THE IDENTIFICATION OF 6α-HYDROXYTETRAHYDROCORTISOL (3α,6α,11β,17α,21-PENTAHYDROXY-5β-PREGNAN-20-ONE) IN URINE

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1. Introduction

Some unidentified highly polar steroids have been recognised as quantitatively important metabolites of cortisol in baboons [1] and newborn infants [2,3]. Using gas chromatography–mass spectrometry (GC–MS) we investigated urinary steroid excretion by baboons [4] and identified these polar steroids as 6-hydroxylated tetrahydrocorticosteroids [5] and tentatively suggested the configuration of the C-6 hydroxyl to be β-orientated. Nuclear magnetic resonance (NMR) spectroscopy and additional GC–MS data has now shown unequivocally that these steroids are 6α-hydroxylated.

This paper summarises conclusive evidence for the major steroid excreted in the urine of baboons having the structure 3α,6α,11β,17α,21-pentahydroxy-5β-pregnan-20-one (6α-hydroxytetrahydrocortisol).

2. Experimental

The polar steroids in urine from adult baboons (*Papio papio, Papio hamadryas* and *Papio anubis*) were isolated and characterised by GC and GC–MS as in [4,5]. The identification of a steroid was based upon the GC retention time relative to a homologous series of straight chain aliphatic hydrocarbons (C-20 to C-32) using a temperature-programmed operation (MU value), and the mass spectrum of the derivatised steroid.

2.1. *Nuclear magnetic resonance (NMR) spectroscopy*

NMR spectra were determined with a Varian HA 100 spectrometer at 100 MHz, relative to tetramethylsilane as the internal standard. The solvent was perdeuteriopyridine (C_5D_5N) or deuteriochloroform ($CDCl_3$). With small or poorly soluble samples, time averaging was employed to improve specta. Signals due to OH protons were eliminated where necessary by exchange with D_2O.

2.2. *Preparation of the authentic steroid, 6β-hydroxytetrahydrocortisol*

The authentic steroid was prepared from 6β-hydroxycortisol by chemical reduction of the 4-ene bond followed by enzymatic reduction of the 3-oxo group using the 3α-hydroxysteroid dehydrogenase enzyme present in rat liver microsomes (K.D.R.S. et al., to be published).

3. Results

3.1. *GC–MS*

As the methyloxime-trimethylsilyl (MO-TMS) ether derivative the retention time (t_R) of the major polar steroid excreted by the baboon was 30.84 MU. The general structure indicated from the mass spectrum was a pregnanepentolone, with a dihydroxyacetone side-chain [5]. Oxidation of the side-chain with sodium bismuthate gave the 17-oxosteroid. The retention time of the MO-TMS ether derivative was 28.37 MU, and the mass spectrum of this compound was identical to that obtained when the original compound is first reduced with sodium borohydride and then oxidised with periodic acid [5]. The mass spectrum of the TMS ether derivative [5] provided evidence for two

of the hydroxyl groups being in positions C-3 and C-11, but the position of the additional hydroxyl group could not be identified, although there was strong evidence for it being in either the C-6 or C-7 position.

Confirmation of a 6-hydroxy steroid was possible after oxidation with chromic acid, which gave rise to an androstanetetrone and analysis by GC–MS of the *O*-methyloxime (MO) derivative. The GC behaviour of the product produced a single broad peak (t_R 28.47 MU) due to partial separation of the *syn*- and *anti*- forms of the MO derivative of the C-3 carbonyl.

The molecular ion (M^+) in the mass spectrum (fig.1) of this compound was at mass/charge (m/z) 403 which is consistent for a MO derivative of an androstanetetrone structure possessing an underivatised carbonyl group at the C-11 position. The base peak in the spectrum was at m/z 138 which is the principal ion in all spectra analysed in the 5β-series of androstanes, pregnanes and cholestanes with MO groups in positions C-3 and C-6 [6]. In the mass spectra of the MO derivative of the 5β-series of 3,6-dioxygenated steroids, the order of relative intensities of the ions has been shown to be (M-31)>M^+>(M-46), while for the corresponding 5α-series this sequence is M^+>(M-46)>(M-31). The mass spectra show this product to be a 3,6-dioxygenated steroid with the configuration of the C-5 hydrogen being 5β.

To exclude a 3,7-dioxygenated structure for this compound, reference steroids with this configuration were analysed by GC–MS. The mass spectra obtained for the MO derivatives of these, differed completely from those of 3,6-dioxygenated steroids (fig.1) as follows:

(i) The ion at m/z 138, typical for the MO derivative of 3,6-dioxygenated steroids, is absent in the spectrum of 3,7-dioxygenated steroids.
(ii) The relative intensity of ions in 3,7-dioxygenated steroids is in the order (M-46)>(M-31)>M^+.

