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Stable isotope dilution mass spectrometry for the simultaneous determination of cortisol, cortisone, prednisolone and prednisone in plasma

Hiromi Shibasaki, Takashi Furuta and Yasuji Kasuya

Department of Medicinal Chemistry and Clinical Pharmacy, Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-03 (Japan)

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ABSTRACT

A capillary gas chromatographic–mass spectrometric method for the simultaneous determination of cortisol, cortisone, prednisolone and prednisone in human plasma is described. [1,1,19,19,19-$^2H_5$]Cortisol, [1,1,19,19,19-$^2H_5$]cortisone, [1,19,19,19-$^2H_4$]prednisolone and [1,19,19,19-$^2H_4$]prednisone were used as internal standards. Formation of the bismethylenedioxy-3-heptafluoro-*n*-butyryl (BMD-HFB) derivatives made possible the separation of the four corticosteroids with good gas chromatographic behaviour. The new double derivatization has been demonstrated to be of value for sensitive and selective quantification by this technique. Detection was performed by monitoring the molecular ion (M^+) of the BMD-HFB derivatives for cortisone and prednisolone, the $[M - 18]^+$ ion for cortisol, and the $[M - 30]^+$ ion for prednisone. The method requires no complex corrections for contributions and provides good accuracy and precision.

INTRODUCTION

Prednisone and prednisolone, synthetic analogues of cortisone and cortisol, have been used widely for a number of years for the treatment of rheumatic and various other diseases. Prednisone has very little intrinsic glucocorticoid activity [1,2] and has to be converted into prednisolone for therapeutic effect. The administration of prednisone and prednisolone suppresses cortisol production [3]. The possible occurrence of daily variations in the pharmacokinetic behaviour of prednisone and prednisolone during different hours of the day could be correlated with the endogenous cortisol circadian rhythm [4]. Examination of the effect of these steroids on circulating cortisol concentrations has, therefore, significant importance for establishing optimal dosage schedules.

This paper deals with the simultaneous gas chromatographic (GC) assay of cortisol, cortisone, prednisolone and prednisone with detection by stable isotope dilution mass spectrometry (MS), using the respective deuterated analogues ([1,1,19,19,19-$^2H_5$]cortisol (cortisol-d_5), [1,1,19,19,19-$^2H_5$]cortisone (cortisone-d_5), [1,19,19,19-$^2H_4$]prednisolone (prednisolone-d_4) and [1,19,19,19-$^2H_4$]prednisone (prednisone-d_4)) as internal standards (Fig. 1). A new type of derivative (bismethylenedioxy-3-heptafluoro-*n*-butyrate, BMD-HFB) was used for the GC–MS analysis of the four corticosteroids.

Correspondence: Dr. Y. Kasuya, Department of Medicinal Chemistry and Clinical Pharmacy, Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan.

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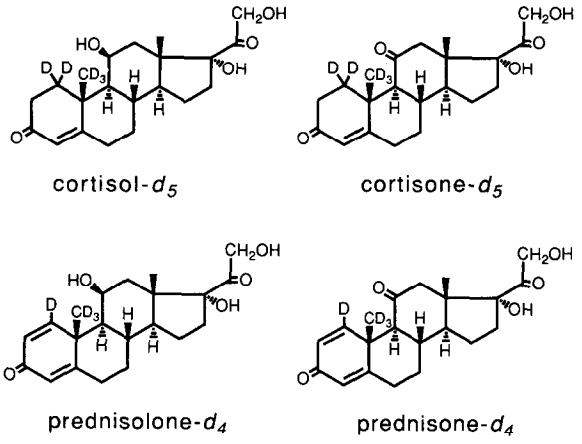

Fig. 1. Structures of the deuterated internal standards.

EXPERIMENTAL

Chemicals and reagents

Cortisol-d_5, cortisone-d_5, prednisolone-d_4 and prednisone-d_4 were synthesized in this laboratory as described elsewhere [5–8]. The isotopic composition of the deuterium-labelled compounds was greater than 98 atom%. Cortisol, cortisone, prednisolone and prednisone were purchased from Sigma (St. Louis, MO, USA). Heptafluoro-*n*-butyric anhydride (HFBA) and paraformaldehyde were purchased from Pierce (Rockford, IL, USA) and Kanto (Tokyo, Japan), respectively. All other chemicals and solvents were analytical-reagent grade and were used without further purification.

