Identification of the fecal metabolites of 17α-methyltestosterone in the dog

ERWIN H. MOSBACH, SARAH SHEFER, and LIESE L. ABELL

The Public Health Research Institute of the City of New York, Inc., and The Bureau of Laboratories, New York City Department of Health; and Columbia University Research Service, Goldwater Memorial Hospital, and the Departments of Biochemistry and Medicine, Columbia University College of Physicians and Surgeons, New York 10027

ABSTRACT 17α-Methyltestosterone-4-^{14}C was fed to two dogs in an experiment to determine tissue localization and metabolic disposition of this hypocholesterolemic steroid. No accumulation of the drug was found in any tissue, although a small amount of radioactivity was detected in the liver and the ileal mucosa of one animal. Most of the administered radioactivity was excreted in urine and feces.

The urinary metabolites consisted largely of highly polar compounds which appeared resistant to glucuronidase treatment or solvolysis procedures. Analysis of the fecal metabolites showed the presence of unchanged methyltestosterone, of four isomeric methylandrostanediols, and of labeled unidentified polar compounds. Of the four identified methylandrostanediols, the predominating fecal diols were 17α-methyl-5α-androstane-3β,17β-diol (45–62%) and 17α-methyl-5β-androstane-3α,17β-diol (12–28%); 17α-methyl-5α-androstane-3α,-17β-diol and the 5β:3β isomer were found in very small amounts only.

KEY WORDS 17α-methyltestosterone · metabolism · dog · excretion · 17α-methylandrostanediols · gas–liquid · column · chromatography

THE SYNTHETIC ANDROGEN 17α-methyltestosterone has been found to lower serum total cholesterol concentrations in human beings (1, 2), rabbits (3), dogs (4, 5), chickens (6, 7), and rats (8). Little is known about the mode of action of this drug, and experiments concerning its biological disposition in laboratory animals have not been reported. Segaloff, Gabbard, Carriere, and Rongone (9) administered 17α-methyltestosterone-4-^{14}C to a human subject and found $^2/_3$ of the recovered radioactivity in the stools and $^1/_3$ in the urine. They isolated 17α-methyl-5α-androstane-3α,17β-diol and its 5β:3α isomer from the nonconjugated and β-glucuronidase-treated fractions of the urine. Unchanged methyltestosterone, 17α-methyl-5α-androstane-3β,-17β-diol, and 17α-methyl-5β-androstane-3α,17β-diol were recovered from the feces. In both urine and stools highly polar labeled material was found which was not identified.

In the present study 17α-methyltestosterone-4-^{14}C was fed to two dogs to obtain information on its possible localization in specific tissues and on its metabolic fate. Four isomeric methylandrostanediols were isolated from the feces and identified. The predominating diol was 17α-methyl-5α-androstane-3β,17β-diol. The 5β-androstane-3α,17β-diol made up 12–28% of the identified material, and the 5α-androstane-3α,17β-diol and the 5β-androstane-3β,17β-diol were recovered in small amounts. The predominating diol was tested in dogs for its hypocholesterolemic activity, and the results are reported in the second paper of this series.

MATERIALS AND METHODS

The two adult mongrel dogs used for the tracer studies (one male, weight 6.8 kg and one female, weight 8.4 kg) were housed in individual cages and maintained on a diet of commercial dog biscuits (Big Red Kibbled Dog Cakes, GFL Marketing Company, Canandaigua, N.Y.). They were weighed weekly and showed no significant weight changes during the experimental period. 17α-

This is paper 1 of a series on the metabolism of 17α-methyltestosterone in the dog. Paper 2 is the accompanying paper (1968. *J. Lipid Res.* **9**: 98).

Address requests for reprints to Dr. E. H. Mosbach, Public Health Research Institute, New York 10009.

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography.

Methyltestosterone-4-^{14}C (50 μc, New England Nuclear Corp.) was cocrystallized with unlabeled methyltestosterone. The purity of this product was established by GLC and TLC.

