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Simultaneous determination of tetrahydrocortisol and tetrahydrocortisone in human plasma and urine by stable isotope dilution mass spectrometry

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Abstract

A capillary gas chromatographic–mass spectrometric method for the simultaneous determination of tetrahydrocortisol (THF, 3α,11β,17α,21-tetrahydroxy-5β-pregnane-20-one), allo-tetrahydrocortisol (allo-THF, 3α,11β,17α,21-tetrahydroxy-5α-pregnane-20-one) and tetrahydrocortisone (THE, 3α,17α,21-trihydroxy-5β-pregnane-11,20-dione) in human plasma and urine is described. [1,2,3,4,5-$^{2}H_{5}$]THF (THF-d_5), allo-[1,2,3,4,5-$^{2}H_{5}$]THF (allo-THF-d_5) and [1,2,3,4,5-$^{2}H_{5}$]THE (THE-d_5) were used as internal standards. A double derivatization (bismethylenedioxypentafluoropropionate, BMD-PFP) made possible the separation of the three tetrahydrocorticoids with good gas chromatographic behavior. Quantitation was carried out by selected-ion monitoring of the characteristic fragment ions ($[M-30]^{+}$) of the BMD-PFP derivatives of THF, allo-THF and THE. The sensitivity, specificity, precision and accuracy of the method were demonstrated to be satisfactory for measuring low concentrations of THF, allo-THF and THE in human plasma and urine. © 1998 Elsevier Science B.V.

Keywords: Tetrahydrocortisol; Tetrahydrocortisone

1. Introduction

Cortisol and cortisone are metabolized to tetrahydrocortisol (THF, 3α,11β,17α,21-tetrahydroxy-5β-pregnane-20-one), allo-tetrahydrocortisol (allo-THF, 3α,11β,17α,21-tetrahydroxy-5α-pregnane-20-one) and tetrahydrocortisone (THE, 3α,17α,21-trihydroxy-5β-pregnane-11,20-dione) by a two-step reduction of the steroid ring A catalyzed by 5α- or 5β-reductase, followed by 3-oxoreductase. The metabolism of cortisol and cortisone has recently been the subject of biochemical and clinical investigations in patients with apparent mineralocorticoid excess (AME) characterized by hypertension, hypokalemia, and low renin levels despite subnormal or normal levels of mineralocorticoids [1–7].

The assay of THF, allo-THF and THE in urine has generally been carried out by a gas chromatographic–mass spectrometric (GC–MS) method using internal standards such as epitetrahydrocortisol and methyltestosterone [1,2,8–11]. However, the employed methoxime-trimethylsilyl (MO-TMS) derivatization does not permit the complete separation of THF and its stereoisomer, allo-THF, by GC. This leads to a difficulty in accurate and selective quantification of low levels of these compounds in biological

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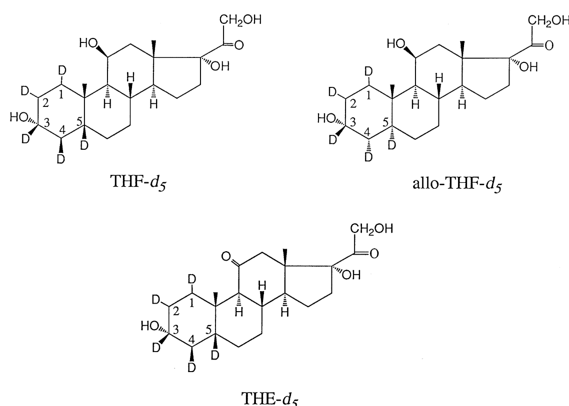

Fig. 1. Structures of the deuterium-labelled internal standards of THF, allo-THF and THE.

fluids. 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 stable isotopically labelled compounds as internal standards [12–14]. This methodology has been applied successfully to pharmacokinetic studies of cortisol and cortisone in humans following administration of stable isotopically labelled cortisol or cortisone [15,16].

The present study describes a stable isotope dilution MS method for the simultaneous determination of THF, allo-THF and THE in human plasma and urine, using the deuterium-labelled analogues [1,2,3,4,5-$^2H_5$]THF (THF-d_5), allo-[1,2,3,4,5-$^2H_5$]THF (allo-THF-d_5) and [1,2,3,4,5-$^2H_5$]THE (THE-d_5) as internal standards [17] (Fig. 1). The advantage of using a double derivatization step (bismethylenedioxypentafluoropropionate, BMD-PFP) for the GC–MS analysis of the three tetrahydrocorticoids is also presented.

