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Simultaneous determination of endogenous and ^{13}C-labelled cortisols and cortisones in human plasma by stable isotope dilution mass spectrometry

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Abstract

This study describes a capillary GC–MS method for the simultaneous determination of endogenous cortisol and cortisone and their ^{13}C-labelled analogues, [1,2,4,19-$^{13}C_4$]cortisol (cortisol-$^{13}C_4$) and [1,2,4,19-$^{13}C_4$]cortisone (cortisone-$^{13}C_4$), in human plasma. [1,2,4,19-$^{13}C_4$,1,1,19,19,19-$^{2}H_5$]Cortisol (cortisol-$^{13}C_4$,$^{2}H_5$) and [1,2,4,19-$^{13}C_4$,1,1,19,19,19-$^{2}H_5$]cortisone (cortisone-$^{13}C_4$,$^{2}H_5$) were used as analytical internal standards. A double derivatization (bismethylenedioxy-pentafluoro-propionate, BMD-PFP) with good GC behavior was employed for the GC–MS analysis of cortisol and cortisone. Quantitation was carried out by selected-ion monitoring of the molecular ions ($[M]^{+\cdot}$) of the BMD-PFP derivatives of cortisol and cortisone. The sensitivity limit of the present GC–MS–SIM method was found to be 150 pg per injection for cortisol ($s/n=5.0$) and 50 pg for cortisone ($s/n=8.1$). The within-day reproducibility in which the amounts of unlabelled and labelled cortisols and cortisones determined were in good agreement with the actual amounts added, the relative errors being less than 3.07%. The inter-assay coefficients of variation (C.V.) were less than 1.80% for unlabelled and labelled cortisols and cortisones.

Keywords: Steroids; Cortisol; Cortisone

1. Introduction

11β-Hydroxysteroid dehydrogenase (11β-HSD) catalyzes the interconversion of cortisol to biological inactive cortisone. The reversible conversion of cortisol and cortisone in human has recently been subjects of biochemical and clinical investigations in connection with patients with low activities of 11β-HSD, characterized by hypertension, hypokalemia, and low renin [1–7].

Stable isotope dilution mass spectrometry is widely accepted as the most accurate and specific method for estimation of the small amounts of endogenous and synthetic steroids in biological fluids [8]. We have previously developed a sensitive and reliable GC–MS or LC–MS method for the simultaneous determination of cortisol and cortisone in plasma using deuterium-labelled internal standards, [1,1,19,19,19-$^{2}H_5$]cortisol (cortisol-$^{2}H_5$) and [1,1,19,19,19-$^{2}H_5$]cortisone (cortisone-$^{2}H_5$) [9–11].

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Stable isotopically labelled cortisol can also be used as a biological internal standard to investigate the pharmacokinetics of cortisol in patients for assessing the in vivo enzyme activities of 11β-HSD. This methodology has been applied to pharmacokinetic studies of cortisol and cortisone in human following administration of cortisol-$^{2}H_5$ or cortisone-$^{2}H_5$ [12,13]. The plasma samples containing endogenous and exogenous (labelled) cortisols and cortisones were analyzed by the double isotope dilution method [12], which requires the two series of plasma samples obtained at each time after the administration. The method, however, is time-consuming and often leads to a drawback in accurate and selective quantification of low levels of these compounds in biological fluids.

The aim of the present study is to develop simultaneous measurement of endogenous and exogenous (stable isotopically labelled) cortisols and cortisones in human plasma by GC–MS, using [1,2,4,19-$^{13}C_4$,1,1,19,19,19-$^{2}H_5$]cortisol (cortisol-$^{13}C_4$,$^{2}H_5$) and [1,1,19,19,19-$^{13}C_4$,1,2,3,4,5-$^{2}H_5$]cortisone (cortisone-$^{13}C_4$,$^{2}H_5$) as analytical internal standards (Fig. 1). The ^{13}C-labelled analogues, [1,2,4,19-$^{13}C_4$]cortisol (cortisol-$^{13}C_4$) and [1,2,4,19-$^{13}C_4$]cortisone (cortisone-$^{13}C_4$) as shown in Fig. 1, were chosen, instead of cortisol-$^{2}H_5$ and cortisone-$^{2}H_5$, for use as a biological internal standard in the in vivo metabolic or pharmacokinetic studies.