The two dogs were given 200 mg/day of labeled methyltestosterone in gelatin capsules five times per week for 3 wk. (Radioactivity administered: 1.77×10^6 cpm/day to the male dog and 3.38×10^6 cpm/day to the female dog). The animals were killed by intravenous injection of Nembutal 24 hr after the last dose of the drug and autopsied. The following organs were removed: liver, lung, heart, kidney, testes (ovaries), adrenals, spleen, mucosa of duodenum–jejunum and ileum; and samples of bile, blood, muscle, and of adipose tissue were taken.

Each organ was weighed, and 2 g of each tissue was cut into small pieces and extracted for 2 min in a Waring Blendor with 40 ml of chloroform–methanol, 2:1. Bile, serum, and red cells (0.5 ml of each) were extracted by the same procedure. After filtration through a Buchner funnel and washing, the solution was concentrated to a volume of 25 ml. One ml aliquots (equal to 80 mg of tissue) were counted for 50 min in a Packard TriCarb scintillation counter, with 10 ml of a solution of 5 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene in 1 liter of toluene as the counting medium.

Isolation of Metabolites

24-hr samples of feces and urine were collected during the 3rd wk of administration of labeled methyltestosterone. The entire 24 hr feces sample was extracted with three 250 ml portions of boiling 95% ethanol and the combined extracts were concentrated to 100 ml. An aliquot (25 ml) was evaporated to dryness under nitrogen, the residue was dissolved in 30 ml of chloroform, 15 ml of methanol was added, and the solution was transferred to a separatory funnel. After it had been washed with 0.1 M NaCl according to Folch, Lees, and Sloane Stanley (10) the chloroform layer was separated, dried over Na_2SO_4, filtered, and taken to dryness undenitrogen. The residue was dissolved in chloroform–benr zene 5:95.

After removal of some insoluble material the radioactivity of the chloroform–benzene solution was determined and it was then chromatographed on a 2×30 cm column containing 50 g of neutral Woelm alumina, grade IV (10% water). The fractions obtained by elution with solvents of increasing polarity were analyzed for radioactivity as described above for the tissue extracts. For further purification the radioactive fractions were combined and rechromatographed on a 2×30 cm column containing 50 g of Woelm alumina activity grade IV (10% water), at 25 ± 1° C, the fractions being eluted with increasing amounts of chloroform in benzene (Table 1). The fractions were assayed for radioactivity in a Unilux scintillation counter (Nuclear-Chicago Corporation). GLC was performed in a Barber-Colman Model 5000 gas-chromatograph, on a 6 ft column packed[1] with 3% QF-1 (methyl fluoroalkyl silicone) and a 4 ft column packed with 1% SE-54 (methylpolysiloxy gum).

An aliquot (38%) of the 24 hr urine sample was processed according to Segaloff et al. (9). After the sample had been extracted with chloroform for 3 days in a continuous liquid–liquid extractor, the chloroform layer was separated, evaporated to dryness under nitrogen, taken up in chloroform–benzene 1:5 and chromatographed on Woelm alumina grade IV as described for the feces samples. The aqueous layer was subjected to hydrolysis with β-glucuronidase and solvolysis (11). The chloroform extract obtained after hydrolysis was chromatographed as described above.

Reference Compounds

17α-methyl-5α-androstane-3β,17β-diol was purchased from G. D. Searle & Co., Chicago, Ill. 17α-methyl-5α-androstane-3α,17β-diol and 17α-methyl-5β-androstane-3α,17β-diol were gifts from R. Bruce Gabbard and A. Segaloff, Division of Endocrine Research, Alton Ochsner Medical Foundation, New Orleans, La. 17α-methyl-5β-androstane-3β,17β-diol was prepared by the Grignard reaction from CH_3MgI and 5β-androstane-3β-ol-17-one (Steraloids, Inc., Pawling, N. Y.) (12) and obtained in 62.5% yield in a chromatographically pure form; mp 179.5–181.5°C. 17α-methyltestosterone was purchased from Organon Inc., West Orange, N. J.

