[Chem. Pharm. Bull. 20(6)1243—1259(1972)]

UDC 547.92.09 : 615.31.015.4.076.9

Investigations on Steroids. XII.[1] Metabolites of Furazabol (17β-Hydroxy-17α-methyl-5α-androstano[2,3-*c*]furazan) administered to Rats

Toshio Takegoshi, Haruo Tachizawa, and Genkichi Ohta

Research Laboratories, Daiichi Seiyaku Co., Ltd.[2]

(Received December 4, 1971)

Isolation and characterization of metabolites of furazabol (17β-hydroxy-17α-methyl-5α-androstano[2,3-*c*]furazan; IIa) following oral or subcutaneous administration of the compound to rats are described. From faeces were obtained the corresponding 18,17β-lactone (I), unchanged furazabol, the 16β-hydroxylated compound (III), the 18-hydroxylated compound (IV), the 17α-hydroxymethyl derivative (V), the 18-carboxylic acid (VII) and a compound assumed to be the 4β,16ξ-dihydroxylated 18,17β-lactone (VI). In the bile the lactone (I) and probably the conjugated metabolites were present. Urinary metabolites were not characterized. The furazan ring of furazabol appeared to be stable *in vivo*. The metabolic significance of the isolated compounds is discussed.

Furazabol (17β-hydroxy-17α-methyl-5α-androstano[2,3-*c*]furazan; IIa) is a heterocyclic steroid possessing a furazan ring fused to the steroidal ring A and by virtue of its favorable biological activities[3] is now used clinically as an anabolic[4a,b] and hypolipidaemic agent.[4c,d] The metabolism of similar heterocyclic steroids has been studied in a few cases. The literature includes studies on the distribution of 17β-hydroxy-17α-methyl-5α-androstano[3,2-*c*]-isoxazole in rats[5a] and of the corresponding 4,4-dimethylandrostano[2,3-*d*]isoxazole in the same animals.[5b,c] An assumption has also been proposed that stanozolol (17β-hydroxy-17α-methyl-5α-androstano[3,2-*c*]pyrazole), an anabolic steroid having a similarly fused pyrazole ring, is metabolized to give the heterocycle-cleaved products which exert the biological activities.[5d] It was therefore the matter of significance to study the metabolic fate of furazabol, especially to determine whether or not the furazan ring is cleaved. The absorption, distribution and excretion of the compound in rats and a brief comment on the metabolites were described previously.[6] This paper reports the isolation and structure elucidation of the metabolites of furazabol administered orally or subcutaneously to rats.

Furazabol labelled with ^{14}C at the 17α-methyl position (^{14}C-furazabol; IIb)[7] was used for administration in several doses. For oral administration a dose of 12.5 mg/kg with a specific activity of 20.4 μCi/mg was employed as a standard. In addition, a moderate dose with a low specific activity (300 mg/kg, 0.55 μCi/mg) for preliminary experiments and a

1) Part XI: G. Ohta, K. Koshi, and K. Obata, *Chem. Pharm. Bull.* (Tokyo), **16**, 1487 (1968).
2) Location: *Minamifunabori-cho, Edogawa-ku, Tokyo.*
3) *a*) A. Kasahara, T. Onodera, M. Mogi, Y. Oshima, and M. Shimizu, *Chem. Pharm. Bull.* (Tokyo), **13**, 1460 (1965); *b*) T. Onodera, M. Ishihara, A. Kasahara, and Y. Oshima, *Pharmacometrics*, **3**, 191 (1969); *c*) T. Onodera, A. Kasahara, M. Ishihara, H. Ogawa, and Y. Oshima, *Nippon Yakurigaku Zasshi*, **66**, 458 (1970).
4) *a*) M. Muroo, *Chiryo*, **49**, 146 (1967); *b*) H. Suzuki, *Shinyaku To Rinsho*, **15**, 31 (1966); *c*) Y. Goto, K. Murakami, A. Horisada, and M. Goto, *Rinsho To Kenkyu*, **43**, 172 (1966); *d*) N. Kimura, A. Nishimoto, Y. Mizuguchi, M. Kitamura, A. Yoshinaga, Y. Fuyuno, T. Fukamizu, H. Nakayama, T. Arima, M. Yamada, Y. Oki, S. Fukano, and Y. Ohtsuka, *ibid.*, **43**, 150 (1966).
5) *a*) N. Ogawa, H. Tazima, T. Kinoshita, and J. Ishiguro, Abstracts of Papers, 90th Annual Meeting of Pharmaceutical Society of Japan, Sapporo, July 1970, p. III-9; *b*) A.S. Goldman, *Endocrinology*, **86**, 678 (1970); *c*) A.S. Goldman and C.Z. Kenneck, *ibid.*, **86**, 711 (1970); *d*) N.J. Doorenbos and C.P. Dorn, *J. Pharm. Sci.*, **54**, 1219 (1965).
6) H. Tachizawa, T. Takegoshi, M. Chonan, T. Akimoto, and A. Kasahara, *Clinical Report*, **4**, 2055 (1970).
7) T. Takegoshi, *Chem. Pharm. Bull.* (Tokyo), **20**, 1260 (1972).

high dose without radioactivity (500 mg/kg) for preparative separation of the metabolites were given orally. For subcutaneous injection a dose of 10 mg/kg with a specific activity of 16.9 μCi/mg was used.

Faecal Metabolites of Orally Given Furazabol

The previous experiments showed that when the standard dose was given about 65% of the total radioactivity was excreted in the faeces.[6] Extraction of the faeces, partition of the extracts between hexane and 70% methanol and removal of the hexane soluble material afforded a concentrated metabolite mixture which contained about 80% of the faecal radioactivity. The thin-layer chromatography (TLC) of the concentrate revealed several radioactive spots (Fig. 1) and the percentage of each radioactivity is shown in Table I. The preparative separation of the metabolites without the isotopic label was guided by this chromatogram and seven compounds were isolated and characterized as described below.

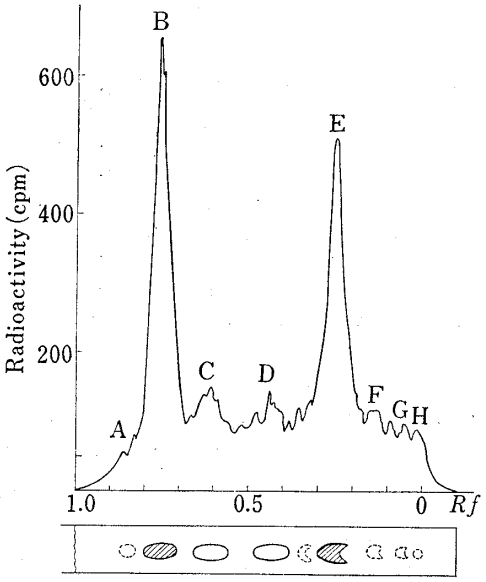

Fig. 1. Radiochromatogram of TLC of the Faecal Metabolite Mixture; the TLC was developed with $CHCl_3$–MeOH (9:1), 12.5 mg/kg, *p.o.*

Adsorption chromatography of the concentrate on silica gel and rechromatography of the non-polar fraction gave two compounds, metabolite I and compound II. From the polar fraction, after acetylation and adsorption chromatography, were obtained metabolite III acetate and a mixture of metabolite III acetate and IV acetate. Hydrolysis of the acetate mixture followed by acetonide formation provided the metabolite III acetonide. Removal of this compound separated the unaffected metabolite IV in a practically pure but not crystalline state.

The more polar fraction was further processed by combination of adsorption chromatography, preparative TLC and conversion into the acetate or the acetonide. The fraction was found to contain acidic materials and from the neutral portion crude metabolite V acetate and pure metabolite VI acetate were obtained. Hydrolysis of the crude acetate followed by acetonide formation made available the pure acetonide of metabolite V. From the acidic fraction, metabolite VII was isolated. In addition to these compounds, there remained extremely polar metabolites which, however, were not isolated successfully. The isolation procedure is shown schematically in Chart 1.

Table I. Percentages of Radiochromatogram Peak Areas of Faecal Metabolites (*cf.* Fig. 1)

Dose	Peak[a] (metabolite) (%)						
	A (I)	B (II)	C (III+IV)	D (V+VI)	E (VII)	F —	G,H —
12.5 mg/kg, *p.o.*	1.7	32.2	11.2	10.9	26.0	7.2	5.2
60 mg/kg, *p.o.*	1.4	40.1	11.5	9.5	13.8	9.8	13.9
10 mg/kg, *s.c.*[b]	2.5	12.5	12.7	14.9	19.8	8.2	29.4
10 mg/kg, *s.c.*[c]	1.3	5.8	7.3	7.8	24.4	14.2	39.2

a) Peaks are designated alphabetically in the order of increasing polarity.
b) Metabolites collected during 24 hr after administration.
c) Metabolites collected during 72 hr after administration.