Stock solutions

Stock solutions of cortisol (10.60 mg per 50 ml), cortisone (12.32 mg per 100 ml), prednisolone (11.96 mg per 100 ml), prednisone (11.78 mg per 100 ml), cortisol-d_5 (667.5 μg per 50 ml), cortisone-d_5 (174.35 μg per 50 ml), prednisolone-d_4 (71.10 μg per 50 ml) and prednisone-d_4 (50.15 μg per 50 ml) were prepared in methanol. All analyses were performed by diluting the stock solutions with methanol.

Gas chromatography with selected-ion monitoring (SIM) mass spectrometry

GC–SIM-MS 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 SP2100 fused-silica capillary column (15 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 (EI) mode at an energy of 70 eV. Helium was used as the carrier gas at 29.4 kPa.

The splitless injector was used at a septum purge flow-rate of 5ml/min and a split flow-rate of 30 ml/min. The purge activation time was 2 min. The initial column temperature was set at 100°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 molecular (M^+) or characteristic fragment ions of the bismethylenedioxy-3-heptafluoro-*n*-butyryl (BMD-HFB) derivatives of the four corticosteroids and their labelled compounds as internal standards, *i.e.*, cortisone (M^+; *m/z* 598), cortisone-d_5 (M^+; *m/z* 603), prednisolone (M^+; *m/z* 598), prednisolone-d_4 (M^+; *m/z* 602), cortisol ($[M - 18]^+$; *m/z* 582), cortisol-d_5 ($[M - 18]^+$; *m/z* 587), prednisone ($[M - 30]^+$; *m/z* 566) and prednisone-d_4 ($[M - 30]^+$; *m/z* 570).

Sample preparation for GC–SIM-MS

Extraction. To 1.0 ml of human plasma were added 133.5 ng of cortisol-d_5, 69.74 ng of cortisone-d_5, 142.2 ng of prednisolone-d_4 and 120.4 ng of prednisone-d_4 dissolved in 10 μl each of methanol and 2 ml of 0.5 *M* orthophosphoric acid solution. The plasma sample (pH 1.5) was applied to a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, USA). The cartridge was washed with 8 ml of distilled water and then eluted with 2 ml of methanol into a 2-ml V-vial. After evaporation to dryness at 70°C under a stream of nitrogen, acetone (3 × 100 μl) was added to the residue and the sample was transferred to a 2-ml V-vial and evaporated to dryness at room temperature under a stream of nitrogen.

Derivatization. To the residue suspended in

chloroform (50 μl) were added 37% paraformaldehyde in water (25 μl) and 37% HCl (25 μl), and the reaction mixture was vigorously stirred at room temperature for 20 min. The mixture was extracted with chloroform (3 × 200 μl) and the chloroform extracts were washed with water (3 × 300 μl). The solvent was evaporated to dryness under a stream of nitrogen at room temperature. To the residue dissolved in acetone (200 μl) were added 50 μl of heptafluoro-*n*-butyric anhydride (HFBA). The reaction mixture was vortex-mixed for 1 min and then left for 1 h at room temperature. The excess reagent was removed under a stream of nitrogen at room temperature, and the residue was dissolved in dichloromethane (10 μl). A 1.0-μl portion of the solution was subjected to GC–MS.

Calibration graphs

To each of six standards containing known amounts of cortisol (4.24, 10.60, 21.20, 106.0, 169.6 and 254.4 ng), cortisone (3.08, 6.16, 12.32, 26.64, 61.60 and 123.2 ng), prednisolone (11.96, 47.84, 119.6, 239.2, 478.4 and 956.8 ng) and prednisone (11.78, 41.23, 117.8, 235.6, 412.3 and 824.6 ng) dissolved in methanol, 133.5 ng of cortisol-d_5, 69.74 ng of cortisone-d_5, 142.2 ng of prednisolone-d_4 and 120.4 ng of prednisone-d_4 were added. Each sample was prepared in triplicate. After evaporation of the solvent to dryness, the samples were derivatized as described above. A 1.0-μl portion of a dichloromethane solution (10 μl) was subjected to GC–MS. The peak-height ratios (*m/z* 582–587, *m/z* 598–603, *m/z* 598–602 and *m/z* 566–570) were determined in triplicate. The calibration graphs were obtained by an unweighted least-squares linear fitting of the peak-height ratios *versus* the mixed molar ratios of cortisol/cortisol-d_5, cortisone/cortisone-d_5, prednisolone/prednisolone-d_4 and prednisone/prednisone-d_4 on each analysis of the standard mixtures.