The mass spectrum of the MO derivative of a typical 3,7-dioxygenated steroid (5β-androstane-3,7,17-trione) is shown in fig.1 for comparison. Our findings indicate the specificity of the fragmentation patterns observed [6] for 3,6-dioxygenated steroids.

The mass spectrometric data obtained from this product, together with previous data, confirm the position of the carbonyl groups in the product from

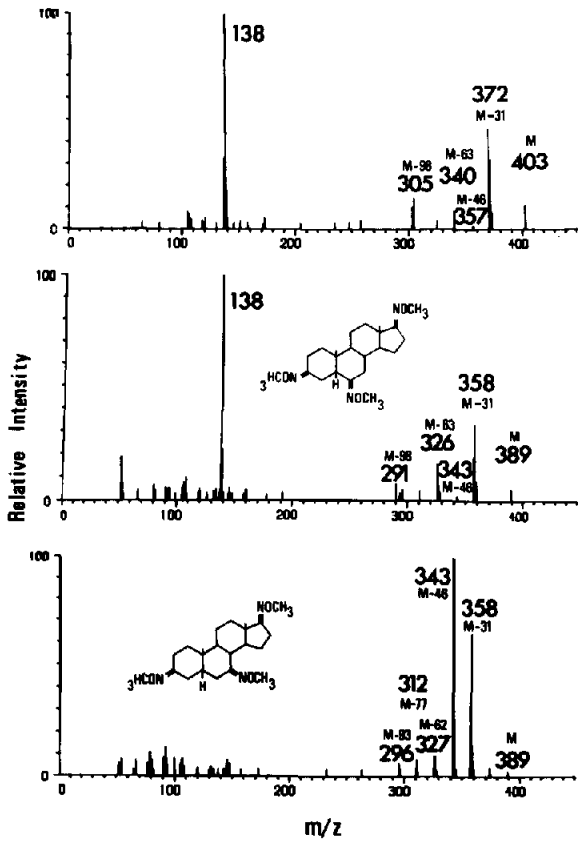

Fig.1. The mass spectrum of the methyloxime derivative of the product formed following total oxidation of the baboon steroid with chromic acid (upper panel). The mass spectrum for the methyloxime derivative of the authentic steroids 5β-androstane-3,6,17-trione (middle panel) and 5β-androstane-3,7,17-trione (lower panel) are shown for comparison of the fragmentation patterns observed in typical 3,6-dioxygenated and 3,7-dioxygenated steroid structures.

total oxidation to be in C-3, C-6, C-11 and C-17, and these data are consistent with the original compound having the structure 3α,6α,11β,17α,21-pentahydroxy-5β-pregnan-20-one.

After our report that this steroid was most probably 6β-hydroxytetrahydrocortisol [5], the authentic steroid was prepared. The mass spectrum of the MO-TMS ether derivative of the authentic steroid 6β-hydroxy-tetrahydrocortisol was indistinguishable from that of the urinary steroid; however, its GC retention time (t_R 30.62 MU) was slightly shorter. These data provided strong evidence for the urinary steroid being

6α-hydroxytetrahydrocortisol since all 6β-hydroxy steroids have shorter retention times than the analogous 6α-hydroxy isomers. Confirmation of this stereochemistry was made using NMR spectroscopy.

3.2. *NMR spectra*

The ^{1}H NMR spectrum of the baboon steroid, 6α-hydroxytetrahydrocortisol (in C_5D_5N), exhibited the expected quartet for the two protons at C-21, an unresolved multiplet for 11α-H, and a broad multiplet for 3β-H. These signals were close to the corresponding signals from tetrahydrocortisol itself (table 1). An additional rather broad band ($W_{1/2} \sim 14$–15 Hz; $\delta = 4.45$ ppm) was assigned to 6β-H, vicinal to the 6α-hydroxy group, since it was absent from the spectrum of tetrahydrocortisol. The spectrum of 3α,6α-dihydroxy-5β-pregnan-20-one, run for comparison, showed the 6β-H signal as a similar poorly resolved multiplet ($W_{1/2} \sim 16$–17 Hz; $\delta = 4.32$ ppm). The width of the 6β-H signal is compatible with a proton of axial conformation, spin–spin coupled with geminal protons and particularly with the axial 7α-H [7]. By contrast, a reference spectrum of 5β-cholestane-3β,6β-diol showed the 6α-H (equatorial)-signal as a much narrower band ($W_{1/2} \sim 7$–8 Hz) as expected [7,8].

