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Syntheses of stable isotope-labeled 6β-hydroxycortisol, 6β-hydroxycortisone, and 6β-hydroxytestosterone

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Abstract

A method is described for the preparation of two types of multi-labeled 6β-hydroxycortisol containing either five deuterium atoms at C-19 methyl and C-1 methylene or four ^{13}C atoms at C-1, C-2, C-4, and C-19 in addition to the five deuterium atoms for use as analytical internal standards for gas chromatography-mass spectrometry (GC-MS). BMD derivatives of [1,1,19,19,19-$^{2}H_5$]cortisone and [1,2,4,19-$^{13}C_4$,1,1,19,19,19-$^{2}H_5$]cortisone (cortisone-$^{2}H_5$-BMD and cortisone-$^{13}C_4$,$^{2}H_5$-BMD) were first synthesized via indan synthon method starting from optical active 11-oxoindanylpropionic acid and labeled 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). The labeled 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. Ultraviolet (UV) irradiated autoxidation at C-6 position of 3-ethyl-3,5-dienol ether derivatives of the labeled 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, as a mixture of 6β- and 6α-epimers in a ratio of 4:1. Separation of 6β- and 6α-epimers by thin-layer chromatography (TLC) and subsequent hydrolysis of the BMD group at C-17 gave pure labeled 6β-hydroxycortisone. After protecting the keto group at C-3 of the labeled 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 (6β-hydroxycortisol-$^{2}H_5$) and 6β-hydroxy-[1,2,4,19-$^{13}C_4$,1,1,19,19,19-$^{2}H_5$] cortisol (6β-hydroxycortisol-$^{13}C_4$,$^{2}H_5$), respectively, as a mixture of 6β- and 6α-epimers (6β:6α = 4.4:1). The isotopic compositions of 6β-hydroxycortisol-$^{2}H_5$ and 6β-hydroxycortisol-$^{13}C_4$,$^{2}H_5$ were 90.9 and 92.1 at.%, respectively. Furthermore, 6β-hydroxy-[1α,16,16,17α-$^{2}H_4$]testosterone was synthesized by the UV irradiated autoxidation at C-6 position of 3-ethyl-3,5-dienol ether derivative of deuterium-labeled testosterone ([1α,16,16,17α-$^{2}H_4$]testosterone) obtained by using catalytic deuteration and hydrogen–deuterium exchange reactions.

Keywords: 6β-Hydroxycortisol; 6β-Hydroxycortisone; 6β-Hydroxytestosterone; Deuterium-label; ^{13}C-Label; Mass spectrometry

1. Introduction

Cortisol and cortisone are metabolized to 6β-hydroxycortisol, 6α-hydroxycortisol, and 6β-hydroxycortisone by cytochrome P450 3A (CYP3A4 and CYP3A5) isozymes in humans [1–3]. The CYP3A isozymes also catalyze the oxidation of testosterone to 6β-hydroxyltestosterone [4]. Because of wide interindividual variations in the CYP3A activity in humans, evaluation of the in vivo 6β-hydroxylation activity of cortisol and testosterone in human has been required for simply and accurately predicting or assessing the CYP3A-metabolizing capacity of an individual in the fields of drug development and clinical research and practice [5–7]. Recently, we have proposed a new and reliable index for phenotyping the in vivo CYP3A activity using cortisol 6β-hydroxylation clearance [24,25]. The 6β-hydroxylation of synthetic and naturally occurring steroid compounds has also drawn attention, due to the physiological significance in the hypertension [8–10]. These findings led us to synthesize stable isotope-labeled 6β-hydroxyl compounds for use as internal standards for mass spectrometry.

Stable isotope dilution mass spectrometry is widely accepted as the most accurate and specific method for estimation of the small amounts of endogenous and

Abbreviations: GC-MS, gas chromatography-mass spectrometry; UV, ultraviolet; TLC, thin-layer chromatography; BMD, bismethylenedioxy; CYP3A, cytochrome P450 3A; MO-TMS, methoxime-trimethylsilyl; NMR, nuclear magnetic resonance; 6β-Hydroxycortisol-$^{2}H_5$, 6β-hydroxy-[1,1,19,19,19-$^{2}H_5$]cortisol; 6β-Hydroxycortisone-$^{2}H_5$, 6β-hydroxy-[1,1,19,19,19-$^{2}H_5$]cortisone; 6β-Hydroxycortisol-$^{13}C_4$,$^{2}H_5$, 6β-hydroxy-[1,2,4,19-$^{13}C_4$,11,19,19,19-$^{2}H_5$]cortisol; Cortisol-$^{2}H_5$, [1,1,19,19,19-$^{2}H_5$] cortisol; Cortisol-$^{13}C_4$, [1,2,4,19-$^{13}C_4$]cortisol; Cortisol-$^{13}C_4$,$^{2}H_5$, [1,2,4,19-$^{13}C_4$,1,1,19,19,19-$^{2}H_5$]cortisol; 6β-Hydroxytestosterone-$^{2}H_4$, 6β-hydroxy-[1α,16,16,17α-$^{2}H_4$]testosterone

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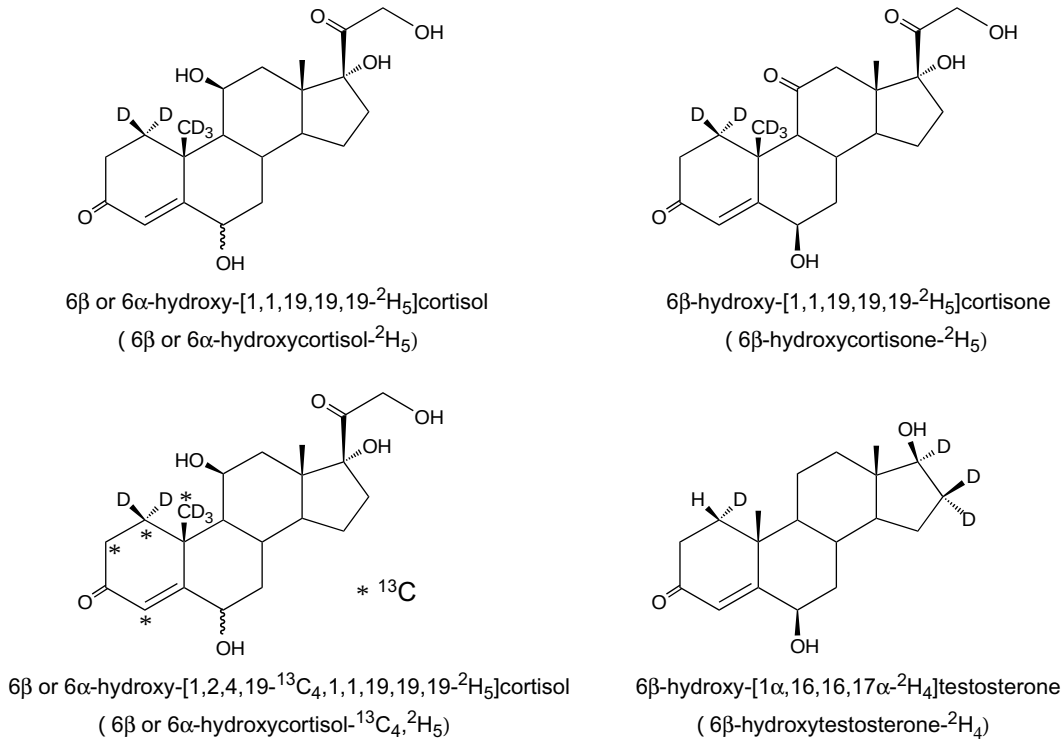


Fig. 1. Structures of stable isotopically labeled 6-hydroxylated steroids.

synthetic steroids in biological fluids [11]. Palermo et al. [12] employed 6β-hydroxy-[1,2-$^{2}H_2$]cortisol as internal standard to determine 6β-hydroxycortisol in urine by gas chromatography-mass spectrometry (GC-MS). 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 trimethylsilyloxy groups at C-6β, C-11β, C-17α, and C-21 of the 3,20-dimethoxime-6β,11β,17α,21-tetra(trimethylsilyl) derivative (MO-TMS derivative) of 6β-hydroxycortisol because of the high abundance of silicon isotopes. The analyte and its labeled internal standard, therefore, should differ by at least four or five mass units to accurately determine 6β-hydroxycortisol. However, there have been no reports for the synthesis of 6-hydroxylated derivatives of cortisol, cortisone, and testosterone labeled with stable isotopes, suitable for use as internal standards in the study of elucidating metabolism of cortisol and testosterone concerning the 6β-hydroxylation in vivo.

