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Simultaneous determination of endogenous and stable isotope-labelled 6β-hydroxycortisols in human urine 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 6β-hydroxycortisol (6β-OHF) and its stable isotope-labelled analogue, 6β-hydroxy-[1,1,19,19,19-$^{2}H_{5}$]cortisol (6β-OHF-$^{2}H_{5}$), in human urine. 6β-Hydroxy-[1,2,4,19-$^{13}C_{4}$,1,1,19,19,19-$^{2}H_{5}$]cortisol (cortisol-$^{13}C_{4}$,$^{2}H_{5}$) was used as an analytical internal standard. The methoxime trimethylsilyl ether (MO-TMS) derivatization was employed for the GC–MS analysis of 6β-OHF. Quantitation was carried out by selected-ion monitoring (SIM) of the characteristic fragment ion ($[M-31]^{+\cdot}$) of the MO-TMS derivative of 6β-OHF. The sensitivity limit of the present GC–MS-SIM method was found to be 25 pg per injection for 6β-OHF (*S*/*N* ratio=5.6). The within-day reproducibility in the amounts of unlabelled and labelled 6β-OHFs determined were in good agreement with the actual amounts added, the relative errors being less than 5.30%. The inter-assay RSDs were less than 4.95% for unlabelled and labelled 6β-OHFs.

Keywords: 6β-Hydroxycortisols

1. Introduction

Cortisol (F) is metabolized to 6β-hydroxycortisol (6β-OHF) catalyzed by cytochrome P450 3A (CYP3A) enzyme, and the metabolite 6β-OHF is then excreted as an unconjugated form in urine [1–3]. The urinary excretion ratio 6β-OHF/F has been used as a useful non-invasive index for evaluating the in vivo activity of this enzyme induced by many xenobiotics including drugs [3–8]. Recently, we have demonstrated that the urinary ratio 6β-OHF/F does not always reflect the in vivo CYP3A activity [9,10]. It is also suggested that endogenous cortisol 6β-hydroxylation clearance, calculated from the amount of urinary excreted 6β-OHF divided by the area under the plasma concentration–time curve (AUC) of cortisol, can be used as a reliable index for the in vivo CYP3A phenotyping [9,10]. The validity of 6β-hydroxylation clearance as an index was confirmed by evaluating the C-6β oxidation of cortisol catalyzed by CYP3A in vivo, following administration of either [1,1,19,19,19-$^{2}H_{5}$]cortisol

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(cortisol-$^{2}H_5$) or [1,2,4,19-$^{13}C_4$]cortisol (cortisol-$^{13}C_4$) as biological internal standard in humans.

We previously developed a capillary gas chromatography–mass spectrometry (GC–MS) method for the determination of cortisol and cortisone in plasma [11] or 6β-hydroxycortisol in urine [12] with good accuracy and precision by using stable isotope dilution MS. After administering stable isotopically labelled cortisol (cortisol-$^{2}H_5$ or cortisol-$^{13}C_4$) as biological internal standard, the plasma concentrations of endogenous cortisol and exogenous cortisol (cortisol-$^{2}H_5$ or cortisol-$^{13}C_4$) were simultaneously determined by GC–MS using [1,2,4,19-$^{13}C_4$,1,1,19,19,19-$^{2}H_5$]cortisol (cortisol-$^{13}C_4$,$^{2}H_5$) as analytical internal standard [13,14]. On the other hand, the concentrations of endogenous 6β-OHF and exogenous (labelled) 6β-hydroxycortisol (6β-OHF-$^{2}H_5$ or 6β-OHF-$^{13}C_4$) excreted in urine were determined by the double isotope dilution method using the GC–MS technique [10,12]. The method, however, required two series of urine sample obtained after dosing, because there was no available internal standard for the simultaneous GC–MS measurement of endogenous and exogenous 6β-hydroxycortisols. The double isotope dilution method is generally time-consuming and requires great care and technical skill in preparing the samples for the GC–MS analysis, especially in measuring low levels of unlabelled and labelled substances of interest in biological fluids.

The aim of the present study is to develop the simultaneous measurement of endogenous and exogenous (stable isotopically labelled) 6β-hydroxycortisols in human urine by GC–MS, using 6β-hydroxy-[1,2,4,19-$^{13}C_4$,1,1,19,19,19-$^{2}H_5$]cortisol (6β-OHF-$^{13}C_4$,$^{2}H_5$) as analytical internal standard (Fig. 1). The method can be applied to the in vivo metabolic studies associated with the determination of cortisol 6β-hydroxylation clearance as an index for phenotyping the in vivo CYP3A activity in humans.