2. Experimental

2.1. Chemicals and reagents

Tetrahydrocortisol (THF, 3,11β,17α,21-tetrahydroxy-5β-pregnane-20-one), allo-tetrahydrocortisol (allo-THF, 3α,11β,17α,21-tetrahydroxy-5α-pregnane-20-one) and tetrahydrocortisone (THE, 3α,17α,21-trihydroxy-5β-pregnane-11,20-dione) were purchased from Sigma (St. Louis, MO, USA). [1,2,3,4,5-$^2H_5$]THF (THF-d_5), allo-[1,2,3,4,5-$^2H_5$]THF (allo-THF-d_5) and [1,2,3,4,5-$^2H_5$]THE (THE-d_5) for use as internal standards were synthesized in this laboratory [17]. The isotopic compositions of the deuterium-labelled compounds were 86.2 atom% (THF-d_5), 74.5 atom% (allo-THF-d_5) and 81.5 atom% (THE-d_5), respectively. Pentafluoropropionic anhydride (PFPA) (GL Sciences, Tokyo, Japan), heptafluoro-*n*-butyric anhydride (HFBA) (Pierce, Rockford, IL, USA) 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.

2.2. Preparation of standards

Stock solutions of THF (10.25 mg per 100 ml), allo-THF (10.32 mg per 100 ml), THE (10.05 mg per 100 ml), THF-d_5 (6.129 mg per 100 ml), allo-THF-d_5 (4.590 mg per 100 ml) and THE-d_5 (3.375 mg per 100 ml) were prepared in methanol. All analyses were performed by diluting the stock solutions with methanol.

2.3. Gas chromatography–mass spectrometry-selected ion monitoring

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 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 5 ml/min and a split flow-rate of 30 ml/min. The purge activation time was 2 min. The initial column temperature was set to 100°C. After the sample injection, it was maintained for 2 min and was increased at 20°C/min to 250°C, maintained 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 to 280°C.

The multiple-ion detector was focused on the characteristic fragment ions ($[M-HCHO]^+$) at m/z 670 for the bismethylenedioxy-3,11-dipentafluoropropionyl (BMD-diPFP) derivatives of THF and allo-THF and at m/z 522 for the bismethylenedioxy-3-pentafluoropropionyl (BMD-monoPFP) derivative of THE, and at m/z 675 for the corresponding derivatives of THF-d_5 and allo-THF-d_5 and at m/z 527 for THE-d_5, respectively.

2.4. Sample preparation for GC–MS-SIM

2.4.1. Extraction

To 1.0 ml of human plasma or urine was added 55.17 ng of THF-d_5, 55.08 ng of allo-THF-d_5 and 50.63 ng of THE-d_5 as the internal standards dissolved in 10 μl each of methanol. The plasma and urine samples were 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 70°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) were 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 in cyclohexane (10 μl). A 1.0-μl volume of the solution was subjected to GC–MS.

2.5. Recovery

To 1.0 ml of plasma or urine was added 55.17 ng of THF-d_5, 55.08 ng of allo-THF-d_5 and 50.63 ng of THE-d_5 as the internal standards. The sample was then carried through the sample preparation procedure described above. Another set of plasma or urine sample (1.0 ml each) was first subjected to the extraction procedures using a Sep-Pak C_{18} cartridge, and then 55.17 ng of THF-d_5, 55.08 ng of allo-THF-d_5 and 50.63 ng of THE-d_5 were added to the processed samples. The recoveries of THF, allo-THF and THE were calculated by comparing the peak area ratios of the endogenous compounds versus the corresponding internal standards before and after the extraction procedures.

2.6. Calibration

To each of six standards containing known amounts of THF (1.025, 2.563, 5.125, 10.25, 25.63 and 51.25 ng), allo-THF (1.032, 2.580, 5.160, 10.32, 25.80 and 51.60 ng) and THE (1.005, 2.513, 5.025, 10.05, 25.13 and 50.25 ng) dissolved in methanol, 55.17 ng of THF-d_5, 55.08 ng of allo-THF-d_5 and 50.63 ng of THE-d_5 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 cyclohexane solution (10 μl) was subjected to GC–MS. The peak-area ratios (m/z 670 to 675 for THF and allo-THF and m/z 522 to 527 for THE) were determined in triplicate. The calibration graphs were obtained by an unweighted least-squares linear fitting of the peak-area ratios versus the mixed molar ratios of THF/THF-d_5, allo-THF/allo-THF-d_5 and THE/THE-d_5 on each analysis of the standard mixtures.