We have previously developed a method for a concise 11-step total synthesis of optically pure (+)cortisol by using the chiral hydrindan as a starting material and applied it to the introduction of five deuterium atoms at C-19 methyl and C-1 methylene and/or four ^{13}C atoms at C-1, C-2, C-4, C-19 of cortisol and cortisone, i.e. [1,1,19,19,19-$^{2}H_5$]cortisol, [1,1,19,19,19-$^{2}H_5$]cortisone, [1,2,4,19-$^{13}C_4$,1,1,19,19,19-$^{2}H_5$]cortisol, and [1,2,4,19-$^{13}C_4$,1,1,19,19,19-$^{2}H_5$]cortisone [13,14]. In this study, we synthesize two types of multi-labeled 6β- and 6α-hydroxycortisols, i.e. 6β- and 6α-hydroxy-[1,1,19,19,19-$^{2}H_5$]cortisol and 6β- and 6α-hydroxy-[1,2,4,19-$^{13}C_4$,1,1,19,19,19-$^{2}H_5$]cortisol, based on the use of the corresponding labeled cortisone. 6β-Hydroxy-[1,1,19,19,19-$^{2}H_5$]cortisone was also prepared by this procedure. Furthermore, 6β-hydroxy-[1α,16,16,17α-$^{2}H_4$] testosterone was synthesized from deuterium-labeled testosterone obtained by using catalytic deuteration and hydrogen–deuterium exchange reactions. By using the labeled internal standards, we recently developed a capillary GC-MS method for the simultaneous determination of 6β- and 6α-hydroxycortisols and 6β-hydroxycortisone in urine with good accuracy and precision [15]. The structures of 6-hydroxylated corticosteroids and testosterone labeled with ^{13}C and ^{2}H are given in Fig. 1.

2. Experimental

^{1}H NMR spectra were determined on Bruker DPX-400 400-MHz and Bruker DPX-500 500-MHz spectrometers for samples in $CDCl_3$, CD_3OD, and [$^{2}H_5$]pyridine with tetramethylsilane as internal reference. Capillary gas chromatographic-mass spectrometric (GC-MS) analysis was done on a Shimadzu QP1000EX GC-MS equipped with a data-processing system. GC-MS employed 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).

6β-Hydroxycortisol (6β,11β,17α,21-tetrahydroxypregn-4-ene-3,20-dione) and 6α-hydroxycortisol (6α,11β,17α,21-tetrahydroxypregn-4-ene-3,20-dione) were purchased from Steraloids (Wilton, NH). 6β-Hydroxycortisone (6β,17α,21-trihydroxypregn-4-ene-3,11,20-trione) was purchased from Sigma (St. Louis, MO). 6β-Hydroxytestosterone (Ultrafine, Manchester, UK), testosterone (Tokyo Kasei Kogyo, Tokyo, Japan), and androsta-1,4-diene-3,17-dione (Tokyo Kasei Kogyo) were obtained commercially. Sodium borodeuteride (NaB^2H_4) (99 at.% ^2H) and 30% sodium deuteroxide (NaOD) (99 at.% ^2H) were purchased from Acros (Fair Lawn, NJ). Deuterium gas (99.5 at.% ^2H, Showa Denko, Tokyo, Japan), benzene-$^2H_6$ (99.5 at.% ^2H, Merck, Darmstadt, Germany), CH_3OD (99.5 at.% ^2H, Aldrich, Milwaukee, WI), and deuterium chloride (99.5 at.% ^2H, Aldrich) were obtained commercially. Preparative thin-layer chromatography (TLC) was performed on glass plates coated with a 0.25-mm layer of silica gel 60 F_{254} (Merck).

17α,20;20,21-Bismethylenedioxy-[1,1,19,19,19-$^2H_5$]pregn-4-ene-3,11-dione (cortisone-$^2H_5$-BMD), 17α,21-dihydroxy-[1,1,19,19,19-$^2H_5$]pregn-4-ene-3,11,20-trione (cortisone-$^2H_5$), and 11β,17α,21-trihydroxy-[1,1,19,19,19-$^2H_5$]pregn-4-ene-3,20-dione (cortisol-$^2H_5$) were synthesized in our laboratory [13]. 17α,20;20,21-Bismethylenedioxy-[1,2,4,19-$^{13}C_4$,1,1,19,19,19-$^2H_5$]pregn-4-ene-3,11-dione (cortisone-$^{13}C_4$,$^2H_5$-BMD), 17α,21-dihydroxy-[1,2,4,19-$^{13}C_4$,1,1,19,19,19-$^2H_5$]pregn-4-ene-3,11,20-trione (cortisone-$^{13}C_4$,$^2H_5$), and 11β,17α,21-trihydroxy-[1,2,4,19-$^{13}C_4$,1,1,19,19,19-$^2H_5$]pregn-4-ene-3,20-dione (cortisol-$^{13}C_4$,$^2H_5$) were also synthesized in our laboratory [14].

2.1. Syntheses of 6β- and 6α-hydroxycortisols and 6β-hydroxycortisone labeled with ^{13}C and ^2H

2.1.1. 17α,20;20,21-Bismethylenedioxy-3-ethoxy-[1,1,19,19,19-$^2H_5$]pregna-3,5-diene-11-one (cortisone-$^2H_5$-BMD-3-ethyl-3,5-dienol ether) (**2a**)

To a suspension of 17α,20;20,21-bismethylenedioxy-[1,1,19,19,19-$^2H_5$]pregn-4-ene-3,11-dione (cortisone-$^2H_5$-BMD) (**1a**; ca. 8 mg, 20 μmol) in dioxane (300 μl) were added triethyl orthoformate (100 μl, 0.60 mmol) and absolute ethanol (5 μl), followed by addition of 20 μl of H_2SO_4/dioxane (1:20, v/v). The mixture was then stirred vigorously for 10 min at room temperature. To the dark reaction mixture were added pyridine (10 μl) and water (0.5 ml). The reaction mixture was extracted with $CHCl_3$ (5 × 1 ml), the $CHCl_3$ extract washed with water, and the solvent evaporated to dryness to give the crude cortisone-$^2H_5$-BMD-3-ethyl-3,5-dienol ether (**2a**); *m/z* 435 (M^+). Unlabeled compound; *m/z* 430 (M^+).

2.1.2. 17α,20;20,21-Bismethylenedioxy-3-ethoxy-[1,2,4,19-$^{13}C_4$,1,1,19,19,19-$^2H_5$]pregna-3,5-diene-11-one (cortisone-$^{13}C_4$,$^2H_5$-BMD-3-ethyl-3,5-dienol ether) (**2b**)

According to the procedure for the preparation of cortisone-$^2H_5$-BMD-3-ethyl-3,5-dienol ether (**2a**), cortisone-$^{13}C_4$,$^2H_5$-BMD-3-ethyl-3,5-dienol ether (**2b**) was synthesized from ca. 6.75 mg of cortisone-$^{13}C_4$,$^2H_5$-BMD (**1b**). Cortisone-$^{13}C_4$,$^2H_5$-BMD-3-ethyl-3,5-dienol ether (**2b**); *m/z* 439 (M^+). Unlabeled compound; *m/z* 430 (M^+).

