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Simultaneous determination of 6β- and 6α-hydroxycortisols and 6β-hydroxycortisone in human urine by stable isotope dilution mass spectrometry

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Abstract

A capillary gas chromatographic–mass spectrometric method for the simultaneous determination of 6β-hydroxycortisol (6β-OHF, 6β,11β,17α,21-tetrahydroxypregn-4-ene-3,20-dione), 6α-hydroxycortisol (6α-OHF, 6α,11β,17α,21-tetrahydroxypregn-4-ene-3,20-dione) and 6β-hydroxycortisone (6β-OHE, 6β,17α,21-trihydroxypregn-4-ene-3,11,20-trione) in human urine is described. Deuterium-labelled compounds, 6β-[1,1,19,19,19-$^{2}H_{5}$]OHF (6β-OHF-d_{5}), 6α-[1,1,19,19,19-$^{2}H_{5}$]OHF (6α-OHF-d_{5}) and 6β-[1,1,19,19,19-$^{2}H_{5}$]OHE (6β-OHE-d_{5}) were used as internal standards. Quantitation was carried out by selected-ion monitoring of the characteristic fragment ions ($[M-31]^{+}$) of the methoxime–trimethylsilyl (MO–TMS) derivatives of 6β-OHF, 6α-OHF and 6β-OHE. The sensitivity, specificity, precision and accuracy of the method were demonstrated to be satisfactory for measuring 6β-OHF, 6α-OHF and 6β-OHE in human urine.

Keywords: Hydroxycortisol; Hydroxycortisone

1. Introduction

Cortisol and cortisone are metabolized to 6β-hydroxycortisol (6β-OHF, 6β,11β,17α,21-tetrahydroxypregn-4-ene-3,20-dione), 6α-hydroxycortisol (6α-OHF, 6α,11β,17α,21-tetrahydroxypregn-4-ene-3,20-dione) and 6β-hydroxycortisone (6β-OHE, 6β,17α,21-trihydroxypregn-4-ene-3,11,20-trione) by cytochrome P450 (CYP3A) in the endoplasmic reticulum of hepatocytes [1,2]. The metabolism of cortisol to 6β-hydroxycortisol has been evaluated as a useful non-invasive index for the activity of this enzyme induced by many xenobiotics including drugs [3–7]. Both the 24-h excretion of 6β-hydroxycortisol and the ratio of 6β-hydroxycortisol to cortisol as unconjugated or free form in urine have been used to evaluate the activities of CYP3A in vivo. The 6β-hydroxylation has recently drawn attention, due to the physiological significance in the hypertension [8–10].

The ratio of 6β-hydroxycortisol/cortisol as the index of the 6β-hydroxycortisol production has the advantage for adjusting the day-to-day variation in adrenal cortisol production. The lack of circadian

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change on the ratio of 6β-hydroxycortisol to cortisol allows the indirect assessment of the CYP3A activities in spot urine samples without a 24-h collection [6,11,12]. However, cortisol is also metabolized extensively to many compounds other than 6β-hydroxycortisol, such as cortisone, tetrahydrocortisol (THF), allo-tetrahydrocortisol (allo-THF), tetrahydrocortisone (THE), etc. The day-to-day variation of urinary excretion of cortisol should be caused by both the adrenal production and the metabolism. Stable isotopically labelled cortisol and cortisone can be used as biological internal standards for directly assessing the oxidative conversions of cortisol and cortisone to 6β-OHF, 6α-OHF and 6β-OHE, catalyzed by CYP3A in vivo.

The concentration of 6β-OHF in urine has been determined by various immunochemical techniques such as radioimmunoassay (RIA) [13–15], enzyme immunoassay [16]. These methods are highly sensitive, but they lack selectivity for 6β-OHF. The HPLC technique [12,17–22] offers the advantage that 6β-OHF can be analyzed selectively, but it lacks sensitivity. We have previously developed sensitive and reliable GC–MS and LC–MS methods for the simultaneous determination of cortisol and cortisone [23–25] and the tetrahydrocorticoid metabolites (THF, allo-THF and THE) [26] in plasma and urine using their stable isotopically labelled analogues as internal standards. This methodology has been applied to pharmacokinetic and metabolic studies of cortisol and cortisone to evaluate the interconversion of cortisol to cortisone catalyzed by 11β-hydroxysteroid dehydrogenase in vivo, following administration of stable isotopically labelled cortisol or cortisone to humans [27,28].