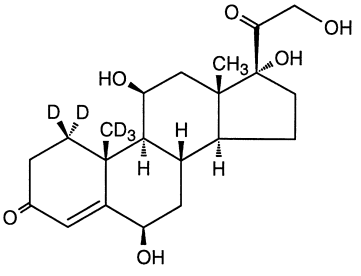
2. Experimental

2.1. Chemicals and reagents

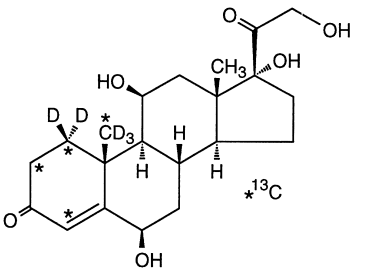
6β-OHF (6β,11β,17α,21-tetrahydroxypregn-4-ene-3,20-dione) was purchased from Steraloids (Wilton, NH, USA). Methoxyamine hydrochloride (Eastman Kodak, Rochester, NY, USA) and *N*,*O*-bis-(trimethylsilyl)acetamide (BSA) (Tokyo Chemicals, Tokyo, Japan) were obtained commercially. All other chemicals and solvents were of analytical-reagent grade and were used without further purification.

2.2. Stable isotopically labelled 6β-hydroxycortisol

[1,1,19,19,19-$^{2}H_5$]Cortisone-BMD and [1,2,4,19-$^{13}C_4$,1,1,19,19,19-$^{2}H_5$]cortisone-BMD were first synthesized via the indan synthon method starting from optically active 11-oxoindanylpropionic acid



6β-hydroxy-[1,1,19,19,19-$^{2}H_5$]cortisol
(6β-OHF-$^{2}H_5$)



6β-hydroxy-[1,2,4,19-$^{13}C_4$,1,1,19,19,19-$^{2}H_5$]cortisol
(6β-OHF-$^{13}C_4$,$^{2}H_5$)

Fig. 1. Structures of stable isotopically labelled 6β-hydroxycortisols; 6β-hydroxy-[1,1,19,19,19-$^{2}H_5$]cortisol (6β-OHF-$^{2}H_5$) and 6β-hydroxy-[1,2,4,19-$^{13}C_4$,1,1,19,19,19-$^{2}H_5$]cortisol (6β-OHF-$^{13}C_4$,$^{2}H_5$).

and labelled isopropenyl anion ([1,1,3,3,3-$^{2}H_5$]- or [1,3-$^{13}C_2$,1,1,3,3,3-$^{2}H_5$]isopropenyl anion) [15,16]. The labelled isopropenyl anion was prepared from commercially available [1,1,1,3,3,3-$^{2}H_6$]- or [1,3-$^{13}C_2$,1,1,1,3,3,3-$^{2}H_6$]acetone. UV irradiated autoxidation at C-6 position of 3-ethyl-3,5-dienol ether derivatives of the labelled cortisone-BMDs gave 6β-hydroxy-[1,1,19,19,19-$^{2}H_5$]cortisone-BMD and 6β-hydroxy- [1,2,4,19-$^{13}C_4$,1,1,19,19,19-$^{2}H_5$]cortisone-BMD, respectively. After protecting the keto group at C-3 of the labelled 6β-hydroxycortisone-BMD as semicarbazone, reduction of 11-keto group with $NaBH_4$ and subsequent removal of the C-3 and C-17 protecting groups gave 6β-hydroxy-[1,1,19,19,19-$^{2}H_5$]cortisol and 6β-hydroxy-[1,2,4,19-$^{13}C_4$,1,1,19,19,19-$^{2}H_5$]cortisol, respectively, as a mixture of 6β and 6α epimers (6β: 6α=4.4:1). The isotopic compositions of 6β-OHF-$^{2}H_5$ and 6β-OHF-$^{13}C_4$,$^{2}H_5$ were 90.9 and 92.1 atom%, respectively.

2.3. Preparation of standards

Stock solutions of 6β-OHF (5.37 mg per 100 ml), 6β-OHF-$^{2}H_5$ (2.107 μg/ml), and 6β-OHF-$^{13}C_4$,$^{2}H_5$ (830.40 ng/ml) were prepared in methanol. All analyses were performed by diluting the stock solutions with methanol.