2.1.3. 17α,20;20,21-Bismethylenedioxy-[1,1,19,19,19-$^2H_5$]pregn-4-ene-6β-ol-11-one (6β-hydroxycortisone-$^2H_5$-BMD) (**3a**)

A solution of crude cortisone-$^2H_5$-BMD-3-ethyl-3,5-dienol ether (**2a**) in absolute ethanol (10 ml) placed in a 50 ml round-bottomed flask was irradiated with a 100 W high pressure mercury arc lamp through a Pyrex filter for 4 h at room temperature [16]. The light source was placed at a distance of 10 cm from the reaction vessel. The reaction was monitored by TLC. Evaporation of the solvent under reduced pressure at room temperature gave a crude labeled compound as a mixture of 6β- and 6α-hydroxycortisones-$^2H_5$-BMD (6β:6α = 4:1, determined by GC-MS). Separation of the epimers by preparative TLC ($CHCl_3$/CH_3OH = 100:6, v/v) gave pure 6β-hydroxycortisone-$^2H_5$-BMD (**3a**) in a 25% yield (ca. 2.1 mg); *m/z* 425 (M^+). Unlabeled 6β-hydroxycortisone-BMD; *m/z* 420 (M^+). TLC: R_F 0.31 for 6β-hydroxycortisone-$^2H_5$-BMD and R_F 0.26 for 6α-hydroxycortisone-$^2H_5$-BMD.

2.1.4. 17α,20;20,21-Bismethylenedioxy-[1,2,4,19-$^{13}C_4$,1,1,19,19,19-$^2H_5$]pregn-4-ene-6β-ol-11-one (6β-hydroxycortisone-$^{13}C_4$,$^2H_5$-BMD) (**3b**)

According to the procedure for the preparation of 6β-hydroxycortisone-$^2H_5$-BMD (**3a**), 6β-hydroxycortisone-$^{13}C_4$,$^2H_5$-BMD (**3b**) was synthesized from ca. 6.75 mg of cortisone-$^{13}C_4$,$^2H_5$-BMD (**1b**). Separation of 6β- and 6α-epimers (4:1) by preparative TLC ($CHCl_3$/CH_3OH = 100:6, v/v) gave pure 6β-hydroxycortisone-$^{13}C_4$,$^2H_5$-BMD (**3b**) in a 24% yield (ca. 1.6 mg); *m/z* 429 (M^+). Unlabeled 6β-hydroxycortisone-BMD; *m/z* 420 (M^+). TLC: R_F 0.31 for 6β-hydroxycortisone-$^{13}C_4$,$^2H_5$-BMD and R_F 0.26 for 6α-hydroxycortisone-$^{13}C_4$,$^2H_5$-BMD.

2.1.5. 6β,17α,21-Trihydroxy-[1,1,19,19,19-$^2H_5$]pregn-4-ene-3,11,20-trione (6β-hydroxycortisone-$^2H_5$) (**4a**)

A solution of pure 6β-hydroxycortisone-$^2H_5$-BMD (**3a**) (ca. 85 μg) in ethanol/tetrahydrofuran (0.2 ml, 1:1, v/v) was added dropwise to 46% HF (0.6 ml) in a polyethylene test-tube at 0 °C with stirring. The mixture was stirred vigorously at 0 °C for 8 h and then kept at 2–5 °C for 15 h. The mixture was neutralized to pH ca. 7 by careful addition of chilled saturated Na_2CO_3 solution and then extracted with ethyl acetate (4 × 10 ml). The extracts were washed with water and the solvent was evaporated under reduced pressure. Purification of the residue by TLC (R_F 0.31, $CHCl_3$/CH_3OH = 100:6, v/v) gave pure 6β-hydroxycortisone-$^2H_5$ (**4a**) in a 34.8% yield (26.6 μg). ^1H NMR spectrum of (**4a**) was not measured. The labeled compound (**4a**) was converted to and characterized as 3,20-dimethoxime-6β,17α,21-tri(trimethylsilyl) derivative (MO-triTMS derivative); *m/z*

655 (M^+) [15]. Unlabeled 6β-hydroxycortisone; *m/z* 650 (M^+) as MO-triTMS derivative [15]; δ_H (400 MHz, $CDCl_3$): 0.67 (3H, s, 13-CH_3), 1.62 (3H, s, 10-CH_3), 4.33–4.34 (1H, t, 6α-CH), and 5.83 (1H, s, 4-CH).

*2.1.6. 6β (or 6α),11β,17α,21-Tetrahydroxy-[1,1,19,19,19-$^2H_5$]pregn-4-ene-3,20-dione (6β- and 6α-hydroxycortisols-$^2H_5$) (**6a**)*

The conversion of cortisone-BMD into cortisol was achieved by a known route [13,14]. The 3-keto group of 6β-hydroxycortisone-$^2H_5$-BMD (**3a**) (ca. 2.1 mg) was protected with semicarbazide and the 11-keto group was then reduced with $NaBH_4$. Removal of the protecting group at C-3 with pyruvic acid, followed by hydrolysis of the BMD protecting group with 46% HF afforded crude 6β- and 6α-hydroxycortisols-$^2H_5$ (**6a**). The epimers of the labeled compounds (**6a**) (R_F 0.20) could not be separated by TLC. Purification of the crude materials by TLC (R_F 0.20, $CHC1_3/CH_3OH$ = 9:1, v/v) gave 6β- and 6α-hydroxycortisols-$^2H_5$ (**6a**) (ca. 25 μg) as a mixture of two epimers (6β:6α = 4.4:1, determined by GC-MS). ^1H NMR spectra of 6β- and 6α-hydroxycortisols-$^2H_5$ (**6a**) were not measured. The labeled compounds (**6a**) were converted to and characterized as 3,20-dimethoxime-6β,11β,17α,21-tetra(trimethylsilyl) derivatives (MO-tetraTMS derivatives); *m/z* 730 (M^+) [15]. Unlabeled 6β-hydroxycortisol; *m/z* 725 (M^+) as MO-tetraTMS derivative [15]. δ_H (400 MHz, $CDCl_3$): 0.90 (3H, s, 13-CH_3), 1.62 (3H, s, 10-CH_3), 4.39 (1H, s, 11α-CH), 4.25 (1H, t, 6α-CH), and 5.74 (1H, s, 4-CH).

*2.1.7. 6β (or 6α),11β,17α,21-Tetrahydroxy-[1,2,4,19-$^{13}C_4$,1,1,19,19,19-$^2H_5$]-pregn-4-ene-3,20-dione (6β- and 6α-hydroxycortisol-$^{13}C_4$,$^2H_5$) (**6b**)*

According to the procedure for the preparation of 6β-hydroxycortisol-$^2H_5$, 6β-hydroxycortisol-$^{13}C_4$,$^2H_5$ was synthesized from ca. 1.62 mg of 6β-hydroxycortisone-$^{13}C_4$,$^2H_5$-BMD (**3b**). The resulting material was subjected to preparative TLC (R_F 0.20, $CHC1_3/CH_3OH$ = 9:1, v/v), giving 6β- and 6α-hydroxycortisols-$^{13}C_4$,$^2H_5$ (**6b**) (ca. 27 μg) as a mixture of two epimers (6β:6α = 4.4:1, determined by GC-MS). ^1H NMR spectra of 6β- and 6α-hydroxycortisols-$^{13}C_4$,$^2H_5$ (**6b**) were not measured. The labeled compounds (**6b**) were converted to and characterized as MO-tetraTMS derivatives; *m/z* 734 (M^+) [15]. Unlabeled 6β-hydroxycortisol; *m/z* 725 (M^+) as MO-tetraTMS derivative.

2.2. Synthesis of 6β-hydroxytestosterone labeled with ^2H

*2.2.1. [1α,2α-$^2H_2$]Androst-4-ene-3,17-dione (androstenedione-$^2H_2$) (**10**)*

A solution of chlorotris(triphenylphosphine)rhodium (I) (250 mg) in benzene-$^2H_6$ (19.5 ml) was stirred for 1 h at room temperature under the deuterium atmosphere. To the solution was added dropwise 1 g (3.5 mmol) of androsta-1,4-diene-3,17-dione (**9**) dissolved in benzene-$^2H_6$ (19.5 ml) for 10 min. The mixture was stirred for 1 h under the deuterium atmosphere. After the reaction (absorption of 78.4 ml (3.5 mmol) of deuterium gas), the solution was evaporated to dryness, and the residue was taken up in a mixture of petroleum ether and $CHCl_3$ (1:1, v/v) and filtered through alumina B (50 mg). Evaporation of the solvent gave 910.2 mg of androstenedione-$^2H_2$ (**10**) in a 90.2% yield. Androstenedione-$^2H_2$; *m/z* 288 (M^+). Unlabeled compound; *m/z* 286 (M^+).

