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COMPARISON OF THE USE OF MASS SPECTROMETRY AND METHYLENE UNIT VALUES IN THE DETERMINATION OF THE STEREOCHEMISTRY OF ESTRANEDIOL, THE MAJOR URINARY METABOLITE OF 19-NORTESTOSTERONE IN THE HORSE

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SUMMARY

The stereochemistry of an isomer of 5-estrane-3,17α-diol, the major metabolite of 19-nortestosterone in horse urine has been established by the use of methylene unit (MU) values. The empirical MU values of the bis-trimethylsilyl (TMS) derivatives of the eight available isomers of 5-androstane-3,17-diol and four isomers of 5-estrane-3,17β-diol were determined by capillary gas chromatography using three different columns. From this data the theoretical MU values for the bis-TMS derivatives of the four 5-estrane-3,17α-diol isomers were predicted. Comparison of the experimentally determined MU value of the urinary metabolite with those of the theoretical values established the correct stereochemistry of the steriod. This method has been compared with the use of gas chromatography–mass spectrometry in the determination of the stereochemistry of unknown metabolites.

INTRODUCTION

Studies related to the metabolism of 19-nortestosterone (estr-4-en-17β-ol-3-one), **1**, in the horse have demonstrated that an isomer of estrane-3,17-diol, **2**, is the major urinary metabolite excreted as a glucuronic acid conjugate[1]. Initial studies demonstrated that the hydroxyl function at the 17- position of this major metabolite had an α-configuration[1], a minor estrane-3,17-diol metabolite was also isolated with a 17β-OH configuration. Due to the unavailability of the isomers of estrane-3,17α-diol as reference steroids it was not possible to determine the stereochemistry at the 3- and 5- positions of the major metabolite by a direct comparison of gas chromatographic (GC) retention time data.

Gas chromatographic–mass spectrometric (GC–MS) studies[2] have shown differences in the relative intensities of some of the fragment ions in the mass spectra of

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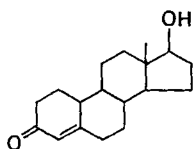
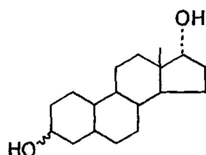


1
19–Nortestosterone



2
Estrane–3,17–diol

the trimethylsilyl (TMS) derivatives of the four isomeric 5α-androstanediols and considerable differences in the mass spectrum of 5β-androstane-3α,17β-diol bis-TMS when compared to the mass spectra of derivatives of the 5α-series. To investigate whether GC–MS could be used in determining the stereochemistry of the estrane-3,17α-diol metabolite a study of the mass spectra of the TMS derivatives of the eight isomers of androstane-3,17-diol and the four isomers of estrane-3,17β-diol has been undertaken and the results are reported in this paper.

It has been demonstrated that the retention behaviour of a steroid on GC analysis is a property of the steroid nucleus and additive contributions due to the substituent groups attached to the nucleus[3]. Thus changes brought about by a chemical reaction or a stereochemical change in structurally related compounds alters the retention time by a constant factor provided there is no interference from neighbouring groups[4].

Using the same series of twelve reference steroids, the additive contributions to the MU values[5] due to changes in the steroid nucleus and stereochemistry have been used to predict the MU values of the four isomers of estrane-3,17α-diol bis-TMS derivatives. The MU value of the TMS derivative of the estrane-3,17α-diol metabolite was compared with these predicted values and the stereochemistry at the 3- and 5-positions has been established.

EXPERIMENTAL

Solvents and chemicals

The even numbered hydrocarbons n-C_{16}–n-C_{32}, N,O-bis(trimethylsilyl) acetamide (BSA), trimethylchlorosilane (TMCS), 5α-androstane-3α,17β-diol, 5α-androstane-3β,17β-diol, 5β-androstane-3α,17β-diol, 5β-androstane-3β,17β-diol and undecane were obtained from Sigma (Poole, U.K.). 5α-Androstane-3α,17α-diol, 5α-androstane-3β,17α-diol, 5β-androstane-3α,17α-diol, 5β-androstane-3β,17α-diol and 5α-estrane-3β,17β-diol were obtained from the M.R.C. Steroid Reference collection. 5α-Estrane-3α,17β-diol, 5β-estrane-3α,17β-diol and 5β-estrane-3β,17β-diol were gifts from Organon (Oss, The Netherlands).