2. Experimental

2.1. Chemicals and reagents

Cortisol and cortisone were purchased from Sigma (St. Louis, MO, USA). Stable isotopically labelled compounds, i.e., [1,2,4,19-$^{13}C_4$]cortisol (cortisol-$^{13}C_4$), [1,2,4,19-$^{13}C_4$]cortisone (cortisone-$^{13}C_4$), [1,2,4,19-$^{13}C_4$,1,1,19,19,19-$^{2}H_5$]cortisol (cortisol-$^{13}C_4$,$^{2}H_5$), and [1,2,4,19-$^{13}C_4$,1,1,19,19,19-$^{2}H_5$]cortisone (cortisone-$^{13}C_4$,$^{2}H_5$) for use as biological and analytical internal standards were synthesized in this laboratory [14]. The isotopic compositions of the labelled compounds were >97.3 atom% for cortisol-$^{13}C_4$ and >96.7 atom% for cortisol-$^{13}C_4$,$^{2}H_5$, respectively. Pentafluoropropionic anhydride (PFPA) (GL Sciences, Tokyo, Japan) and paraformaldehyde (Kanto, Tokyo, Japan) were obtained commercially. Benzene was redistilled before use and all other chemicals and solvents were of analytical-reagent grade and were used without further purification.

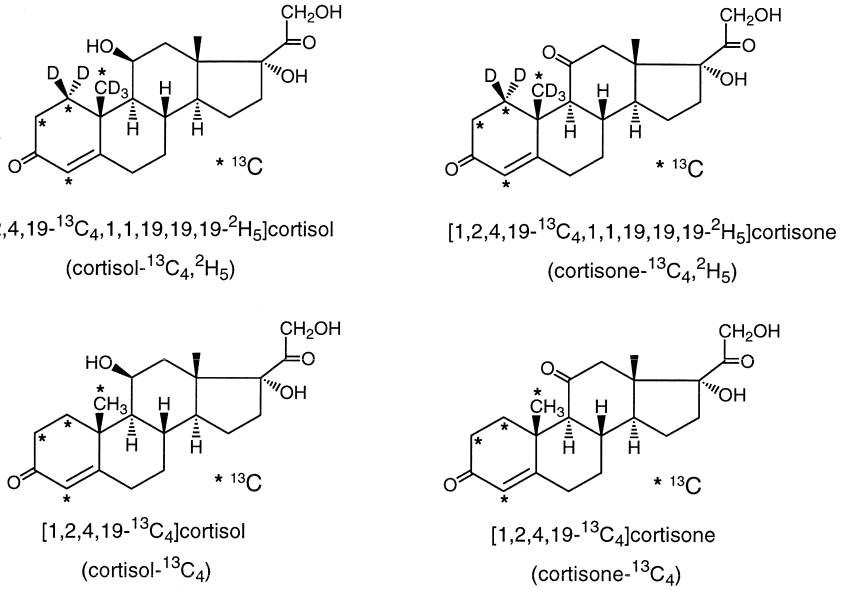

Fig. 1. Structures of stable isotopically labelled cortisols and cortisones (cortisol-$^{13}C_4$, cortisone-$^{13}C_4$, cortisol-$^{13}C_4$,$^{2}H_5$ and cortisone-$^{13}C_4$,$^{2}H_5$).

2.2. Preparation of standards

Stock solutions of cortisol (1.046 ng/μl), cortisone (1.042 ng/μl), cortisol-$^{13}C_4$ (0.6520 ng/μl), cortisone-$^{13}C_4$ (1.206 ng/μl), cortisol-$^{13}C_4$,$^{2}H_5$ (1.608 ng/μl) and cortisone-$^{13}C_4$,$^{2}H_5$ (1.201 ng/μl) were prepared in methanol.

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

Capillary GC–MS–SIM analysis was carried out on a QP1000EX gas chromatograph mass spectrometer equipped with a data processing system (Shimadzu, Kyoto, Japan). Gas chromatography was performed on an SPB-1 fused-silica capillary column (15 m×0.25 mm I.D.) with the stationary phase coated at 0.25-μm film thickness (Supelco, Bellefonte, PA, USA). Helium was used as the carrier gas at a column head pressure of 40 kPa.

A split–splitless injection system (Shimadzu SPL-14) operated in the splitless mode was used with a septum purge flow-rate of 10 ml/min and a split flow-rate of 40 ml/min. The purge activation time was 2 min. The initial column temperature was set at 100°C. After the sample injection, it was maintained for 2 min and was increased at 20°/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. The mass spectrometer was operated in the electron-impact mode at an energy of 70 eV and the ion source temperature was set at 280°C.

The multiple-ion detector was focused on the molecular ions ($[M]^{+\cdot}$) at m/z 696/700 for the bismethylenedioxy-3, 11-dipentafluoropropionyl (BMD-diPFP) derivatives of cortisol/cortisol-$^{13}C_4$, and at m/z 548/552 for the bismethylenedioxy-3-pentafluoropropionyl (BMD-monoPFP) derivatives of cortisone/cortisone-$^{13}C_4$, and at m/z 705 for the corresponding derivative of cortisol-$^{13}C_4$,$^{2}H_5$ and at m/z 557 for cortisone-$^{13}C_4$,$^{2}H_5$ as analytical internal standards, respectively.