2.4. GC–MS with selected-ion monitoring (SIM)

Capillary GC–MS analysis was done on a Shimadzu GCMS-2010 system equipped with a data-processing system (Shimadzu, Kyoto, Japan). GC 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 50 kPa.

A split–splitless injection system operated in the splitless mode was used with a septum purge flow-rate of 6 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 50 °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 200 °C.

The multiple-ion detector was focused on the characteristic fragment ions ($[M-OMe]^{+\cdot}$) at m/z 694/699 for the 3,20-dimethoxime-6β,11β,17α,21-tetra(trimethylsilyl) (MO-tetraTMS) derivatives of 6β-OHF/6β-OHF-$^{2}H_5$ and at m/z 703 for the corresponding derivative of 6β-OHF-$^{13}C_4$,$^{2}H_5$.

2.5. Sample preparation for GC–MS-SIM

2.5.1. Extraction

To 0.5 ml of human urine was added a methanol solution (50 μl) containing 41.52 ng of 6β-OHF-$^{13}C_4$,$^{2}H_5$ as analytical internal standard. The 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 60 °C under a stream of nitrogen, acetone (200 μl×2) was added to the residue and the sample was transferred to a 0.3-ml micro product V-vial (Wheaton, Millville, NJ, USA) and then evaporated to dryness at room temperature under a stream of nitrogen.

2.5.2. Derivatization

The methoxime trimethylsilyl ether (MO-TMS) derivatization was performed according to the general procedures employed for the GC and GC–MS analyses of corticosteroids in biological fluids [17]. To the residue containing labelled and unlabelled 6β-hydroxycortisols in a 0.3-ml micro product V-vial were 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 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 (Waters), which was eluted with 7 ml of *n*-hexane. The eluate was evaporated under a stream of nitrogen and the residue was dissolved in 25 μl of chloroform. A

1.0-μl portion of the solution was subjected to GC–MS.

2.6. Calibration graphs

To each of six standards containing known amounts of 6β-OHF (10.74, 21.48, 53.70, 107.4, 268.5, and 537.0 ng) and 6β-OHF-$^{2}H_5$ (10.09, 20.17, 40.34, 80.68, 121.0 and 201.7 ng) dissolved in methanol, 41.52 ng of 6β-OHF-$^{13}C_4$,$^{2}H_5$ 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 chloroform solution (25 μl) was subjected to GC–MS. The peak-area ratios (m/z 694 and m/z 699 to m/z 703) 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 and 6β-OHF-$^{2}H_5$ 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 of 6β-OHF (107.4 ng) and 6β-OHF-$^{2}H_5$ (40.34 ng), using 6β-OHF-$^{13}C_4$,$^{2}H_5$ (41.52 ng) as internal standard. After preparation of the sample for GC–MS-SIM as described above, the peak-area ratios (6β-OHF and 6β-OHF-$^{2}H_5$/6β-OHF-$^{13}C_4$,$^{2}H_5$) were measured.

3. Results and discussion

Stable isotope methodology has widely been accepted for investigating the pharmacokinetics and metabolism of steroids in humans. The stable isotope-labelled (exogenous) analogue can be easily distinguished from naturally occurring (endogenous) steroid by using the mass spectrometric technique. The endogenous and exogenous substances and their metabolites in plasma and urine are often analyzed by using the double isotope dilution method. This method requires two sets of each sample obtained at various time intervals after the administration of labelled compound. A known amount of either unlabelled or labelled substance (metabolite) is added to one set of the sample as internal standard, and to another sample is not. Peak-area or height ratios of the two samples are then measured to calculate the levels of endogenous and exogenous (labelled) substances in plasma or urine. The method is time-consuming. Furthermore, it is often the case that several different amounts of either unlabelled or labelled substance have to be tested to choose an adequate amount to be added. This led us to synthesize another type of stable isotope-labelled 6β-hydroxyl compound for use as analytical internal standard for MS.