2.7. Accuracy

Accuracy was determined by assaying six preparations of 1.0-ml portions of human plasma or urine

spiked with 10.25 ng of THF (55.17 ng of THF-d_5 as internal standard), 10.32 ng of allo-THF (55.08 ng of allo-THF-d_5) and 10.05 ng of THE (50.63 ng of THE-d_5). After preparation of the sample for GC–MS-SIM as described above, the peak-area ratios (THF/THF-d_5, allo-THF/allo-THF-d_5 and THE/THE-d_5) were measured.

3. Results and discussion

The use of stable isotopically labelled internal standards for the GC–MS analysis offers major advantage that they behave in almost identical manner to the analyte through all steps in the isolation and chromatographic procedures, thereby allowing procedural losses to be disregarded [10,18]. Successful application of stable isotope dilution MS to the pharmacokinetic and metabolic investigations is dependent upon the availability of compounds labelled at predesigned positions that are chemically inert. We have synthesized multi-labelled tetrahydrocortisol (THF-d_5), allo-tetrahydrocortisol (allo-THF-d_5) and tetrahydrocortisone (THE-d_5) containing five nonexchangeable deuterium atoms at C-1, C-2, C-3, C-4 and C-5 for use as internal standards (Fig. 1). Detailed discussion concerning the syntheses of THF-d_5, allo-THF-d_5 and THE-d_5 will be described elsewhere [17].

The simultaneous determination of THF, allo-THF and THE by GC–MS requires the GC separation of these compounds because of their close or identical mass numbers. A methoxime trimethylsilyl ether (MO-TMS) derivative has usually been employed for the GC–MS assay of THF, allo-THF and THE in urine [1,2,8–11]. However, the derivative does not permit good resolution of the two stereoisomeric tetrahydrocortisols, THF and allo-THF, in GC. We previously described a new type of derivative (bismethylenedioxy-3-heptafluoro-*n*-butyrate, BMD-HFB) for the GC–MS assay of cortisol, cortisone, prednisolone and prednisone in plasma, which has been shown to result in good resolution, peak shape and sensitivity [13]. The MO-TMS derivatives of cortisol and cortisone are not separable from their synthetic analogues, prednisolone and prednisone, respectively, and elute as *syn–anti* isomer pairs of each derivative [10,11]. The bismethylenedioxy (BMD) derivative imparts chemical and thermal stability to the 17-dihydroxyacetone side chain of THF, allo-THF and THE. The fluoroacylation of the hydroxyl groups at C-3 and/or C-11 positions provides a sharp peak, leading to the GC separation of these compounds as described below.

3.1. BMD-HFB derivatization

The bismethylenedioxy-3-heptafluoro-*n*-butyryl (BMD-monoHFB) derivatives of THF and allo-THF ($[M]^+$, *m/z* 604) were obtained by treating the respective BMD derivatives of THF and allo-THF with 20 μl of heptafluoro-*n*-butyric anhydride (HFBA) in benzene (1 ml) at room temperature for 60 min. The diheptafluoro-*n*-butyration (BMD-diHFB) of the hydroxyl groups at C-3 and C-11 positions of THF and allo-THF ($[M]^+$, *m/z* 801) was achieved by heating the respective BMD derivatives of THF and allo-THF with 50 μl of HFBA in benzene (50 μl) at 70°C for 30 min. Although these derivatives gave sharp peaks, the BMD-monoHFB and BMD-diHFB derivatives of THF were not well separable from those of allo-THF.

The use of acetone instead of benzene as a solvent for the HFB derivatization has been shown to proceed with acid-catalyzed β-elimination of the hydroxyl group at C-11, forming the double bond in the positions at C-9, C-11 or C-11, C-12 [19,20]. The reaction in acetone led to the partial hydrolysis of BMD group with loss of HCHO for THF and allo-THF, respectively, whereas no hydrolysis of the BMD group was observed for THE and gave the BMD-monoHFB derivative ($[M]^+$, *m/z* 602) quantitatively.