2.4. Sample preparation for GC–MS–SIM

2.4.1. Extraction

To 0.5 ml of human plasma was added, 80.42 ng of cortisol-$^{13}C_4$,$^{2}H_5$ and 48.04 ng of cortisone-$^{13}C_4$,$^{2}H_5$ as the internal standards dissolved in methanol. The plasma sample was applied to a Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA). The cartridge was washed with 8 ml of distilled water and then eluted with 4 ml of ethyl acetate into a conical centrifuge tube (100×13 mm I.D.). After evaporation to dryness at 50°C under a stream of nitrogen, acetone (200 μl×2) was added to the residue and the sample was transferred into a 2-ml micro product V-vial (Wheaton, Millville, NJ, USA) and then evaporated to dryness at room temperature under a stream of nitrogen.

2.4.2. Derivatization

To the residue suspended in chloroform (50 μl) was added, 12% paraformaldehyde in 25.7% HCl (50 μl), and the reaction mixture was vigorously stirred at room temperature for 20 min. A solution of 12% paraformaldehyde in 25.7% HCl was freshly prepared by dissolving paraformaldehyde (1.68 g) in water (4 ml) and concentrated HCl (10 ml). The reaction mixture was extracted with chloroform (300 μl×4) and the extracts were washed with water (300 μl×3). The solvent was evaporated to dryness under a stream of nitrogen at room temperature. To the residue dissolved in benzene (50 μl) was added 50 μl of pentafluoropropionic anhydride (PFPA). The reaction mixture was vortexed for 1 min and then heated for 30 min at 70°C. After evaporating the excess reagent under a stream of nitrogen at room temperature, the residue was dissolved with cyclohexane (10 μl). A 1.0-μl portion of the solution was subjected to GC–MS. The derivatives were stable at room temperature for at least two months.

2.5. Calibration graphs

To each of six standards containing known amounts of cortisol (5.23, 10.46, 31.38, 62.76, 125.5 and 251.0 ng), cortisone (5.21, 10.42, 20.84, 41.68, 52.10 and 83.36 ng), cortisol-$^{13}C_4$ (6.52, 13.04, 26.08, 52.16, 97.80 and 195.6 ng) and cortisone-$^{13}C_4$ (6.03, 12.06, 24.12, 36.18, 54.27 and 72.36 ng) dissolved in methanol, 80.42 ng of cortisol-$^{13}C_4$,$^{2}H_5$ and 48.04 ng of cortisone-$^{13}C_4$,$^{2}H_5$ were added. Each sample was prepared in duplicate. After evaporation of the solvent to dryness, the samples were derivatized as described above. A 1.0-μl portion of a

cyclohexane solution (10 μl) was subjected to GC–MS. The peak-area ratios (*m/z* 696 and *m/z* 700 to *m/z* 705 for cortisol and *m/z* 548 and *m/z* 552 to *m/z* 557 for cortisone) were determined in triplicate. The calibration graphs were obtained by an unweighted least-squares linear fitting of the peak-area ratios versus the concentrations (ng/ml) of cortisol, cortisol-$^{13}C_4$, cortisone and cortisone-$^{13}C_4$ on each analysis of the standard mixtures.

2.6. Accuracy

Accuracy was determined by assaying six preparations of 0.5-ml portions of human plasma spiked with 41.84 ng of cortisol and 32.60 ng of cortisol-$^{13}C_4$ (80.42 ng of cortisol-$^{13}C_4$,$^{2}H_5$ as internal standard), and 20.84 ng of cortisone and 24.12 ng of cortisone-$^{13}C_4$ (48.04 ng of cortisone-$^{13}C_4$,$^{2}H_5$ as internal standard). After preparation of the sample for GC–MS–SIM as described above, the peak-area ratios (cortisol/cortisol-$^{13}C_4$,$^{2}H_5$, cortisol-$^{13}C_4$/cortisol-$^{13}C_4$,$^{2}H_5$, cortisone/cortisone-$^{13}C_4$,$^{2}H_5$, and cortisone-$^{13}C_4$/cortisone-$^{13}C_4$,$^{2}H_5$) were measured.

3. Results and discussion

Stable isotope methodology has widely been accepted for investigating the pharmacokinetics and metabolism of steroids in humans. One of the major advantages of the methodology coupled with mass spectrometry is that endogenous and exogenous compounds with the same basic structure can be differentiated easily by using the stable isotopically labelled compound as biological internal standard.