Accuracy

The accuracy was determined by assaying six preparations of 1.0-ml portions of human plasma spiked with 21.20 or 42.40 ng of cortisol (133.5 ng of cortisol-d_5 as internal standard), 12.32 or 24.64 ng of cortisone (69.74 ng of cortisone-d_5), 35.88 or 71.76 ng of prednisolone (142.2 ng of prednisolone-d_4) or 35.34 or 70.68 ng of prednisone (120.4 ng of prednisone-d_4). After preparation of the sample for GC–SIM-MS as described above, the peak-height ratios were measured.

RESULTS AND DISCUSSION

GC–SIM-MS is widely accepted as the most accurate and specific method for the estimation of small amounts of endogenous and synthetic steroids in biological fluids [9–13]. The use of stable isotopically labelled analogues as internal standards in the GC–MS analysis offers major advantages of compensating for losses of material at all stages of sample handling, which may not be the case with internal standards possessing a different structure from the compound under investigation. This methodology, however, has not as yet been applied successfully to the determination of prednisolone and prednisone in plasma. Complicated corrections for contributions from potential interferences are required, and the desired accurate quantification has not been achieved [14]. Successful application of stable isotope dilution MS to pharmacokinetic investigations is dependent on the availability of compounds labelled at predesignated positions that are chemically inert. We have synthesized multi-labelled cortisol, cortisone, prednisolone and prednisone containing four or five non-exchangeable stable isotopes with high isotopic purity [5–8].

The simultaneous determination of the four corticosteroids by GC–MS requires the GC separation of these compounds because of their close mass numbers. In the GC or GC–MS analysis of corticosteroids, it is usual to employ derivatives in which some or all of the original functional groups are protected. The C-17 dihydroxyacetone side-chain of corticosteroids undergoes thermal decomposition in the injection port. Thermal stabilization of corticosteroids in the GC and GC–MS analyses has been achieved

most successfully by formation of methoxime trimethylsilyl ether (MO-TMS) derivatives and analogous compounds such as methoxime-*tert.*-butyldimethylsilyl (MO-TBDMS) [15,16] and pentafluorobenzyloxime-trimethylsilyl (PFBO-TMS) ethers [17]. We have previously developed the stable isotope dilution MS method to be sensitive, specific and accurate enough to quantify

