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THE EXCRETION OF STEROIDS BY THE ADULT MARMOSET MONKEY (*CALLITHRIX JACCHUS*)

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SUMMARY

The qualitative and quantitative aspects of steroid excretion of five adult marmoset monkeys (3 male, 2 female) has been studied using the technique of combined gas chromatography–mass spectrometry. The major metabolites of cortisol present in urine have an unchanged 3-oxo-4-ene group, for example, cortisone, 20-dihydrocortisone and 20α (and β)-dihydrocortisol. Compared to the situation in man, tetrahydrocortisone and tetrahydrocortisol were found to be relatively minor metabolites. Androsterone, 11-oxo-androsterone and 11β-hydroxyandrosterone were the major C-19 steroids identified and compounds with a 3α-hydroxy-5β group were not detected. The major pregnancy urinary steroids 6β-hydroxypregnanolone and 16α-hydroxypregnanolone were also important progesterone metabolites in both male and non-pregnant female animals.

INTRODUCTION

The marmoset (*Callithrix jacchus*) is a species of New World monkey of the family Callitricadae [1]. Because of their small size (average weight about 400 g) these animals are conveniently kept in captivity and are suitable primates for use in biochemical investigations.

In a recent publication by the author describing the excretion of steroids by pregnant animals of this species [2], relatively high levels of progesterone metabolites (principally 3α,6β-dihydroxy-5α-pregnan-20-one and oestradiol) were reported. No other reports of the steroid excretion of this species have been found in the literature and before the marmoset monkey may be used as an animal model for pharmacological or metabolic studies relating to man, it is necessary to know the normal metabolism of cortisol and other steroids. This communication describes the identification of metabolites of cortisol and other C-21 and C-19 steroids in urine from adult male and female animals.

EXPERIMENTAL

Materials

Gas chromatograph–mass spectrometer. Varian-Mat 731, Varian GmbH, Bremen, Germany; Gas chromatograph, Model 409, Packard–Becker, Delft, Netherlands; Sephadex LH-20, Pharmacia AB, Uppsala, Sweden; Lipidex 5000, Packard–Becker, Delft, Netherlands; Helix Pomatia, L'Industrie Biologique Francaise, Gennevilliers, Seine, France; Hexamethyldisilazane (HMDS), Trimethylchlorosilane (TMCS), Applied Science Laboratories, State College, PA, USA; Trimethylsilylimidazole (TSIM), (Pierce Chemicals), Phase Separations, Queensferry, Great Britain, Methoxyamine hydrochloride, Eastman Organic Chemicals, Rochester, New York, U.S.A.; Pyridine (Analar), BDH, Poole, England (distilled and stored over sodium hydroxide); Cyclohexane (Analar), BDH, Poole, England (charcoal washed and double distilled); Reference steroids, obtained from Medical Research Council, Steroid Reference Collection, Westfield College, England.

Methodology

During the period of the urine collection the animals were housed in metabolism cages. Three male and 2 female animals were studied, 24 h urine samples being obtained from each animal. It was not known at what period of the reproductive cycle the urine samples were collected from the female animals.

The volume of urine excreted by the animals was between 10 and 25 ml. This volume was diluted to 25 ml with water and 2·5 ml 5 M acetate buffer was added. 0·5 ml of 10% barium chloride was added, followed by 1 ml of *Helix pomatia* digestive juice. The hydrolysis was carried out at 40°C for 48 h and the freed steroids were extracted on columns of Amberlite XAD-2 resin (15 g wet wt.). A group separation of the steroids was carried out by Sephadex LH-20 chromatography according to the method of Setchell and Shackleton[3]. Six g columns of Sephadex LH-20 were prepared using the solvent system cyclohexane–ethanol, 4:1 v/v. Following addition of the sample and the eluting solvent, 2 eluant fractions were collected: Fraction 1 (12 to 55 ml) and Fraction 2 (55 to 160 ml).

These fractions were dried under vacuum and transferred to vials with 2 ml of ethanol. To a 250 μl portion of the ethanol solution was added 10 μg of the internal standards cholesteryl butyrate and 5α-androstane-3α,17α,-diol. The sample was then dried

and dissolved in 100 μl of methoxyamine hydrochloride in pyridine (1%) and left at 60° for 1 h. For the silylation of Fraction 1 100 μl of HMDS–TMCS, 30:1 v/v was used, the silylation being allowed to proceed overnight at room temperature. The derivative was dried under nitrogen and transferred to a vial with 1 ml cyclohexane. For the silylation of the oxime derivative of Fraction 2, TSIM (100 μl) was used, the silylation taking place at 100° for 2 h. The derivative was purified on small Lipidex 5000 columns (250 μg) using the solvent system cyclohexane–HMDS–Pyridine, 98:1:1 by vol. This is an adaption of the method of Axelson *et al.* [4].