We have previously used multi-labelled cortisol and cortisone with five deuterium atoms at C-1 and C-19 with high isotopic purity (>98 atom% ^{2}H) [15,16] for the pharmacokinetic studies [12,13]. The deuterium labels at C-1 and C-19 are chemically and metabolically stable. The kinetic isotope effect derived from deuteriums at C-1 and C-19 could be minor in assessing the 11β-hydroxydehydrogenation of cortisol to cortisone catalyzed by 11β-HSD. ^{13}C-Labelled compounds, however, should preferably be used as biological internal standards, since they generally offer major advantages to metabolic or pharmacokinetic studies in vivo because of the stability of the label and the avoidance of isotope effects [17]. Recently, we have synthesized ^{13}C-labelled cortisol and cortisone (cortisol-$^{13}C_4$ and cortisone-$^{13}C_4$) containing four ^{13}C at C-1, C-2, C-4 and C-19 with high isotopic purity (>97.3 atom%^{13}C) for use as biological internal standards. We have also synthesized multilabelled cortisol and cortisone (cortisol-$^{13}C_4$,$^{2}H_5$ and cortisone-$^{13}C_4$,$^{2}H_5$) with five deuterium atoms at C-1 and C-19 in addition to the four ^{13}C atoms (>96.7 atom%^{2}H, ^{13}C) for use as the analytical internal standards. Multi-labelled cortisol and cortisone with ^{13}C and ^{2}H were prepared via the indan synthon method, starting from chiral 11-oxoindanylpropionic acid. [1,3-$^{13}C_2$]Acetone was used for the syntheses of cortisol-$^{13}C_4$ and cortisone-$^{13}C_4$, and [1,3-$^{13}C_2$,1,1,1,3,3,3-$^{2}H_6$]acetone was for cortisol-$^{13}C_4$,$^{2}H_5$ and cortisone-$^{13}C_4$,$^{2}H_5$. Detailed discussion concerning the syntheses of the labelled corticosteroids is described elsewhere [14]. The structures of these labelled compounds are given in Fig. 1.

For the GC–MS analysis of natural and synthetic glucocorticoids, we previously developed a new type of derivative (bismethylenedioxy-heptafluoro-*n*-butyrate, BMD-HFB) to simultaneously measure cortisol, cortisone, prednisolone and prednisone in plasma, which has been shown to result in good resolution, peak shape and sensitivity [10]. The BMD–HFB derivatization, however, was found not to be suitable for the analysis of tetrahydrocortisol (THF), allo-tetrahydrocortisol (allo-THF) and tetrahydrocortisone (THE) in human plasma and urine, since the BMD-3-monoHFB and BMD-3,11-diHFB derivatives of THF were not well separable from those of allo-THF [18]. The separation of the three tetrahydrocorticoids was achieved by the bismethylenedioxy-pentafluoropropionyl (BMD–PFP) derivatization [18]. The BMD-PFP derivatives of cortisol and cortisone have been confirmed to be separable from their synthetic analogues, prednisolone and prednisone, respectively. In the present study, cortisol and cortisone were simultaneously derivatized by the two-step reaction to give the BMD-3,11-dipentafluoropropionyl (BMD-diPFP) derivative of cortisol and the BMD-3-pentafluoropropionyl (BMD-monoPFP) derivative of cortisone (Fig. 2).

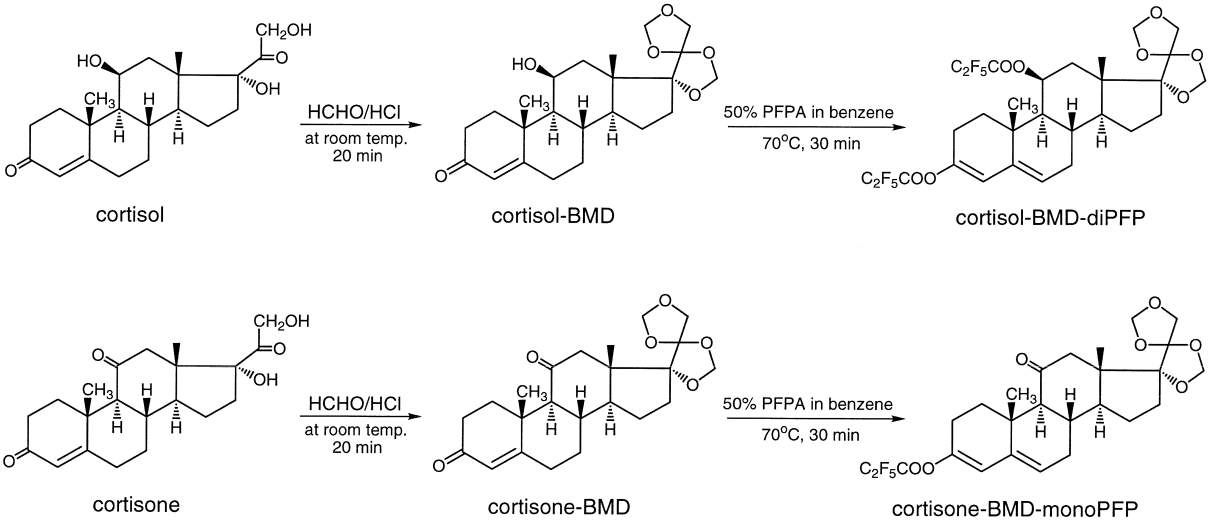

Fig. 2. BMD-PFP Derivatization for the GC–MS analysis of cortisol and cortisone.