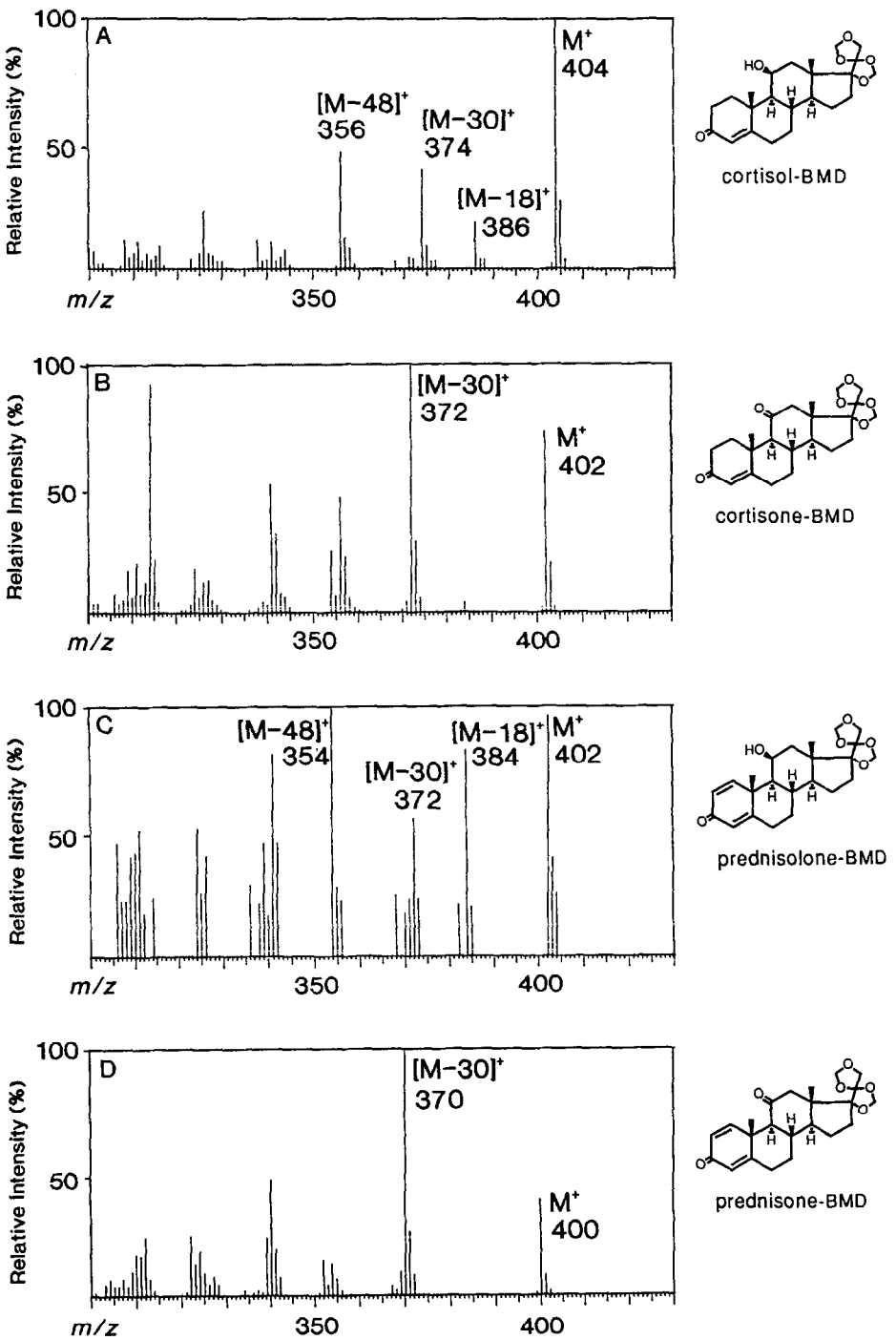

Fig. 2. Electron-impact mass spectra of BMD derivatives of cortisol (A), cortisone (B), prednisolone (C) and prednisone (D).

plasma cortisol and cortisone, using cortisol-d_5 and cortisone-d_5 as internal standards [18,19]. The MO-TMS derivatives of cortisol and cortisone employed are not separable from their synthetic analogues, prednisolone and prednisone, respectively, and are eluted as *syn–anti* isomer pairs of each derivative [20]. There have been no satisfactory methods available for the GC separation of cortisol, cortisone, prednisolone and prednisone. We have investigated other methods for derivatizing these synthetic steroids for analysis by GC–MS.

Among the types of derivative tested, the bismethylenedioxy (BMD) derivatives, which introduce chemical and thermal stability to the 17-dihydroxyacetone side-chain [21], seemed to be of promise for the GC separation of prednisolone and prednisone from the endogenous corticosteroids. The EI mass spectra illustrated in Fig. 2 show that the BMD derivatives gave the molecular ions M^+ at *m/z* 400 (prednisone), 402 (cortisone and prednisolone) and 404 (cortisol). The characteristic $[M - 30]^+$ fragment ions were attributed to the loss of HCHO, being observed for the four corticosteroids. The loss of H_2O (C-11) or HCHO or both from cortisol-BMD and prednisolone-BMD resulted in the formation of the ions $[M - 18]^+$ and $[M - 48]^+$. As shown in Fig. 3, SIM of the molecular ions of these BMD derivatives demonstrated the GC separation of the four corticosteroids.

In our previous paper, we described stable isotope dilution MS for the simultaneous determination of plasma testosterone and androstenedione [22]. The heptafluoro-*n*-butyryl (HFB) derivatization employed has been shown to result in good resolution, peak shape and sensitivity in GC–MS. In order to improve the chromatographic and mass resolutions of the BMD derivatives of the corticosteroids, the double derivatization of corticosteroids, BMD-3-heptafluoro-*n*-butyrylation (BMD-HFB), was attemped. Mass spectra of the BMD-HFB derivatives in the high mass region (ions above *m/z* 480) are shown in Fig. 4. Molecular ions (M^+) were prominent in the BMD-HFB derivatives of cortisone and prednisolone, but cortisol-BMD-HFB failed to give

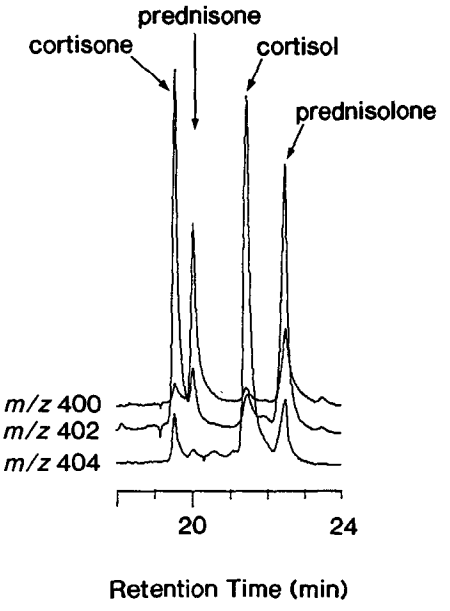

Fig. 3. Selected-ion recordings of BMD derivatives of cortisol (*m/z* 404), cortisone (*m/z* 402), prednisolone (*m/z* 402) and prednisone (*m/z* 400).