*2.2.2. [1α,2,2,4,6,6,16,16-$^2H_8$]Androst-4-ene-3,17-dione (androstenedione-$^2H_8$) (**11**)*

Androstenedione-$^2H_2$ (10) (200 mg, 0.7 mmol) was dissolved in CH_3OD (6 ml) in order to replace the hydroxyl protons of trace H_2O contained in crystals with deuterium. The solvent was immediately evaporated under reduced pressure at room temperature. To a solution of androstenedione-$^2H_2$ (**10**) in 3.0 ml of CH_3OD/D_2O (5:1, v/v) was added 12.5% NaOD in D_2O (650 μl), and the mixture was heated at 65 °C for 24 h under the argon atmosphere [17]. After the reaction, the mixture was neutralized with 7% DCl (pD 6–7) at room temperature, and the solvent was evaporated under reduced pressure. The residue was dissolved in D_2O (5 ml) and then extracted with $CHCl_3$ (4 × 10 ml). The organic solvent was dried over anhydrous sodium sulfate, and then removed under reduced pressure to give 127 mg of androstenedione-$^2H_8$ (**11**) as colorless crystals in a 63.5% yield. Androstenedione-$^2H_8$; *m/z* 294 (M^+). Unlabeled compound; *m/z* 286 (M^+).

*2.2.3. [1α,2,2,4,6,6,16,16,17α-$^2H_9$]Androst-4-en-17β-ol-3-one (testosterone-$^2H_9$) (**12**)*

Androstenedione-$^2H_8$ (**11**) (102 mg) was dissolved in CH_3OD (3 ml) in order to replace the hydroxyl protons of trace H_2O in crystals with deuterium. The solvent was immediately evaporated under reduced pressure at room temperature. To a solution of androstenedione-$^2H_8$ (**11**) in 15 ml of *n*-C_3H_7OD/D_2O (4:1, v/v) was added approximately 10 mg of NaB^2H_4, and the mixture was stirred at 0 °C for 2 h. After the reaction, the mixture was neutralized with 2% CH_3COOD in D_2O (pD 6–7), and the solvent was evaporated under reduced pressure. The residue was dissolved in D_2O (5 ml) and then extracted with $CHCl_3$ (3 × 8 ml). The organic solvent was dried over anhydrous sodium sulfate, and then removed under reduced pressure to give 102 mg of testosterone-$^2H_9$ (**12**) as colorless crystals in a 99% yield. Testosterone-$^2H_9$; *m/z* 297 (M^+). Unlabeled compound; *m/z* 288 (M^+).

*2.2.4. [1α,16,16,17α-$^2H_4$]Androst-4-en-17β-ol-3-one (testosterone-$^2H_4$) (**13**)*

Testosterone-$^2H_9$ (**12**) (100 mg) dissolved in a 5% methanolic solution of H_2SO_4 (2 ml) was heated at 55 °C for 48 h with constant stirring. After the reaction, the mixture was neutralized to pH 7–8 with 4.6 ml of 2.5% KOH in H_2O at room temperature, and then extracted with $CHCl_3$

(4 × 6 ml). The organic solvent was dried over anhydrous sodium sulfate, and then removed under reduced pressure. The residue was subjected to column chromatography on silica gel. The column (60 cm × 2.5 cm, i.d.) was eluted with toluene, followed by *n*-hexane/$CHCl_3$/acetone (5:4:1, v/v/v) to give 57.6 mg of [1α,16,16,17α-$^2H_4$]androst-4-en-17β-ol-3-one (testosterone-$^2H_4$) (**13**) as colorless crystals in a 58.5% yield. Testosterone-$^2H_4$; *m/z* 292 (M^+). ^1H NMR (400 MHz, $CDCl_3$) proton signals at 1.48 ppm (16β-CH), 1.70 ppm (1α-CH), 2.05 ppm (16α-CH), and 3.65 ppm (17α-CH) disappeared, and ^{13}C NMR δ (400 MHz, $CDCl_3$): 30.42 ppm (s, 16-CH_2), 35.71 ppm (s, 1-CH_2), and 81.58 ppm (s, 17α-CH) disappeared. Unlabeled testosterone; *m/z* 288 (M^+). δ_H (500 MHz, $CDCl_3$): 1.48 (1H, m, 16β-CH), 1.70 (1H, m, 1α-CH), 2.05 (1H, m, 16α-CH), and 3.65 (1H, t, 17α-CH). ^{13}C NMR δ (500 MHz, $CDCl_3$): 30.42 (s, 16-CH_2), 35.71 (s, 1-CH_2), and 81.58 (s, 17α-CH).

*2.2.5. [1α,16,16,17α-$^2H_4$]Androst-4-en-6β,17β-diol-3-one (6β-hydroxytestosterone-$^2H_4$) (**15**)*

To a solution of 39 mg (0.13 mmol) of testosterone-$^2H_4$ (**13**) in dioxane (2.1 ml) was added triethyl orthoformate (390 μl) and absolute ethanol (40 μl). After adding a solution of 4.8% H_2SO_4 in dioxane (200 μl; 10:200, v/v), the reaction mixture was stirred vigorously for 20 min. After adding pyridine (40 μl) dropwise, followed by H_2O (1 ml) at room temperature, the mixture was extracted with diethyl ether (4 × 4 ml). The extracts were washed with water and the solvent was evaporated under reduced pressure to give a crude testosterone-$^2H_4$-3-ethyl-3,5-dienol ether (**14**). A solution of the crude compound (**14**) in ethanol (30 ml) was irradiated with a 100 W high-pressure mercury arc lamp through a Pyrex filter for 1 h [16]. After the reaction, the solvent was evaporated under reduced pressure, and the residue was subjected to column chromatography on silica gel. The column (20 cm × 2.5 cm, i.d.) was eluted with $CHCl_3$/CH_3OH (9:1, v/v) to give 0.46 mg of 6β-hydroxytestosterone-$^2H_4$ (**15**) as colorless crystals in a 1.1% yield. 6β-Hydroxytestosterone-$^2H_4$; *m/z* 308 (M^+). Neither ^1H nor ^{13}C NMR spectra of labeled compound (**15**) were measured. Unlabeled 6β-hydroxytestosterone; *m/z* 304 (M^+). δ_H (500 MHz, pyridine-$^2H_5$): 1.59–1.65 (2H, m, 1α-CH and 15α-CH), 1.77 (1H, m, 16β-CH), 2.07 (1H, m, 16α-CH), and 3.91 (1H, m, 17α-CH). ^{13}C NMR δ (500 MHz, $CDCl_3$): 31.09 (s, 16-CH_2), 35.68 (s, 1-CH_2), and 81.35 (s, 17α-CH).