The derivatives were analysed by gas chromatography and GC–MS. Semi-quantitation of the steroids was obtained by relating the peak heights to the height of a line joining the tops of the internal standards. Although no internal standard was added to the urine to monitor recovery of steroids through the procedure it has been found previously that the mean recoveries of free dehydroepiandrosterone and tetrahydrocortisone were 94 and 82% respectively. Other steroids are likely to give similar values.

RESULTS

For a steroid to be considered identified it had to have a mass spectrum and retention time value (MU) identical to the appropriate reference compound. Most of the relevant mass spectra have been published in other communications and discussion will be limited to those of most interest. Gas chromatograms illustrating the separation of steroids in Fractions 1 and 2 are illustrated in Fig. 1. The results of the semi-quantitative estimation are given in Table 1, together with the retention time values for individual steroids.

Excretion of C-19 steroids

Androsterone, 11-oxo-androsterone, 11β-hydroxyandrosterone and an androstane-3,16,17-triol were the major C-19 steroids excreted by adult marmosets. There was no evidence for the excretion of aetiocholanolone or its derivatives.

In addition, a compound was found which gives mass spectra indicative of a hydroxyandrosterone. The base peak in the mass-spectrum of the oxime-silyl ether was formed by loss of 103 mass units from the parent ion (*m/e* 479) suggesting the presence of a primary trimethylsilyl group. This primary hydroxyl group cannot be at position 18 since the compound had a different spectrum from that given by reference 18-hydroxyandrosterone. It seems probable that the compound was 19-hydroxyandrosterone but identification must await the availability of the appropriate reference compound.

Excretion of oestrogens

Oestrone, oestradiol and oestriol were all detected in urine from the female animals, oestradiol being the major urinary oestrogen.

Excretion of cortisol metabolites

Cortisol. The mass spectrum of the oxime-silyl ether of cortisol is illustrated in Fig. 2 (top). The parent ion is at *m/e* 636 and base peak at *m/e* 605 (M-31). Other important peaks in the mass spectrum are at *m/e* 103, 246, 425 and 515.

Cortisone. In the mass spectrum of the oxime-silyl ether of urinary cortisone (Fig. 2) the parent ion is at *m/e* 562 and base peak at *m/e* 531, other important peaks are at *m/e* 459 (M-103) and *m/e* 441 [M-(31 + 90)].

20-Dihydrocortisone. Also in Fig. 2 is the mass spectrum of urinary 20-dihydrocortisone. The parent ion is at *m/e* 607 and base peak at *m/e* 402, a fragment (M-205) formed by loss of the side chain. The loss of 103 mass units (*m/e* 504) is due to the removal of the primary trimethylsilyl group at C-21. The urinary steroid had a slightly shorter retention time than reference 20β-dihydrocortisone so it may be assumed that the urinary steroid was 20α-dihydrocortisone.

A metabolite of 20-dihydrocortisone reduced at position 5 was also detected but this compound was excreted in low amounts. The mass spectrum was similar to that of 20-dihydrocortisone, the major ions being two mass units higher.

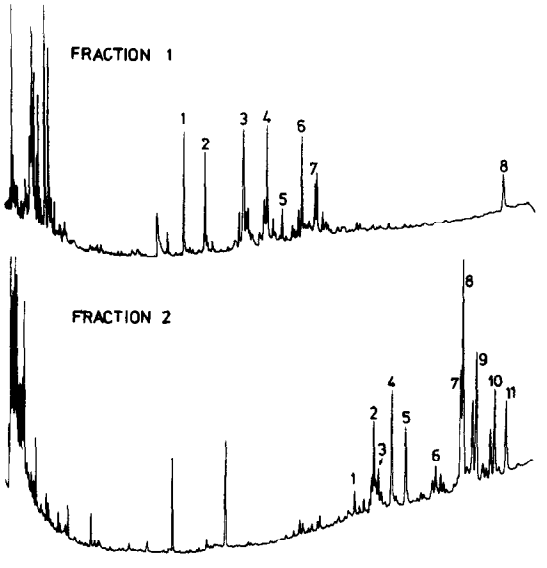

Fig. 1. *Upper chromatogram.* Gas chromatogram separation of the less polar C-19 and C-21 steroids. The major steroids are numbered—**1**, 5α-androstane-3α,17α-diol (internal standard); **2**, androsterone; **3**, 11-oxo-androsterone; **4**, 11β-hydroxyandrosterone; **5**, androstane-3,16,17-triol; **6**, 3α,6β-dihydroxy-5α-pregnan-20-one; **7**, unidentified 6-hydroxypregnanolone and 3α,16α-dihydroxy-5α-pregnan-20-one; **8**, cholesteryl butyrate (internal standard). *Lower chromatogram.* Gas chromatographic separation of the oxime-silyl ether derivatives of the cortisol metabolites excreted by an adult marmoset. The major steroids are numbered—**1**, tetrahydrocortisone; **2**, tetrahydrocortisol; **3**, 5-epitetrahydrocortisol; **4**, cortolone-20β; **5**, unidentified cortolone; **6**, cortisone; **7**, cortolone; **8**, 20-dihydrocortisone; **9**, 20β-dihydrocortisol (two peaks); **10**, 20α-dihydrocortisol (two peaks); **11**, cholesteryl butyrate (internal standard). The 2 early peaks are formed by impurities present in the TSIM reagent.