Isolation and purification of urinary metabolites

The administration of 19-nortestosterone to cross-bred gelded ponies, the collection of urine and the determination of urinary excretion have been reported previously[1].

For one animal, 70% of the administered dose was excreted in the first 24 h. The metabolites were isolated from this urine (900 ml) by lyophilisation, enzyme hydrolysis of the residue reconstituted in acetate buffer, pH 4.5 and solvent extraction. Purification by repeated chromatography on Kieselgel H[1] yielded a fraction which, following derivatisation and GC–MS analysis, was shown to contain two isomers of estrane-3,17-diol.

Derivatisation of steroids

The reference steroids (5–10 μg) were treated with BSA (50 μl) and TMCS (25 μl) and heated at 60°C for 2 h. The excess derivatisation reagents were removed in a stream of nitrogen at 40°C and the residue dissolved in undecane (100 μl) for analysis by GC and GC–MS. Aliquots of the purified fraction containing the estranediol metabolites were similarly derivatised.

Gas chromatography

GC was carried out using a Hewlett-Packard 5890A gas chromatograph with a HP3392A integrator. The steroid derivatives were analysed on three different columns, a 1701 (OV-1701), a BP5 (SE-S4) and a BP1 (SE-30) (25 m × 0.3 mm I.D.); the columns were purchased from S.G.E.

The analysis was carried out in the splitless injection mode with undecane as solvent. The initial temperature of 150°C was ramped to 300°C at 5°C/min; hydrogen was used as carrier gas (linear gas velocity approximately 40 cm/s).

The steroid derivatives were individually coinjected with the even numbered hydrocarbon mixture n-C_{16}–n-C_{32} in undecane. For each analysis a plot of retention time (min) *vs.* carbon number of the hydrocarbons was obtained and the methylene unit (MU) values for the steroids determined from linear regression analysis. To check the reproducibility of the injections replicate analysis ($n = 7$) of the bis-TMS derivative of 5β-androstane-3β,17β-diol with the hydrocarbon mixture were made.

Capillary column GC–MS

Mass spectra were obtained on the Hewlett-Packard 5970 mass selective detector interfaced to a HP 5890A gas chromatograph. A bonded polydimethylsiloxane fused-silica column (18 m × 0.25 mm I.D.) was used with helium as carrier gas. Splitless injections were made in undecane at 150°C, the oven temperature was ramped to 180°C at 10°C/min then to 280°C at 5°C/min; mass spectra were recorded over the range m/z 100–500.

RESULTS AND DISCUSSION

The relative intensities of the relevant ions in the mass spectra of the bis-TMS derivatives of the eight isomers of androstane-3,17-diol are shown in Table I. The mass spectra were similar in that they showed molecular ions ($M^{+\cdot}$, m/z 436) with fragment ions a m/z 421 $[M - 15]^{+}$; m/z 346 $[M - 90]^{+\cdot}$; m/z 331 $[M - (90 + 15)]^{+}$; m/z 256 $[M - (90 + 90)]^{+\cdot}$; m/z 241 $[M - (90 + 90 + 15]^{+}$ and m/z 129 (D-ring fragment).

TABLE I

MASS SPECTRAL DATA FOR THE TMS DERIVATIVES OF THE EIGHT ANDROSTANE-3,17-DIOL ISOMERS

Androstanediol isomer	*Relative intensities (%)*							
	Molecular ion $M^{+\cdot}$ *(m/z 436)*	*Fragment ions*						
		$[M-15]^+$ *(m/z 421)*	$[M-90]^{+\cdot}$ *(m/z 346)*	$[M-(90+15)]^+$ *(m/z 331)*	$[M-(90+90)]^{+\cdot}$ *(m/z 256)*	$[M-(90+90+15)]^+$ *(m/z 241)*	*m/z 215*	*m/z 129*
5α-A-3β,17β-diol	29	70	54	37	35	70	37	100
5β-A-3β,17β-diol	14	18	52	27	100	67	40	97
5α-A-3α,17β-diol	24	18	35	45	65	100	47	65
5β-A-3α,17β-diol	9	15	45	10	100	65	38	52
5α-A-3β,17α-diol	34	80	60	40	40	77	26	100
5β-A-3β,17α-diol		8	50	30	100	54	26	62
5α-A-3α,17α-diol	29	20	46	56	70	100	43	67
5β-A-3α,17α-diol	6	2	39	12	100	62	34	40