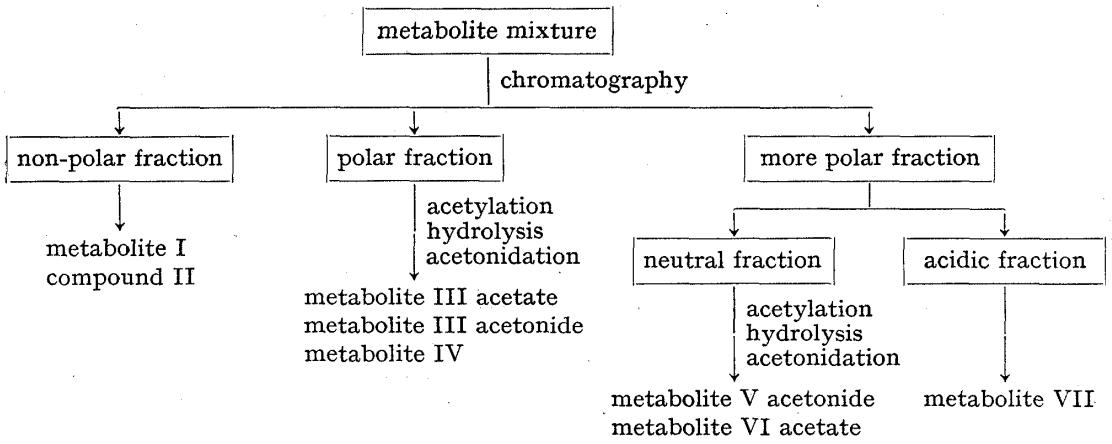

Chart 1. Scheme of Preparative Separation of Faecal Metabolites

Metabolite I revealed in the infrared (IR) and ultraviolet (UV) spectra the characteristic absorptions (1492, 1208, 1003, 770 cm^{-1} and 217 mμ) to androstano[2,3-*c*]furazan.[8] The IR spectrum also contained a band at 1785 cm^{-1} indicating the presence of a lactone ring which was proved by hydrolysis to an acid. The TLC of the acid showed it to be identical with metabolite VII. An attempted lactonization of the acid under usual acidic conditions gave the unchanged material. The lactone ring was therefore assumed to be a β-lactone but not a γ-lactone, although the above IR band at 1785 cm^{-1} appeared at lower frequencies than those reported for β-lactones (1800—1820 cm^{-1}).[9] In the mass spectrum, the highest mass peak was observed at *m/e* 314 and the subsequent peak at *m/e* 298. The difference of 16 mass units indicated the absence of the molecular ion peak. The *m/e* 298 peak corresponded to a fragment possessing the steroidal furazan skeleton and having lost carbon dioxide (M-CO_2) and consequently the *m/e* 314 peak was assigned to the M–CO fragment. These and another fragment observed at *m/e* 283 (M–CO_2–CH_3) coincided with the presence of a lactone ring. Although the nuclear magnetic resonance (NMR) spectrum was not available due to the scarcity of material, consideration of the above results and of the structure of metabolite VII described below led to the assignment of the

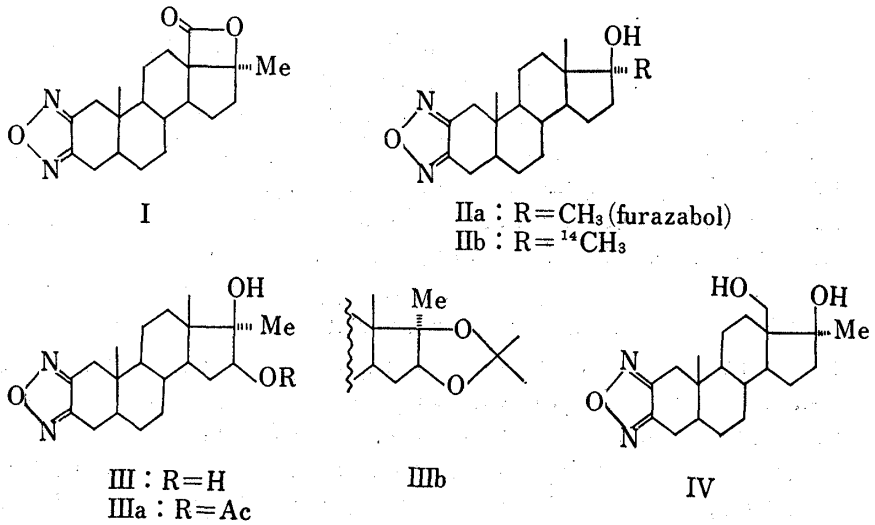

Chart 2

8) G. Ohta, T. Takegoshi, K. Ueno, and M. Shimizu, *Chem. Pharm. Bull.* (Tokyo), **13**, 1445 (1965).
9) Y. Etienne and N. Fischer, "The Chemistry of Heterocyclic Compounds," Vol. 19, ed. by A. Weissberger, Interscience Publishers, Inc., New York, N.Y., 1964, p. 729.

18,17β-lactone structure (I) for metabolite I. This was proved by comparison with an authentic sample prepared by the established procedure.[7]

The identity of compound II with furazabol (IIa) was suggested by TLC at an early stage of the separation and established, after isolation, by direct comparison of the two compounds. A considerable amount of furazabol was recovered when a high dose was administered.

Metabolite III was isolated as its acetate whose mass spectrum exhibited the molecular ion peak at *m/e* 388 corresponding to the formula of an acetoxylated derivative of furazabol. Hydrolysis of the acetate regenerated metabolite III and the formation of its acetonide disclosed the presence of vicinal hydroxyl groups for which were assignable the 16β- and 17β-hydroxyl groups or the 17β-hydroxy and 17α-hydroxymethyl groups. The former assignment was supported by the NMR spectrum of the acetate with the signals at 0.78 (C-18 methyl), 0.90 (C-19 methyl), 1.24 (17α-methyl), 2.09 (methyl of acetate) and 4.69 ppm (16α-H). The last signal (1-H, quartet, J=5.9 Hz) corresponds to the α-proton at the 16-position bearing the acetoxyl group and the angular methyl signals accord with those observed for furazabol. The IR spectrum of the acetate showed bands at 1710 ($\nu_{C=O}$) and 1268 cm^{-1} (ν_{C-O}) due to the acetoxyl group. The observed shifts of position of $\nu_{C=O}$ band to lower frequencies and of ν_{C-O} band to higher frequencies from the respective positions of the usual acetate band are attributable to the interaction between the *cis* vicinal hydroxy and acetoxy groups. Structure III, IIIa, and IIIb were assigned respectively for metabolite III, its acetate and its acetonide, and confirmed by comparison with the corresponding authentic samples.[7]

Metabolite IV was obtained as a crude material which, nevertheless, exhibited the characteristic IR and UV absorptions to androstano[2,3-*c*]furazan. Acetylation followed by examination of the product by TLC indicated the presence of an acylable hydroxy function. Oxidation of the crude metabolite IV with chromium trioxide in acetic acid gave a mixture, the TLC of which revealed two spots corresponding to metabolite I, the lactone, and metabolite VII, the acid. Although the β-lactone formation by oxidation seems exceptional, the above results can be interpreted as indicating metabolite IV to be the 18-hydroxy compound (IV). Scarcity of material prevented further examination and therefore an authentic sample was synthesized.[7] Comparison of the two samples by means of TLC and gas liquid chromatography (GLC) proved the structure IV. The chemistry of this compound will be described in the following paper.[7]

Metabolite V was isolated as its acetonide which indicated the presence of vicinal hydroxy groups. In the mass spectrum of the acetonide, the molecular ion peak at *m/e* 386 was the same as that of metabolite III acetonide, showing that two compounds are the positional isomers. Since metabolite III was proved to be the 16β,17β-diol, it followed that metabolite V is the 17β-hydroxy-17α-hydroxymethyl compound (V). Oxidation of crude metabolite V with chromium trioxide in acetic acid gave 17-oxo-5α-androstano[2,3-*c*]furazan (Vc) identical with an authentic sample. The fact is explained as the result of oxidation to the 17α-carboxylic acid, followed by decarboxylation, and supports the assigned structure (V). The structure was established by comparison with an authentic sample.[7]

The structure of metabolite VI was not completely determined; however the 4β,16ξ-dihydroxy-18,17β-lactone structure (VI) was proposed on the basis of the observations which follow. The compound was isolated as its acetate. The IR spectrum of the acetate showed the bands at 1814 cm^{-1} corresponding to a lactone, at 1738 and 1220 cm^{-1} to an acetoxyl group, and at 1490, 1008, 780, and 759 cm^{-1} to the furazan ring. The shift of the position of the β-lactone band to the higher frequencies than the position observed for metabolite I (1785 cm^{-1}) is consistent with the presence of the electronegative 16ξ-acetoxyl group in the proximity of the lactone. The NMR spectrum of the acetate showed the presence of two acetoxyl groups (2.08, 2.18 ppm), two, instead of three, tertiary methyl groups (1.00, 1.49 ppm), one methylene group adjacent to the furazan ring (2.29, 3.39 ppm, AB doublet,

J=17 Hz) assignable for the C-1 methylene, and two protons each adjacent to an acetoxyl group (4.80 ppm, broad; 6.32 ppm, doublet, J=5 Hz). Comparison with the spectrum of furazabol indicated that the signal of 1.00 ppm assignable for the C-19 methyl protons shifted to downfield. The shift is probably resultant from the deshielding effect of the 4β-acetoxyl function which is situated in the 1,3-diaxial relation to the C-19 methyl group. The assignment of the 4β-acetoxyl configuration was supported by the signal at 6.32 ppm of the 4α-proton which is deshielded by the effect of the furazan ring and which couples vicinally with the *cis* 5α-proton, giving the coupling constant of 5 Hz. The signal at 1.49 ppm was assigned for the 17α-methyl protons and shifted also the downfield probably due to the effect of the lactone ring. The broad signal at 4.80 ppm was ascribed to the proton at the acetoxy-bearing C-16 carbon atom but the position and configuration could not be determined precisely. In the mass spectrum of the acetate, the molecular ion peak (mol. wt. 458) was not observable. The highest mass peak at m/e 416 (M-42) corresponded to a fragment formed by the expulsion of ketene from the molecule. The M-42 fragment, which was also observed in the spectrum of the 16β-acetate (IIIa), afforded evidence for the presence of 16ξ-acetoxy group, since the fragment from the simple acetoxy compound is usually M-60 ion arising through 1,2-elimination of acetic acid.[10] The above M-42 fragmentation can be explained as resulting from fission of the 16—17 linkage followed by abstraction of a hydrogen-atom from the acetoxyl group (VIb→VIc). The base peak at m/e 354 (M-CO_2-CH_3COOH) corresponded to a fragment formed by the loss of a lactone and of an acetic acid, being compatible with the assigned structure. In the lower mass range, fragments which were assumed to contain the furazan ring and steroidal ring A were found at m/e 147 and 159. The same