3.2. BMD-PFP derivatization

The BMD derivatives of THF and allo-THF were treated with 20 μl of pentafluoropropionic anhydride (PFPA) in benzene (1 ml) at room temperature for 30 min. As shown in Fig. 2A, the two BMD-3-pentafluoropropionyl (BMD-monoPFP) derivatives ($[M]^+$, *m/z* 554) were found to be completely separable. The derivatization for THF, however, was accompanied by 5–20% of a stereoisomeric by-product, the peak of which appeared at the same retention time (13.54–13.56 min) of the BMD-monoPFP derivative

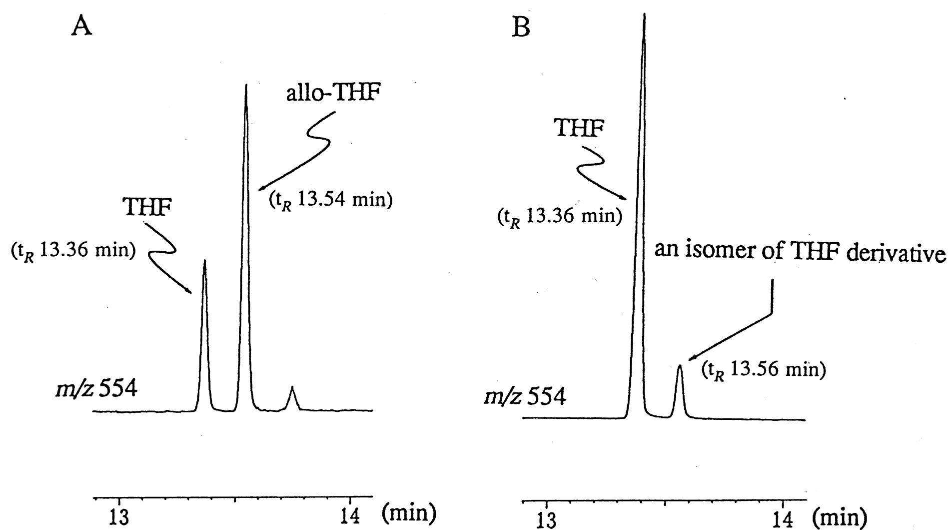

Fig. 2. Selected-ion recordings of BMD-monoPFP derivatives of THF plus allo-THF (A) and THF (B) (m/z 554).