The inability of MS to unequivocally identify all stereoisomers of androstane-3,17-diol bis-TMS derivatives is illustrated in Table I. However some structural information could be obtained from the mass spectral data. In both the 17β-series and the 17α-series it is possible to differentiate between the 5β- and 5α-isomers on the basis of the ions at *m/z* 256 and *m/z* 241. In the 5β-isomers the ion at 256 is of greater intensity than the ion at 241 as previously reported by Vihko[2]; for the 5α-isomers the reverse is true. For the 5α-isomers it is possible to establish the stereochemistry at C_3 on the basis of the intensity of the molecular ion ($M^{+\cdot}$, *m/z* 436) and that of the fragment ion at *m/z* 421. The 5α,3β-isomers show a prominent fragment ion at *m/z* 421 of greater intensity than the molecular ion; for the 5α,3α-isomers this pattern is reversed.

For the 5α,3α/5α,3β-isomers differences, although less prominent, are also observed in the ratios of the ions at *m/z* 346 and *m/z* 331. It was not possible to distinguish between 5β,3α/5β,3β-isomers nor between the 17α/17β-isomers.

The structures of the reference steroids are shown in Fig. 1. The ability of MS to distinguish between steroid isomers at the 5-position has been reported previously[6]. The stereochemical change 5α- to 5β-results in the loss of planarity in the steroid nucleus producing a marked change in its shape (Fig. 2). Following loss of two moles of trimethylsilanol in the androstanediol series $[M-(90+90)]^{+\cdot}$ and a methyl radical it would appear that the planar ring system of the 5α isomers is better able to stablize the ion at *m/z* 241. A study of the fragmentation pathways of stereoisomeric androstane-3,17-diol bis-*tert.*-butyldimethylsilyl ethers by linked field scanning has demonstrated the value of a combination of conventional electron impact (EI) data and parent and daughter ion spectra in distinguishing between stereoisomers[7].

The mass spectra of the bis-TMS derivatives of the four isomers of estrane-3,17β-diol are shown in Fig. 3. The spectra show weak molecular ions ($M^{+\cdot}$, *m/z* 422)

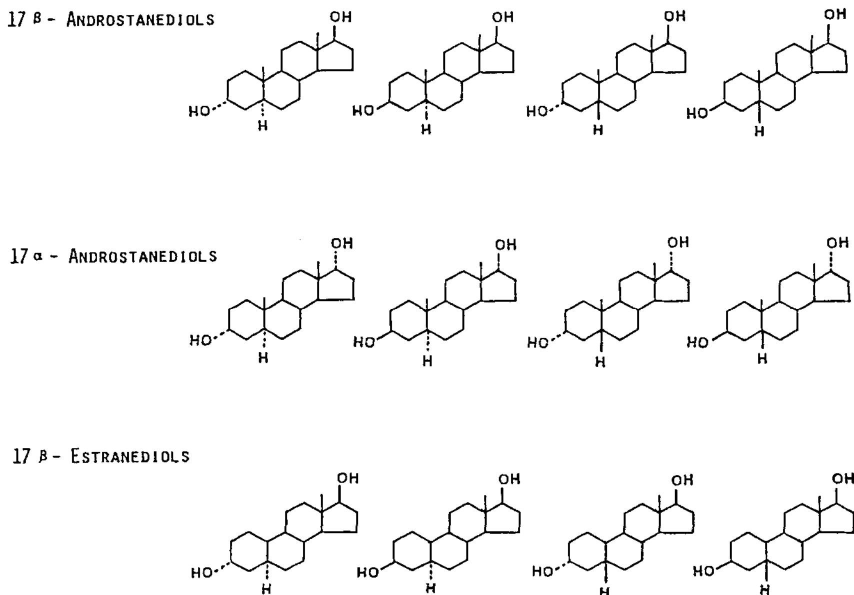


Fig. 1. Structures of reference steroids.