We then synthesized 6β-OHF-$^{13}C_4$,$^{2}H_5$ containing four ^{13}C and five deuterium atoms. The labelled internal standard can be used for the simultaneous GC–MS measurement of endogenous 6β-OHF and exogenous 6β-hydroxycortisol (6β-OHF-$^{2}H_5$; 6β-hydroxy-[1,1,19,19,19-$^{2}H_5$]cortisol or 6β-OHF-$^{13}C_4$; 6β-hydroxy-[1,2,4,19-$^{13}C_4$]cortisol) in human urine obtained after administering stable isotopically labelled cortisol, either cortisol-$^{2}H_5$ or cortisol-$^{13}C_4$. Synthetic oxidation at C-6β of cortisol was achieved by UV irradiated autoxidation of 3-ethyl-3,5-dienol ether derivative of labelled cortisone ([1,2,4,19-$^{13}C_4$,1,1,19,19,19-$^{2}H_5$]cortisone), followed by reduction of 11-keto group to give 6β-OHF-$^{13}C_4$,$^{2}H_5$ as a mixture of 6β and 6α epimers (6β:6α=4.4:1). 6β-OHF-$^{2}H_5$ was synthesized by the same procedure as above. The isotopic compositions of 6β-OHF-$^{2}H_5$ and 6β-OHF-$^{13}C_4$,$^{2}H_5$ were 90.9 and 92.1 atom%, respectively. Detailed discussion concerning the synthesis of the labelled 6β-hydroxycortisols will be described elsewhere [18]. The structures of these labelled compounds are given in Fig. 1.

We previously employed the MO-TMS derivatizations for the simultaneous GC–MS measurements of endogenous 6β- and 6α-hydroxycortisols and 6β-hydroxycortisone in urine using the corresponding deuterium-labelled internal standards, 6β-OHF-$^{2}H_5$ and 6α-OHF-$^{2}H_5$ and 6β-OHE-$^{2}H_5$ [14]. In the present study, the MO-TMS derivatizations were also used for the GC–MS measurement of unlabelled 6β-OHF and labelled 6β-hydroxycortisols (6β-OHF-$^{2}H_5$ and 6β-OHF-$^{13}C_4$,$^{2}H_5$) in urine. The MO-TMS derivatization produced the *syn-anti* isomer pair, giving two peaks at t_R 17.25 min and t_R 17.54 min. The peak-area ratio of the isomers at t_R 17.54 and t_R

17.25 was 3.7±1.3 (n=47). The variability in the ratio of the *syn-anti* isomer formation can be ignored for the measurement of 6β-OHF, because 6β-OHF is concurrently subjected to the MO-TMS derivatization with stable isotope-labelled analogue as internal standard. The larger peak at t_R 17.54 min was chosen for the GC–MS measurement of 6β-OHF in urine. Fig. 2 shows the electron impact (EI) mass spectra of the MO-TMS derivatives of unlabelled (6β-OHF) and labelled 6β-hydroxycortisols (6β-OHF-$^2H_5$ and 6β-OHF-$^{13}C_4$,$^2H_5$). The MO-TMS derivatives gave the molecular ions $[M]^{+\cdot}$ at m/z 725 for 6β-OHF, at m/z 730 for 6β-OHF-$^2H_5$, and at m/z 734 for 6β-OHF-$^{13}C_4$,$^2H_5$. The characteristic $[M-31]^{+\cdot}$ fragment ions derived from the loss of the OMe group were observed for the three 6β-hydroxycortisols. The fragment ions $[M-31]^{+\cdot}$ in the mass spectra (Fig. 2) for 6β-OHF/6β-OHF-$^2H_5$/6β-OHF-$^{13}C_4$,$^2H_5$ (m/z 694/699/703) were chosen for the SIM of the MO-TMS 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 25 pg per injection (S/N ratio=5.6) (Fig. 3). The MO-TMS derivative provides sufficient sensitivity to measure low levels (ca. 3 ng) of 6β-OHF in urine. The sensitivity limit (25 pg) in the present EI ionization mode was not compared with those in other ionization modes, i.e., chemical ionization (CI), on the MO-TMS derivative of 6β-OHF.

The MO-TMS derivatives do not allow one to measure cortisol simultaneously with 6β-OHF in urine, because the retention times of the *syn-anti* isomer peaks of derivatized cortisol (t_R 17.16–17.26 min) were identical or close to that of a smaller isomer peak of the MO-TMS derivative of 6β-OHF (t_R 17.25 min). Therefore, the molecular (m/z 636) and fragment ions (e.g., m/z 605, 515, etc.) of the MO-TMS derivative of cortisol (Fig. 4) are interfered with the high natural abundance distributions of silicone isotopes at m/z 636, 605, and 515 derived from the MO-TMS derivative of 6β-OHF present in urine. On the other hand, 6β-OHF in urine was not interfered with the MO-TMS derivative of cortisol, because a larger isomer peak of 6β-OHF monitored (t_R 17.54 min) eluted later than the *syn-anti* isomer peaks of cortisol (t_R 17.16–17.26 min). In the case of the measurement of cortisol in plasma by GC–MS, cortisol is not interfered with the MO-TMS derivative of 6β-OHF, because the concentrations of 6β-OHF in plasma are negligibly low (<1 ng/ml) [19,20], compared to those of cortisol (20–160 ng/ml plasma) [21].