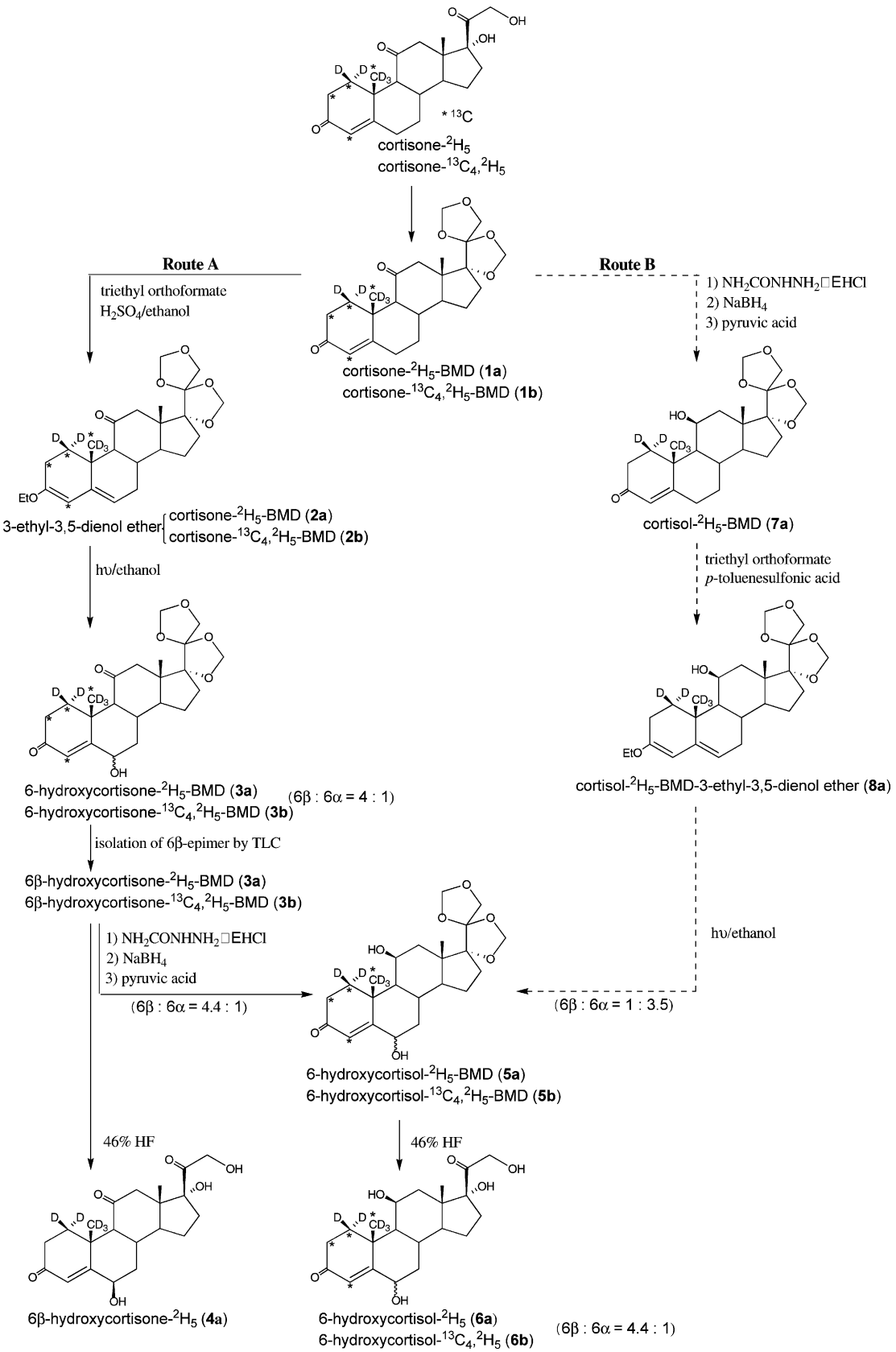
3. Results and discussion

3.1. Syntheses of 6β-hydroxycortisol and 6β-hydroxycortisone labeled with ^{13}C and ^2H

The present study was designed to synthesize two types of labeled 6β-hydroxycortisols, 6β-hydroxy-[1,1,19,19,19-$^2H_5$]cortisol (6β-hydroxycortisol-$^2H_5$) and 6β-hydroxy-[1,2,4,19-$^{13}C_4$,1,1,19,19,19-$^2H_5$]cortisol (6β-hydroxycortisol-$^{13}C_4$,$^2H_5$) shown in Fig. 1. Deuterium-labeled 6β-hydroxycortisone, 6β-hydroxy-[1,1,19,19,19-$^2H_5$]cortisone (6β-hydroxycortisone-$^2H_5$), was also synthesized. A very short synthesis of multiply labeled 6β-hydroxycortisone would be possible if an oxidative step of introducing a hydroxyl group at C-6 could be set after the preparation of labeled cortisone [13,14], i.e. [1,1,19,19,19-$^2H_5$]cortisone (cortisone-$^2H_5$) and [1,2,4,19-$^{13}C_4$,1,1,19,19,19-$^2H_5$]cortisone (cortisone-$^{13}C_4$,$^2H_5$) (Scheme 1). In this study, oxidation of C-6 was performed by using a photochemically induced autoxidation reported by Gardi and Lusignani [16].

Dihydroxyacetone moiety of cortisone-$^2H_5$ was first protected as bismethylenedioxy (BMD) with 37% HCHO to give cortisone-$^2H_5$-BMD (**1a**) in 70–75% yields. The introduction of a hydroxyl group at C-6 of cortisone-BMD was conducted by ultraviolet (UV)-irradiated autoxidation of the 3-ethyl-3,5-dienol ether (**2a**) in ethanol to give a mixture of 6α- and 6β-hydroxycortisones-$^2H_5$-BMD (**3a**) (route A, Scheme 1). The ratio of 6β- and 6α-epimers was approximately 4:1, determined by GC-MS. Purification and separation of the epimers by TLC (R_F 0.31, $CHCl_3$/CH_3OH = 100:6, v/v) and subsequent hydrolysis of the BMD acetals with 46% HF afforded pure 6β-hydroxycortisone-$^2H_5$ (**4a**). 6β-Hydroxycortisol-$^2H_5$ (**6a**) was then prepared via the reduction of the 11-keto group of 6β-hydroxycortisone-$^2H_5$-BMD (**3a**). The 3-keto group of pure 6β-hydroxycortisone-$^2H_5$-BMD (**3a**) was protected with semicarbazide and the 11-keto group was then reduced with $NaBH_4$. Removal of the protecting group at C-3 with pyruvic acid followed by hydrolysis of the BMD acetals with 46% HF afforded a mixture of 6β- and 6α-hydroxycortisols-$^2H_5$ (**6a**) (6β:6α = 4.4:1), accompanied by the epimerization of hydroxyl group at C-6. The ratio of 6β- and 6α-epimers (4.4:1) was determined by GC-MS. It is likely that 6α-hydroxylated compound is thermodynamically more stable than the corresponding 6β-hydroxylated steroid, where the axially positioned 6β group is sterically hindered by the 10β-methyl group [18]. The β- and α-epimers of 6-hydroxycortisol could not be separated by TLC (R_F 0.20, $CHCl_3$/CH_3OH = 9:1, v/v), and the mixture was then used as analytical internal standards for simultaneously measuring 6β- and 6α-hydroxycortisols in human urine by GC-MS [15].

6β-Hydroxy-[1,2,4,19-$^{13}C_4$,1,1,19,19,19-$^2H_5$]cortisol (6β-hydroxycortisol-$^{13}C_4$,$^2H_5$) (**6b**) was synthesized via route A (Scheme 1) starting from [1,2,4,19-$^{13}C_4$,1,1,19,19,19-$^2H_5$]cortisone-BMD (**1b**), since autoxidation of cortisol-BMD-3-ethyl-3,5-dienol ether (**8a**) (route B, Scheme 1) resulted in less formation of 6β-hydroxycortisol (**5a**) (6β:6α = 1:3.5, determined by GC-MS). The labeled 6β-hydroxycortisol (**6b**) has been used as an analytical internal standard [26] for simultaneously determining endogenous and exogenous (labeled) 6β-hydroxycortisols in urine after the administration of stable isotopically labeled cortisol (cortisol-$^2H_5$ or cortisol-$^{13}C_4$) as biological internal standard (tracer) in human subjects [24,25].



Scheme 1. Synthesis of stable isotopically labeled 6-hydroxylated corticosteroids (6β- and 6α-hydroxycortisols-$^{2}H_5$, 6β- and 6α-hydroxycortisols-$^{13}C_4$,$^{2}H_5$, and 6β-hydroxycortisone-$^{2}H_5$).