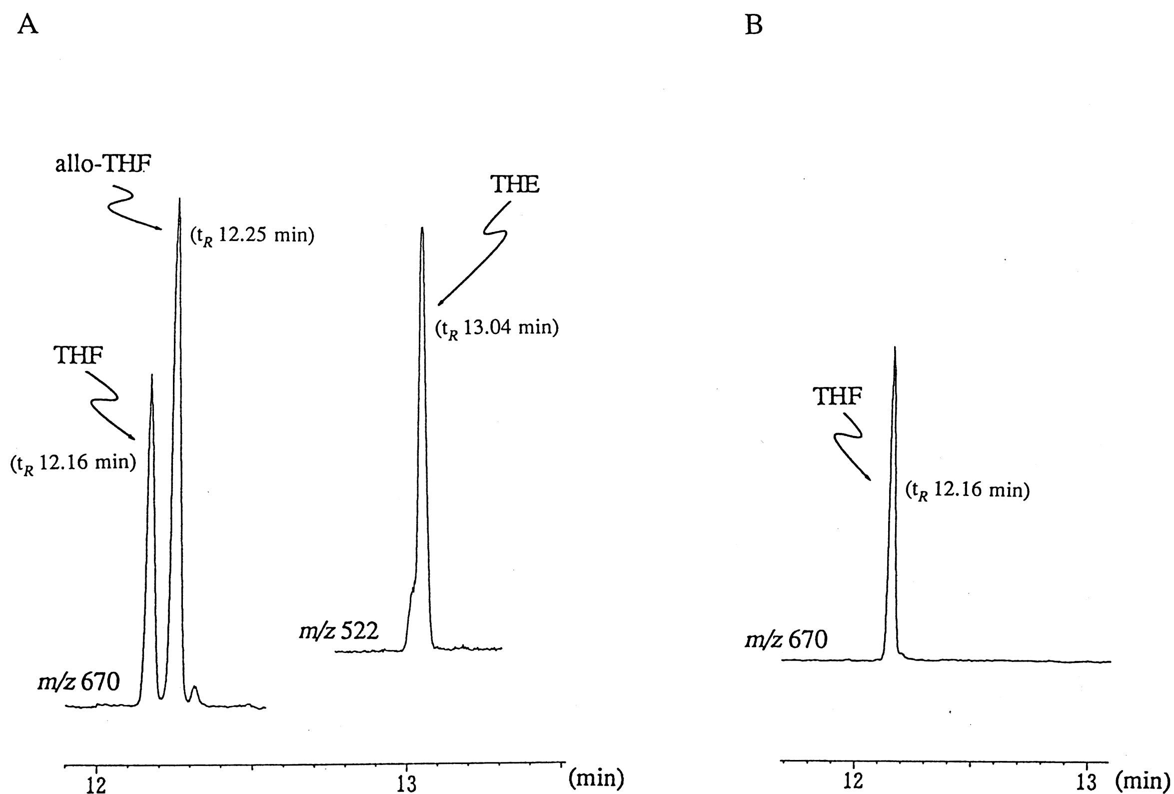

Fig. 3. Selected-ion recordings of BMD-diPFP derivatives of THF and allo-THF (m/z 670) and BMD-monoPFP derivative of THE (m/z 522).

of allo-THF (Fig. 2B). When the purified BMD compound of THF ($[M]^+$, m/z 408) by recrystallization was allowed to react with PFPA (20 μl) in benzene (1 ml), the interfering peak was not observed. Although the structural assignment of the interfering stereoisomer was not performed, it is possible that this stereoisomer could be associated with the isomer formation ($[M]^+$, m/z 408) during the BMD derivatization. The formation of the interfering isomer was found to be ~5% under the optimal conditions for the BMD derivatization tested.

The simultaneous derivatization of THF, allo-THF and THE applied to the present GC–MS assay was carried out by the following two-step reaction to give the BMD-3,11-dipentafluoropropionyl (BMD-diPFP) derivatives of THF and allo-THF, and the BMD-3-pentafluoropropionyl (BMD-monoPFP) derivative of

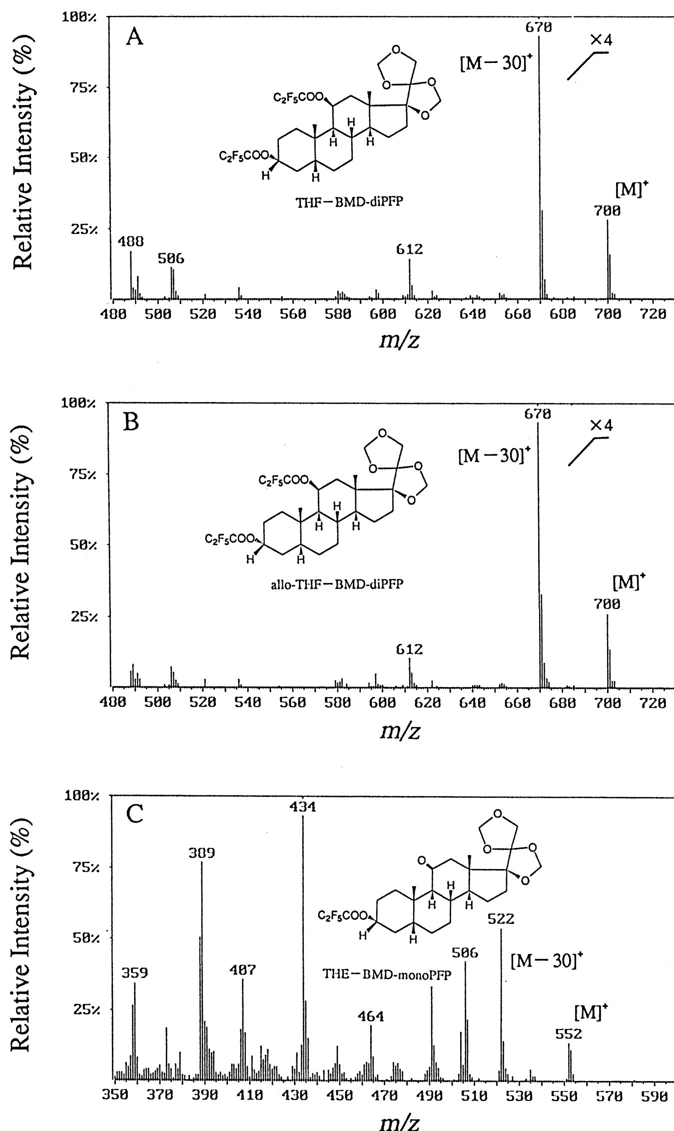

Fig. 4. Electron-impact mass spectra of BMD-diPFP derivatives of THF (A) and allo-THF (B), and BMD-monoPFP derivative of THE (C).