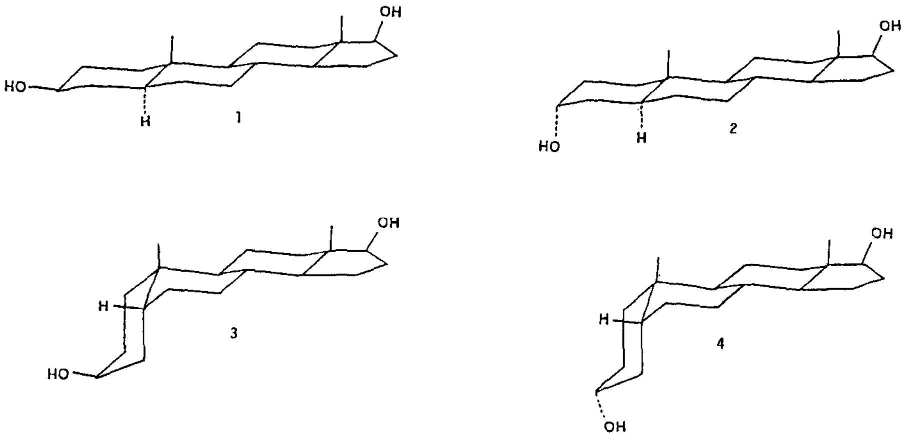


Fig. 2. Perspective views of 5α- and 5β-androstane-3,17β-diols. (1) 5α-Androstane-3β,17β-diol, (2) 5α-androstane-3α,17β-diol, (3) 5β-androstane-3β,17β-diol and (4) 5β-androstane-3α,17β-diol.

with fragment ions at m/z 407 $[M-15]^+$; m/z 332 $[M-90]^{+\cdot}$; m/z 242 $[M-(90+90)]^{+\cdot}$ and m/z 129. As the loss of a methyl radical from the fragment ions m/z 332 and 242 was of little significance, the characteristic differences observed between the mass spectra of the 5α/5β androstane-3,17-diol bis-TMS derivatives were not apparent in the spectra of the derivatised estrane-3,17β-diols and no conclusion could be drawn with regard to stereochemistry. A similar conclusion has been drawn for a series of

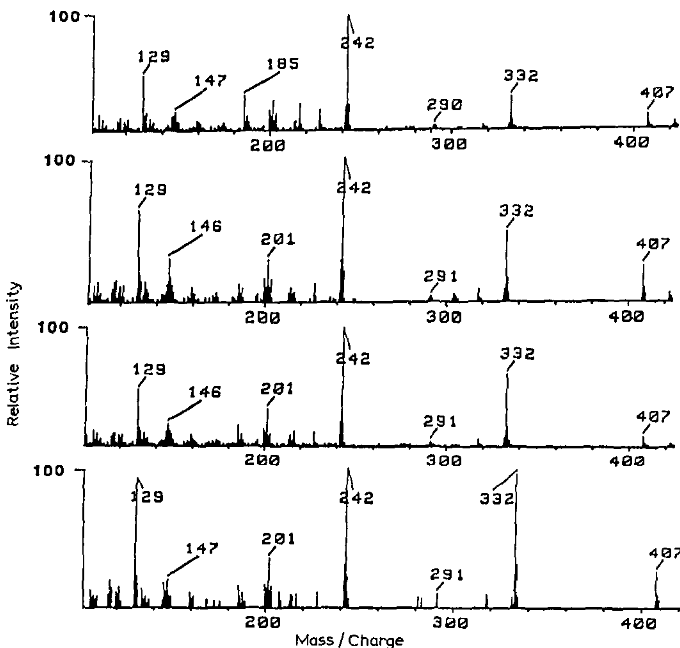


Fig. 3. Mass spectra of the bis-TMS derivatives of the four isomers of estrane-3,17β-diol. From top to bottom: 5β,3α,17β; 5α,3α,17β; 5β,3β,17β; 5α,3β,17β.

5α/5β androstanetriols where mass spectra of the TMS derivatives were dominated by rearrangement ions[8]. The mass spectrum (Fig. 4) of the bis-TMS derivative of the major estrane-3,17-diol metabolite was similar to those of the estrane-3,17β-diol isomers (Fig. 3) and stereochemical distinction on the basis of MS was impossible. On the basis of relative intensities of the ions, the spectrum was different to that reported previously[1], presumably due to the use of a different instrument. MS analysis of the TMS derivatives of androstane-3,17-diol and estrane-3,17β-diol isomers therefore failed to provide sufficient characteristic information to unequivocally determine the stereochemistry of the metabolite.