Table 1. Quantitation of the major neutral steroids excreted by adult marmosets

	Methylene unit value	Steroid excretion μg/24 h				
		M_1	M_2	M_3	F_1	F_2
Androsterone	25·07	18	26	42	13	41
11-oxo-androsterone	26·21	22	17	28	76	20
11β-Hydroxyandrosterone	26·96	25	15	22	40	37
19-Hydroxyandrosterone*	26·80	12	8	6	35	16
Androstane-3,16,17-triol	27·43	7	6	6	<3	<3
6β-Hydroxypregnanolone	28·07	18	24	23	27	77
16α-Hydroxypregnanolone	28·50	11	11	16	9	12
Cortisone	32·00	5	18	15	10	20
Cortisol	32·61	26	53	5	31	90
Tetrahydrocortisone	29·54	5	8	5	5	10
Tetrahydrocortisol	30·11	16	28	26	26	56
5-*epi*-Tetrahydrocortisol	30·15	12	30	25	26	40
Cortolone-20α + cortol-20α†	30·25	8	15	10	7	7
Cortolone-20β + cortol-20β†	30·67	49	84	40	31	56
Unidentified cortolone	31·10	31	55	43	35	58
17α,20α,21-Trihydroxy-4-pregnene-3,11-dione	32·75	35	86	80	48	184
11β,17α,20α,21-Tetrahydroxy-4-pregnene-3-one	32·93, 33·00‡	89	114	153	114	172
11β,17α,20β,21-Tetrahydroxy-4-pregnene-3-one	33·47, 33·60‡	33	89	98	59	140
Total excretion of identified cortisol metabolites		309	580	500	392	833

* This steroid was only tentatively identified.

† These steroids could not be separated on the column used.

‡ Two peaks were given for this compound.

20α and 20β-Dihydrocortisol. Both these steroids were identified in the urine extracts. Unfortunately the *syn-* and *anti-* forms of the oxime derivatives were separable by gas chromatography on open-tubular columns resulting in 2 peaks being given for each compound (Fig. 1). The first doublet corresponded in retention time to 20β-dihydrocortisol and the second to 20α-dihydrocortisol.

The mass spectrum of 20α-dihydrocortisol is illustrated in Fig. 2. The parent ion is at *m/e* 681 and base peak at *m/e* 296 [M-(205 + 90 + 90)]. Other important peaks are seen at *m/e* 578 (M-103), *m/e* 488 [M-(90 + 103)], *m/e* 476 (M-205), *m/e* [M-(205 + 90)] and *m/e* 243. The mass spectrum of 20β-dihydrocortisol is similar but the base peak is at *m/e* 243 rather than *m/e* 296.

"Tetrahydro-" metabolites of cortisol. Three steroids corresponding in retention times and mass spectra to tetrahydrocortisone, tetrahydrocortisol and 5-*epi*-tetrahydrocortisol were identified.

Cortolones (Steroids with the general structure 5-ξ-pregnane-3ξ,17α,20ξ,21-tetrol-11-one). Three cortolones were detected:

(a) A peak with retention time 30·25 MU corresponds to cortolone-20α or its 5α-epimer since these steroids are not resolved on the column used.