THE; an initial BMD derivatization by 12% paraformaldehyde in 25.7% HCl (50 μl) at room temperature for 20 min, followed by pentafluoroacylation with 50 μl of PFPA in benzene (50 μl) at 70°C for 30 min. As shown in Fig. 3A, a good GC resolution of the BMD-PFP derivatives of THF, allo-THF and THE was achieved. The BMD-diPFP derivative of THF appeared as a single peak on the chromatogram (Fig. 3B). The interfering isomer formation as seen in BMD-monoPFP derivatization of THF did not cause any problem in the simultaneous analysis of THF and allo-THF.

Fig. 4 shows the electron-impact (EI) mass spectra of the BMD-diPFP derivatives of unlabelled THF and allo-THF, and the BMD-monoPFP derivative of unlabelled THE. The BMD-PFP derivatives gave the molecular ions $[M]^+$ at m/z 700 for THF and allo-THF and at m/z 552 for THE. The charac-

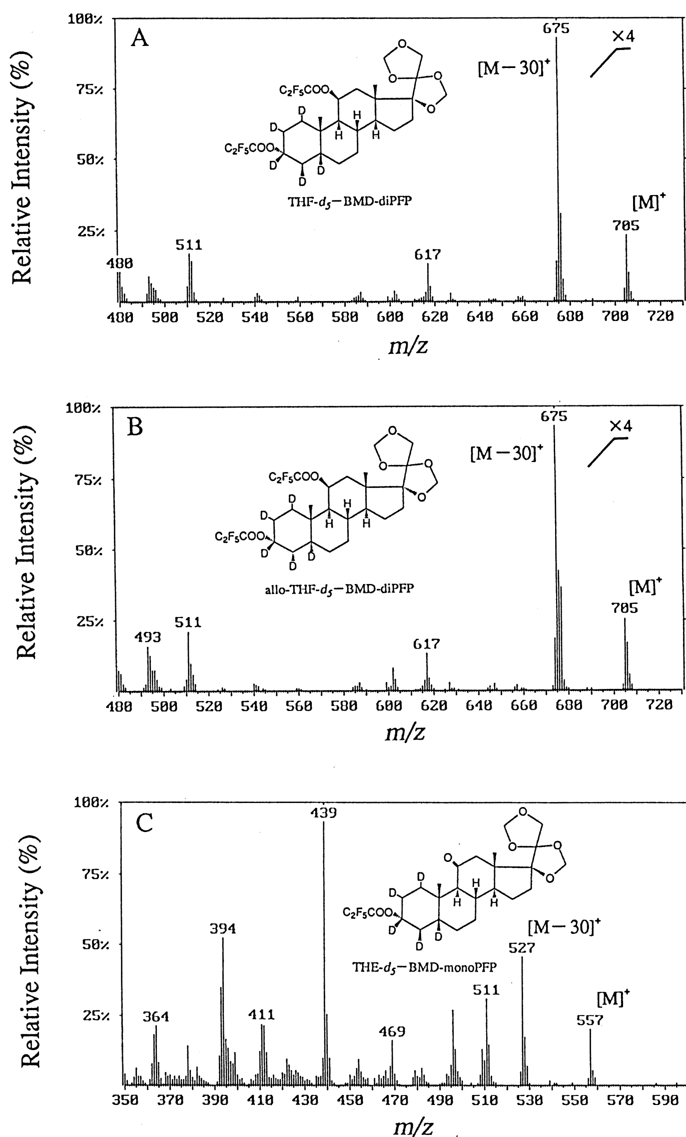

Fig. 5. Electron-impact mass spectra of BMD-diPFP derivatives of THF-d_5 (A) and allo-THF-d_5 (B), and BMD-monoPFP derivative of THE-d_5 (C).

teristic $[M-30]^+$ fragment ions derived from the loss of HCHO were observed for the three tetrahydrocorticoids.

Fig. 5 shows the EI mass spectra of BMD-PFP derivatives of deuterium-labelled THF, allo-THF and THE (THF-d_5, allo-THF-d_5 and THE-d_5). The isotopic purities were calculated to be 86.2% for THF-d_5, 74.5% for allo-THF-d_5 and 81.5% for THE-d_5, based on the ion intensities in the region of the molecular ion of each compound. The characteristic ions in the mass spectra (Figs. 4 and 5), i.e., the