RESULTS

The extracts obtained from the tissues of the male dog receiving 1.77×10^6 cpm/day contained no appreciable radioactivity; counting rates were less than 25% above background. Only the extracts obtained from the liver and the ileal mucosa of the female dog, which had received 3.38×10^6 cpm/day, showed radioactivity significantly higher than background [total radioactivity: 2.9×10^4 cpm in liver (wet weight 172 g) and 0.76×10^4 cpm in ileal mucosa (wet weight 55 g)]. The total radioactivity of a 24 hr urine sample of the female dog was 2.1×10^6 cpm.

The chloroform-extractable material from this sample weighed 260 mg and contained 40% of the radioactivity. When this extract was chromatographed, more than 90% of the radioactivity applied to the column was

[1] All column packings were purchased from Applied Science Laboratories Inc., State College, Pa.

TABLE 1 ALUMINA CHROMATOGRAPHIC SEPARATION OF IDENTIFIED FECAL METABOLITES OF 17α-METHYLTESTOSTERONE IN FEMALE DOG

Solvent	Vol. Eluate*	Weight	Total radio-activity	Compound	Pro-portion of Total
		mg	*cpm*		%
Chloroform–benzene					
5:95	2	—	—		
10:90	2	—	—		
15:85	2.2	63.7	50,600	MET† + cholesterol	11
	2.0	2.0	26,500	5β:3β,17β-diol‡	5
	3	3.6	47,800	5α:3α,17β-diol	10
20:80	3.6	8.3	303,000	5α:3β,17β-diol	62
	3.6	1.6	56,200	5β:3α,17β-diol	12
Methanol	2.0	*40.1*	*488,000*		
Eluted		119.3	972,100		
Applied to column		150.5	1,101,000		

* Given in multiples of the retention volume of the alumina column used (50 ml).
† MET, 17α-methyltestosterone.
‡ The following abbreviations are used:
5β:3β,17β-diol = 17α-methyl-5β-androstane-3β,17β-diol.
5β:3α,17β diol = 17α-methyl-5β-androstane-3α,17β-diol.
5α:3α,17β-diol = 17α-methyl-5α-androstane-3α,17β-diol.
5α:3β,17β-diol = 17α-methyl-5α-androstane-3β,17β-diol.

recovered in the methanol eluate. This eluate, which contained the highly polar material from the urine, was not identified further. The aqueous fraction after extraction with chloroform contained 60% of the urinary radioactivity. Solvolysis procedures (11) failed to liberate any chloroform-soluble radioactive material either from a sample of the above-mentioned aqueous portion of the urine or from the glucuronidase-treated material. This is in accordance with the findings of Segaloff et al. in man (9), that most of the urinary metabolites of methyltestosterone resided in an unidentified polar fraction.

Since the 24 hr fecal extract corresponding to this urine sample contained 1.1×10^6 cpm (see Table 1), a total of 3.2×10^6 cpm were recovered during this 24 hr collection period. Thus 97.5% of the administered dose (3.38×10^6 cpm) of labeled methyltestosterone was recovered.

Table 1 presents results obtained upon rechromatography on alumina of a 24 hr fecal extract of the female dog. Unchanged methyltestosterone was eluted first, followed by the "axial" diols; as expected, the "equatorial" diols were eluted later. 50% of the recovered radioactivity resided in the polar fraction, eluted with methanol. The radioacive fractions were monitored by GLC (see Table 2 for retention times of reference compounds), first on a column packed with 3% QF-1 as the stationary phase. Two of the four diols, 17α-methyl-5β-androstane-3α,17β-diol and 17α-methyl-5α-androstane-3α,17β-diol, were not separated on this column. Cholesterol and 17α-methyltestosterone had much longer retention times relative to 5α-androstane and did not interfere with the identification of the diols. The two diols that were not resolved on QF-1 could be separated on 1% SE-54. Thus, preliminary identification of all four diols by GLC was feasible.