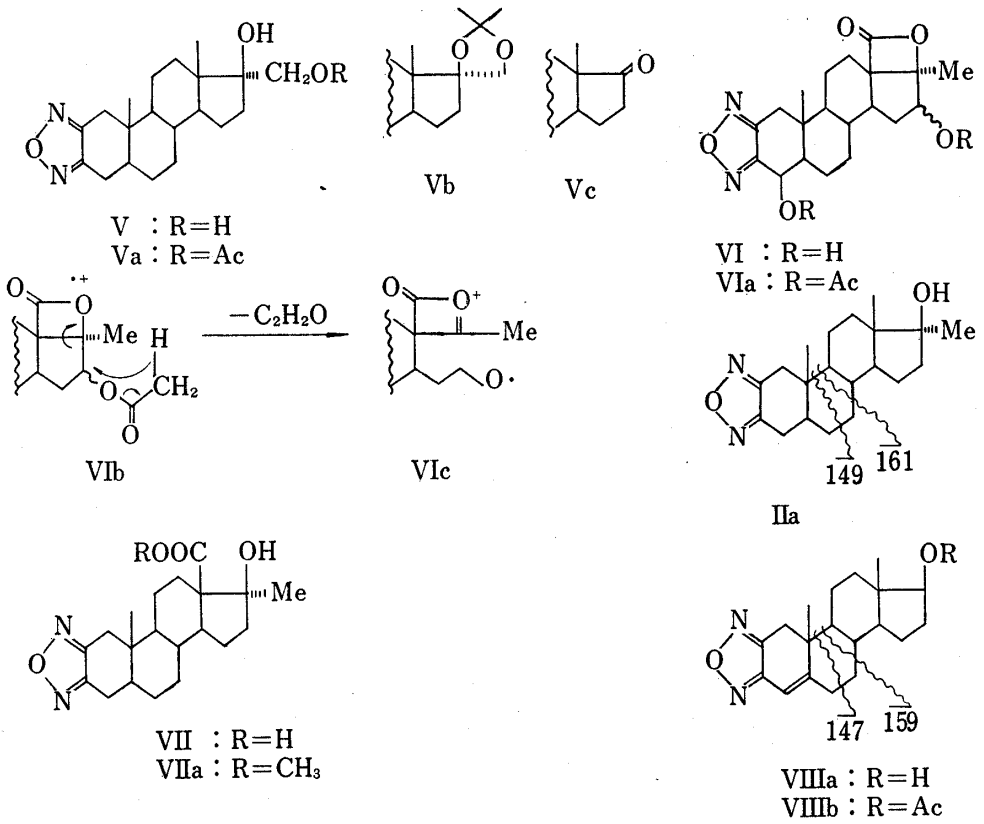

Chart 3

10) H. Budzikiewicz, C. Djerassi, and D.H. Williams, "Mass Spectrometry of Organic Compounds," Holden-Day, Inc., San Fransisco, 1967, p. 468.

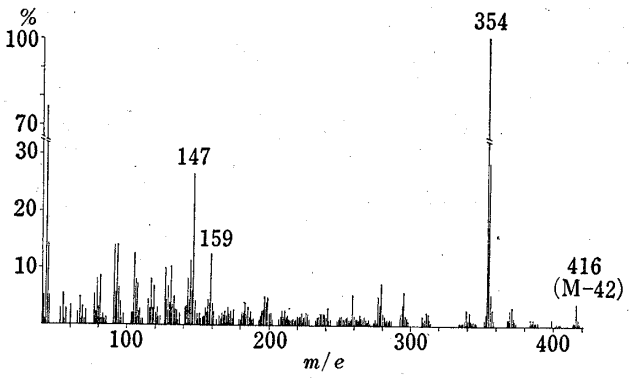

Fig. 2. Mass Spectrum of Metabolite VI

fragments appeared in the spectra of androst-4-eno[2,3-*c*] furazans (VIIIa, VIIIb),[7] whereas in the spectra of saturated androstano[2,3-*c*] furazans, such as furazabol (IIa), metabolite I and the acetates or the acetonides of metabolite III and V, were observed 2 mass units higher *m/e* 149 and 161 fragments. This is consistent with the proposed structure (VI) possessing the 4β-acetoxy group.

Structure VII was assigned for metabolite VII on the basis of the following evidence. The acidic character of metabolite VII was indicated by the separation procedure. The IR spectrum showed the presence of a hydroxyl group (3430 cm^{-1}) and a carboxyl group (2650, 1708, 945 cm^{-1}). The acid was convertible into the methyl ester whose mass spectrum revealed the molecular ion peak at *m/e* 374, indicating that one of the *tert.* methyl groups originally contained in furazabol was replaced by a methoxycarbonyl group. In the NMR spectrum of the acid the signal due to the C-18 methyl group was absent and the signals at 0.68 and 1.32 ppm corresponded, respectively, to the C-19 methyl and 17α-methyl groups. The signal observed at 5.27 ppm for two protons disappeared after deuteration and attributed to the protons of the 17β-hydroxy and the 13-carboxy groups. The interrelation of metabolite VII with metabolite I and with metabolite IV described above was also consistent with the assigned structure. Finally, comparison with an authentic sample of the 18-oic acid (VII)[7] established the structure.

Faecal Metabolites of Subcutaneously Given Furazabol

About 55% of the radioactivity of the given dose was found in the faeces collected during 7 days after administration.[6] Extraction of the faeces followed by concentration afforded a metabolite mixture which showed a similar pattern of TLC as in the case of oral administration, except that the spot corresponding to furazabol was small. A difference was also observed between the metabolites of 24 hr's faeces and those of 72 hr's faeces (Table I). In the latter the amount of polar metabolites increased. The metabolite mixture was separated into the acidic and neutral portions, and further fractionated by preparative TLC. Identification of the metabolite was performed by comparison with the authentic sample by the use of TLC, together with comparison of the derivative of the metabolite with the corresponding authentic derivative. Furazabol was identified by an isotopic dilution method. The presence of the lactone (I), furazabol (IIa), the 16β-hydroxy- (III), 18-hydroxy- (IV), and 17α-hydroxymethyl- (V) derivatives, 4β,16ξ-dihydroxy-18,17β-lactone (VI) and the 18-oic acid (VII) was thus established.

Biliary Metabolites

About 6% of the given radioactivity was excreted in the bile during 6 hr after administration of the standard dose by way of duodenum. During 24 hr after subcutaneous injection the biliary excretion was 37%.[6] No marked difference due to the administration route was observed on the pattern of TLC of the bile extract, which indicated the presence of metabolite I (about 15—30%) and extremely polar metabolites as shown in Fig. 3 (a). The ratio of metabolite I to the total metabolites in the bile was greater than that observed in the faeces. Since the biliary metabolites were assumed to be mainly conjugated, the bile extract was hydrolyzed in a sodium hydroxide solution and subsequently solvolyzed in aqueous tetrahydrofuran in the presence of perchloric acid. The radiochromatogram of TLC of the hydrolyzate showed that the peak corresponding to the metabolite I disappeared

and instead the peak due to metabolite VII appeared. In addition, the amount of the most polar metabolites decreased and less polar compounds were newly detected. Solvolysis of the hydrolyzate further decreased the amount of the polar metabolites, increasing the ratio of the less polar compounds. In the reversed procedure, when the extract was solvolyzed first the metabolite I was unaffected and the most polar metabolites diminished significantly. Subsequent hydrolysis of the solvolyzate gave the similar final result to that of hydrolysis followed by solvolysis (Fig. 3). The fraction containing metabolite I was separated by preparative TLC, and hydrolyzed to give a fraction which on TLC proved to contain metabolite VII. These results indicated that the biliary metabolites consisted of metabolite I and other conjugated metabolites.