Fig. 3 shows the electron-impact (EI) mass spectra of the BMD-diPFP derivatives of unlabelled and stable isotopically labelled cortisols. The BMD-diPFP derivatives gave the molecular ions $[M]^{+\cdot}$ at m/z 696 for cortisol, at m/z 700 for cortisol-$^{13}C_4$ and at m/z 705 for cortisol-$^{13}C_4$,$^{2}H_5$. The characteristic $[M\text{-}91]^+$ ($M\text{-}3CH_2O+H$) and $[M\text{-}164]^+$ ($M\text{-}C_2F_5COOH$) fragment ions were observed for the unlabelled and labelled cortisols, respectively.

Fig. 4 shows the EI mass spectra of BMD-monoPFP derivatives of unlabelled and stable isotopically labelled cortisones. The BMD-monoPFP derivatives gave the molecular ions $[M]^{+\cdot}$ at m/z 548 for cortisone, at m/z 552 for cortisone-$^{13}C_4$ and at m/z 557 for cortisone-$^{13}C_4$,$^{2}H_5$. The molecular ions $[M]^{+\cdot}$ in the mass spectra (Figs. 3 and 4), i.e., the $[M]^{+\cdot}$ ions for cortisol/cortisol-$^{13}C_4$/cortisol-$^{13}C_4$,$^{2}H_5$ (m/z 696/700/705) and cortisone/cortisone-$^{13}C_4$/cortisone-$^{13}C_4$,$^{2}H_5$ (m/z 548/552/557) were chosen for the selected ion monitoring of the BMD-PFP derivatives. When a signal-to-noise (s/n) ratio of 5.0 or greater was used as a criterion for a significant response, the sensitivity limit of the present GC–MS–SIM method was found to be 150 pg per injection for cortisol ($s/n=5.0$) and 50 pg for cortisone ($s/n=8.1$).

The present derivatization method was then applied to the simultaneous determination of endogenous and labelled cortisols and cortisones (cortisol-$^{13}C_4$ and cortisone-$^{13}C_4$) in human plasma, using their respective labelled internal standards (cortisol-$^{13}C_4$,$^{2}H_5$ and cortisone-$^{13}C_4$,$^{2}H_5$). Fig. 5 shows the selected ion-recordings of the BMD–PFP derivatives of unlabelled and labelled cortisols and cortisones after processing from spiked plasma. Blank plasma sample contained no interfering substances.

Administration of relatively too large a dose of tracer will cause the endogenous cortisol secretion to be lowered by negative feedback. Conversely administration of too little will lead to an unmeasurable concentration of labelled cortisol and cortisone [19]. We have previously measured plasma concentrations of endogenous and exogenous (deuterium-labelled) cortisols and cortisones in humans following administration of cortisol-$^{2}H_5$ or cortisone-$^{2}H_5$ [12,13]. There was no significant suppression of the endogenous cortisol secretion in two healthy volunteers after receiving a single 5-mg oral dose of cortisol-$^{2}H_5$ or cortisone-$^{2}H_5$. The plasma levels were in the range 16.13–125.6 ng/ml of endogenous cortisol and 5.64-43.98 ng/ml of endogenous cortisone. Maximum plasma concentrations of labelled cortisol and cortisone were 137.1 ng/ml and 15.57 ng/ml, respectively.

Calibration graphs were then prepared in the range 5.23–251.0 ng/ml of endogenous cortisol, 6.52–195.6 ng/ml of cortisol-$^{13}C_4$, 5.21–83.36 ng/ml of endogenous cortisone, and 6.03–72.36 ng/ml of