The present study describes a stable isotope dilution MS method for the simultaneous determination of 6β-hydroxycortisol (6β-OHF), 6α-hydroxycortisol (6α-OHF) and 6β-hydroxycortisone (6β-OHE) in human urine, using the deuterium-labelled analogues 6β-[1,1,19,19,19-$^2H_5$]OHF (6β-OHF-d_5), 6α-[1,1,19,19,19-$^2H_5$]OHF (6α-OHF-d_5) and 6β-[1,1,19,19,19-$^2H_5$]OHE (6β-OHE-d_5) as internal standards (29) (Fig. 1).

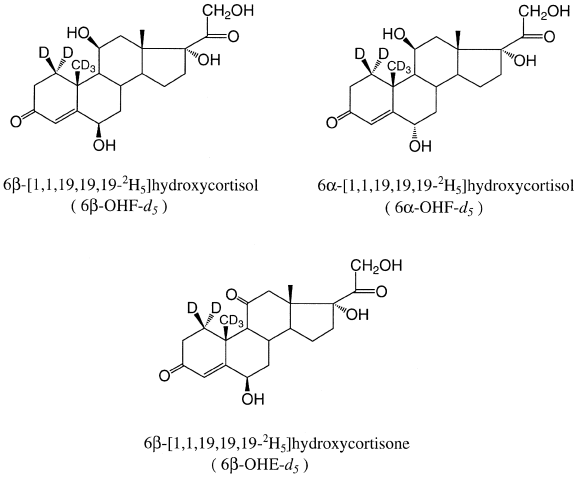

Fig. 1. Structures of the deuterium-labelled internal standards of 6β-OHF, 6α-OHF and 6β-OHE.

2. Experimental

2.1. Chemicals and reagents

6β-Hydroxycortisol (6β-OHF, 6β,11β,17α,21-tetrahydroxypregn-4-ene-3,20-dione) and 6α-hydroxycortisol (6α-OHF, 6α,11β,17α,21-tetrahydroxypregn-4-ene-3,20-dione) were purchased from Steraloids (Wilton, NH, USA). 6β-Hydroxycortisone (6β-OHE, 6β,17α,21-trihydroxypregn-4-ene-3,11,20-trione) was purchased from Sigma (St. Louis, MO, USA). Deuterium-labelled internal standards, 6β-[1,1,19,19,19-$^2H_5$]OHF (6β-OHF-d_5), 6α-[1,1,19,19,19-$^2H_5$]OHF (6α-OHF-d_5) and 6β-[1,1,19,19,19-$^2H_5$]OHE (6β-OHE-d_5) were synthesized in this laboratory [29]. The labelled 6-hydroxycortisols were a mixture of 6β-OHF-d_5 (77.2%) and 6α-OHF-d_5 (22.8%), while 6β-OHE-d_5 was isolated as a pure compound. The isotopic composition of 6β-OHF-d_5 was 90.9 atom%D. Methoxyamine hydrochloride (Eastman Kodak, Rochester, NY, USA) and *N,O*-bis(trimethylsilyl)acetamide (BSA) (Tokyo Chemicals, Tokyo, Japan) were obtained commercially. All other chemicals and solvents were of analytical-reagent grade and were used without further purification.

2.2. Preparation of standards

Stock solutions of 6β-OHF (5.37 mg per 100 ml), 6α-OHF (5.00 mg per 100 ml), 6β-OHE (5.04 mg per 100 ml), 6β-OHF-d_5 (42.79 μg per 100 ml), 6α-OHF-d_5 (12.67 μg per 100 ml) and 6β-OHE-d_5

(106.50 μg 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 (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 a 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°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. 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 characteristic fragment ions ($[M\text{-}OMe]^+$) at m/z 694 for the 3,20-dimethoxime-6β,11β,17α,21-tetra(trimethylsilyl) (MO-tetraTMS) derivatives of 6β-OHF and 6α-OHF and at m/z 619 for the 3,20-dimethoxime-6β,17α,21-tri(trimethylsilyl) (MO-triTMS) derivatives of 6β-OHE, and at m/z 699 for the corresponding derivatives of 6β-OHF-d_5 and 6α-OHF-d_5 and at m/z 624 for 6β-OHE-d_5, respectively.

2.4. Sample preparation for GC–MS-SIM

2.4.1. Extraction

Extraction procedure from urine samples was performed according to the procedures employed for the GC–MS analyses of cortisol, cortisone and their tetrahydrocorticoid metabolites in plasma and urine [23–26]. To 0.5 ml of human urine was added a methanol solution (240 μl) containing 102.7 ng of 6β-OHF-d_5 and 30.40 ng of 6α-OHF-d_5 and a methanol solution (50 μl) containing 53.25 ng of 6β-OHE-d_5 as the internal standards. The urine samples were 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 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 0.5 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