one, yielding an intense peak at *m/z* 582 ($[M - H_2O]^+$). Prednisone-BMD-HFB gave only a very weak molecular ion at *m/z* 596, and a notable feature of this spectrum was the peak at *m/z* 566 ($[M - HCHO]^+$). The corresponding fragment ion was observed at *m/z* 568 ($[M - HCHO]^+$) in the spectrum of the BMD-HFB derivative of prednisolone. The individual fragmentation processes were different among the four BMD-HFB derivatives in spite of their close structural similarities. No characteristic ions were observed in the molecular ion region (*m/z* 700–800) of the di-HFB derivatives of cortisol-BMD and prednisolone-BMD. This precluded the possibility of HFB formation at C-11 of cortisol and prednisolone. Fig. 5 shows mass spectra of the BMD-HFB derivatives of cortisol-d_5, cortisone-d_5, prednisolone-d_4 and prednisone-d_4. The isotopic composition of the deuterium-labelled compounds was greater than 98 atom%.

The characteristic ions in the mass spectra (Fig. 4), *i.e.*, the M^+ ions for cortisone (*m/z* 598) and prednisolone (*m/z* 598), the $[M - 18]^+$ ion for cortisol (*m/z* 582) and the $[M - 30]^+$ ion for prednisone (*m/z* 566) were chosen for SIM of the

BMD-HFB derivatives. As shown in Fig. 6, a marked improvement in the GC resolution of the BMD-HFB derivatives of prednisone, prednisolone, cortisol and cortisone was achieved. The selected-ion recordings indicate that, in all cases, a single product was obtained with no interference