The present MO-TMS derivatization method was applied to the simultaneous determination of endogenous 6β-OHF and exogenous (labelled) 6β-hydroxycortisol (6β-OHF-$^2H_5$) in human urine, using labelled internal standard (6β-OHF-$^{13}C_4$,$^2H_5$). Extraction of 6β-hydroxycortisol from urine samples was performed according to the procedures employed for the GC–MS and liquid chromatography (LC)–MS analyses for cortisol and its metabolites in plasma and urine [11,14,22,23]. Fig. 5 shows a typical example of the selected-ion recordings of the MO-TMS derivatives of unlabelled 6β-OHF and labelled 6β-hydroxycortisols (6β-OHF-$^2H_5$ and 6β-OHF-$^{13}C_4$,$^2H_5$) after processing from spiked urine. As described above, a larger peak (t_R 17.54 min) of the *syn-anti* isomer pair was monitored for the GC–MS measurement of 6β-OHF in urine. Blank urine sample contained no significant interfering substances.

Calibration graphs were prepared in the ranges 10.74–537.0 ng of 6β-OHF and 10.09–201.7 ng of 6β-OHF-$^2H_5$ with 6β-OHF-$^{13}C_4$,$^2H_5$ (41.52 ng) as the internal standard for the GC–MS assay. The mixture was analysed as the MO-TMS derivatives of 6β-OHF by monitoring the $[M-31]^{+\cdot}$ ion intensities at m/z 694 for 6β-OHF, m/z 699 for 6β-OHF-$^2H_5$ and m/z 703 for 6β-OHF-$^{13}C_4$,$^2H_5$. The peak-area ratios were plotted against the mixed molar ratios of 6β-OHF or 6β-OHF-$^2H_5$ to the corresponding labelled internal standard (6β-OHF-$^{13}C_4$,$^2H_5$). A good correlation was found between the observed peak-area ratios (y) and the molar ratios (x). Unweighted least-squares regression analysis gave typical regression lines $y=0.7278x-0.0113$ ($r=0.9997$) for 6β-OHF and $y=0.8573x+0.0411$ ($r=0.9983$) for 6β-OHF-$^2H_5$.

The accuracy of measurements was determined for 6β-OHF and 6β-OHF-$^2H_5$ added to 0.5-ml aliquots of pooled urine containing endogenous 6β-OHF (286.0±7.97 ng/ml urine). To the urine sample were added 41.52 ng of 6β-OHF-$^{13}C_4$,$^2H_5$ as the internal standard and known amounts of 6β-OHF (107.4 ng) and 6β-OHF-$^2H_5$ (40.34 ng). Table 1 shows the