The MO-TMS derivatization was performed according to the general procedures employed for the GC and GC–MS analyses of corticosteroids in biological fluids [23]. To the residue containing 6α- and 6β-hydroxycorticoids in a 0.5-ml micro product V-vial (Wheaton, Millville, NJ, USA) was added 100 μl of 5% (w/v) methoxyamine in pyridine. The reaction mixture was sealed under a nitrogen atmosphere and heated at 100°C for 30 min. After removal of the solvent under a stream of nitrogen, 50 μl of *N,O*-bis(trimethylsilyl)acetamide (BSA) were added. The reaction mixture was sealed under a nitrogen atmosphere and heated at 100°C for 2 h. After cooling and evaporating under a stream of nitrogen 200 μl of *n*-hexane were added to the residue. The solution was applied to a Sep-Pak silica cartridge, which was eluted with 7 ml of *n*-hexane. The eluate was evaporated to dryness at room temperature under a gentle stream of nitrogen and the residue was dissolved with cyclohexane (10 μl). A 1.0-μl portion of the solution including 6α- and 6β-hydroxycorticoids as MO-TMS derivative was subjected to GC–MS.

2.5. Recovery

To 0.5 ml of urine was added 102.7 ng of 6β-OHF-d_5, 30.40 ng of 6α-OHF-d_5 and 53.25 ng of 6β-OHE-d_5 as the internal standards. The sample was then carried through the sample preparation procedure described above. Another different set of urine sample (0.5 ml) was first subject to the extraction procedures using a Sep-Pak C_{18} cartridge, and then 102.7 ng of 6β-OHF-d_5, 30.40 ng of 6α-

OHF-d_5 and 53.25 ng of 6β-OHE-d_5 were added to the processed samples. The recoveries of 6β-OHF, 6α-OHF and 6β-OHE 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 graphs

To each of six standards containing known amounts of 6β-OHF (10.74, 53.70, 107.4, 214.8, 537.0 and 805.5 ng), 6α-OHF (10.00, 20.00, 30.00, 40.00, 70.00 and 100.0 ng) and 6β-OHE (10.08, 20.16, 30.24, 40.32, 70.56 and 100.8 ng) dissolved in methanol, 102.7 ng of 6β-OHF-d_5, 30.40 ng of 6α-OHF-d_5 and 53.25 ng of 6β-OHE-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 694 to 699 for 6β-OHF and 6α-OHF and m/z 619 to 624 for 6β-OHE) 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 6β-OHF/6β-OHF-d_5, 6α-OHF/6α-OHF-d_5 and 6β-OHE/6β-OHE-d_5 on each analysis of the standard mixtures.

2.7. Accuracy and reproducibility

Accuracy was determined by assaying six preparations of 0.5-ml portions of human urine spiked with 6β-OHF (107.4 ng), 6α-OHF (20.00 ng) and 6β-OHE (20.16 ng), using 6β-OHF-d_5 (102.7 ng), 6α-OHF-d_5 (30.40 ng) and 6β-OHE-d_5 (53.25 ng) as internal standards. After preparation of the sample for GC–MS-SIM as described above, the peak-area ratios (6β-OHF/6β-OHF-d_5, 6α-OHF/6α-OHF-d_5 and 6β-OHE/6β-OHE-d_5) were measured.

3. Results and discussion

3.1. Derivatization for GC–MS analysis

We have previously developed a new type of derivatives, i.e., bismethylenedioxy-heptafluoro-*n*-butyrate (BMD-HFB) and bismethylenedioxy-pentafluoropropionate (BMD-PFP) to simultaneously measure cortisol, cortisone, prednisolone and prednisone in plasma, which have been shown to result in good resolution, peak shape and sensitivity [24]. The simultaneous GC–MS analysis of tetrahydrocortisol (THF), allo-tetrahydrocortisol (allo-THF) and tetrahydrocortisone (THE) was also achieved by the BMD-PFP derivatization [26]. The BMD-HFB and -PFP derivatizations, however, appeared to be inappropriate for the analysis of 6β-hydroxycortisol (6β-OHF) and 6α-hydroxycortisol (6α-OHF). The derivatives do not permit the distinction between 6β-OHF and 6α-OHF, due to the isomerization of the hydroxyl to the keto group at C-6 to give 6-keto-5α-dihydrocorticoids during the C-17 protection with BMD [30,31].