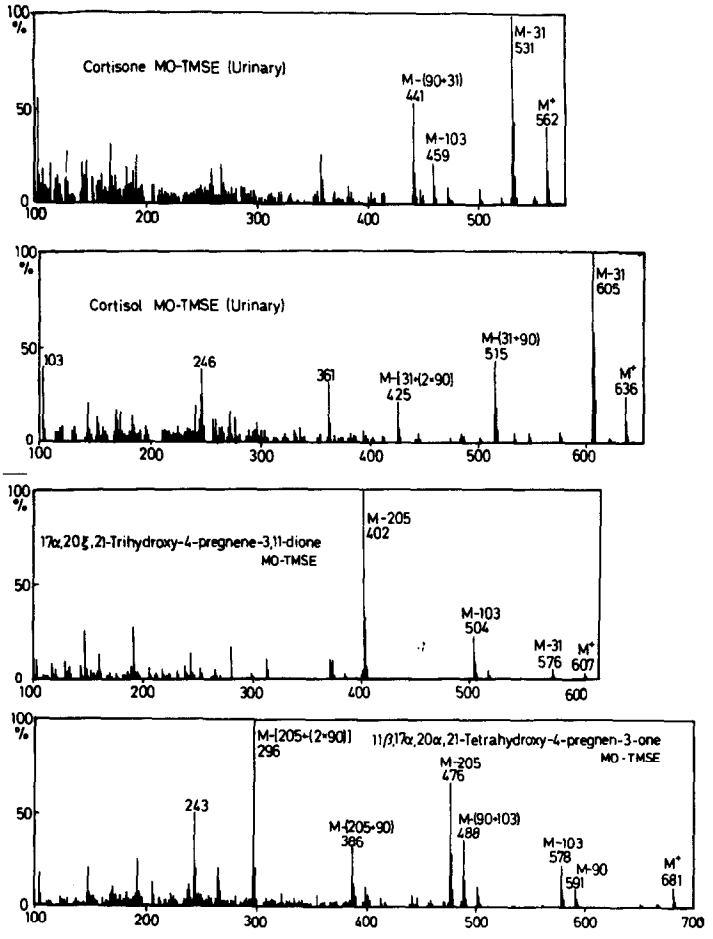

Fig. 2. Mass spectra of the oxime–silyl ether derivatives of the major cortisol metabolites excreted by adult marmosets.

(b) A peak with retention time of 30·67 MU corresponds to cortolone-20β or its 5α-epimer.

(c) A peak with the long retention time of 31·10 MU suggests a compound with a 3β-hydroxyl group. Further structural studies were not carried out.

The mass spectra of each of the cortolones were very similar, the major peaks in the spectrum of cortolone-20β being: *m/e* 654, M^+ (6%); *m/e* 551, M-103 (40%); *m/e* 449, M-205 (100%); *m/e* 359 (38%); *m/e* 269 (18%) and *m/e* 243 (40%).

Cortols (*Steroids with general structure 5ξ-pregnane-3ξ,11β,17α,20ξ,21-pentol*): Two cortols were detected with retention times corresponding to cortol-20α and cortol-20β. However, the 5α-epimers cannot be excluded, due to similarity of the retention times of 5α and 5β epimers.

The principal fragment ions in the mass spectrum of cortol-20α trimethylsilyl ether were: *m/e* 728, M^+ (18%); *m/e* 638, M-90 (17%); *m/e* 625, M-103 (25%); *m/e* 548 (13%); *m/e* 535 (55%); *m/e* 523, M-205 (70%); *m/e* 445 (40%); *m/e* 433 (30%); *m/e* 343 (72%); *m/e* 253 (73%) and *m/e* 243 (100%).

Other C-21 steroids. The major steroids found in the urine of pregnant marmosets (3α,6β-dihydroxy-5α-pregnan-20-one and 3α,16α-dihydroxy-5α-pregnan-20-one) were also detected in urine from both male and non-pregnant female animals. In addition a pregnane-3,17,20-triol a pregnane-3,16-20 triol and metabolites of corticosterone were also detected but their identification was not completed.

DISCUSSION

In contrast to man the marmoset excretes C-19 steroids principally with the 3α-hydroxy-5α A-ring configuration since aetiocholanolone and its 11-oxygenated metabolites were not detected. This is also true for the major progesterone metabolites 3α,6β-dihydroxy-5α-pregnan-20-one and 3α,16α-dihydroxy-5α-pregnan-20-one.

The metabolism of cortisol by the marmoset is also different from that of man, relatively more steroid being excreted with a hydroxy group at position 20 yet with unchanged 3-oxo-4-ene configuration. The excretion of the major human metabolites tetrahydrocortisone and tetrahydrocortisol was relatively low and no evidence was obtained for the presence of 6β-hydroxycortisol, although this is an important cortisol metabolite in *Cebus albifrons*, another species of New World monkey [5]. Unlike the situation in the macaque monkey, relatively little cortisol is metabolised to 11-oxygenated C-19 steroids [6]. Even if it is assumed that all the 11-oxygenated 17-oxosteroid originates as cortisol, this would still only account for about 12% of the total C-21 steroid metabolites.

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REFERENCES

1. Napier J. R. and Napier P. H.: *A Handbook of Living Primates*. Academic Press, New York (1972).
2. Shackleton C. H. L.: *J. steroid Biochem.* **5** (1974) 597–600.
3. Setchell K. D. R. and Shackleton C. H. L.: *Clin. chim. Acta* **47** (1973) 381–388.
4. Axelson M. and Sjövall J.: *J. steroid Biochem.* **5** (1974) 733–738.
5. Birchall K., O'Day W. G., Fajer A. B. and Burstein S.: *Gen. Comp. Endocr.* 7 (1966) 352–362.
6. Setchell K. D. R. and Shackleton C. H. L.: *Acta endocr., Copenh.* **78** (1975) 91–109.