To confirm the position of the extra hydroxy function, and to eliminate any possibility that it might be located at the 7α-position, comparison was made with the spectrum of 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid (cholic acid) in C_5D_5N. The 7β-H (equatorial) signal from cholic acid was the expected [8] narrow unresolved multiplet ($W_{1/2} \sim 8$ Hz; $\delta = 4.07$ ppm), distinguished from the other methine protons, at C-3 and C-12, by reference to the spectrum of deoxycholic acid, which lacks the 7α-hydroxy function.

Further evidence corroborating the 6α-hydroxy- rather than the 6β-hydroxytetrahydrocortisol structure for the baboon metabolite came from the chemical shifts of signals for the protons of the angular methyl groups (C-18 and C-19). The ^{1}H chemical shift at C-19 is a particularly sensitive indicator of the configuration of substituent groups known to be at C-6. 6β-Substituents, which are in a 1,3-diaxial relationship to C-19, cause significant downfield shifts of C-19 proton signals, while 6α-substituents have almost no effect at C-19 [9]. Chemical shifts of methyl protons, calculated for 6α-hydroxytetrahydrocortisol and 6β-hydroxytetrahydrocortisol, show excellent agreement with experimental values obtained from the baboon product and the synthetic 6β-isomer, respectively (table 2). Group increments appropriate for $CDCl_3$ as solvent were taken from the data [9–11]. Corrections for the use of C_5D_5N are from our own data (D.N.K. and R.E.M., to be published) with a series of reference compounds of the 5β-pregnane series.

4. Discussion

The work emphasises the need for more than one physical technique for accurate identification, especially where the stereochemical configuration may be in doubt and the relevant authentic steroids are unavailable. In this instance NMR spectroscopy provided a complementary technique to GC–MS in the definitive identification of 3α,6α,11β,17α,21-pentahydroxy-5β-pregnan-20-one in baboon urine.

Table 1
^{1}H NMR data for tetrahydrocortisol and its 6α-hydroxy derivative

Compound	21-CH_2 (q)	11α-H(m)	6-H(m)	3β-H(m)	19-CH_3 (s)	18-CH_3 (s)
Tetrahydrocortisol	5.32 5.14 5.02 4.84	4.6 (~ 10–12)	–	3.90 (~ 28)	1.49	1.29
6α-Hydroxytetrahydrocortisol (Baboon steroid)	5.39 5.19 4.91 4.72	4.67 (~ 10)	4.45 (14–15)	3.90 (18–20)	1.54	1.30

100 MHz; solvent, C_5D_5N
Chemical shifts, δ (ppm) ($W_{1/2}$ in parentheses), from Me_4Si as internal standard

Table 2
^{1}H NMR: Calculation of chemical shifts for methyl protons in tetrahydrocortisol and its 6-hydroxy derivatives

	δ (ppm)	
	19-H	18-H
Shift increments for $CDCl_3$ solutions[a]		
5β-Androstane	0.925	0.692
3α-OH	0.008	0.008
11β-OH	0.258	0.242
17α-OH, β-$CO.CH_2OH$	−0.018	−0.070
Calculated for tetrahydrocortisol ($CDCl_3$)	1.173	0.872
Additional increments for C_5D_5N solution		
3α-OH[b]	0.00	0.00
11β-OH[b]	0.27	0.25
17α-OH, β-$CO.CH_2OH$[c]	0.025	0.12
Calculated for tetrahydrocortisol (C_5D_5N)	1.468	1.242
Found for authentic tetrahydrocortisol (C_5D_5N)	1.49	1.29
6-Hydroxy derivatives		
6α-OH increment ($CDCl_3$)[a]	−0.008	0.008
Correction for C_5D_5N[b]	0.02	0.00
Calculated for 6α-hydroxytetrahydrocortisol (C_5D_5N)	1.48	1.25
Found for baboon steroid (C_5D_5N)	1.54	1.30
6β-OH increment ($CDCl_3$)[a]	0.19	0.04
Correction for C_5D_5N[b]	0.03	0.00
Calculated for 6β-hydroxytetrahydrocortisol (C_5D_5N)	1.958	1.282
Found for authentic 6β-hydroxytetrahydrocortisol (C_5D_5N)	1.98	1.32

[a] [9]
[b] [14]
[c] D.N.K. and R.E.M., to be published

This steroid has also been identified in significant amounts in the urine of pregnant women [12] and other 6-hydroxylated polar corticosteroids have been shown to be important metabolites of cortisol in newborn infants [13]. The significance of these steroids in pregnancy is presently under investigation.

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