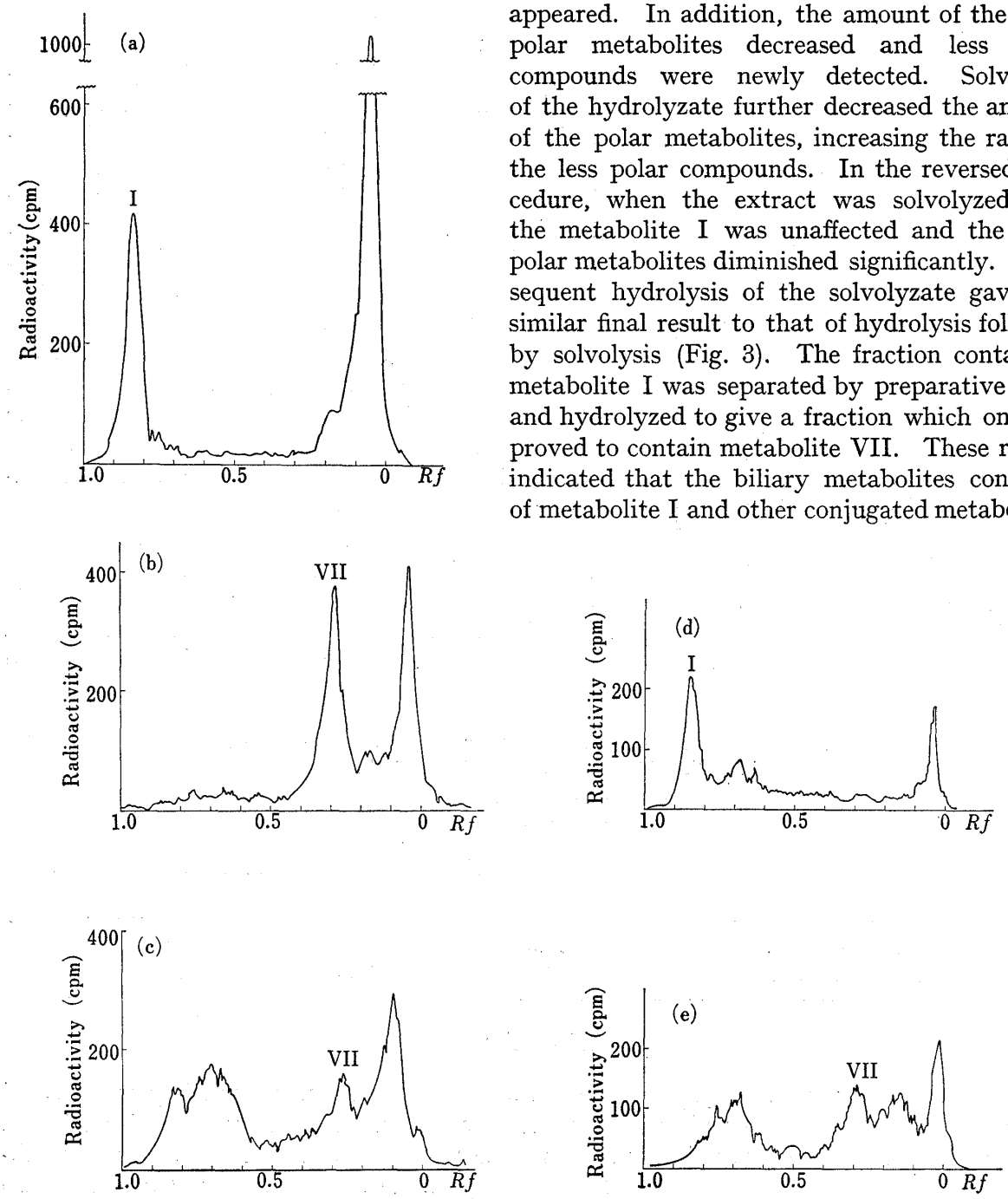

Fig. 3. Radiochromatograms of TLC of Biliary Metabolites, developed with $CHCl_3$–MeOH (9: 1)

(a): biliary extract
(b): after hydrolysis with 15% NaOH
(c): after hydrolysis with 15% NaOH, followed by solvolysis with 0.1N $HClO_4$
(d): after solvolysis with 0.1N $HClO_4$
(e): after solvolysis with 0.1N $HClO_4$, followed by hydrolysis with 5% NaOH

Urinary Metabolites

It was shown previously that, after oral or subcutaneous administration, about 20—23% of the given dose was excreted in the urine during 72 hr.[6] The TLC of urinary metabolites showed that they are strongly polar substance and not influenced by the administration route. The pattern of the twice developed chromatogram is shown in Fig. 4 and Table II.

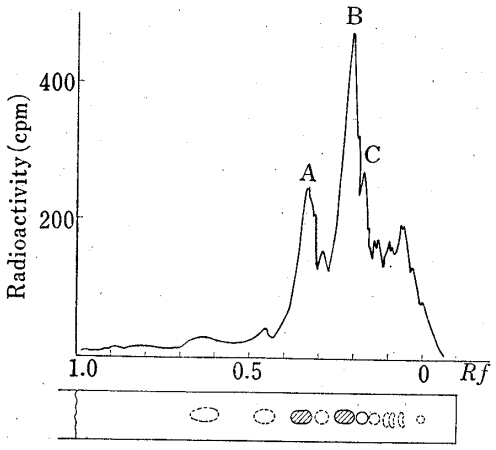

Fig. 4. Radiochromatogram of TLC of Urine excreted during 16 hr after Oral Administration of ^{14}C-Furazabol (12.5 mg/kg); the TLC was developed twice with $CHCl_3$–MeOH (9: 1)

The metabolites of the orally given furazabol were separated according to the usual method of separation of urinary steroids; extraction of the urine gave the free steroid fraction in 66% yield calculated from the total urinary radioactivity, hydrolysis of the remaining urinary compounds with β-glucuronidase gave the fraction from the glucuronides in 20% yield and subsequent solvolysis gave the fraction from the sulfate conjugates in 7% yield. Since the TLC of each fraction was similar to that of the original urine and the polar metabolites were not readily extractable, it was also probable that the fractions from the glucuronides and from the sulfates contained mainly the free steroids which had remained through the preceding incomplete extraction. For the preparative separation of the metabolites various methods were attempted, employing the unlabelled metabolites of orally given furazabol, but no homogenous compound was isolated. A fraction of the metabolite mixture obtained by column chromatography, followed by TLC revealed the UV absorption characteristic to androstano[2,3-*c*]furazan (λ_{max}: 217 mμ), indicating that the furazan ring was still retained in the metabolites. An attempted GLC-Mass spectrum measurement of this fraction also failed.

Table II. Percentages of Radiochromatogram Peak Areas of Metabolites in Urine excreted during 72 hr after Administration (*cf.* Fig. 4)

Dose	Peak (%)		
	A	B	C
12.5 mg/kg, *p.o.*	19.5	24.6	10.3
10.0 mg/kg, *s.c.*	20.8	23.0	16.8

Discussion

It would appear from the present experiments that the furazan ring moiety of furazabol is not affected *in vivo*. This view is supported by the chemistry of the 3,4-disubstituted furazan which has proved that the compound is chemically stable.[11] After the oral or subcutaneous administration of furazabol to rats, its presence in the blood and liver has also been observed.[6] It is therefore most likely that the anabolic and hypolipidaemic activities of furazabol are effected without cleavage of the furazan ring. This is in contrast to the assumption proposed for stanozolol.[5d]

The difference between the biliary metabolites and the faecal metabolites as evidenced in the TLC is probably related to the effect of intestinal microorganisms. A series of studies on the faecal steroidal metabolites obtained from conventional rats and germ free rats has shown that free steroids are found in the faeces from conventional rats whereas conjugated

11) *a*) J.H. Boyer, "Heterocyclic Compounds," Vol. 7, ed. by R.C. Elderfield, John Wiley and Sons, Inc., New York, N.Y., 1961, p. 462; *b*) L.C. Behr, "Hetrocyclic Compounds," Vol. 17, ed. by R.H. Wiley, Interscience Publishers, Inc., New York, N.Y., 1962, p. 283.

steroids are the main metabolites in those from germ free rats.[12] The metabolites of testosterone in the bile of human have been shown to be in a conjugated form while a greater part of the faecal metabolites has been found in the free form.[13a] These results have been ascribed to the deconjugating activity of intestinal microorganisms. In the study of C_{19}- and C_{20}-steroidal metabolites in human faeces, the decojugation and other modifications due to the activities of microorganisms have also been suggested.[13b,c] The faecal metabolites characterized in the present study are all free steroids and the results obtained by hydrolysis and solvolysis of biliary metabolites indicate that they are conjugates except the lactone (I). Accordingly microbiological deconjugation and other transformation are to be considered.

The 18,17β-lactone (I) was found in the bile as a main product and in the faeces as a minor product. Therefore, the compound is the direct metabolite by rats and assumed to be converted by intestinal microorganisms. This type of structure containing the four membered lactone has not been reported in the field of steroidal metabolites and rarely found in natural products.[14] The formation of such a strained ring is of interest and probably by way of the 18-ol (IV). Since chemical oxidation of IV proved to give I,[7] the same mechanism is inferred to be operative biologically.

Furazabol (IIa) was recovered from the faeces after either oral or subcutaneous administration. A considerable amount was obtained when a high dose was given orally, indicating that the major part of furazabol was excreted without being absorbed in this case. In the bile furazabol was not detectable but its presence in the faeces after subcutaneous administration indicates that furazabol was excreted in the bile as the conjugate, which was then deconjugated by intestinal microorganisms.

The 16β-ol (III) was not found in the biliary metabolites. Thus the compound was excreted into the bile as its conjugate which was deconjugated in the intestine or it was formed by microbiological hydroxylation of furazabol. Recent reports about the 17β-hydroxy-17α-methylsteroids described the occurrence of their 16β-hydroxylated metabolites in rabbits[15a,b] and human urine,[15c,d] pointing out the effects of the 17α-methyl group on the metabolic hydroxylation.[15] Furazabol has the same grouping at C-17 and 16β-hydroxylation in rat metabolism is compatible with the reported results.

The 18-hydroxy compound (IV) is considered the precursor of the lactone (I) and, therefore, the metabolite by rats. Based on the quantity of I in the bile, it can be assumed that the metabolic route from furazabol *via* IV to I is one of the main routes. The hydroxylation at C-18 in adrenal gland is known and the same hydroxylation in rat liver has recently been reported.[16a,b] In addition, the sulfate of 3α,18-dihydroxy-5α-androstan-17-one has been found in a considerable amount in human bile.[16c] The occurrence of IV is comparable with these recent findings and probably it is conjugated in the bile.

