A SIMPLE, RAPID PROCEDURE FOR THE PREPARATION OF DEUTERIUM-LABELLED TESTOSTERONE

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A simple and rapid procedure for the preparation of [16,16-$^2H_2$]- and [16,16,17-$^2H_3$]testosterone of high isotopic purity is described. [16,16-$^2H_2$]Dehydroepiandrosterone is obtained by base-catalysed exchange; reduction of the 17-keto group using lithium aluminium hydride or lithium aluminium deuteride is followed by selective enzymic oxidation of the 3β-hydroxy function with concomitant Δ^5 to Δ^4 isomerisation. The incorporation of deuterium in intermediates and products is established by gas chromatographic-mass spectrometric analyses of parent compounds and alkylsilyl derivatives. Deuterium labels in the 16 and 17 positions of testosterone are shown to be non-labile under strongly acidic and basic conditions.

Key words : [16,16,17-$^2H_3$]testosterone; deuterium; gas chromatography-mass spectrometry; enzymic oxidation.

INTRODUCTION

Techniques employing gas chromatography-mass spectrometry (GC-MS) with selected ion detection (SID) are increasingly accepted as reference methods for the determination of steroid hormones in physiological fluids (1,2,3). The accuracy and precision achieved by these techniques are in part attributable to the use of appropriate internal standards which are not distinguished from the analyte until the final GC-MS analysis. Analogues of the compounds of interest, labelled with stable isotopes, are frequently the preferred internal standards by virtue of their close chemical similarity. The preparation of analogues with non-labile isotopic labels, however, has often been time-consuming, retarding the otherwise rapid development of GC-MS methods.

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As the first stage in the development of reference methods for the determination of testosterone in blood plasma and other fluids, we have devised a simple and rapid procedure for the preparation of a ^2H-labelled analogue. Procedures for the preparation of deuterium-labelled testosterone have been reported previously. Thus, for example, a relatively elaborate synthesis yields [19,19,19-$^2H_3$]testosterone via the Grignard reaction of 17β-acetoxy-5α,10α-epoxy-estrane-3-cycloethyleneketal with [^2H]methyl magnesium bromide (4). The preparation of [^2H]testosterone by an exchange reaction (5) has the merit of simplicity but the product is of low isotopic purity and the risk of loss of deuterium during sample manipulation is high. In this paper we report the preparation of [16,16,17-$^2H_3$]testosterone and provide evidence for the stability of the deuterium label under acidic and basic conditions.

EXPERIMENTAL

Steroids were obtained from Sigma London (Poole, U.K.). 3β-Hydroxysteroid oxidase, isolated from Brevibacterium sterolicum (6) by Dr. T. Uwajima (Kyowa Hakko Kogyo Co., Machida-shi, Tokyo, Japan), was a gift from Dr. A.G. Smith (Medical Research Council Toxicology Laboratories, Carshalton, Surrey, U.K.). $LiAl^2H_4$, CH_3O^2H and ^2H_2O were obtained from Aldrich Chemical Co. (Gillingham, U.K.).

[16,16-$^2H_2$]Dehydroepiandrosterone (2)

Dehydroepiandrosterone was dissolved in a solution (4 ml; 0.27 M) of sodium deuteroxide in CH_3O^2H and heated under reflux for 48 h. After addition of an equal volume of water, the product was extracted with ethyl acetate (2 x 10 ml).

[16,16,17-$^2H_3$]- and [16,16-$^2H_2$]-5-androstene-3β,17β-diol (3 and 4)

(2) was dissolved in a saturated solution of lithium aluminium deuteride in dry diethyl ether (2 ml). After reaction (1 h) at 20°C, excess reagent was destroyed by addition of ethyl acetate (2 ml) and water (2 ml) and the product (3) extracted with ethyl acetate (2 x 5 ml). (4) was prepared by a similar procedure employing lithium aluminium hydride.