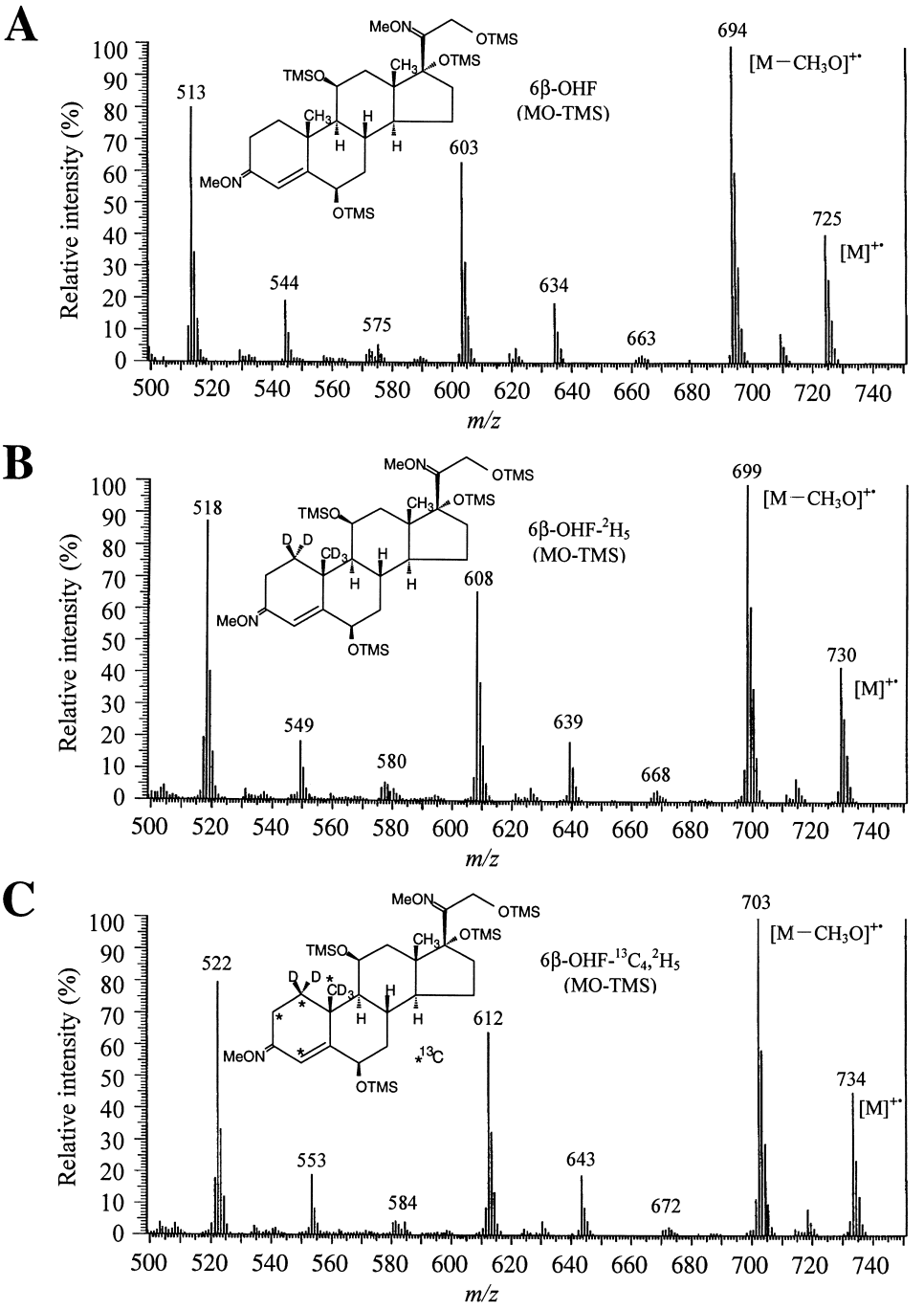


Fig. 2. Electron impact mass spectra of the methoxime trimethylsilyl ether (MO-TMS) derivatives of unlabelled and labelled 6β-hydroxycortisols; 6β-OHF (A), 6β-OHF-$^{2}H_{5}$ (B), and 6β-OHF-$^{13}C_{4}$,$^{2}H_{5}$ (C).

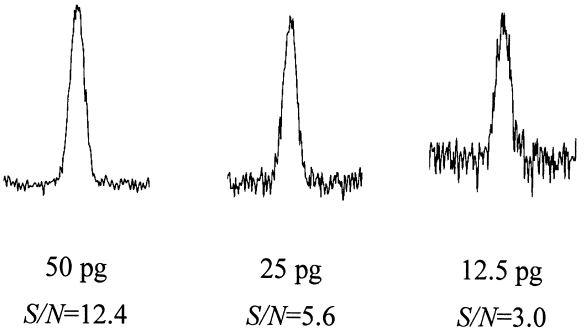


Fig. 3. Sensitivity limits for 6β-hydroxycortisol (6β-OHF).

within-day accuracy and reproducibility in the amounts of unlabelled and labelled 6β-hydroxycortisols determined were in good agreement with the actual amounts added, the relative error being −0.38% for 6β-OHF and 5.30% for 6β-OHF-$^{2}H_5$. The inter-assay RSDs were less than 4.95% for unlabelled and labelled 6β-hydroxycortisols.