The 17α-hydroxymethyl compound (V) was not observed in the bile. Since the hydroxylation of the steroidal 17α-methyl group has not been reported, there is no clue at present to consider whether the hydroxylation is due to rat metabolism or to microbilogical hydroxylation. It should be noted here that further biological oxidation of V to the 17α-carboxylic

12) *a*) B.E. Gustafsson, J.-Å. Gustafsson, and J. Sjövall, *Eur. J. Biochem.*, **4**, 568 (1968); *b*) H. Eriksson, J.-Å. Gustafsson, and J. Sjövall, *ibid.*, **9**, 289 (1969).

13) *a*) A.A. Sandberg and W.R. Slaunwhite, *J. Clin. Invest.*, **35**, 1331 (1956); *b*) H. Eriksson, J.-Å. Gustafsson, and J. Sjövall, *Eur. J. Biochem.*, **12**, 520 (1970); *c*) T. Laatikainen and R. Vihko, *ibid.*, **13**, 534 (1970).

14) D.C. Aldridge, D. Giles, and W.B. Turner, *Chem. Commun.*, **1970**, 639.

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acid followed by decarboxylation would furnish the 17-ketone (Vc) which is not detectable by radioactivity. In the present experiments, however, careful examination of the faecal extract with TLC and GLC failed to find Vc.

Metabolite VI was assumed to be the 4β,16ξ-dihydroxy-18,17β-lactone (VI) which has the multiple groups resulted from metabolism. The metabolic significance of the 18,17β-lactone and the 16β-hydroxy group is discussed above. The 16α-hydroxylation is a common process of metabolism. The position 4 of androstano[2,3-*c*]furazan is neighbouring to the terminal aromatic ring and is similar to the position 6 of estrone. Since the 6β-hydroxylation of estrone is the known metabolic process, it is probable that the similar process takes place in the case of furazabol. Metabolite VI was not detectable in the bile and the effect of micro-organisms on the formation of the compound can not be excluded.

The 18-oic acid (VII) is the main product in the faeces and absent in the bile, whereas the lactone (I) is the main product in the bile and the minor in the faeces. Consequently, the 18-oic acid appears to be mainly derived from the lactone by hydrolyzing effect of micro-organisms.

At the present stage of this investigation the structures of the urinary metabolites are obscure. Only the presence of furazan ring and of the 17α-carbon atom was indicated, respectively, by the UV spectrum and by the radioactivity.

Experimental

General Procedure——Melting points are uncorrected. UV spectra were taken in EtOH, IR spectra in KBr disc and NMR spectra in $CDCl_3$ with tetramethylsilane as internal standard at 100 Mz. Mass spectra were measured on Hitachi RMS-4 spectrometer by direct insertion technique, the ionization potential was 70 eV, the ion source temperature was in the range of 200° to 250° and the results are tabulated in Table V.

Unless otherwise stated, TLC was carried out on glass plates coated with silica gel (Silica Rider, Daiichi Pure Chemicals Co., Ltd.) by using two solvent systems, solvent A: $CHCl_3$–MeOH (9:1) and solvent B: benzene–AcOEt (4:1). Spots were detected by their radioactivities and by spraying a 1% vanilin solution in H_3PO_4–EtOH (1:1). Furazabol was used as a standard and the results are given in Table VI, where *Rf*-values are approximated to the nearest value.

GLC was carried out on Barber–Colman Model 10 with argon ionization detector and glass column 2 m × 6 mm. The column was packed with A) 1% SE-30 on Anakrom ABS and B) 1% OV-17 on Gas Chrom P. The conditions used were as follows: column temperature A) 230°, B) 270°, argon gas flow rate 48—55 ml/min. The trimethylsilyl (TMS) derivative was prepared as follows: a sample (0.5—3 mg) was dissolved in pyridine (0.2 ml), hexamethyldisilazane (3 drops) and trimethylchlorosilane (1 drop) were added, and the solution was kept at room temperature for 30 min. After evaporation under N_2 the residue was dissloved in benzene or acetone and subjected to GLC. Similarly the trifluoroacetate was prepared from a sample (0.5—2 mg), tetrahydrofuran (0.2 ml), pyridine (3 drops) and trifluoroacetic anhydride (3 drops). The results are listed in Table VII. In micro-scale experiments such as described above one drop refers to a drop from a capillary with 1 mm diameter.

Microanalyses and mass spectrum measurements were performed by Mr. B. Kurihara and his associates, and NMR spectrum measurements by Messrs K. Suyama and K. Tomita in these Laboratories.

Labelled Furazabol——17β-Hydroxy-17α-methyl[-^{14}C]-5α-androstano[2,3-*c*]furazan (^{14}C-furazabol, IIb) was prepared in these Laboratories.[7]

Measurement of Radioactivity——The radioactive materials, except faeces, were dissolved in MeOH or EtOH and the solution (0.10 ml) was added to the scintilator (15 ml) prepared from dioxane (900 ml), dimethyl POPOP (0.30 g), PPO (7.0 g) and naphthalene (100 g). Radioactivity was determined with a Kobe Kogyo JSL-112 liquid scintilation spectrometer or with an Aloka LSC-601 liquid scintilation counter. Corrections were made by a channel ratio method in the former and an external channel ratio method in the latter. The radioactivities of faeces were determined by a combution method as described previously.[6] The radioactivities on TLC were measured with an Aloka TLC Scanner Model TLC-2B.

Animals——The animals used were male rats of Donryu-strain with average body weight of 200 g, aged 63—65 days.

Administration of Furazabol and Collection of Faeces and Urine——Furazabol dissolved or suspended in cottonseed oil was administered orally to male rats. As a standard ^{14}C-furazabol (20.4 μCi/mg, 2.5 mg/ml-solution) was given in a dose of 12.5 mg/kg to each of 27 rats. Four or five rats of this group were killed after 2, 6, 16, 24, 48 and 72 hr for the study of distribution in the tissues.[6] Total faeces and urine collected during these periods were 166 g (435 μCi) and 124 ml (164 μCi) respectively. The TLC of the urine excreted for 16 hr is illustrated in Fig. 3 and Table II.

For preliminary experiments, a moderate dose of ^{14}C-furazabol (300 mg/kg; 0.55 μCi/mg, 60 mg/ml-suspension) was administered orally to each of 14 rats. The faeces and the urine were collected for eight days, and 279 g of the faeces (249.5 μCi) and 476 ml of the urine (26 μCi) were collected.

For the preparative separation of the metabolites, a dose of 500 mg/kg of furazabol without radioactivity (50 mg/ml-suspension) was given orally to each of 44 rats. The faeces and urine were collected for 4 days, amounting to 737 g and 1130 ml respectively.

Similarly, ^{14}C-furazabol in sesame oil (25 mg/ml; 16.9 μCi/mg) was given subcutaneously in a dose of 10 mg/kg to each of 27 rats and every three rats were killed after 1, 2, 3, 4, 8, 16, 48 and 72 hr for the study of distribution whilest the faeces and urine were collected. Total 63.5 g (86.0 μCi) of faeces and 220 ml (77.8 μCi) of urine were obtained.

Extraction and Concentration of Faecal Metabolites of Orally Given Furazabol——Radioactive faeces were dried at 50—60°, pulverized and extracted in portions in a Soxhlet apparatus with $CHCl_3$ for 20 hr and then EtOH for 20 hr. The average yield of radioactive extracts was 80%, amounting totally 40 g, 609.1 μCi. A mixture of extracts (6.5 g, 91.2 μCi) was partitioned three times between 70% MeOH and hexane (each 100 ml×1, 50 ml×2). From the methanolic solution a concentrated metabolite mixture (2.43 g, 82.6 μCi) was obtained in a radiochemical yield of 90.6%. In other experiments the yields varied in the range of 75—95%. For the TLC of the mixture see Fig. 1 and Table I.

The faeces without the label (total 737 g) was extracted twice with EtOH (2 liters×2) at room temperature for 2 days, giving 40.6 g of the extracts. Partition of the extracts as described above afforded the concentrates without the label (14.4 g).

Chromatography of the Faecal Metabolites of Orally Given Furazabol——According to the results of preliminary experiments with the labelled materials, non-labelled materials were separated as follows. The concentrated mixture (14.4 g) was dissolved in $CHCl_3$–MeOH and mixed with silica gel (30 g). The solvent was removed and the residue was placed on the top of a column prepared from $CHCl_3$ and silica gel (100 g). Elution with $CHCl_3$ (1100 ml), $CHCl_3$–MeOH (9:1, 1200 ml), $CHCl_3$–MeOH (1:1, 1000 ml) and MeOH (600 ml) gave, respectively, fraction A (6.67 g), fraction B (3.64 g), fraction C (3.38 g) and fraction D (0.51 g). Fraction C and D have not been examined in detail (chromatography-1).

Fraction A was rechromatographed over silica gel (180 g) and separated into 76 fractions of 100 ml each as shown in Table III (chromatography-2). Fraction No. 16—17 contained metabolite I and from fraction No. 22—23 was obtained cholesterol (10.0 mg), mp 145—147°, identical with an authentic sample (mixed mp and IR). Fraction No. 24—28 contained compound II, fraction No. 60—64 contained metabolite III and fraction No. 65—71 contained metabolite III, IV, V, VI and VII.