In the present study, the methoxime trimethylsilyl ether (MO-TMS) derivatization [23,32,33] was chosen for the simultaneous measurement of 6β-OHF, 6α-OHF and 6β-OHE. Fig. 2 shows the mass chromatograms of MO-TMS derivatives of 6β-OHF, 6α-OHF and 6β-OHE. The derivatization procedure produced the *syn-anti* isomer pairs of each derivative [34]. As shown in Fig. 2A, the major peak (peak a) of 6β-OHF(t_R 17.02 min) appeared at the almost same retention time (t_R 17.06 min) of the smaller isomer peak of 6α-OHF (peak b′). The peak-area ratio of the isomers of the MO-TMS derivative of 6α-OHF (peak b′ to peak b) was found to be 0.494 ± 0.075 (mean$\pm$SD, $n=23$) which was tested by the derivatization of 6α-OHF in the range of 10–150 ng. Although no significant interfering peaks were found in the vicinity of the other isomer peak (peak a′) of 6β-OHF, the larger peak (peak a) was chosen for the GC–MS analysis of 6β-OHF in urine to obtain higher sensitivity, accuracy and precision. The corrections were made for the quantitation of 6β-OHF using the formation ratio (peak b′/peak b; 0.50) of the *syn-anti* isomer peaks of 6α-OHF.

3.2. Mass spectrometry

Fig. 3 shows the electron-impact (EI) mass spectra of the MO-TMS derivatives of unlabelled 6β-OHF, 6α-OHF and 6β-OHE. The MO-TMS derivatives gave the molecular ions $[M]^+$ at m/z 725 for 6β-OHF and 6α-OHF and at m/z 650 for 6β-OHE. The

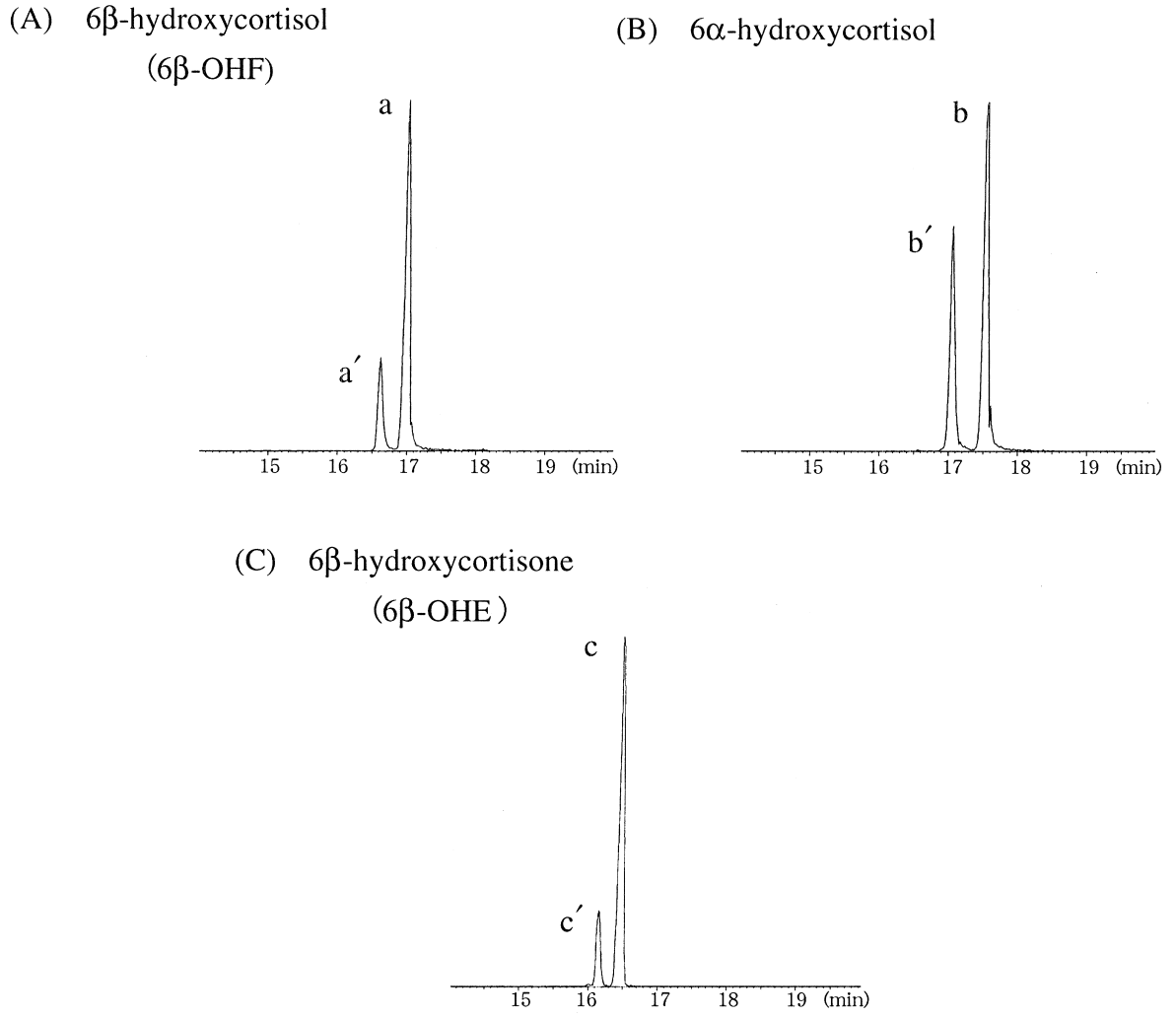

Fig. 2. Mass chromatograms of the MO-TMS derivatives of 6β-OHF (*m*/*z* 694) (A), 6α-OHF (*m*/*z* 694) (B) and 6β-OHE (*m*/*z* 619) (C).

