Cortisol	98.1, 100.3, 99.1, 90.3, 101.3, 94.5	97.3 ± 4.14

Calibration graphs were prepared in the ranges 10–300 ng of cortisol and 1–250 ng of cortisone by using [$^2H_5$]cortisol (162.3 ng) and [$^2H_5$]cortisone (104.6 ng) as internal standards. The peak-

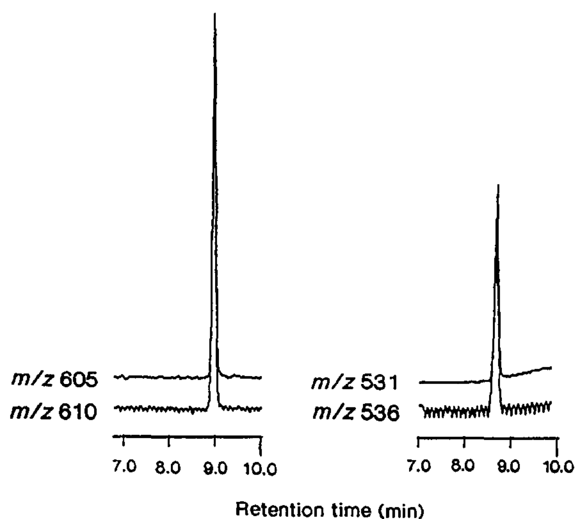

Fig. 2. Selected ion recordings of MO–TMS derivatives of cortisol (*m/z* 605), [$^2H_5$]cortisol (*m/z* 610), cortisone (*m/z* 531) and [$^2H_5$]cortisone (*m/z* 536).

TABLE II

ACCURACY OF GC–MS–SIM DETERMINATION OF CORTISOL AND CORTISONE IN PLASMA

Added (ng/ml)	Expected (ng/ml)	Found (ng/ml)		Relative error (%)	R.S.D. (%)
		Individual values[a]	Mean ± S.D.		
Cortisol					
–	–	60.62, 64.90, 64.91, 59.36, 64.91, 66.88	63.10 ± 2.91	–	4.6
10.27	73.37	79.21, 72.99, 75.40, 70.70, 76.54, 76.58	75.24 ± 3.00	+2.6	4.0
30.81	93.91	95.87, 94.45, 92.04, 90.02, 89.84, 89.12	91.89 ± 7.56	−2.2	3.0
Cortisone					
–	–	27.23, 27.00, 26.20, 26.13, 27.08, 26.98	26.77 ± 0.48	–	1.8
10.80	35.57	37.48, 37.88, 39.27, 36.63, 36.00	37.45 ± 1.25	−0.32	3.3
21.08	47.85	48.88, 47.55, 49.01, 47.07, 48.38, 48.73	48.27 ± 0.79	+0.88	1.6

[a] Each individual value represents the mean of duplicate measurements.

height ratios of the recordings at m/z 605 (cortisol) and 610 ([$^2H_5$]cortisol) and at m/z 531 (cortisone) and 536 ([$^2H_5$]cortisone) were plotted against the mixed molar ratios of cortisol to [$^2H_5$]cortisol and of cortisone to [$^2H_5$]cortisone. A good correlation was found between the observed peak-height ratios (y) and the mxied molar ratios (x). Unweighted least-squares regression analysis gave the regression line $y = 1.015\,x - 0.022$ ($r = 0.9998$) for cortisol and $y = 0.878\,x + 0.034$ ($r = 0.9998$) for cortisone. There was no indication of contributions and interferences at the masses monitored.

The accuracy of measurements was determined for cortisol and cortisone added to 1.0-ml aliquots of pooled plasma. The contents of endogenous cortisol and cortisone in the pooled plasma were 63.10 and 26.77 ng/ml, respectively. To the plasma samples were added fixed amounts of the internal standards (162.3 ng of [$^2H_5$]cortisol and 104.6 ng of [$^2H_5$]cortisone) and different amounts of cortisol (10.27 and 30.81 ng) and cortisone (10.80 and 21.08 ng). Table II shows that the amounts of cortisol and cortisone added were in good agreement with the amounts of cortisol and cortisone measured, the relative error being less than 3% for cortisol and 1% for cortisone. The inter-assay relative standard deviations (R.S.D.s) ($n = 6$) were less than 5% for cortisol and less than 4% for cortisone. The intra-assay R.S.D.s ($n = 3$) were less than 3% for both cortisol (73.37 and 93.91 ng/ml) and cortisone (37.57 and 47.85 ng/ml). The sensitivity of the present GC–MS assay was 200 pg per injection for cortisol and cortisone with a signal-to-noise ratio of about 3.

The described method provides a sensitive and reliable technique for the simultaneous determination of plasma concentrations of cortisol and cortisone. Good accuracy and precision are obtained without complex corrections for contributions by using [$^2H_5$]cortisol and [$^2H_5$]cortisone as internal standards for the GC–MS assay.

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