Table III. Chromatography-2

Fraction No.	Eluent	Weight (mg)
1— 4	light petroleum–benzene (3:7)	59
5— 9	benzene	57
10—14	benzene–ether (95:2)	2
15	benzene–ether (95:5)	26
16—17	benzene–ether (95:5)	70
18—19	benzene–ether (95:5)	70
20—21	benzene–ether (95:5)	69
22—23	benzene–ether (95:5)	70
24—38	benzene–ether (95:5)	2097
39—44	benzene–ether (95:5)	154
45—54	benzene–ether (9:1)	194
55—59	benzene–ether (9:1)	418
60—61	benzene–ether (9:1)	172
62—64	benzene–ether (9:1)	218
65—71	benzene–ether (9:1)	247
72—76	MeOH	2060

Fraction B of chromatography-1 and fraction No. 72—76 were combined and rechromatographed on silica gel (160 g) (chromatography-3; Table IV). Fractions of 100 ml each were collected.

Metabolite I (17α-Methyl-5α-androstano[2,3-*c*]furazan-18,17β-lactone; I)——Crystallization of fraction no. 16 and 17 from MeOH and then from acetone separated metabolite I (1.0 mg) as needles, mp 155—156°. UV λ_{max}: 217 mμ, inflexions at 220, 225 and 234 mμ. IR ν_{max} cm^{-1}: 1785 (lactone), 1492, 1208, 1003, 860, 840, 805, 770, 753. This was identified with I by comparison of the TLC, GLC, mass spectrum and IR spectrum of the respective sample (see Table V, VI and VII).

Table IV. Chromatography-3

Fraction No.	Eluent	Weight (mg)
77— 84	$CHCl_3$	34
85— 89	$CHCl_3$–MeOH (199:1)	trace
90— 96	$CHCl_3$–MeOH (99:1)	56
97—101	$CHCl_3$–MeOH (98:2)	201
102—115	$CHCl_3$–MeOH (97:3)	1091
116—120	$CHCl_3$–MeOH (97:3)	188
121—128	$CHCl_3$–MeOH (95:5)	340
129—135	$CHCl_3$–MeOH (95:5)	194
136—140	$CHCl_3$–MeOH (9:1)	336
141—145	MeOH	2102

To a solution of metabolite I (0.03 mg) in MeOH (1.0 ml) was added a 5% aqueous NaOH solution (1 drop) and the mixture was refluxed for 1 hr. The reaction mixture was acidified with 5% HCl and extracted with AcOEt. The AcOEt solution was washed with H_2O, dried and concentrated. The residue showed in TLC identical *Rf*-value with that of metabolite VII (solvent A, $Rf=0.28$; $CHCl_3$–MeOH–AcOH (28:1:1), $Rf=0.31$). In GLC its RRT 0.38 was identical with that of metabolite VII (see Table VII).

Compound II (Furazabol; IIa)——The solid (2.097 g) of fraction no. 24—38 of chromatography-2 was washed with hexane and crystallized from ether–hexane to yield needles of compound II in two crops (1.42 g and 0.31 g). Recrystallization from aqueous MeOH afforded a pure sample identical with furazabol, mp and mixed mp 154—155°. UV λ_{max}: 217 mμ (ε 4300), inflexions at 220, 225 and 234 mμ. IR ν_{max} cm^{-1}: 3430, 1590, 1500, 1430, 1208, 1006, 934, 885, 876, 768, 740. *Anal.* Calcd. for $C_{20}H_{30}O_2N_2$: C, 72.69; H, 9.15; N, 8.48. Found: C, 72.69; H, 9.07; N, 8.53. The IR spectrum of compound II was identical with that of furazabol.

Metabolite III Acetate (16β-Acetoxy-17β-hydroxy-17α-methyl-5α-androstano[2,3-*c*]furazan; IIIa)——To fraction No. 60 and 61 (172 mg) of chromatography-2 was added benzene and the insoluble materials (3 mg) were removed by filtration. The filtrate was evaporated, and the residue combined with fraction No. 62—64 (218 mg), was acetylated with Ac_2O (0.5 ml) and pyridine (1 ml) at room temperature overnight. The product was chromatographed on silica gel (17 g). The column was eluted with a) benzene (250 ml), benzene–ether (98:2; 250 ml) and b) benzene–ether (95:5; 960 ml). Crystallization of fraction b) from MeOH afforded metabolite III acetate as prisms (25 mg) identical with IIIa, mp and mixed mp 219—221°. UV λ_{max}: 217 mμ (ε 5200), inflexions at 220, 225 and 234 mμ. IR ν_{max} cm^{-1}: 3440, 1710, 1494, 1268, 1008, 878, 769, 738. NMR δ_{ppm}: 0.78 (3H, s, 19-CH_3), 0.90 (3H, s, 18-CH_3), 1.24 (3H, s, 17α-CH_3), 2.09 (3H, s, CH_3 of acetoxyl), 4.69 (1H, quartet, $J=5$ and 9 Hz, 16α-H). *Anal.* Calcd. for $C_{22}H_{32}O_4N_2$: C, 68.01; H, 8.03; N, 7.21. Found: C, 67.75; H, 8.08; N, 7.25. The IR spectrum of metabolite III acetate was identical with that of IIIa. For further identification see Table VI and VII.

Metabolite III (16β,17β-Dihydroxy-17α-methyl-5α-androstano[2,3-*c*]furazan; III)——To the above acetate (5.0 mg) in MeOH (3.0 ml) was added 5% NaOH (5 drops) and the solution was refluxed for 1 hr. The product was crystallized from aqueous MeOH to give metabolite III as plates (4.0 mg), identical with III, mp and mixed mp 221—223°. UV λ_{max}: 217 mμ, inflexions at 220, 225 and 234 mμ. IR ν_{max} cm^{-1}: 3430, 3350, 1485, 1000, 873, 768, 732. The IR spectrum was identical with that of an authentic specimen.

Metabolite III Acetonide——A solution of metabolite III (1.0 mg) in acetone (2 ml) and 70% $HClO_4$ (1 drop) was kept at room temperature for 40 hr. Chromatography of the product in benzene solution over silica gel (0.50 g), followed by crystallization from MeOH afforded the acetonide (0.94 mg), mp 236—238°, identical with 16β,17β-dihydroxy-17α-methyl-5α-androstano[2,3-*c*]furazan 16,17-acetonide (IIIb). Identification was performed by comparison of TLC, GLC and mass spectrum (see Table V, VI and VII).

Hydrolysis of the acetonide (0.02 mg) in a mixture of 90% MeOH (1 ml) and 35% HCl (1 drop) at room temperature overnight regenerated metabolite III identified by comparison of TLC.

Metabolite IV (17β,18-Dihydroxy-17α-methyl-5α-androstano[2,3-*c*]furazan; IV) and Separation of Metabolite V Acetate Fraction——Fraction No. 65—71 (247 mg) of chromatography-2 was acetylated with Ac_2O (0.5 ml) and pyridine (2 ml) at room temperature overnight. The product was chromatographed on silica gel (8 g) and eluted with a) benzene (300 ml), b) benzene–ether (98:2; 400 ml), c) benzene–ether (95:5; 150 ml) and d) benzene–ether (95:5, 250 ml; 9:1, 350 ml; 1:1, 200 ml) and MeOH (100 ml). TLC indicated the presence of metabolite V acetate in fraction b), and of metabolite III acetate and metabolite IV acetate in fraction c). Evaporation of solution b) afforded crude metabolite V acetate (20 mg), TLC: solvent B, $Rf=$ 0.20 (main), 0.12, 0.27 and 0.32.

Solution c) was concentrated and the residue was mixed with MeOH to separate crystals (4 mg), mp 188—209° which was shown to be a mixture by TLC and removed by filtration. The filtrate was concentrated and the residual oil (13 mg) was hydrolyzed in a mixture of MeOH (2 ml) and 5% NaOH (2 drops) at reflux

temperature for 1 hr. The hydrolyzed product was dissolved in acetone (1ml) and 70% $HClO_4$ (1 drop) was added. The solution was kept at room temperature for 24 hr. The product was chromatographed over silica gel. The fraction eluted with benzene contained metabolite III acetonide (2 mg) and fraction eluted with benzene–ether (1:1) afforded metabolite IV as an oil (5.0 mg), UV λ_{max}: 217 mμ with inflexions at 220, 225 and 234 mμ. This was identified with the 17β,18-dihydroxy compound (IV) by comparison of TLC and GLC of the respective sample (*cf.* Table VI and VII).

Oxidation of oily metabolite IV (0.05 mg) in a mixture of AcOH (1.0 ml) and a 0.2% aqueous CrO_3 solution (0.1 ml) at room temperature for 2 hr and separation of the reaction product by means of TLC showed the presence of two compounds corresponding to metabolite I and metabolite VII, TLC: solvent B, Rf=0.60 and 0.04; $CHCl_3$–AcOH (29:1), Rf=0.60 and 0.21 (*cf.* Table VI). GLC (1% SE-30) of the mixture exhibited the peak at RRT 0.38 (see Table VII).