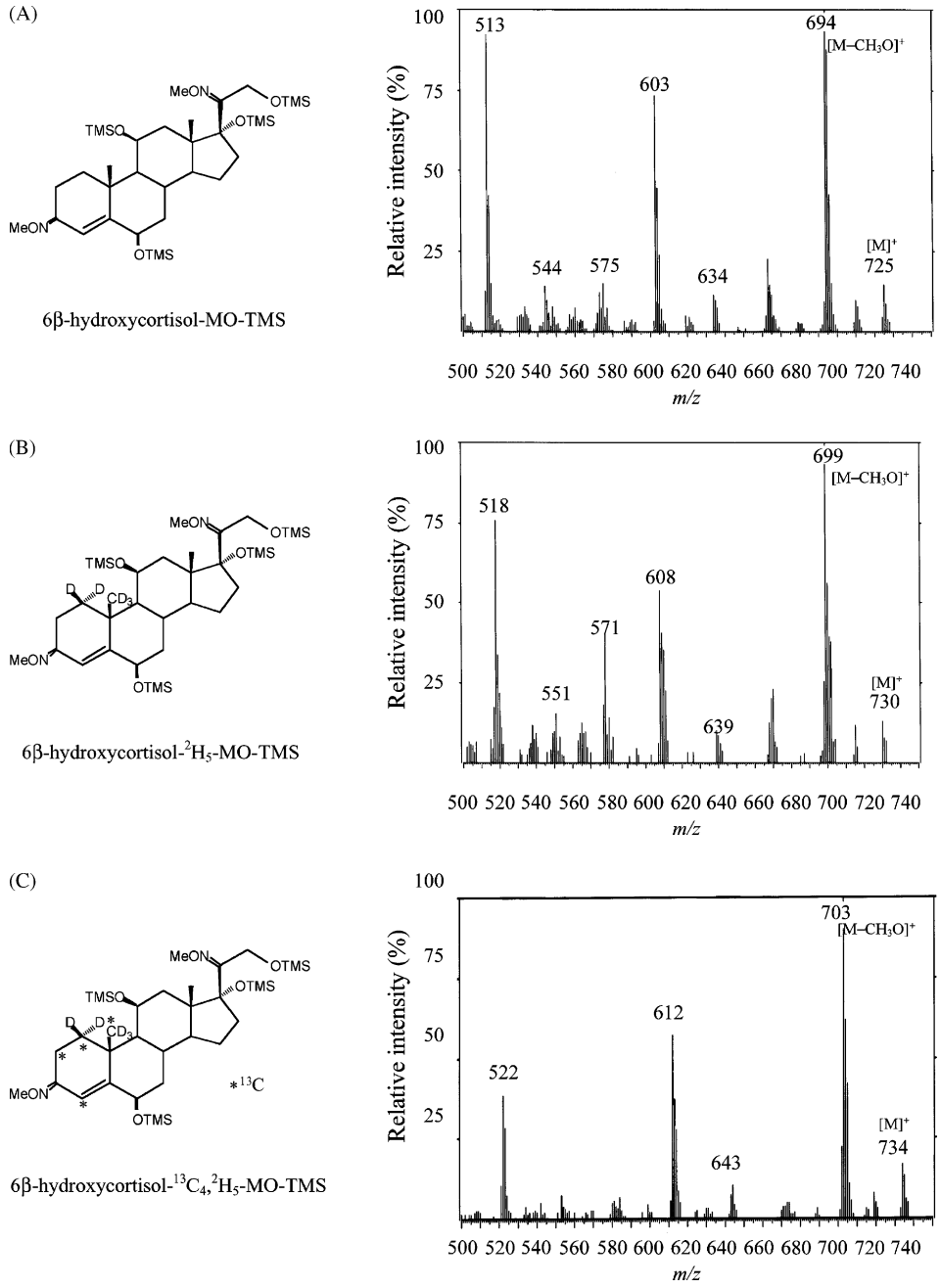
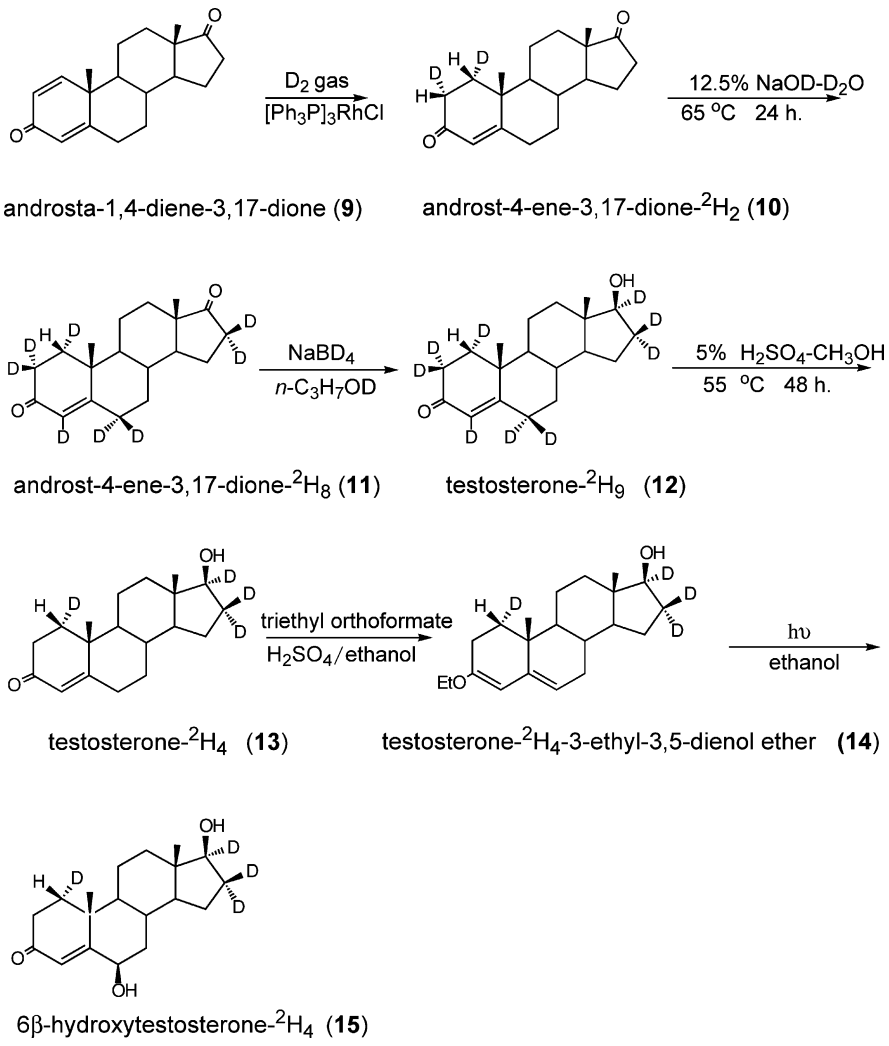


Fig. 2. EI mass spectra of MO-TMS derivatives of unlabeled 6β-hydroxycortisol (A), 6β-hydroxycortisol-$^{2}H_5$ (B), and 6β-hydroxycortisol-$^{13}C_4$,$^{2}H_5$ (C).

Fig. 2 shows the electron-impact (EI) mass spectra of the MO-TMS derivatives of unlabeled 6β-hydroxycortisol and labeled 6β-hydroxycortisols (6β-hydroxycortisol-$^{2}H_5$ and 6β-hydroxycortisol-$^{13}C_4$,$^{2}H_5$) (**6a** and **6b**). The molecular ions of MO-TMS derivatives of 6β-hydroxycortisol-$^{2}H_5$ (*m*/*z* 730) (**6a**) and 6β-hydroxycortisol-$^{13}C_4$,$^{2}H_5$ (*m*/*z* 734) (**6b**) were five and nine mass unit higher, respectively, than the molecular ion (*m*/*z* 725) of unlabeled 6β-hydroxycortisol. In the mass spectra of the MO-TMS derivatives of labeled 6β-hydroxycortisone (6β-hydroxycortisone-$^{2}H_5$) (**4a**), the molecular ion (*m*/*z* 655) was five mass units higher than that (*m*/*z* 650) of unlabeled 6β-hydroxycortisone (the mass spectra are not shown). The isotopic purity was calculated to be >97% for 6β-hydroxycortisol-$^{2}H_5$ (**6a**), 6β-hydroxycortisol-$^{13}C_4$,$^{2}H_5$ (**6b**), and 6β-hydroxycortisone-$^{2}H_5$ (**4a**), based on the ion intensities in the region of the molecular ion of the respective compound.