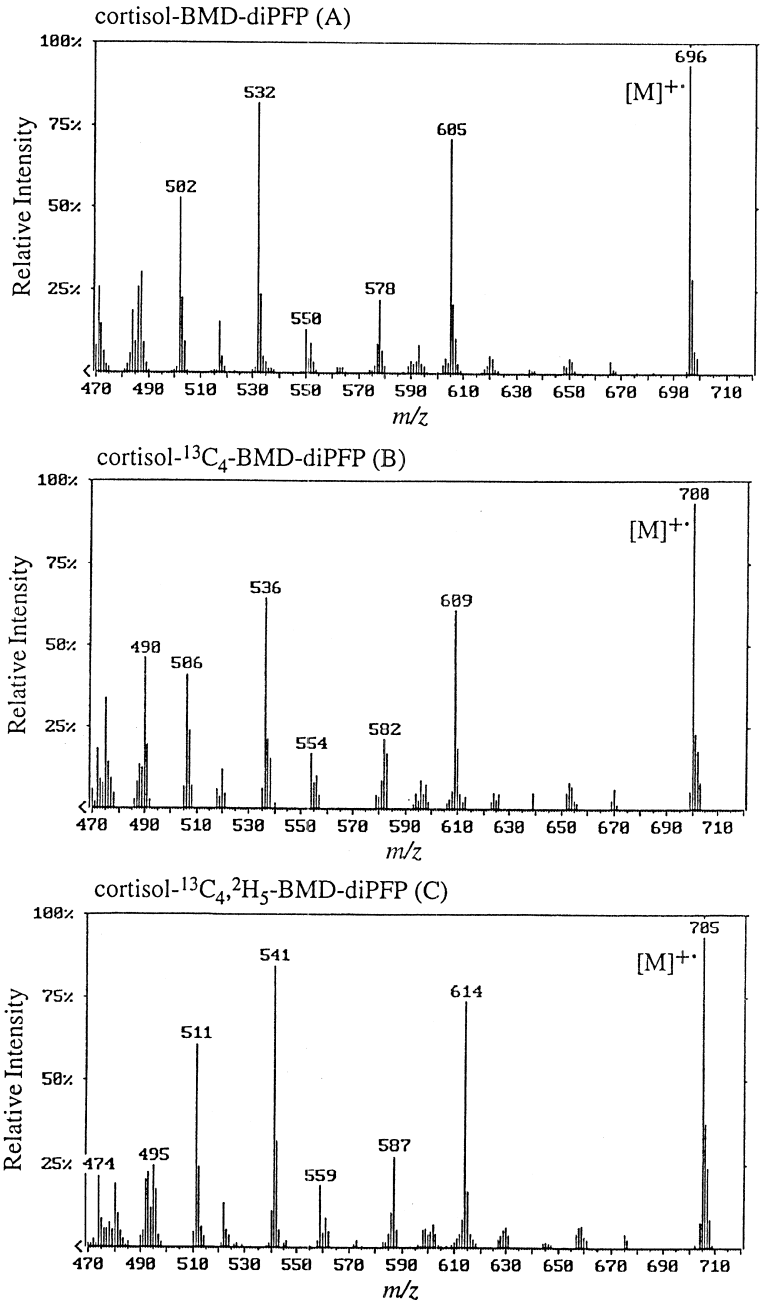

Fig. 3. Electron-impact mass spectra of BMD-diPFP derivatives of cortisol (A), cortisol-$^{13}C_4$ (B) and cortisol-$^{13}C_4$,$^{2}H_5$ (C).

cortisone-$^{13}C_4$ with cortisol-$^{13}C_4$,$^{2}H_5$ (80.42 ng) and cortisone-$^{13}C_4$,$^{2}H_5$ (48.04 ng) as the internal standards for the GC–MS assay. The peak-area ratios (cortisol, cortisol-$^{13}C_4$, cortisone and cortisone-$^{13}C_4$ to the corresponding labelled internal standards; cortisol-$^{13}C_4$,$^{2}H_5$ and cortisone-$^{13}C_4$,$^{2}H_5$) were plotted against the concentrations of unlabelled cortisol and cortisone and their ^{13}C-labelled ana-