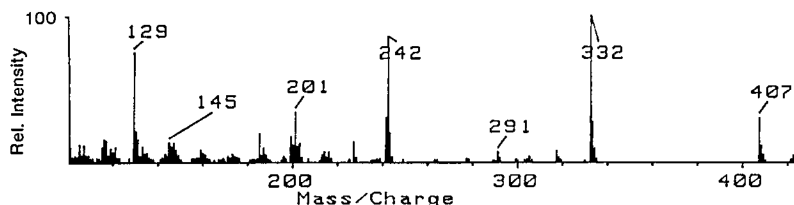


Fig. 4. Mass spectrum of the bis-TMS derivative of the most abundant isomer of estrane-3,17-diol isolated from horse urine following administration of 19-nortestosterone.

Although it is known that GC can separate stereoisomers, the determination of the stereochemistry of the metabolite on the basis of a direct comparison of retention time data was not possible due to the unavailability of the estrane-3,17α-diol isomers as reference steroids. An indirect approach was therefore adopted which depended upon the prediction of the MU values of the estrane-3,17α-diol bis-TMS derivatives and a comparison of these predicted values with the experimentally determined values for the metabolites. The TMS derivatives of the reference steroids were analysed on three different columns. As reported previously the 17β-isomers had longer retention times than the 17α-isomers[9].

To determine MU values a typical GC chromatogram, obtained for coinjection of the bis-TMS derivative of 5β-androstane-3β,17β-diol and the hydrocarbon mixture, is shown in Fig. 5. A plot of the retention time (min) *vs.* carbon number gave a linear relationship over the range n-C_{16}–n-C_{28} (correlation coefficient, 0.9997) (Fig. 6). The MU value 25.71 for 5β-androstane-3β,17β-diol bis-TMS was determined from linear regression analysis. Reanalysis of this mixture ($n = 6$) gave the following MU values for this steroid derivative, 25.70, 25.71, 25.71, 25.71, 25.71 and 25.71. The MU values, determined for the TMS derivatives of the eight isomers of androstane-3,17-diol and four isomers of estrane-3,17β-diol on the three different columns are shown in Table II. The MU values of the derivatised estrane-3,17-diol metabolites are shown in Table III.

MU values allow retention indices to be determined under temperature programmed conditions and can be regarded, for practical purposes, to be equivalent to the Kovats retention indices. Based upon the concept that the paper chromatographic mobility of a compound results from the additive contributions of its components[10], it has been shown that the logarithm of the retention time of a steroid is made up of the additive contributions of the substituents together with that of the nucleus[3,11,12]. For the TMS derivatives of the eight isomers of androstanediol and

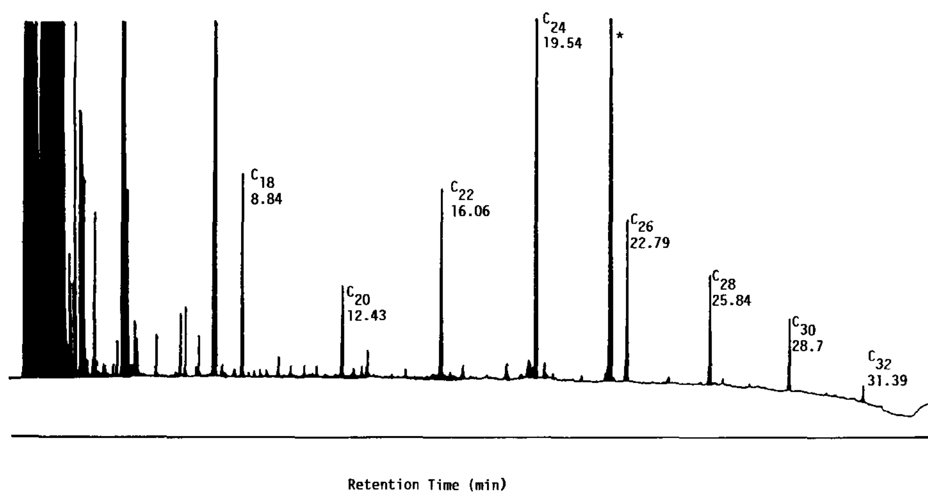


Fig. 5. Capillary column gas chromatogram for coinjection of the hydrocarbons n-C_{16}–n-C_{32} with the bis-TMS derivative of 5β-androstane-3β,17β-diol* (column BP5).