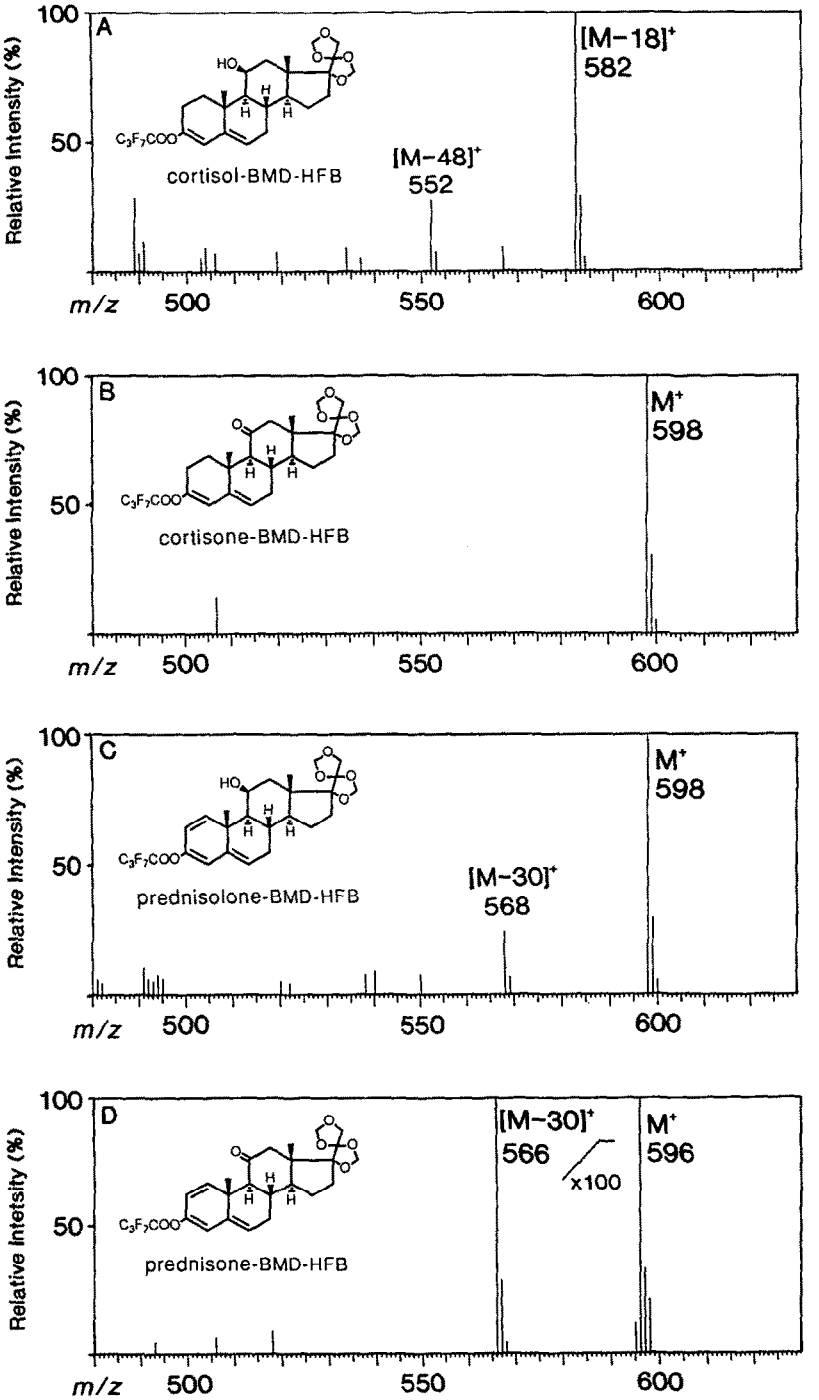

Fig. 4. Electron-impact mass spectra of BMD-HFB derivatives of cortisol (A), cortisone (B), prednisolone (C) and prednisone (D).

by ions from other compounds in the sample. When a signal-to-noise (S/N) ratio of 2.5 or greater was used as a criterion for a significant response, the sensitivity limit of the present GC–SIM-MS method was found to be 10 pg per injection (S/N = 2.7) for cortisone, 100 pg (S/N =