Metabolite VI Acetate and Separation of Metabolite V Acetate Fraction——Fraction No. 97—101 (201 mg) and No. 102—110 (879 mg) of chromatography-3 were combined, dissolved in benzene and adsorbed on a column of silica gel (30 g). The chromatogram was eluted with benzene (300 ml), benzene–AcOEt (9:1, 400 ml; 8:2, 800 ml; 7:3, 1050 ml; 6:4, 400 ml), AcOEt (150 ml) and MeOH (100 ml). The fractions which were eluted with benzene–AcOEt (8:2 and 7:3) and which revealed the Rf value of 0.44 on TLC (solvent B) were collected. The combined material (147 mg) was subjected to preparative TLC on 4 plates (20×20 cm) coated with Kiesel Gel HF_{254} (Merck). The plates were developed with solvent B by descending method for 5 hr and the main band was extracted with MeOH to give an oil (61 mg). This was acetylated with Ac_2O and pyridine at room temperature overnight. The product (65 mg) in benzene solution was chromatographed on silica gel (4 g) and eluted with benzene (100 ml), benzene–ether (98:2, 150 ml; 95:5, 450 ml; 9:1, 200 ml) and MeOH (50 ml). The fraction eluted with benzene–ether (98:2) was crystallized from MeOH to give metabolite VI acetate (2.0 mg), mp 191—194° (decomp.). UV λ_{max}: 213—217 mμ. IRν_{max} cm^{-1}: 1814 (lactone), 1738 (acetate), 1490, 1220, 1008, 780, 759 (furazan). NMR δ_{ppm}: 1.00 (19-CH_3), 1.49 (17α-CH_3), 2.08 (AcO), 2.13 (AcO), 2.29 (doublet, J=17 Hz, 1α-H), 3.25 (doublet, J=17Hz, 1β-H) and 6.32 (doublet, J=5 Hz, 4α-H). The NMR spectrum was taken in a solution (1 mg/0.3 ml) of $CDCl_3$ by use of a computer of averaged transients and chemical shifts were approximated to the nearest ppm. Mass Spectrum *m/e* (relative abundance; assignment): 416 (3.8; M-CH_2CO), 354 (100; M-CH_3COOH-CO_2); see Fig. 2. This compound was assigned as 4β,16ξ-diacetoxy-17α-methyl-5α-androstano[2,3-*c*]furazan-18,17β-lactone.

The fraction eluted with benzene–ether (95:5) gave crude metabolite V acetate as an oil (23 mg), TLC: solvent B, Rf=0.20 (main) and others (minor).

Metabolite VII (17β-Hydroxy-17α-methyl-5α-androstano[2,3-*c*]furazan-18-oic Acid; VII)——Fraction no. 116—228 of chromatography-3 were combined (528 mg), dissolved in AcOEt (20 ml) and extracted successively with 5% $NaHCO_3$ (10 ml×3), 5% Na_2CO_3 (10 ml×3) and 2% NaOH (10 ml×3). Concentration of the AcOEt solution gave neutral substance (260 mg) which was reserved for separation of metabolite V (see below). Acidification of each aqueous solution with 5% HCl, followed by extraction with AcOEt afforded acidic materials (92 mg from the $NaHCO_3$ solution, 61 mg from the Na_2CO_3 solution and 12 mg from the NaOH solution), which were crystallized from aqueous MeOH to separate metabolite VII as plates (25 mg), mp 200—205° (decomp.). Recrystallization gave a pure sample, mp 217—218° (decomp.). UV λ_{max}: 217 mμ (ε 4800), inflexions at 220, 225 and 234 mμ. IR ν_{max} cm^{-1}: 3430, 2650, 1708, 1615, 1492, 1002, 945, 870, 768, 740. NMR δ_{ppm}: 0.68 (19-CH_3), 1.32 (17α-CH_3), 5.27 (OH, COOH). Metabolite VII was identical with VII in IR, NMR and mass spectra (see Table V).

Methylation of metabolite VII in ether with diazomethane and crystallization of the product from aqueous MeOH furnished the methyl ester, mp 164—165°. IR ν_{max} cm^{-1}: 3495, 3460, 3400, 1724, 1490, 1006, 877, 766, 747.

Metabolite V (17β-Hydroxy-17α-hydroxymethyl-5α-androstano[2,3-*c*]furazan; V), Its Acetate (Va) and Acetonide (Vb)——The neutral substances (260 mg) described in the preceding paragraph were acetylated with Ac_2O (0.5 ml) and pyridine (3 ml) at room temperature for 18 hr. The acetate in benzene solution was chromatographed on silica gel (10 g). The column was eluted with benzene (120 ml), benzene–ether (98:2, 240 ml; 95:5, 400 ml; 9:1, 120 ml) and MeOH (60 ml). The fractions which were eluted with benzene–ether (98:2 and 95:5) and which exhibited the TLC Rf-value of 0.20 (solvent B) were collected to give an oily material (60 mg). This was subjected to preparative TLC (Kiesel Gel HF_{254}) and developed twice with solvent B. The band of Rf 0.30—0.40 was collected and extracted with MeOH. The extract was dissolved in benzene, insoluble materials were removed, and the solvent was evaporated to give crude metabolite V acetate as an oil (21 mg). This and other lots of metabolite V acetate described above gave the same Rf-value in TLC (*cf.* Table VI).

The oily acetate (5.0 mg) was hydrolyzed in a mixture of 5% NaOH (3 drops) and MeOH (2 ml) at reflux temperature for 1 hr. To the mixture AcOEt (10 ml) was added and the solution was washed with H_2O, dried and concentrated to give crude metabolite V as an oil. The main peak of GLC of the trimethylsilyl derivative of this material had identical retention time with that of an authentic sample of V (Table VII). The oil was dissolved in acetone (3 ml), 70% $HClO_4$ (2 drops) was added and the mixture was kept at room temperature for 16 hr. Benzene (10 ml) was added and the solution was washed with 5% $NaHCO_3$ and H_2O, dried and evaporated. The residue was chromatographed over silica gel (1 g) and eluted with light

petroleum–benzene (1:1; 50 ml) and benzene (50 ml). The fraction obtained from the latter eluent was crystallized twice from aqueous MeOH to separate needles of metabolite V acetonide (0.35 mg), identical with an authentic sample of Vb in TLC, GLC and mass spectra (see Table V, VI and VII).

Crude metabolite V acetate (0.05 mg) was converted into metabolite V as described above. This was dissolved in AcOH (1.0 ml), a 0.2% aqueous CrO_3 solution (0.1 ml) was added and the mixture was kept at room temperature for 2 hr. The product, isolated by means of AcOEt, was identical with 17-oxo-5α-androstano[2,3-*c*]furazan in TLC (solvent B, $Rf=0.45$) and GLC (1% SE-30, RRT=0.89).

Separation of Faecal Metabolites of Subcutaneously Given Furazabol——Similarly as described in the case of oral administration, the pulverized faeces (total, 6.38 g; 86.0 μCi) were extracted with EtOH at room temperature overnight and the extraction was repeated. The filtered solution was evaporated and the residue (81.0 μCi) was partitioned between 70% MeOH and hexane (1:1). The hexane phase (3.7 μCi) was removed and the methanolic phase (77.2 μCi) was concentrated to give a metabolite mixture, the TLC of which is shown in Table I. The mixture (78.5 μCi) dissolved in AcOEt was extracted with 5% $NaHCO_3$ to separate the neutral and the acidic materials. Evaporation of the AcOEt solution gave the neutral materials (34.9 μCi) which still contained some acidic materials. The aqueous solution was acidified with 10% HCl and extracted with AcOEt to give the acidic materials (18.6 μCi). Extremely polar materials (19.5 μCi) remained in the aqueous solution.

The neutral materials (20.1 μCi) were separated by preparative TLC on 4 plates (20×20 cm: Kiesel Gel HF_{254}, Merck) with solvent A. Eight radioactive bands appeared and each band was scraped and extracted to give the radioactive material as follows: band 1 (the most non-polar band), 0.31 μCi; band 2, 2.14 μCi; band 3, 2.51 μCi; band 4, 2.64 μCi; band 5, 2.78 μCi; band 6, 1.87 μCi; band 7 and 8 (combined), 3.35 μCi, and the compounds at the starting line, 0.41 μCi. In a similar manner the acidic materials (4.23 μCi) were separated to give one radioactive band (band 9), 3.31 μCi and the compounds at the starting line of TLC, 0.53 μCi.

For identification by TLC, the metabolites of each band and the corresponding authentic sample were developed on the same plate, the former being detected by the radioactivity and the latter by colouration with 1% vanilin in H_3PO_4–EtOH (1:1). For the *Rf*-values see Table VI.

The metabolite of band 1 was identical with the lactone (I) in TLC with solvent A, solvent B or cyclohexane–ether (1:1) ($Rf=0.52$). The material of band 1 was hydrolyzed as described for metabolite I and the product was identified with the 18-oic acid (VII) by TLC using solvent A or $CHCl_3$–MeOH–AcOH (27:2:1) ($Rf=0.56$).

Band 2 material was identified with furazabol (IIa) by the isotope dilution method as follows. A portion of the material (0.707 μCi) mixed with IIa (112.6 mg) was crystallized from ether–hexane to give crystals, mp 151.5—153° with specific activity of 4.31×10^{-3} μCi/mg. The second crystallization from aqueous MeOH gave crystals, mp 152—153°; specific activity, 3.53×10^{-3} μCi/mg and the third crystallization from the same solvent gave the unchanged crystals, mp 152—153°; specific activity, 3.54×10^{-3} μCi/mg.

The material of band 3 showed, in TLC with solvent A, a streak indicative of III and IV and with solvent B, one spot corresponding to III and IV (both the same *Rf*-value). After acetylation as described for metabolite III acetate, the product was identified as a mixture of the 16β-acetate (IIIa) and the 18-ol acetate (IV acetate) by TLC developed twice with solvent B (IIIa, $Rf=0.51$; IV acetate, $Rf=0.35$) or developed firstly with benzene–MeOH (14:1) and secondly with solvent B (IIIa, $Rf=0.49$; IV acetate, $Rf=0.37$). After acetonide formation, the product exhibited newly a spot with the same *Rf*-value as that of IIIb in TLC developed with solvent B or cyclohexane–ether (3:1) ($Rf=0.35$).