[16,16,17-$^2H_3$]- and [16,16-$^2H_2$]testosterone (5 and 6)

3β-Hydroxysteroid oxidase (1 mg) was dissolved in NaH_2PO_4-Na_2HPO_4 buffer (50 mM; pH 7.0; 2 ml). The solution was extracted with diethyl ether (7 ml) and the extract discarded. (3) or (4) was

dissolved in diethyl ether (8 ml) and the solution shaken with the aqueous enzyme solution at 20°C until oxidation was judged, by TLC (silica; chloroform/acetate (185/15), mobile phase), to be complete (approximately 48 h). The ether layer was recovered and the aqueous layer extracted with further portions (2 x 5 ml) of diethyl ether. The extracts were combined, washed with water (4 ml) and evaporated to dryness.

In an alternative procedure (7), applicable to small quantities of substrate, steroid (0.1 mg) was dissolved in propan-2-ol (50 µl) and mixed with NaH_2PO_4 - Na_2HPO_4 buffer (3 ml), containing 3β-hydroxysteroid oxidase (300 µg), to form a homogeneous system. After reaction (24 h) at 20°C, steroid was recovered by extraction with ethyl acetate (2 x 3 ml).

Preparation of derivatives

Trimethylsilyl ether (TMS) derivatives were prepared by dissolving the steroids in bis-trimethylsilylacetamide (Pierce and Warriner, Chester, U.K.) and standing at 20° overnight. Excess reagent was removed under a stream of nitrogen. t-Butyldimethylsilyl (TBDMS) ethers were similarly prepared using t-butyldimethylchlorosilane/imidazole/dimethylformamide (1/1/10, by wt.; Applied Science Laboratories, State College, PA 16801, U.S.A.). Excess reagent was removed by passage through a short column of Sephadex LH-20, as previously described (8).

Assessment of stability of deuterium label

Aliquots (0.5 mg) of (5) and (6) were dissolved in 0.3 M HCl in 95% methanol or 0.3 M NaOH in 95% methanol (3 ml) and heated under reflux for 1 h. After addition of water (2 ml), the steroid was extracted with hexane (2 x 5 ml), derivatised and analysed by GC-MS.

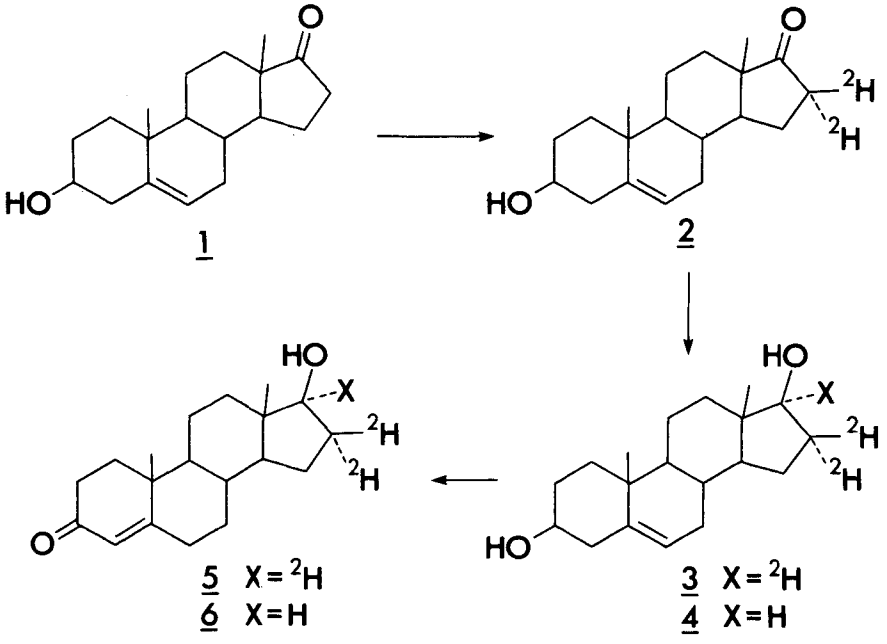
Gas chromatography and combined gas chromatography-mass spectrometry (GC-MS)