characteristic $[M-31]^+$ fragment ions derived from the loss of the OMe group were observed for the three 6-hydroxycorticoids.

Palermo and co-workers [34] utilized 6β-[1,2-$^2H_2$]hydroxycortisol as internal standard to determine 6β-OHF in urine by GC–MS. The investigators reported that the use of the dideutero internal standard showed significant disadvantage, because there was a major ion contribution by the analyte to the internal standards and vice versa. The extents of the ion contributions were augmented by the four trimethylsilyl groups of the MO-TMS derivative of 6β-OHF because of the high abundance of silicon isotopes. The analyte and its labelled internal standard, therefore, should differ by at least four or five mass units to determine 6β-OHF without complex corrections for the ion contributions.

In the present study, stable isotopically labelled analogues, i.e., 6β-[1,1,19,19,19-$^2H_5$]OHF (6β-OHF-d_5), 6α-[1,1,19,19,19-$^2H_5$]OHF (6α-OHF-d_5) and 6β-[1,1,19,19,19-$^2H_5$]OHE (6β-OHE-d_5) (Fig. 1), were used as internal standards for the GC–MS analysis. Fig. 4 shows the EI mass spectra of the MO-TMS derivatives of deuterium-labelled 6β-OHF, 6α-OHF and 6β-OHE (6β-OHF-d_5, 6α-OHF-d_5 and 6β-OHE-d_5). The MO-TMS derivatives gave the