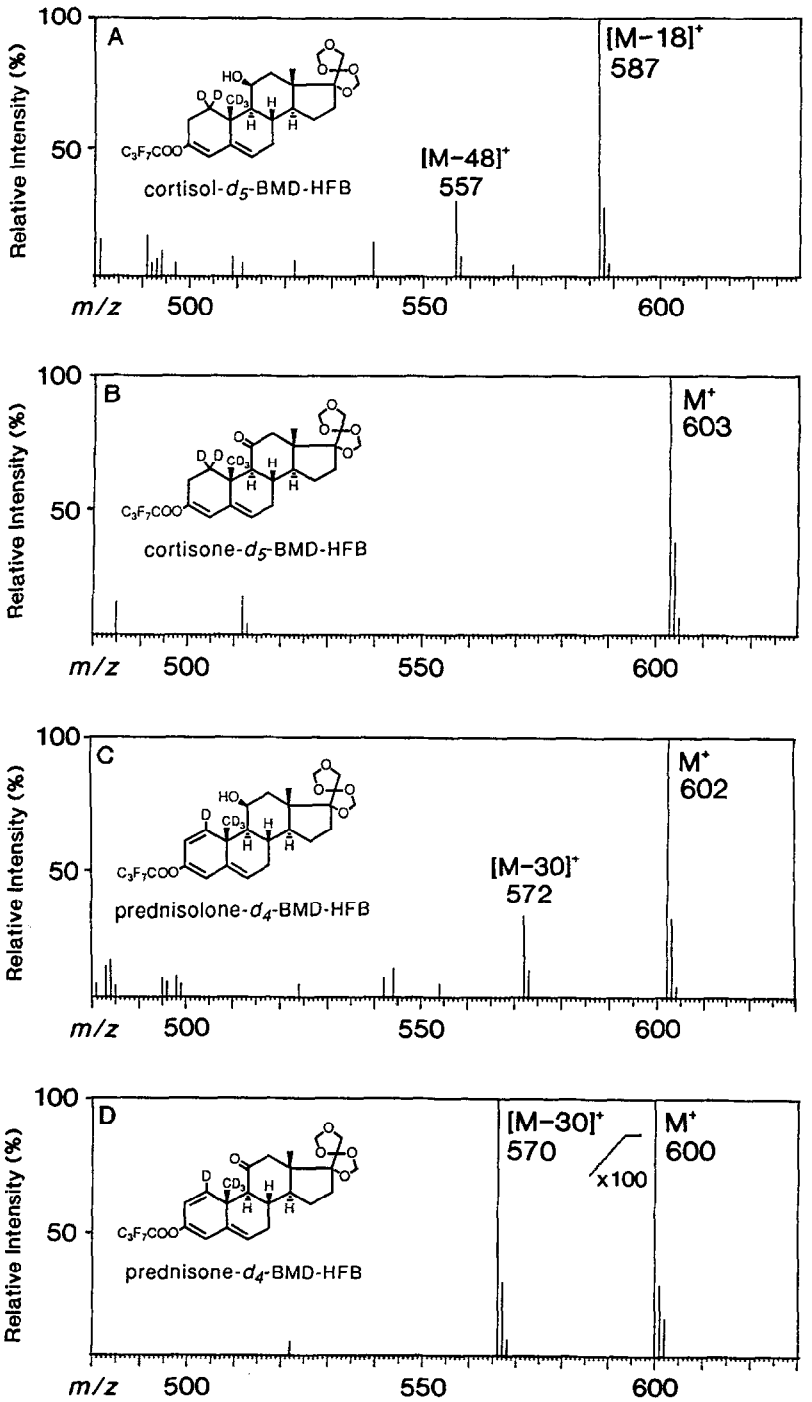

Fig. 5. Electron-impact mass spectra of BMD-HFB derivatives of cortisol-d_5 (A), cortisone-d_5 (B), prednisolone-d_4 (C) and prednisone-d_4 (D).

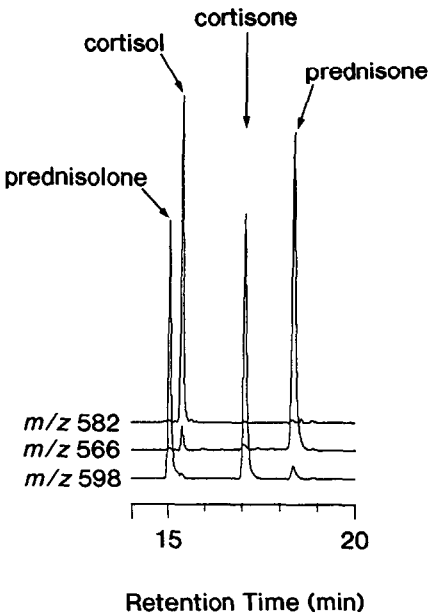

Fig. 6. Selected-ion recordings of BMD-HFB derivatives of cortisol (*m/z* 582), cortisone (*m/z* 598), prednisolone (*m/z* 598) and prednisone (*m/z* 566).

4.4) for prednisolone and 250 pg for cortisol (S/N = 2.7) and prednisone (S/N = 3.4) (Fig. 7).

We have applied the present derivatization method to the simultaneous determination of cortisol, cortisone, prednisolone and prednisone in human plasma, using their respective deuterium-labelled internal standards. Fig. 8 shows selected-ion recordings of BMD-HFB derivatives of unlabelled and labelled corticosteroids after processing from spiked plasma. Blank plasma samples contained no interfering substances derived from plasma.

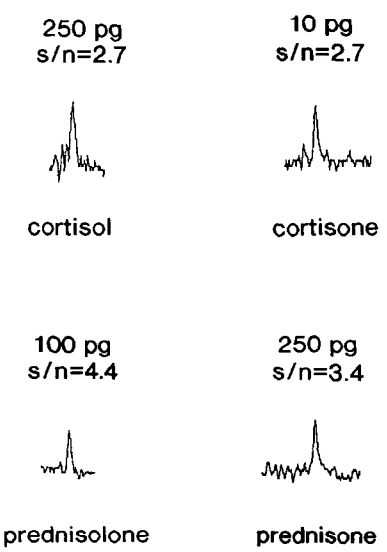

Fig. 7. Sensitivity limits for the four corticosteroids.

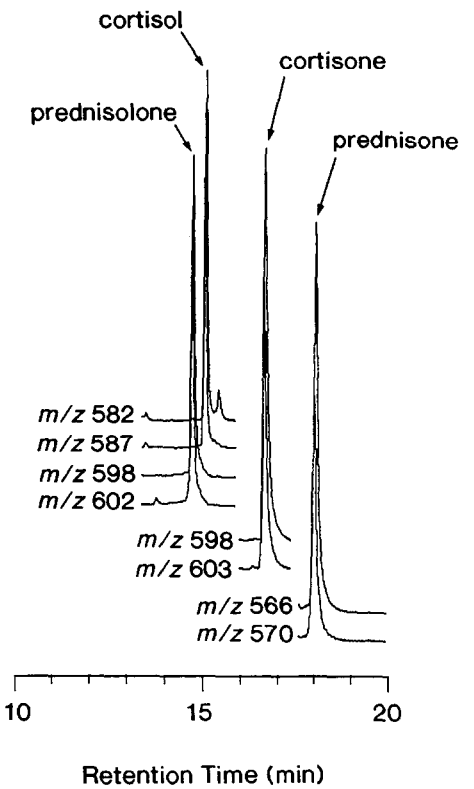

Fig. 8. Selected-ion recordings of BMD-HFB derivatives of cortisol/cortisol-d_5 (*m/z* 582 and 587), cortisone/cortisone-d_5 (*m/z* 598 and 603), prednisolone/prednisolone-d_4 (*m/z* 598 and 602) and prednisone/prednisone-d_4 (*m/z* 566 and 570) after processing from spiked plasma.