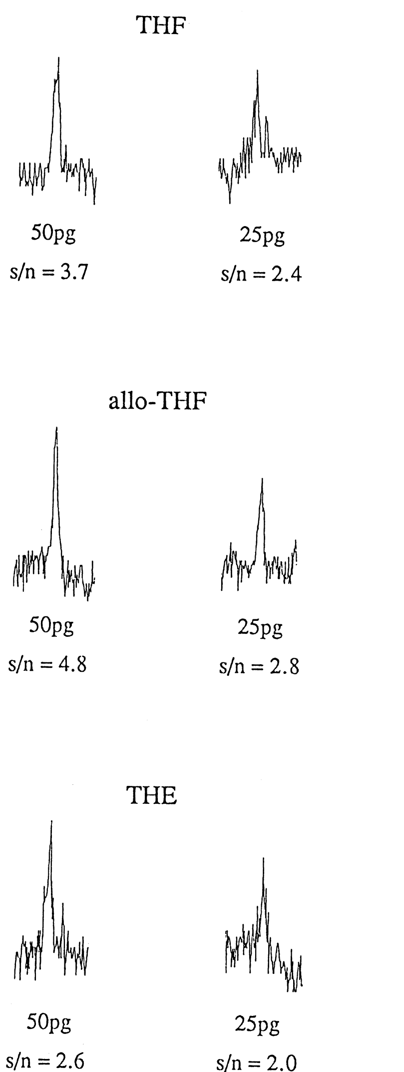

Fig. 6. Detection limits for the three tetrahydrocorticoids (THF, allo-THF and THE).

$[M-30]^+$ ions for THF/THF-d_5 (*m/z* 670/675), allo-THF/allo-THF-d_5 (*m/z* 670/675) and THE/THE-d_5 (*m/z* 522/527) were chosen for the selected ion monitoring of the BMD-PFP derivatives. When a signal-to-noise (*S/N*) ratio of 2.0 or greater was used as a criterion for a significant response, the detection limit of the present GC–MS-SIM method was found to be 25 pg per injection for THF (*S/N*=2.4), allo-THF (*S/N*=2.8), and the (*S/N*=2.0) (Fig. 6).

The present derivatization method was then applied to the simultaneous determination of THF, allo-THF and THE in human plasma and urine, using their respective deuterium-labelled internal standards. Fig. 7 shows the selected ion-recordings of the BMD-PFP derivatives of unlabelled and labelled tetrahydrocorticoids after processing from spiked plasma and urine. Neither blank plasma nor urine sample contained interfering substances. The efficiencies for extracting THF, allo-THF and THE

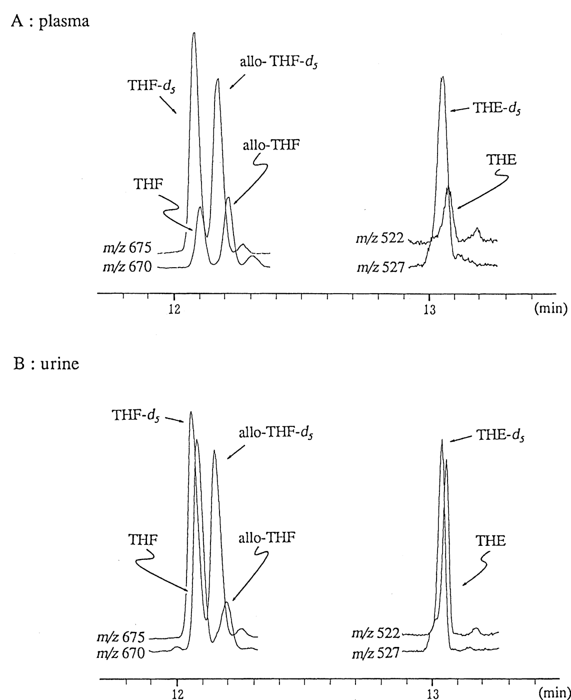

Fig. 7. Selected-ion recordings of BMD-PFP derivatives of THF/THF-d_5 (*m/z* 670 and 675), allo-THF/ allo-THF-d_5 (*m/z* 670 and 675) and THE/THE-d_5 (*m/z* 522 and 527) after processing from plasma (A) and urine (B) spiked with THF/THF-d_5 (10.25 ng and 55.17 ng), allo-THF/allo-THF-d_5 (10.32 ng and 55.08 ng) and THE/ THE-d_5 (10.05 ng and 50.63 ng).

from plasma and urine using a Sep-Pak C_{18} cartridge were 73.7–98.9% ($n=3$) for plasma and 98.7–100.4% ($n=3$) for urine, respectively.