4. Conclusions

By using the labelled 6β-hydroxycortisol (6β-OHF-$^{13}C_4$,$^{2}H_5$) as analytical internal standard, the present method provides a sensitive and reliable technique for the simultaneous determination of endogenous 6β-hydroxycortisol (6β-OHF) and its stable isotope-labelled analogue (6β-OHF-$^{2}H_5$) in urine with good accuracy and precision. The method can be applied to pharmacokinetic and metabolic studies by stable isotope methodology with a particular interest in evaluating the C-6β oxidation of cortisol catalyzed by CYP3A in vivo.

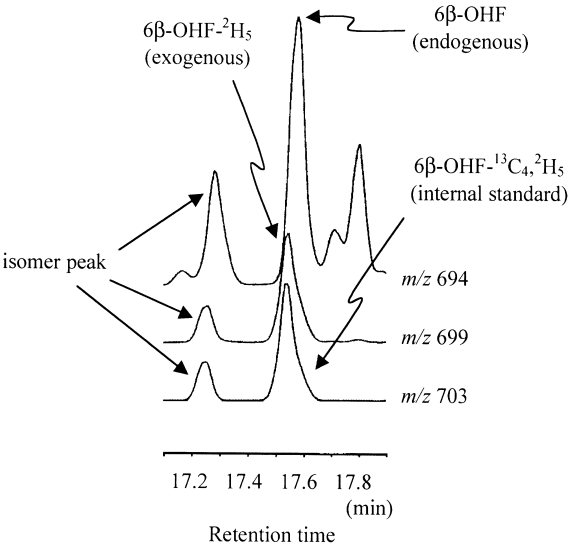


Fig. 5. Selected-ion recordings of the methoxime trimethylsilyl ether (MO-TMS) derivatives of 6β-OHF/6β-OHF-$^{2}H_5$/6β-OHF-$^{13}C_4$,$^{2}H_5$ (m/z 694, 699, and 703) after processing from 0.5 ml of urine spiked with 6β-OHF-$^{2}H_5$ (40.34 ng) and 6β-OHF-$^{13}C_4$,$^{2}H_5$ (41.52 ng) as the internal standard. The MO-TMS derivatization produced the *syn-anti* isomer pair, giving two peaks at t_R 17.25 min and t_R 17.54 min. The larger isomer peak at t_R 17.54 min was monitored for the GC–MS measurement of 6β-OHF in urine.

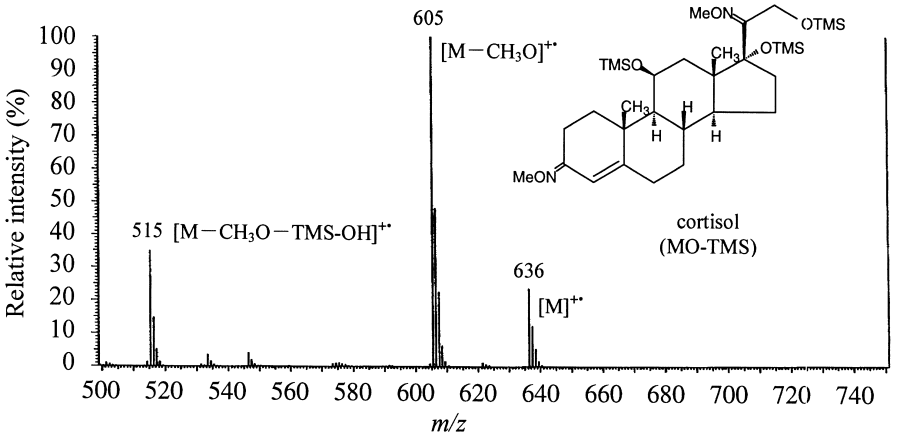


Fig. 4. Electron impact mass spectrum of the methoxime trimethylsilyl ether (MO-TMS) derivative of unlabelled cortisol.

Table 1
Accuracy of GC–MS-SIM determination of endogenous and labelled 6β-hydroxycortisols (6β-OHF and 6β-OHF-$^2H_5$) in human urine

Added (ng/ml) 6β-OHF	Expected (ng/ml)	Found (ng/ml) Individual values[a]						Mean±SD	Relative error (%)	RSD (%)
–	–	274.0	286.7	297.3	290.7	281.2	286.2	286.0±7.97	–	2.79
214.8	500.8	513.6	485.9	476.5	500.7	505.5	511.4	498.9±14.8	−0.38	2.96
6β-OHF-$^2H_5$										
80.68		77.47	83.72	84.67	86.41	88.58	88.91	84.96±4.21	+5.30	4.95

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

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