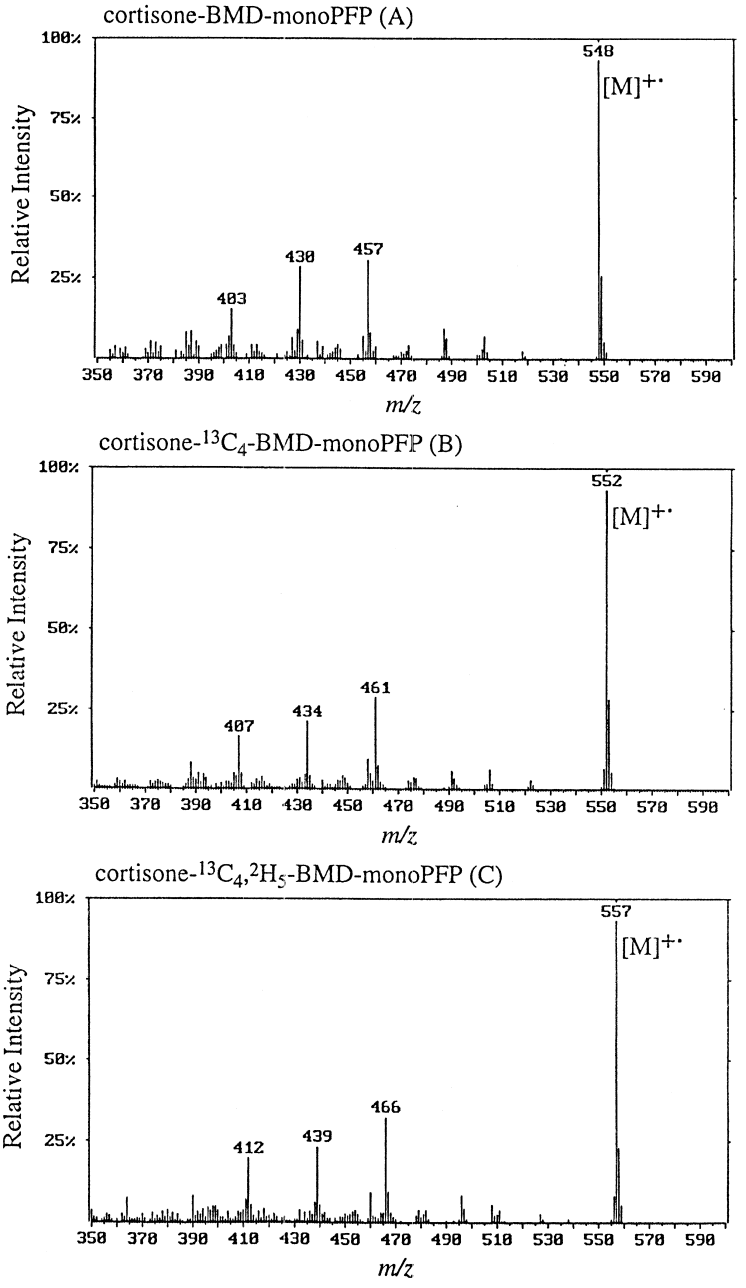

Fig. 4. Electron-impact mass spectra of BMD-monoPFP derivatives of cortisone (A), cortisone-$^{13}C_4$ (B), and cortisone-$^{13}C_4$,$^{2}H_5$ (C).

logues (cortisol-$^{13}C_4$ and cortisone-$^{13}C_4$). A good correlation was found between the observed peak-area ratios (y) and the plasma concentrations (x). Unweighted least-squares regression analysis gave typical regression lines $y=0.0136x-0.0265$ ($r=0.9998$) for cortisol, $y=0.0263x-0.0103$ ($r=0.9999$) for cortisone, $y=0.0124x+0.0033$ ($r=1.000$) for cortisol-$^{13}C_4$, and $y=0.0256x-0.0244$ ($r=0.9998$)