3.2. Synthesis of 6β-hydroxytestosterone labeled with ^{2}H

Synthesis of multiply labeled 6β-hydroxytestosterone was carried out by the oxidation of C-6β position of deuterium-labeled testosterone by using a photochemically



Scheme 2. Synthesis of deuterium-labeled 6β-hydroxytestosterone (6β-hydroxytestosterone-$^2H_4$).

induced autoxidation. We synthesized [1α,16,16,17α-$^2H_4$] testosterone (**15**) containing four non-exchangeable deuterium atoms at C-1, C-16, and C-17, starting from androsta-1,4-diene-3,17-dione (**9**). As shown in Scheme 2, selective reduction of the 1,2-double bond of steroidal 1,4-diene-3-one (**9**) in benzene-$^2H_6$ with chlorotris(triphenylphosphine)rhodium (I) as catalyst under the deuterium atmosphere gave [1α,2α-$^2H_2$]androst-4-ene-3,17-dione (androstenedione-$^2H_2$) (**10**). The stereochemistry of the deuterium atoms was established by Djerassi and Gutzwiller [19], who reported that reduction of the C-1,2 double bond of the steroidal 1,4-diene-3-one with homogenous rhodium catalyst occurred from the α side, while heterogeneous palladium-catalyzed tritiation [20] or deuteration [21] proceeds from the β-side.

The introduction of deuterium at C-16 and C-17 of testosterone was then achieved according to the procedure reported by Sanaullah and Bowers [17] for synthesizing [16,16,17-$^2H_3$]testosterone using a hydrogen–deuterium exchange reaction. Treatment of [1α,2α-$^2H_2$]androst-4-ene-3,17-dione (androstenedione-$^2H_2$) (**10**) with 12.5% NaOD at 65 °C for 24 h gave [1α,2,2,4,6,6,16,16]androstenedione (androstenedione-$^2H_8$) (**11**) by displacing active hydrogens at C-2, C-4, C-6, and C-16 with deuteriums. Reduction of the C-17 keto group of androstenedione-$^2H_8$ (**11**) with $NaBD_4$ at 0 °C for 2 h gave [1α,2,2,4,6,6,16,16,17α]testosterone (testosterone-$^2H_9$) (**12**). Subsequent back-exchange of five deuterium atoms at C-2, C-4, and C-6 of testosterone-$^2H_9$ (**12**) with a 5% methanolic solution of H_2SO_4 at 55 °C for 48 h gave [1α,16,16,17α-$^2H_4$]androst-4-en-17β-ol-3-one (testosterone-$^2H_4$) (**13**). Proton signals at 1.48 ppm (16β-CH), 1.70 ppm (1α-CH), 2.05 ppm (16α-CH), and 3.65 ppm (17α-CH) disappeared in the ^1H NMR spectrum of testosterone-$^2H_4$ (Fig. 3). EI mass spectra of labeled testosterone (testosterone-$^2H_4$) (**13**) (the mass spectra are not shown) showed that the molecular ion (*m*/*z* 292) was four mass units higher than that (*m*/*z* 288) of unlabeled testosterone.

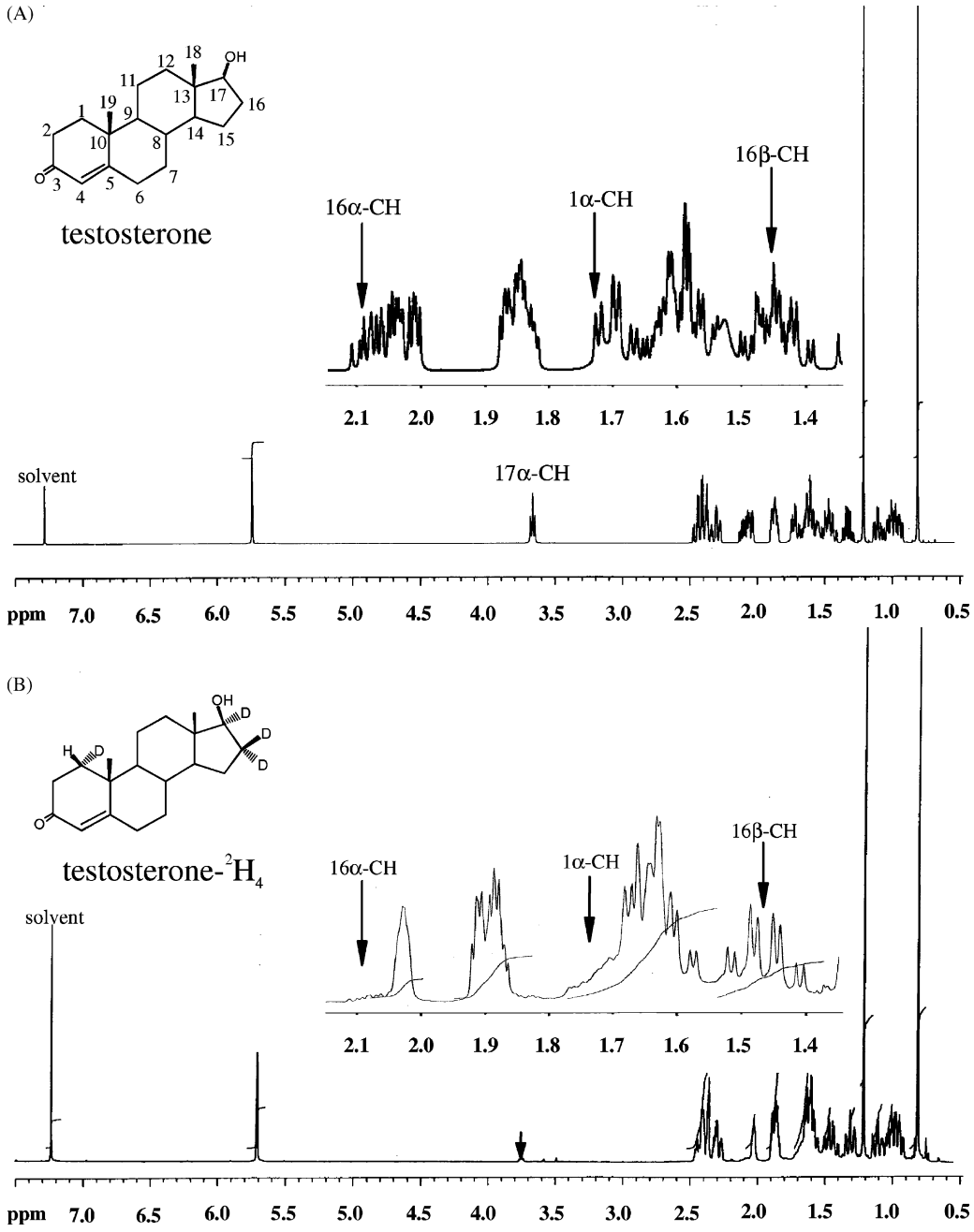


Fig. 3. ^1H NMR spectra of unlabeled testosterone (A) and testosterone-$^2H_4$ (B).

The introduction of a hydroxyl group at C-6β of testosterone-$^2H_4$ (**13**) was conducted according to the procedure for 6β-hydroxylation of cortisone and cortisol. The 3-ethyl-3,5-dienol ether (**14**) was first prepared by treatment of testosterone-$^2H_4$ (**13**) in dioxane (2.1 ml) containing 40 μl of absolute ethanol with triethyl orthoformate (390 μl), followed by adding a solution of H_2SO_4/dioxane (200 μl; 10:200, v/v). The reaction led to the formation of testosterone-3-ethyl-3,5-dienol ether (*m/z* 316; M^+) (**14**) and its 17β-ethoxy derivative (*m/z* 344; M^+) in a ratio of 2:1. Attempts to purify testosterone-3-ethyl-3,5-dienol ether by recrystallization or by column chromatography were unsuccessful because of instability of the 3-ethyl-3,5-dienol ether. A solution of crude testosterone-$^2H_4$-3-ethyldienol ether (**14**) thus obtained in ethanol (30 ml) was irradiated with a 100 W high-pressure mercury arc lamp through a Pyrex filter for 1 h. Purification by column chromatography gave [1α,16,16,17α-$^2H_4$]androst-4-en-6β,17β-diol-3-one (6β-hydroxytestosterone-$^2H_4$) (**15**) as colorless crystals. The 6α-epimer was not detected by GC-MS.