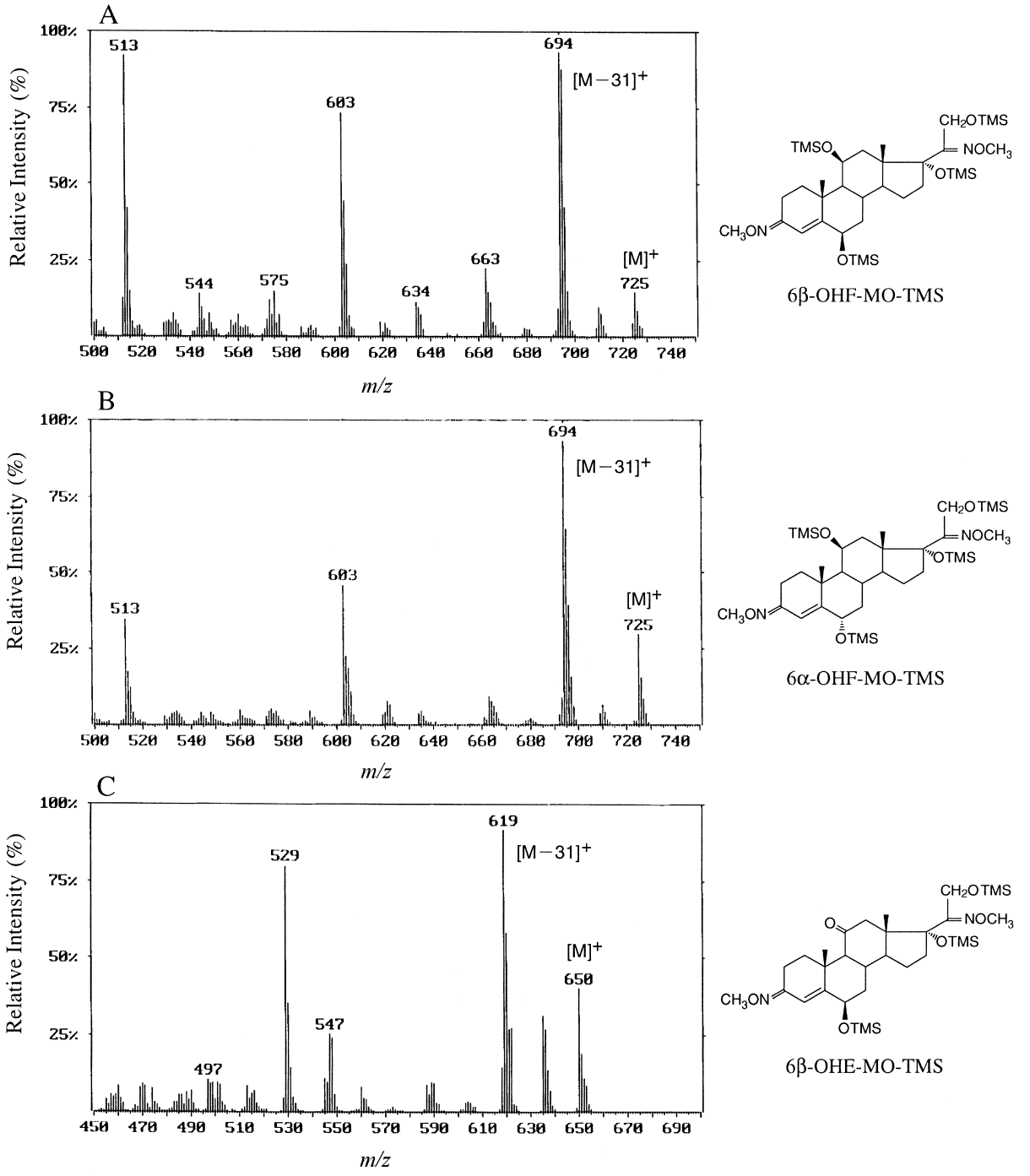

Fig. 3. Electron-impact mass spectra of the MO-TMS derivatives of 6β-OHF (A) and 6α-OHF (B), and 6β-OHE (C).