Gas chromatography employed a Varian 3700 instrument equipped with a glass column (2m x 0.3cm, i.d.) of 1% OV-17 on Gas Chrom Q (100-120 mesh) with helium as carrier gas. GC-MS was performed using a Varian 2700 gas chromatograph coupled via a Watson-Biemann separator to a Varian MAT 731 double-focussing mass spectrometer. The electron energy was 70 eV and the ion source temperature was 200°C. Separations were performed on glass columns (2m x 0.3cm, i.d.) of 1% OV-1 or 1% OV-17 on Gas Chrom Q (100-120 mesh). For the

acquisition of mass spectra, recorded at low resolution (m/Δm 1000, 10% valley definition), the mass spectrometer was on-line to a Varian Spectrosystem 100MS data system. The isotopic composition of deuterium-labelled testosterone was determined by GC-MS/single ion detection analyses of t-butyldimethylsilyl (TBDMS) ether derivatives with monitoring of $[M-C_4H_9]^+$ ions. The mass spectrometric resolution was 8,500. Epitestosterone TBDMS was employed as internal standard (9).

RESULTS AND DISCUSSION

The experimental procedure, outlined in Scheme 1, employed a conventional base-catalysed exchange reaction (10) for the introduction of deuterium adjacent to the 17-keto group of dehydroepiandrosterone (1). Subsequent reduction with lithium aluminium hydride or lithium aluminium deuteride afforded predominantly the 17β-hydroxy isomer (11). Conversion of the intermediate 5-androstenediol (3,4) to testosterone (5,6) was achieved in high yield under mild conditions using a 3β-hydroxysteroid oxidase.



Scheme 1

Cholesterol oxidases are widely used in the clinical determination of cholesterol (reviewed in 12) and have been applied to the microanalysis of steroids, notably by GC and GC-MS (e.g. 7,13,14, 15,16). Despite an enumeration of the advantages of cholesterol oxidases as preparative reagents (12), however, their potential in this respect has not been fully exploited. The few applications in this area include convenient preparations of 7α-hydroxy-[4-^{14}C]-cholest-4-en-3-one (17) and of 20α-[20-^{18}O]hydroxypregn-4-en-3-one and its 20β isomer (18). In the present work, the 3β-hydroxysteroid oxidase from Brevibacterium sterolicum was used in preference to cholesterol oxidase from Nocardia sp. since steroids lacking a side-chain are poor substrates for the latter enzyme (12). The facility, demonstrated here, of effecting oxidation in a heterogeneous system further extends the utility of these oxidases for preparative purposes.

The chemical purity of the products, (5) and (6), as judged by GC and GC-MS, was approximately 93%. The presence of minor reaction products was attributed to the formation of 17α-hydroxy isomers during lithium aluminium hydride reduction and to incomplete enzymic oxidation. The yields of (5) and (6) were approximately 65%.

The incorporation of deuterium in intermediates and products was assessed by GC-MS analyses of parent compounds and alkylsilyl derivatives. GC-MS data for labelled and unlabelled analogues are shown in Table 1. The spectra of (2), as the free compound and the trimethylsilyl derivative, are consistent with the incorporation of two deuterium atoms into the steroid D ring. Thus, for example, ions $[M-129]^+$ (19,20) and $[M-56]^{+\cdot}$ (21,22), both of which arise by loss of ring A fragments from the TMS derivatives, appear 2 mass units higher in the spectrum of the labelled analogue than in the spectrum of the unlabelled compound. The spectra of the diol intermediates (3) and (4), and the corresponding TMS derivatives, similarly indicate the expected mass shifts. For the TMS ethers, loss of trimethylsilanol is attributed to 1, 3 or 1, 4 elimination (23) : the mass shifts observed for these ions in the spectra of the TMS derivatives of (3) and (4) (Table 1) are accordingly consistent with incorporation of deuterium at C16 and C17.

The spectra of the deuterated testosterone products, (5) and (6), indicate, by comparison with the unlabelled analogue (Table 1), that fragment ions retaining the C and D rings are shifted by 2 and 3 mass units, respectively. Thus, for example, ions of m/z 203 and