TABLE 2 RELATIVE RETENTION TIMES OF METHYLANDROSTANEDIOLS DURING GLC*

Compound	Stationary Phase: 3% QF-1 at 205°C	Stationary Phase: 1% SE-54 at 210°C
17α-Methyl-5α-androstane-3α,17β-diol	9.6	4.8
17α-Methyl-5α-androstane-3β,17β-diol	11.0	5.0
17α-Methyl-5β-androstane-3α,17β-diol	9.6	4.4
17α-Methyl-5β-androstane-3β,17β-diol	8.7	4.3
17α-Methyltestosterone	25.3	—
Cholesterol	17.1	—

* Relative to 5α-androstane = 0.4 min.

The equatorial diols (17α-methyl-5α-androstane-3β,-17β-diol and 17α-methyl-5β-androstane-3α,17β-diol) were further identified as follows. (*a*) 17α-methyl-5α-androstane-3β,17β-diol. All column fractions, which by GLC monitoring contained this material, were combined and recrystallized from acetone–water to constant melting point, 212.5–213°C. When the sample was mixed with an authentic sample of diol, its melting point was not depressed (211.5–212°C); the IR spectrum was identical with that of the reference compound. The radioactive compound eluted from the column was diluted with 20 times its weight of authentic material and recrystallized twice from acetone–water and once from ethanol–water. The specific radioactivity remained constant (2170, 2150, 2190 cpm/μmole). The compound

was then oxidized with chromic acid to form 17α-methyl-5α-androstan-3-on-17β-ol. The ketone was recrystallized twice from acetone–water and once from ethanol–water. The specific activity remained constant during this procedure (2250, 2280, 2300 cpm/μmole). Within the precision of measurement the ketone had the same specific radioactivity as the diol. (*b*) 17α-methyl-5β-androstane-3α,17β-diol. The combined column fractions containing this material were recrystallized from ethanol–water to a constant melting point (162–163.5°C). This melting point was not depressed when the reference compound was added. The IR spectrum of the metabolite was identical with that of the authentic sample.

The axial diols, 17α-methyl-5α-androstane-3α,17β-diol and 17α-methyl-5β-androstane-3β,17β-diol, were present in the feces in very low concentrations and were not further identified.

Table 3 presents the distribution of the identified labeled steroids in the 24-hr feces samples of the two dogs studied. About 50% of the administered radioactivity was found to reside in a polar fraction, which has not been examined further. 11–23% of identified material was unchanged 17α-methyltestosterone; the major fecal metabolite was 17α-methyl-5α-androstane-3β,17β-diol, which was present in slightly larger amounts in the feces of the female dog. The male dog excreted approximately twice as much of the 5β : 3α isomer as the female dog, whereas the female dog excreted more of the 17α-methyl-5α-androstane-3α,17β-diol than the male dog. A small amount of 17α-methyl-5β-androstane-3β,17β-diol was found in the feces of the female dog only.

DISCUSSION

Our findings concerning the metabolic fate of methyltestosterone in dogs are similar in many respects to the results reported by Segaloff et al. (9) in man. In both species, much of the methyltestosterone is converted to unidentified polar products which account for a large proportion of the administered radioactivity. The major identified fecal products in both species were 17α-methyl-5α-androstane-3β,17β-diol, 17α-methyl-5β-androstane-3α,17β-diol, and unchanged methyltestosterone. In man, small amounts of methylandrostanediols (17α-methyl-5β-androstane-3α,17β-diol and 17α-methyl-5α-androstane-3α,17β-diol) were isolated from the urine as both free steroids and glucuronides; in the dog the urinary metabolites appeared to be present in the form of highly polar compounds that could not be hydrolyzed by procedures known to deconjugate most steroid glucuronides or sulfates. Since the polar metabolites in urine and feces accounted for most of the administered methyltestosterone it would be desirable to isolate them in pure form in order to determine their structure and biological activity.