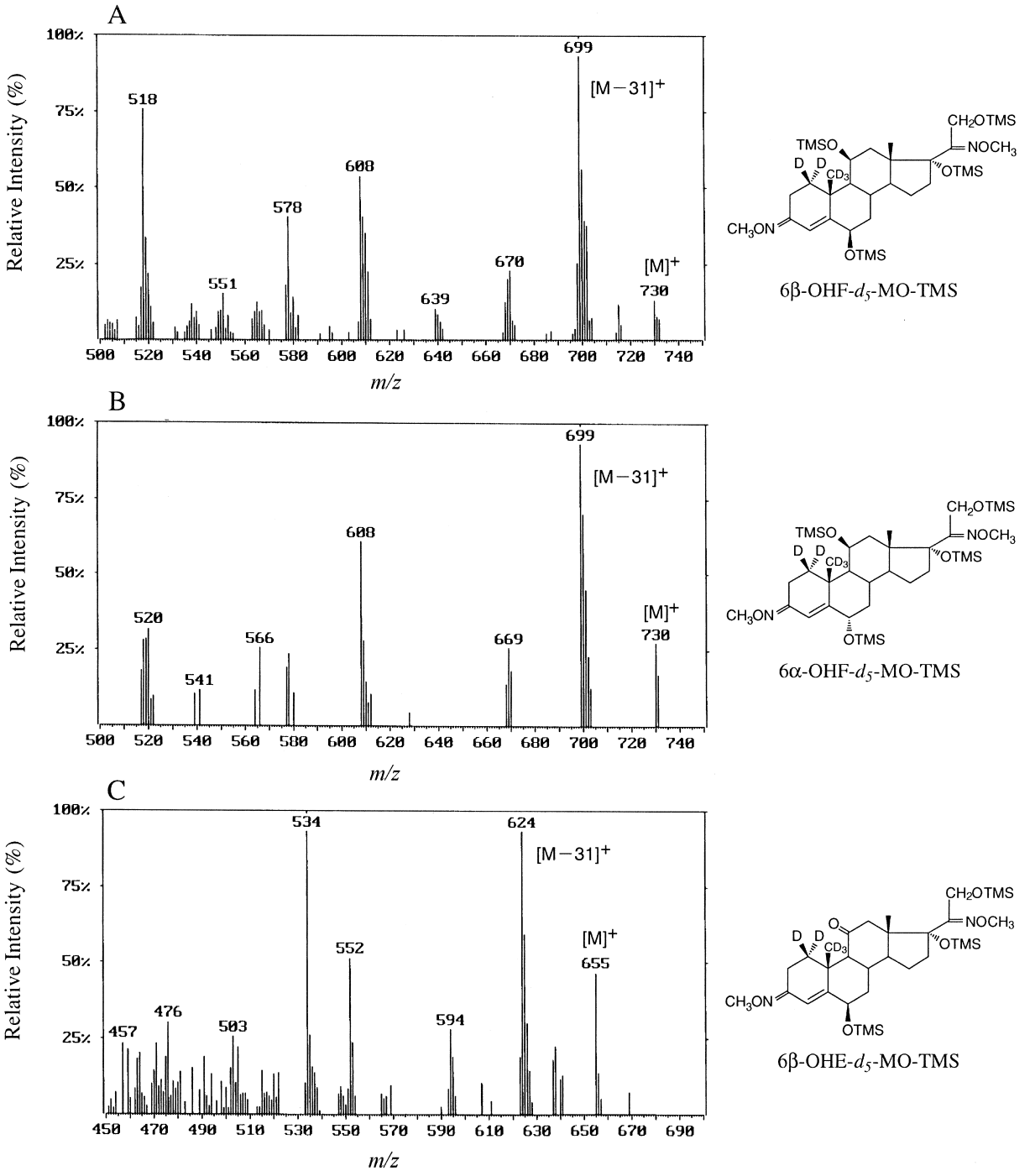

Fig. 4. Electron-impact mass spectra of the MO-TMS derivatives of 6β-OHF-d_5 (A) and 6α-OHF-d_5 (B) and 6β-OHE-d_5 (C).

molecular ions $[M]^+$ at m/z 730 for 6β-OHF-d_5 and 6α-OHF-d_5 and at m/z 655 for 6β-OHE-d_5. The characteristic $[M-31]^+$ (M-OMe) fragment ions were also observed for these labelled 6-hydroxycorticoids, respectively. The isotopic purity was calculated to be 90.9% for 6β-OHF-d_5, based on the ion intensities in the region of the molecular ion of the compound.

3.3. Sensitivity

The characteristic ions in the mass spectra (Figs. 3 and 4), i.e., the $[M-31]^+$ ions for 6β-OHF/6β-OHF-d_5 (m/z 694/699), 6α-OHF/6α-OHF-d_5 (m/z 694/699) and 6α-OHE/6α-OHE-d_5 (m/z 619/624) were chosen for the selected ion monitoring of the MO-TMS derivatives. When a signal-to-noise (S/N) ratio of 2.0 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 250 pg per injection for 6β-OHF ($S/N=2.7$), 6α-OHF ($S/N=2.0$) and 6α-OHE ($S/N=2.1$) (Fig. 5).

3.4. Selected ion-recordings

The derivatization was then applied to the simultaneous determination of 6β-OHF, 6α-OHF and 6α-OHE in human urine, using their respective deuterium-labelled internal standards. Fig. 6 shows the selected ion-recordings of the MO-TMS derivatives of unlabelled and labelled 6-hydroxycorticoids after processing from pooled urine. The recordings showed no significant interfering peaks derived from other endogenous compounds present in urine. The efficiencies for extracting 6β-OHF, 6α-OHF and

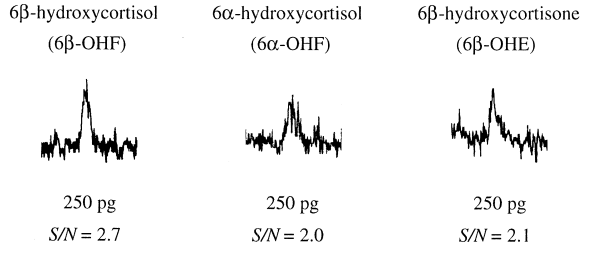

Fig. 5. Sensitivity limits for the three 6-hydroxycorticoids (6β-OHF, 6α-OHF and 6β-OHE).

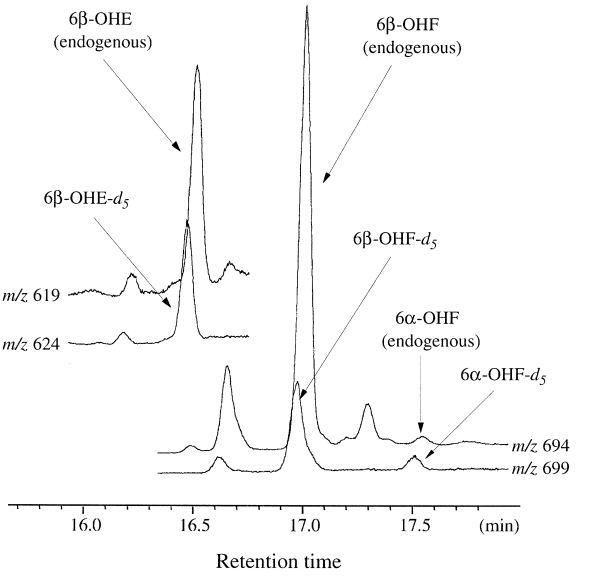

Fig. 6. Selected-ion recordings of the MO-TMS derivatives of 6β-OHF/6β-OHF-d_5 (m/z 694 and 699), 6α-OHF/6α-OHF-d_5 (m/z 694 and 699) and 6β-OHE/6β-OHE-d_5 (m/z 619 and 624) after processing from 0.5 ml of urine spiked with 6β-OHF-d_5 (102.7 ng), 6α-OHF-d_5 (30.40 ng) and 6β-OHE-d_5 (53.25 ng) as internal standards.