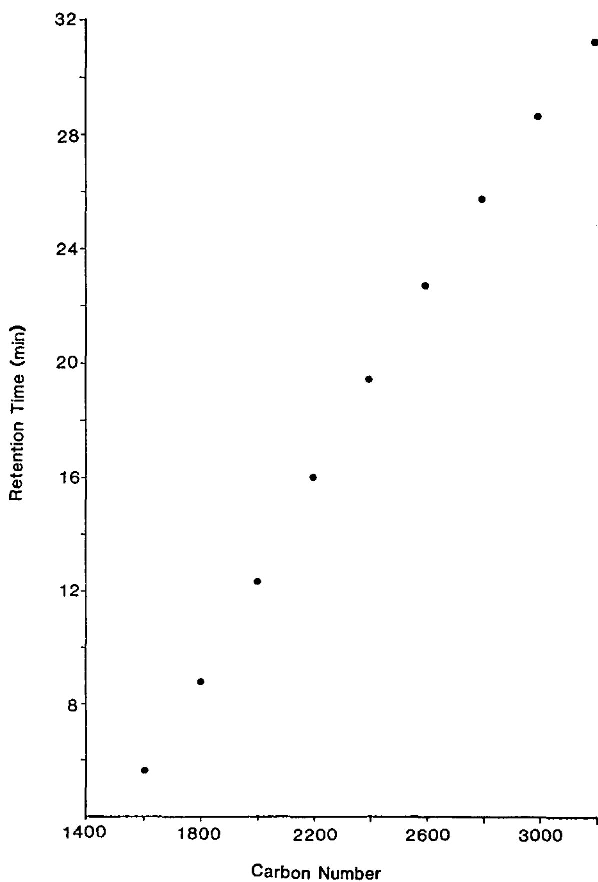


Fig. 6. Plot of retention time *vs.* carbon number for hydrocarbons n-C_{16}–n-C_{32} (column BP5; correlation coefficient, 0.9996 n-C_{16}–n-C_{28}; regression equation $y = 58.6x + 1269$).

TABLE II

MU VALUES FOR THE REFERENCE STEROIDS ON THREE COLUMNS

A = Androstanediol; E = estranediol.

Steroid	*MU values*		
	1701	*BP5*	*BP1*
5α-A-3α,17α	24.98	24.89	24.74
5α-A-3β,17α	26.29	26.12	25.97
5β-A-3α,17α	24.84	24.63	24.45
5β-A-3β,17α	24.92	24.86	24.69
5α-A-3α,17β	26.12	25.80	25.70
5α-A-3β,17β	26.80	26.64	26.49
5β-A-3α,17β	26.04	25.81	25.70
5β-A-3β,17β	25.74	25.71	25.60
5α-E-3α,17β	25.03	25.02	24.91
5α-E-3β,17β	25.87	25.73	25.58
5β-E-3α,17β	25.66	25.50	25.37
5β-E-3β,17β	25.47	25.44	25.32

TABLE III

MU VALUES FOR THE URINARY ESTRANE-3,17 DIOL METABOLITES OF 19-NORTESTOSTERONE IN THE HORSE

	1701	*BP5*	*BP1*
Major metabolite	25.38	25.11	25.13
Minor metabolite	25.86	25.84	25.56

four isomers of estrane-3,17β-diol this principle has been used to calculate the contributions to the MU values of the stereochemical change (17β-OH to 17α-OH) and also a change in the steroid nucleus (androstane to estrane).

For isomers of androstane-3,17β-diol and androstane-3,17α-diol having the same stereochemistry at the 3- and 5- positions, subtraction of the MU values gives the contribution to the retention behaviour of the stereochemical change from 17β-OH to 17α-OH (Table IV). Subtraction of these contributions from the MU values of the

TABLE IV

CONTRIBUTION TO THE MU VALUES FOR THE CONVERSION 17β-OH TO 17α-OH

A = Androstanediol.