TABLE 3 DISTRIBUTION OF IDENTIFIED FECAL METABOLITES OF METHYLTESTOSTERONE*

Metabolite	% of Identified Radioactivity	
	Expt. I (Male)	Expt. II (Female)
Unchanged 17α-methyltestosterone	23	11
17α-Methyl-5β-androstane-3β,17β-diol	—	5
17α-Methyl-5α-androstane-3α,17β-diol	3	10
17α-Methyl-5α-androstane-3β,17β-diol	45	62
17α-Methyl-5β-androstane-3α,17β-diol	28	12

* Isolated from 24-hr feces collections (see Methods). Approximately 50% of the fecal radioactivity was present in a more polar, unidentified fraction.

The participation of the intestinal microorganisms in the production of the identified and unidentified metabolites cannot be excluded. Exposure of methyltestosterone or its tissue metabolites to the intestinal flora during absorption or enterohepatic circulation might well lead to the formation of secondary metabolites. The design of the present studies does not allow definite conclusions as to the origin of the metabolites.

Although the dogs used in the tracer studies received relatively large amounts of methyltestosterone and radioactivity, the specific radioactivities of the administered drug (8850 cpm/mg and 16,900 cpm/mg) were too low to obtain adequate data on tissue localization. However, since more than 95% of the administered daily dose of radioactivity was recovered in urine and feces, appreciable tissue storage could not be expected. Nevertheless, a major proportion of the administered drug must have been absorbed, as evidenced by the large amount of label recovered in the urine. It can be calculated from the data on the female dog (the lower amount of radioactivity injected precluded this calculation for the male) that the liver—wet weight 172 g—contained only about 1.7 mg of the labeled material, and the ileal mucosa (wet weight 55 g) about 0.45 mg. The dog had received 200 mg of labeled methyltestosterone 24 hr prior to autopsy, and the presence of radioactivity in these tissues may merely reflect passage of the steroid through these organs during absorption, metabolism, and excretion. Our inability to detect appreciable amounts of ^{14}C-containing material in the tissues seems to imply that in the dog, methyltestosterone must be metabolized and excreted at a rapid rate. Definite demonstration of binding by specific tissues and cellular or subcellular sites will require administration of labeled steroid of very high specific activity and the use of smaller species such as rats or mice.

This work was supported by grants AM 05222 and HE 00052 of the United States Public Health Service and by grant U-1562 of the Health Research Council of the City of New York.

Manuscript received 31 July 1967; accepted 6 October 1967.

References

1. Furman, R. H., R. P. Howard, L. N. Norcia, and E. C. Keaty. 1958. *Am. J. Med.* **24:** 80.
2. Robinson, R. W., R. J. LeBeau, and W. D. Cohen. 1964. *J. Clin. Endocrinol. Metab.* **24:** 708.
3. Fillios, L. C., and G. V. Mann. 1956. *Circulation Res.* **4:** 406.
4. Furman, R. H., L. N. Norcia, C. W. Robinson, and I. E. Gonzalez. 1957. *Am. J. Physiol.* **191:** 561.
5. Abell, L. L., E. H. Mosbach, and F. E. Kendall. 1962. *Circulation Res.* **10:** 846.
6. Pick, R., J. Stamler, S. Rodbard, and L. N. Katz. 1959. *Circulation Res.* **7:** 202.
7. Raiford, R. L., and H. Y. C. Wong. 1962. *Circulation Res.* **11:** 753.
8. Abell, L. L., and E. H. Mosbach. 1962. *J. Lipid Res.* **3:** 88.
9. Segaloff, A., R. B. Gabbard, B. T. Carriere, and E. L. Rongone. 1965. *Steroids Suppl.* **1:** 149.
10. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. *J. Biol. Chem.* **226:** 497.
11. Burstein, S., and S. Lieberman. 1958. *J. Biol. Chem.* **233:** 331.
12. Schering-Kahlbaum AG Brit. Pat. 479,252. Issued 1938. U.S. Pat. 2,238,936. Issued 1941.