Calibration graphs were prepared in the ranges 4.24–254.4 ng of cortisol, 3.08–123.2 ng of cortisone, 11.96–956.8 ng of prednisolone and 11.78–824.6 ng of prednisone, with cortisol-d_5 (133.5 ng), cortisone-d_5 (69.74 ng), prednisolone-d_4 (142.2 ng) and prednisone-d_4 (120.4 ng) as the internal standards for the GC–MS assays. The mixture was assayed as BMD-HFB derivatives by monitoring the ion intensities at *m/z* 582 and 587 for cortisol, *m/z* 598 and 603 for cortisone, *m/z* 598 and 602 for prednisolone and *m/z* 566

TABLE I

ACCURACY OF GC–SIM-MS DETERMINATION OF CORTICOSTEROIDS IN PLASMA

Added (ng/ml)	Expected (ng/ml)	Found (ng/ml)							Relative error (%)	R.S.D. (%)
		Individual values[a]						Mean ± S.D.		
Cortisol										
–	–	77.85	74.31	76.77	76.30	77.51	77.79	76.76 ± 1.34	–	1.8
21.20	97.96	96.90	93.86	96.97	97.27	93.36	–	95.67 ± 1.90	−2.3	2.0
42.40	119.2	109.2	109.0	121.3	113.9	117.8	119.0	115.0 ± 5.2	−3.5	4.5
Cortisone										
–	–	20.38	20.48	20.97	21.10	20.82	20.43	20.70 ± 0.31	–	1.5
12.32	33.02	33.43	33.51	32.34	33.45	33.42	32.35	33.08 ± 0.57	+0.18	1.7
24.64	45.34	46.16	46.98	42.74	44.54	44.87	42.75	44.50 ± 1.50	−1.9	3.4
Prednisolone										
35.88	–	34.92	33.07	35.21	33.82	36.81	34.72	34.76 ± 1.28	−2.2	3.7
71.76	–	71.18	71.74	75.36	70.91	73.15	75.12	72.91 ± 1.96	+1.6	2.7
Prednisone										
35.34	–	33.76	34.42	34.25	34.79	33.34	32.87	33.91 ± 0.72	−4.0	2.1
70.68	–	67.77	71.10	69.27	76.64	74.03	72.81	71.94 ± 3.24	+1.8	4.5

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

and 570 for prednisone. The peak-height ratios of the recordings were plotted against the mixed molar ratios of unlabelled corticosteroids to the corresponding deuterium-labelled compounds. A good correlation was found between the observed peak-height ratios (y) and the mixed molar ratios (x). Unweighted least-squares regression analysis gave the regression lines $y = 1.1515x + 0.0095$ ($r = 0.999$) for cortisol, $y = 1.0147x - 0.0120$ ($r = 0.998$) for cortisone, $y = 1.1358x - 0.0577$ ($r = 0.999$) for prednisolone and $y = 1.3535x - 0.1977$ ($r = 0.998$) for prednisone. There was no indication of contributions and interferences at the masses monitored.

The accuracy was determined for cortisol, cortisone, prednisolone and prednisone added to 1.0-ml aliquots of pooled plasma containing endogenous cortisol (76.76 ± 1.34 ng/ml) and cortisone (20.70 ± 0.31 ng/ml). To the plasma samples were added fixed amounts of cortisol-d_5 (133.5 ng), cortisone-d_5 (69.74 ng), prednisolone-d_4 (142.2 ng) and prednisone-d_4 (120.4 ng) as the internal standards, and different amounts of cortisol (21.20 or 42.40 ng), cortisone (12.32 or 24.64 ng), prednisolone (35.88 or 71.76 ng) and prednisone (35.34 or 70.68 ng). Table I shows that the amounts of the four corticosteroids determined were in good agreement with the actual amounts added, the relative error being less than 3.5% for cortisol, 1.9% for cortisone, 2.2% for prednisolone and 4.0% for prednisone. The inter-assay relative standard deviations (R.S.D.) were less than 4.5% for all four corticosteroids.

The present method provides a sensitive and reliable technique for the simultaneous determination of plasma concentrations of the four corticosteroids with good accuracy and precision. The method can be applied to pharmacokinetic studies of prednisolone and prednisone, in which there is a particular interest in examining the effect of the administration of the synthetic corticosteroids on changes in the secretion and elimination of endogenous cortisol and cortisone.

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