Fig. 4 shows the EI mass spectra of unlabeled 6β-hydroxytestosterone and labeled 6β-hydroxytestosterone

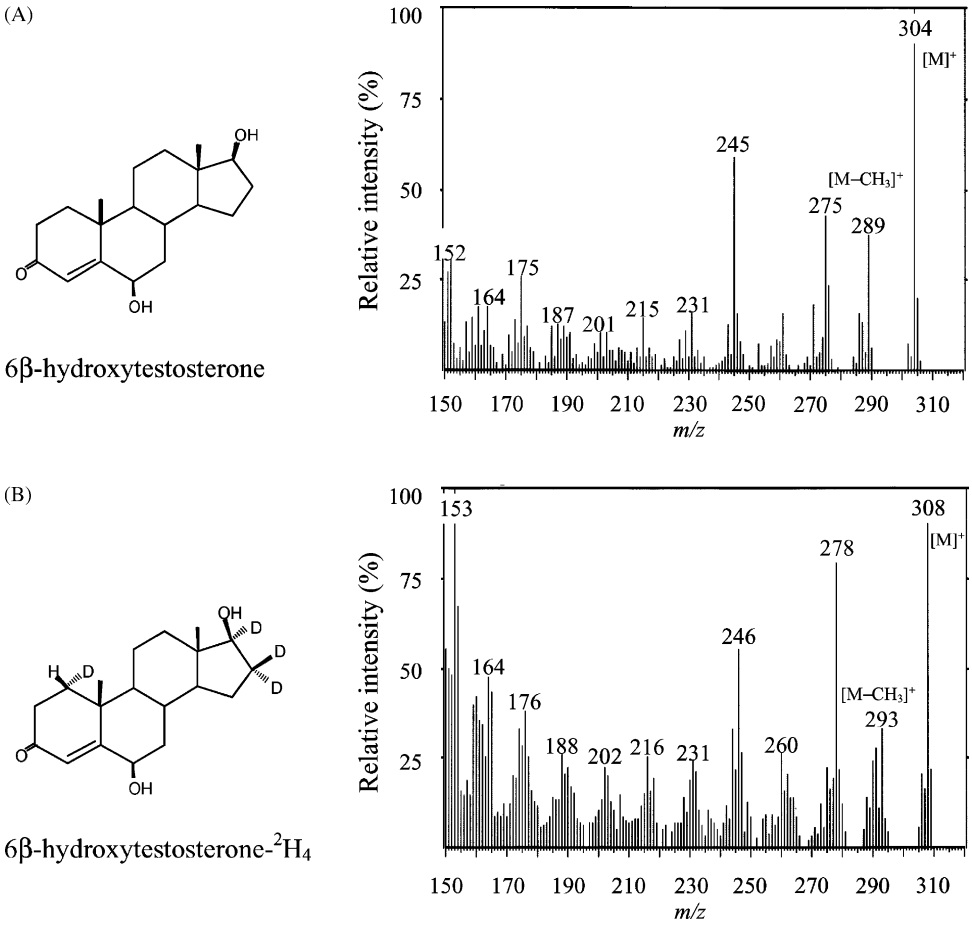


Fig. 4. EI mass spectra of unlabeled 6β-hydroxytestosterone (A) and 6β-hydroxytestosterone-$^{2}H_4$ (B).

(6β-hydroxytestosterone-$^{2}H_4$) (**15**). The molecular ions of (**15**) (*m*/*z* 308) was four mass units higher than that (*m*/*z* 304) of authentic unlabeled 6β-hydroxytestosterone. The isotopic purity was calculated to be 78%, based on the ion intensities in the region of the molecular ion of the compound. The NMR spectral data of labeled 6-hydroxylated compounds were not obtainable in this study because of a small scale of the reactions employed. Assignments for the labeled positions of 6-hydroxylated compounds (6β-hydroxycortisone, 6β- and 6α-hydroxycortisols, and 6β-hydroxytestosterone) were based on the NMR data of the corresponding labeled precursors (labeled cortisone and cortisol [13,14], and labeled testosterone shown in Fig. 3). The chemical shift data for the full assignment of ^{13}C and ^{1}H NMR spectra of unlabeled 6β-hydroxytestosterone and 6β-hydroxycortisol are summarized in Table 1.

In general, the yield of the 6β-hydroxylated compound is much higher than that of 6α-hydroxylated isomer on autoxidation of 3,5-dien-3-ol ether of steroidal 4-ene-3-keto compounds [16], and the ratio between 6β- and 6α-epimers (6β/6α) was 8–10 [22,23]. Preferential oxygenation at C-6 from the β side is probably due to the planarity of the C-5,6 double bond of ring B resulting from the steroidal 3,5-dienol moiety. The sp^2 carbon at C-6 of ring B could be tilted downward away from the β angular methyl group at C-10 and toward the α axial hydrogen at C-9. Oxygenation at C-6 may be hindered more by the C-9 hydrogen on the α-side than by the methyl group on the β-side. In the present study, 6α-hydroxycortisol was predominantly formed (6β:6α = 1:3.5) on autoxidation of cortisol-BMD-3-ethyl-3,5-dienol ether, whereas 6β-hydroxycortisone possessing an 11-keto group was predominantly formed (6β:6α = 4:1) In the case of cortisol possessing a hydroxyl group at C-11β, non-bonding interaction between the 11β-hydroxyl and the 10β-methyl group may force the latter to bend toward the C-6 of the B-ring, preventing oxygenation of C-6 from the β-side.

The stable isotope methodology coupled with mass spectrometry would provide a useful tool for the pharmacokinetic and metabolic studies of cortisol, cortisone, and testosterone with a particular interest in evaluating 6β- or 6α-hydroxylation in the metabolism in human. The present study demonstrated a synthesis of multi-labeled 6-hydroxylated cortisol, cortisone, and testosterone with ^{13}C and ^{2}H for use as internal standards for GC-MS for study of phenotyping the in vivo CYP3A activity in humans.

Table 1
500 MHz (δ) ^{13}C and ^{1}H NMR data of 6β-hydroxycortisol in CD_3OD and 6β-hydroxytestosterone in [$^{2}H_5$]pyridine

Position	6β-Hydroxycortisol			6β-Hydroxytestosterone		
	^{13}C NMR	^{1}H NMR		^{13}C NMR	^{1}H NMR	
1	37.83	α	1.83	37.68	α	1.60
		β	2.24		β	1.91
2	34.92	α	2.33	34.92	α	2.42
		β	2.62		β	2.56
3	203.21			199.75		
4	125.32		5.74	126.00		6.05
5	173.00			169.00		
6	73.18	α	4.25	72.64	α	4.55
7	40.77	α	1.33	39.46	α	1.26
		β	2.12		β	2.13
8	28.28		2.35	30.57		2.29
9	57.74		0.92	54.54		0.89
10	40.13			38.67		
11	68.46	α	4.39	21.19	α	1.50
					β	1.48
12	40.86	α	2.02	37.42	α	1.15
		β	1.62		β	2.09
13	48.30			43.63		
14	53.40		1.76	51.05		1.01
15	24.71	α	1.82	23.93	α	1.60
		β	1.45		β	1.34
16	34.59	α	1.47	31.09	α	2.07
		β	2.72		β	1.77
17	90.37			81.35	α	3.91
18	17.83		0.90	11.90		1.00
19	22.96		1.62	19.76		1.55
20	212.93					
21	67.67		4.26, 4.63			

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