Table 1. Gas chromatographic-mass spectrometric data

	Retention index	$M^{+\cdot}$	Mass spectrum+ base peak			Other††		
dehydroepiandrosterone (1)	2525*	288(100)	288	255(65)	270(52)	121(50)	203(47)	177(47)
1 TMS	2595**	360(17)	129	73(33)	304(17)	231(15)	270(14)	255(7)
2	2525*	290(100)	290	257(73)	271(51)	205(43)	179(40)	121(30)
2 TMS	2600**	362(24)	129	73(28)	306(24)	233(19)	272(17)	257(11)
5-androstene-3β,17β-diol	2525*	290(100)	290	272(73)	179(43)	257(42)	205(42)	161(39)
5-androstene-3β,17β-diol TMS	2510**	432(27)	73	129(89)	215(56)	344(44)	239(44)	254(36)
3	2525*	292(100)	293	275(57)	182(43)	208(42)	260(38)	164(23)
3 TMS	2515**	437(21)	73	129(69)	218(38)	242(35)	257(28)	347(33)
4	2525*	292(100)	292	274(62)	181(46)	259(42)	163(39)	207(21)
4 TMS	2515**	436(15)	73	129(60)	217(38)	241(31)	346(30)	256(27)
testosterone	2630*	288(63)	124	246(45)	147(36)	203(21)	228(12)	213(4)
testosterone TMS	2705**	360(74)	129	73(85)	270(48)	147(37)	345(22)	226(22)
5	2630*	291(77)	124	249(55)	150(24)	149(24)	206(22)	231(13)
5 TMS	2710**	363(97)	131	73(94)	273(59)	150(32)	348(29)	229(26)
6	2630*	290(84)	124	248(53)	149(31)	205(22)	230(15)	215(5)
6 TMS	2710**	362(86)	73	130(90)	272(55)	149(44)	347(28)	228(23)

* OV-1, 210°; ** OV-17, 230° (helium carrier gas)

\+ Ions (other than base peak) cited as : m/z (rel. intens.)

†† Five most intense ions (other than the molecular ion, the base peak and those ions solely attributable to the natural abundance of ^{13}C, ^{29}Si and ^{30}Si).

246 in the spectrum of testosterone (24,25) appear at m/z 206 and 249 in the spectrum of 5. M/z 147 in the spectrum of testosterone is associated with loss of water and fragmentation across the B ring with retention of the C and D rings (23,24); corresponding ions of m/z 149 and 150 in the spectrum of (5) are attributed to the same fragmentation with partial loss of deuterium. Analogous fragmentation patterns are observed in the spectra of the TMS ethers. In addition, the base peak of m/z 129, $[CH_2CHCHOSi(CH_3)_3]^+$, in the spectrum of testosterone TMS is shifted by 2 and 1 mass units, respectively, in the spectra of the TMS derivatives of (5) and (6) (Table 1). The mechanism of formation of this ion, proposed by Diekmann and Djerassi (19), involves fragmentation of the D ring with loss of a single hydrogen from C16 in the charge-retaining fragment. The present data are therefore consistent with deuterium incorporation at C16 and C17.

The isotopic purity of the deuterated products, (5) and (6), was determined by GC-MS analyses of t-butyldimethylsilyl ether derivatives with selected ion detection of $[M-C_4H_9]^+$ ions (9). Results are shown in Table 2. In view of the importance of the stability of the isotopic label when deuterated analogues are employed as internal standards in quantitative GC-MS, the stability of the deuterium label in (5) and (6) was assessed by heating acid and basic solutions under reflux (see Experimental). No significant difference in isotopic composition before and after treatment was observed (Table 2).

In summary, a simple procedure has been described for the preparation of deuterium-labelled testosterone, suitable for use as an internal standard in the quantification of testosterone by GC-MS/SID. The isotopic purity is high and the deuterium label is not removed under strongly acid or basic conditions.

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Table 2. Incorporation of deuterium in testosterone

Sample*	Deuterium incorporation+			
	$^2H_0$	$^2H_1$	$^2H_2$	$^2H_3$
5	0.49	2.70	4.21	92.60
5 (after acid treatment)	0.60	3.26	3.83	92.31
5 (after base treatment)	0.36	1.99	5.02	92.63
6	0.62	7.23	92.15	-
6 (after acid treatment)	0.50	7.04	92.46	-
6 (after base treatment)	0.64	7.84	91.51	-

* See Experimental section. 5 : [16,16,17-$^2H_3$]testosterone ; 6 : [16,16-$^2H_2$]testosterone.

\+ Assessed by GC-MS with selected ion detection of $[M-C_4H_9]^+$ ions of TBDMS ether derivatives. The percentage of the major isotopic species was determined with a precision of 0.8%.

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