	Column		
	1701	*BP5*	*BP1*
(5α-A-3α,17β) → (5α-A-3α,17α)	1.14	0.91	0.96
(5α-A-3β,17β) → (5α-A-3β,17α)	0.51	0.52	0.52
(5β-A-3α,17β) → (5β-A-3α,17α)	1.20	1.18	1.25
(5β-A-3β,17β) → (5β-A-3β,17α)	0.82	0.85	0.91

TABLE V

CALCULATED MU VALUES FOR THE FOUR ESTRANE-3,17α-DIOL ISOMERS

	Column		
	1701	*BP5*	*BP1*
5α-Estrane-3α,17α-diol	23.89	24.11	23.95
5α-Estrane-3β,17α-diol	25.36	25.21	25.06
5β-Estrane-3α,17α-diol	24.26	24.32	24.12
5β-Estrane-3β,17α-diol	24.65	24.59	24.41

TMS derivatives of the isomers of estrane-3,17β-diol having the corresponding stereochemistries at the 3- and 5- positions gives the predicted MU values for the isomers of estrane-3,17α-diol-bis-TMS derivatives (Table V).

The contribution of the C-10 methyl group can be obtained by subtracting the MU values of the TMS derivatives of the estrane-3,17β-diol isomers from those of the androstane-3,17β-diol isomers having the same stereochemistries at the 3- and 5-positions (Table VI). Subtraction of this contribution from the MU values of the TMS derivatives of isomers of androstane-3,17α-diol having the corresponding stereochemistries at the 3- and 5-positions again gave the predicted MU values shown in Table V. In predicting the MU values of the TMS derivatives of the estrane-3,17α-diol isomers the assumption is made that the contribution due to the change in stereochemistry at the 17-position is independent of the presence/absence of the methyl group at C-10 or, this is equivalent to stating the contribution due to the presence/absence of the methyl group at C-10 is independent of the stereochemistry at C-17. This assumption is thus dependent upon the non-interaction of the methyl group at C-10 with the hydroxy function at C-17; due to the remoteness of the two groups any such interaction is improbable. The non-interaction of the methyl group at C-10 with substituents at C-17 has been demonstrated for a series of 4-en-3-one steroids for which, irrespective of the substituent at C-17 the contribution to retention time data of this methyl group was constant[3].

The above assumption can only be applied when the steroid 3,17-diols have the same stereochemistry at the 3- and 5-positions. The fact that the contributions to MU

VALUES VI

CONTRIBUTION TO THE MU VALUES FOR THE C_{10} METHYL GROUP

A = Androstanediol; E = estranediol.

	Column		
	1701	*BP5*	*BP1*
(5α-A-3α,17β) → (5α-E-3α,17β)	1.09	0.78	0.79
(5α-A-3β,17β) → (5α-E-3β,17β)	0.93	0.91	0.91
(5β-A-3α,17β) → (5β-E-3α,17β)	0.38	0.31	0.33
(5β-A-3β,17β) → (5β-E-3β,17β)	0.27	0.27	0.28

values of the 17β-OH to 17α-OH conversion (Table IV) and the methyl group at C-10 (Table VI) are not constant for different isomers demonstrates their dependence upon the stereochemistry at these positions. The predicted MU values for the TMS derivatives of the four estrane-3,17α-diol isomers are shown in Table V. Comparison of the experimentally determined MU values on the three columns for the major estrane-3,17α-diol metabolite (Table III) with the predicted MU values (Table V) established the configurations at the 3- and 5-positions to be β and α, respectively. This configuration was later confirmed by custom synthesis of 5α-estrane-3β,17α-diol and comparison of retention time data with that of the metabolite. Comparison of the MU values of the TMS derivative of the minor metabolite with those of the reference steroids confirmed the configuration to be 5α,3β,17β.

CONCLUSIONS

The value of GC–MS in the identification of a variety of steroids, isolated from biological samples, and their derivatives has been well documented over the past two decades. On the basis of MS alone it is not normally possible to differentiate between steroid stereoisomers. However, stereoisomers of most steroids can be resolved by capillary GC and thus the combination of GC–MS can yield full structure elucidation provided the appropriate reference steroids are available for direct comparison of mass spectral and retention time data. In determining the stereochemistry of estrane-3,17α-diol alternative approaches had to be considered as it was not possible to obtain the four isomers of estrane-3,17α-diol as reference steroids.

The results demonstrate that MU values can be used for the elucidation of the stereochemistry of steroid isomers when the appropriate reference compounds are unavailable.

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