Calibration graphs were prepared in the range 1–50 ng of THF, allo-THF and THE with THF-d_5 (55.17 ng), allo-THF-d_5 (55.08 ng) and THE-d_5 (50.63 ng) as the internal standards for the GC–MS assay. The mixture was analyzed as the BMD-PFP derivatives of THF, allo-THF and THE by monitoring $[M-30]^+$ ion intensities at m/z 670 (THF and allo-THF), m/z 675 (THF-d_5 and allo-THF-d_5), m/z 522 (THE) and m/z 527 (THE-d_5). The peak-area ratios 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-area ratios (y) and the mixed molar ratios (x). Unweighted least-squares regression analysis gave typical regression lines $y=0.959x+0.002$ ($r=1.000$) for THF, $y=0.850x+0.000$ ($r=1.000$) for allo-THF and $y=1.017x+0.002$ ($r=1.000$) for THE. There was no indication of contributions and interferences at the masses monitored. The calibration curves of the method were linear up to approximately 500 ng of THF, allo-THF and THE with the high correlation coefficients ($r=1.000$).

The accuracy of measurements was determined for THF, allo-THF and THE added to 1.0-ml aliquots of pooled plasma and urine containing endogenous THF (5.083±0.10 ng/ml plasma, 43.64±0.60 ng/ml urine), allo-THF (15.43±0.33 ng/ml plasma, 7.262±0.33 ng/ml urine) and THE (3.016±0.16 ng/ml plasma, 26.70±0.50 ng/ml urine). To the plasma or urine sample were added fixed amounts of THF-d_5 (55.17 ng), allo-THF-d_5 (55.08 ng) and THE-d_5 (50.63 ng) as the internal standards and known amounts of THF (10.25 ng), allo-THF (10.32 ng) and THE (10.05 ng). Table 1 shows the within-day reproducibility in which the amounts of the three tetrahydrocorticoids determined were in good agreement with the actual amounts added, the relative error being 1.89% (plasma) and 0.91% (urine) for

Table 1
Accuracy of GC–MS-SIM determination of THF, allo-THF and THE in human plasma and urine

Added (ng/ml)	Expected (ng/ml)	Found (ng/ml)							Relative error (%)	C.V.[b] (%)
		Individual values[a]						Mean±S.D.		
Plasma										
THF										
–	–	5.251	5.142	5.052	4.957	5.049	5.045	5.083±0.10	–	1.99
10.25	15.33	15.49	15.65	15.69	15.94	15.51	15.45	15.62±0.18	+1.89	1.16
Allo-THF										
–	–	15.60	15.86	15.55	15.02	15.04	15.50	15.43±0.33	–	2.15
10.32	25.75	25.82	25.43	26.23	25.99	26.03	25.28	25.80±0.37	+0.19	1.43
THE										
–	–	3.170	2.876	3.145	3.060	2.829	–	3.016±0.16	–	5.16
10.05	13.07	12.73	12.56	13.02	13.11	13.25	12.25	12.82±0.38	−1.91	2.94
Urine										
THF										
–	–	42.73	43.73	44.15	43.54	43.32	44.39	43.64±0.60	–	1.37
10.25	53.89	43.81	53.67	55.45	53.06	52.09	52.34	53.40±1.21	−0.91	2.27
Allo-THF										
–	–	7.068	6.886	7.362	7.185	7.217	7.856	7.262±0.33	–	4.57
10.32	17.58	17.43	17.19	18.80	16.95	17.06	16.54	17.33±0.78	−1.42	4.48
THE										
–	–	26.89	25.92	27.22	27.17	26.33	26.69	26.70±0.50	–	1.89
10.05	36.75	37.02	35.57	35.65	36.28	35.00	34.98	35.75±0.79	−2.72	2.20

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

THF, 0.19% (plasma) and 1.42% (urine) for allo-THF, and 1.91% (plasma) and 2.72% (urine) for THE. The inter-assay coefficients of variation (C.V.) were less than 5.16% for the three tetrahydrocorticoids. The isomer formations in the BMD-mono-PFP derivatization of THE and THE-d_5 (Fig. 3A Fig. 7) did not cause any problem in analyzing THE.

The present method provides a sensitive and reliable technique for the simultaneous determination of THF, allo-THF and THE in plasma and urine with good accuracy and precision. The method can be applied to pharmacokinetic and metabolic studies of cortisol and cortisone with a particular interest in evaluating the ring A reduction in the metabolism of cortisol and cortisone in patients with AME.

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