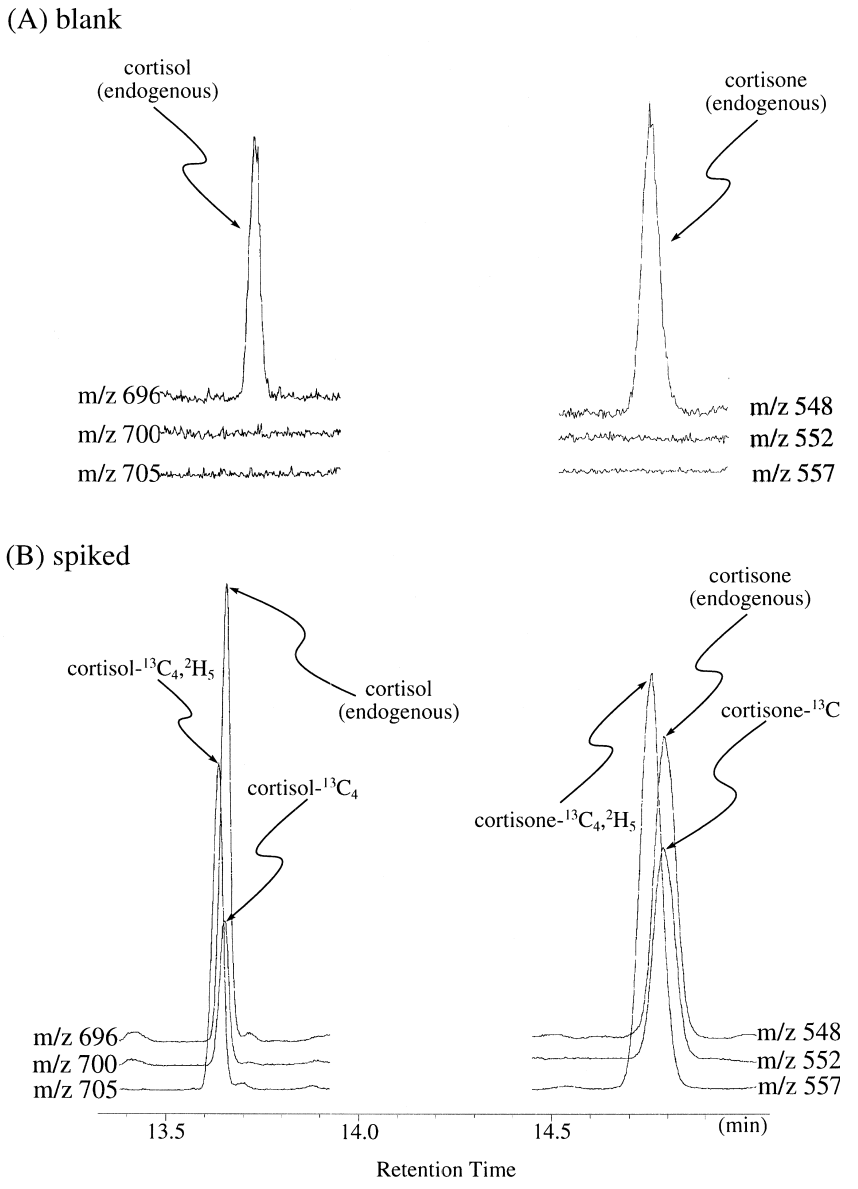

Fig. 5. Selected-ion recordings of BMD–PFP derivatives of cortisol/cortisol-$^{13}C_4$/cortisol-$^{13}C_4$,$^2H_5$ (*m/z* 696, 700 and 705) and cortisone/cortisone-$^{13}C_4$/cortisone-$^{13}C_4$,$^2H_5$ (*m/z* 548, 552 and 557) after processing from 0.5 ml of blank (A) and spiked plasma (B) with cortisol-$^{13}C_4$/cortisol-$^{13}C_4$,$^2H_5$(32.60 ng and 80.42 ng) and cortisone-$^{13}C_4$/cortisone-$^{13}C_4$,$^2H_5$ (24.12 ng and 48.04 ng).

for cortisone-$^{13}C_4$. There was no indication of contributions and interferences at the masses monitored.

The accuracy of measurements were determined for cortisol and cortisone added to 0.5-ml aliquots of pooled plasma containing endogenous cortisol (113.9±0.93 ng/ml plasma) and cortisone (22.74±0.41 ng/ml plasma). To the plasma sample were added fixed amounts of cortisol-$^{13}C_4$,$^2H_5$ (80.42 ng) and cortisone-$^{13}C_4$,$^2H_5$ (48.04 ng) as the internal standards and known amounts of cortisol (41.84 ng), cortisone (20.84 ng), cortisol-$^{13}C_4$

Table 1
Accuracy of GC–MS–SIM determination of cortisol, cortisone, cortisol-$^{13}C_4$ and cortisone-$^{13}C_4$ in human plasma

Added (ng/ml)	Expected (ng/ml)	Found (ng/ml)							Relative error (%)	C.V. (%)
		Individual values[a]						Mean±SD		
Cortisol										
–	–	113.6	113.6	116.7	113.1	113.7	113.7	113.9±0.93	–	0.81
83.68	197.6	193.11	197.2	195.9	194.7	195.3	194.9	195.2±1.35	−1.20	0.69
Cortisone										
–	–	22.25	22.59	23.28	23.18	22.44	22.69	22.74±0.41	–	1.80
41.68	64.42	64.77	62.39	63.12	63.24	62.53	61.85	62.98±1.01	−2.24	1.60
Cortisol-$^{13}C_4$										
65.20		62.90	64.64	65.21	64.08	65.06	64.75	64.44±0.85	−1.17	1.32
Cortisone-$^{13}C_4$										
48.24		48.07	46.63	46.02	47.07	46.19	46.60	46.76±0.74	−3.07	1.58

[a] Each individual value is the mean of triplicate measurements.

(32.60 ng), and cortisone-$^{13}C_4$ (24.12 ng). Table 1 shows the within-day reproducibility in which the amounts of unlabelled and labelled cortisols and cortisones determined were in good agreement with the actual amounts added, the relative error being −1.20% (cortisol), −2.24% (cortisone), −1.17% (cortisol-$^{13}C_4$) and −3.07% (cortisone−$^{13}C_4$). The inter-assay coefficients of variation (C.V.) were less than 1.80% for unlabelled and labelled cortisols and cortisones.

The present method provides a sensitive and reliable technique for the simultaneous determination of cortisol and cortisone and their stable isotopically labelled counterparts in plasma with good accuracy and precision. The method can be applied to pharmacokinetic and metabolic studies of cortisol with a particular interest in evaluating the interconversion of cortisol and cortisone catalyzed by 11β-HSD in vivo.

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