6β-OHE from urine using a Sep-Pak C_{18} cartridge were 87.4–94.5% ($n=4$).

3.5. Calibration graphs

Calibration graphs were prepared in the range 10.74–805.5 ng of 6β-OHF, 10.00–100.0 ng of 6α-OHF and 10.08–100.8 ng of 6β-OHE with 6β-OHF-d_5 (102.7 ng), 6α-OHF-d_5 (30.40 ng) and 6β-OHE-d_5 (53.25 ng) as the internal standards for the GC–MS assay. The mixture was analysed as the MO-TMS derivatives of 6β-OHF, 6α-OHF and 6β-OHE by monitoring the $[M-31]^+$ ion intensities at m/z 694 (6β-OHF and 6α-OHF), m/z 699 (6β-OHF-d_5 and 6α-OHF-d_5), m/z 619 (6β-OHE) and m/z 624 (6β-OHE-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 molar ratios (x). Un-

weighted least-squares regression analysis gave typical regression lines $y=0.9891x+0.0712$ ($r=0.9998$) for 6β-OHF, $y=0.7741\ x-0.1087$ ($r=0.9988$) for 6α-OHF and $y=1.0476\ x-0.0349$ ($r=0.9935$) for 6β-OHE.

3.6. Accuracy and reproducibility

The accuracy of measurements were determined for 6β-OHF, 6α-OHF and 6β-OHE added to 0.5-ml aliquots of pooled urine containing endogenous 6β-OHF (593.1±11.7 ng/ml urine), 6α-OHF (46.17±1.91 ng/ml urine) and 6β-OHE (45.18±0.50 ng/ml urine). To the urine sample were added fixed amounts of 6β-OHF-d_5 (102.7 ng), 6α-OHF-d_5 (30.40 ng) and 6β-OHE-d_5 (53.25 ng) as the internal standards and known amounts of 6β-OHF (107.4 ng), 6α-OHF (20.00 ng) and 6β-OHE (20.16 ng). Table 1 shows the within-day accuracy and reproducibility in which the amounts of the three 6-hydroxycorticoids determined were in good agreement with the actual amounts added, the relative error being −1.99% for 6β-OHF, −5.69% for 6α-OHF, and −5.72% for 6β-OHE. The inter-assay coefficients of variation (C.V.) were less than 5.78% for the three 6-hydroxycorticoids.

In the measurements of 6β-OHF, 6α-OHF and 6β-OHE in urine, the ratio (0.50) of *syn-anti* isomer peak of 6α-OHF (peak b′/peak b, in Fig. 2B) was used for the correction to measure 6β-OHF. The ratio was obtained from the MO-TMS derivative of authentic 6α-OHF. Although the *syn-anti* ratio of 6α-OHF tested was varied in the range 0.36–0.62 (0.494±0.075), the resulting errors in the measurement of 6β-OHF were small, being within±1.7% even when the *syn-anti* ratio of 6α-OHF varied from 0.20 to 0.80.

4. Conclusions

The present method provides a sensitive and reliable technique for the simultaneous determination of 6β-OHF, 6α-OHF and 6β-OHE in 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 C-6 oxidations of cortisol and cortisone in vivo, catalyzed by CYP3A, by administering stable isotopically labelled cortisol as tracer to humans. The measurements of both endogenous and exogenous (labelled) 6β-OHF, 6α-OHF and 6β-OHE in urine are now in progress by using the present method.

Table 1
Accuracy and reproducibility of GC–MS-SIM determination of 6β-OHF, 6α-OHF and 6β-OHE in human urine

Added (ng/ml)	Expected (ng/ml)	Found (ng/ml)							Relative Error (%)	C.V.[b] (%)
		Individual Values[a]						Mean±SD		
6β-OHF										
–	–	614.6	591.0	591.0	591.2	592.0	578.8	593.1±11.7	–	1.97
214.8	807.9	787.4	766.4	759.4	863.4	792.6	781.8	791.8±37.3	−1.99	4.71
6α-OHF										
–	–	45.90	45.56	47.76	–	43.44	48.20	46.17±1.91	–	4.13
40.00	86.17	–	81.16	83.22	87.44	77.66	76.86	81.27±4.31	−5.69	5.31
6β-OHE										
–	–	44.98	45.54	44.70	44.54	45.68	45.62	45.18±0.50	–	1.11
40.32	85.50	75.44	77.40	78.08	88.04	80.98	83.72	80.61±4.66	−5.72	5.78

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

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