The material of band 4 was proved to be a mixture of the 17α-hydroxymethyl compound (V) and the 4β,16ξ-dihydroxy-18,17β-lactone (VI) in the TLC developed with solvent A or with AcOEt-ether (1:1) (V, $Rf=0.55$; VI, $Rf=0.60$). The acetylated product was identified as a mixture of Va and VIa in the TLC developed with solvent A or benzene–MeOH (14:1) (Va, $Rf=0.44$; VIa, $Rf=0.54$).

The metabolites of band 5 and of band 9 were identical with the 18-oic acid (VII) in TLC developed with solvent A, solvent B or $CHCl_3$–MeOH–AcOH (27:2:1) ($Rf=0.56$). After methylation with diazomethane, the product was identified with the methyl 18-oate (VIIa) by TLC developed with solvent A ($Rf=0.67$) or solvent B ($Rf=0.25$).

The metabolites of band 6, 7 and 8 were not identified.

Biliary Metabolites——a) The standard dose administered by way of duodenum to each of two rats whose bile duct was cannulated. The bile was collected for 6 hr and portions were used for determination of radioactivity. The combined bile was diluted with H_2O to 10 ml (2.0×10^5 dpm), acidified with 10% HCl and extracted with AcOEt to give the extract in a radiochemical yield of 88.9%. A portion of the extract was solvolyzed in a 0.1 N solution of $HClO_4$ prepared from 70% $HClO_4$ and tetrahydrofuran at 50° for 11.5 hr, and the solvolyzed product was isolated by means of AcOEt. This was hydrolyzed in a 5% solution of NaOH in 80% MeOH at 50° for 4 hr and after acidification with 10% HCl the hydrolyzed product was extracted with AcOEt.

In another experiment, the extract was first hydrolyzed with 15% aqueous NaOH at 100° for 12 hr and then solvolyzed as described above at 50° for 7 hr. The patterns of TLC of the extract, solvolyzed product and hydrolyzed product are shown in Fig. 3.

From another portion of the extract, the less polar radioactive material was separated by preparative TLC developed with solvent A, and identified as the lactone (I) in a similar manner as described for faecal metabolites of subcutaneously given furazabol. This material was hydrolyzed with 5% NaOH in MeOH at reflux temperature for 1 hr, and the hydrolyzed product was identified with the 18-oic acid (VII) in TLC.

b) After subcutaneous administration of ^{14}C-furazabol, the bile was collected similarly for 24 hr. The extract of the bile gave the same pattern of TLC as that described above.

Separation of Urinary Metabolites——a) After oral administration of furazabol, the radioactive urine (600 ml; 190 μCi) was concentrated to 280 ml and extracted 3 times with ether using a continuous extractor. The extract (extract 1; 422 mg, 124.7 μCi) corresponded to the free steroid fraction and 65.5% of the total

Table V. Comparison of the Mass Spectrum of the Metabolite or Its Derivative with that of the Reference Compound

Peak (*m/e*)	Compound											
	Metab. I	I	Metab. III acetate	IIIa	Metab. III acetonide	IIIb	Metab. V acetonide	Vb	Metab. VII	VII	Metab. VII Me-ester	VIIa
	Relative abundance (%)											
388			2.2	2.3								
386					100	100	27.6	50.2				
374											3.0	3.0
373			1.4	1.3								
371					17.6	15.3	13.8	24.8				
370			1.3	1.3								
359											0.6	0.4
356											0.6	0.5
346			74.4	78.6								
343					2.6	2.5						
342									1.5	1.3	1.6	1.6
329					2.3	2.3	3.5	6.0				
328			31.1	36.3	1.6	1.8						
327									0.7	0.6	0.8	0.8
325					2.8	2.8						
324									1.0	0.7	1.3	1.0
314	3.3	3.1							100	100	100	100
313			26.9	13.4								
311					27.0	30.9	33.0	55.6				
310			17.8	15.9								
303			18.7	14.7							37.1	29.9
301					0.7	1.0						
299					1.5	1.8						
298	100	100			0.7	1.4	3.4	3.8	15.4	15.1	7.2	5.1
297											11.1	8.7
295			8.7	7.4	1.2	1.3	4.3	5.7				
289									14.9	14.3		
287							7.0	6.7				
285			19.4	18.1	1.4	1.5						
284									23.6	23.4	20.6	19.9
283	61.8	60.5			1.1	1.4					3.4	2.5
273			13.0	14.2			3.6	4.3				
271									8.4	10.9	5.7	5.1
270			49.5	46.1	4.7	5.1	8.2	11.9				
258			30.4	33.6	5.7	7.7						
257					7.6	9.2	2.6	4.3				
256	3.8	2.3							10.7	10.1	10.2	10.0
255					3.4	4.8	6.5	9.5				
243			51.7	46.5	24.2	33.8	3.4	4.3				
161	9.5	3.4	21.4	26.9	5.9	10.2	12.1	15.5	3.0	3.7	2.8	2.9
149	35.6	14.2	5.4	10.8	4.0	2.2	100	100	8.8	9.9	6.6	3.5
71			24.1	15.1	5.3	15.5	29.3	27.1	12.7	8.3	5.7	6.7
43	9.5	3.7	100	100	8.1	50.5	36.3	58.6	25.8	27.5	26.7	52.3

urinary radioactivity. The aqueous solution was adjusted to pH 7.0, to which β-glucuronidase (bacterial powder, Sigma; 12500 units) was added, and the mixture was incubated at 37° for 39 hr. The mixture was extracted twice with AcOEt for 18 hr in a continuous extractor. The extract (extract 2; 38.5 μCi, 20.3%) corresponded to the steroids of the glucuronide fraction. The remaining aqueous solution was adjusted to pH 1, and after addition of $(NH_4)_2SO_4$ (0.6 g/ml) the mixture was extracted with an equal volume of tetrahydrofuran. To the tetrahydrofuran solution was added 70% $HClO_4$ to make a 0.01N $HClO_4$ solution and the solution was heated at 50° for 3 hr. After neutralization with an aqueous NaOH solution the mixture was extracted with AcOEt continuously for 18 hr to give the extract (extract 3; 6.5 μCi, 3.4%) corresponding to the steroids of the sulfate fraction. The radioactivity of the remaining aqueous solution was 12.9 μCi (6.8%). The patterns of TLC of extract 1, 2 and 3 were, respectively, similar to that of the original urine.

Table VI. *Rf* values of TLC of Faecal Metabolites and Reference Compounds

Metabolite (Reference compound)	*Rf* value	
	Solvent A[a]	Solvent B[b]
Metabolite I (I)	0.75	0.61
Compound II (IIa)	0.72	0.36
Metabolite III (III)	0.60	0.13
-acetate (IIIa)	—	0.34
-acetaonide (IIIb)	—	0.54
Metabolite IV (IV)	0.63	0.13
Metabolite V (V)	0.53	0.09
-acetonide (Vb)	—	0.57
Metabolite VII (VII)	0.28	0.04
-methyl ester (VIIa)	—	0.21

Standard=furazabol
a) solvent A: $CHCl_3$–MeOH (9:1), *b*) solvent B: benzene–AcOEt (4:1)

Table VII. Results of GLC Analysis of Faecal Metabolites and Reference Compounds (furazabol=1.00)

Compound	RRT			
		1% SE-30 (TMS)	(TFA)	1% OV-17 (TMS)
Metabolite I	0.38			
17α-Methylandrostano[2,3-*c*]furazan-18,17β-lactone (I)	0.37[a]			
Metabolite III		1.29	1.21	1.48
-acetonide	1.55			
16β,17β-Dihydroxy-17α-methylandrostano[2,3-*c*]furzan (III)		1.29	1.20	1.48
-acetonide (IIIb)	1.55			
Metabolite IV		1.49	1.40	1.19
17β,18-Dihydroxy-17α-methylandrostano[2,3-*c*]furazan (IV)		1.51	1.40	1.19
Metabolite V		1.37	2.03	1.08
-acetonide	1.60			
17β-Hydroxy-17α-hydroxymethylandrostano[2,3-*c*]furazan (V)		1.38	2.02	1.10
-acetonide (Vb)	1.60			
Metabolite VII	0.38	1.87		
17β-Hydroxy-17α-methylandrostano-[2,3-*c*]furazan-18-oic acid (VII)	0.38[a]	1.87		

a) The same RRT of I and VII indicated that the RRT corresponds to an identical degradation product.

The urine (1130 ml) without radioactivity was separated as above giving the free steroid fraction (1.021 g) and the fraction from the glucuronides (0.702 g). Adsorption chromatography and preparative TLC failed to give any crystalline or homogeneous metabolite; however following fractions were obtained: a) an oil corresponding to peak A of TLC shown in Fig. 4 (11 mg), b) a mixture of peak B and C (2 mg) and c) a mixture of peak A, B and C (25 mg). Each fraction revealed qualitatively in UV spectrum a maximum at 217 mμ with inflexions at 220, 225 and 234 mμ.

b) After subcutaneous administration of furazabol, the urine was concentrated and subjected to TLC whose pattern was similar to that in the case of oral administration of furazabol. The metabolites have not been examined in detail.

Acknowledgement The authors thank Dr. T. Ishiguro, the president of the Company, and Dr. M. Shimizu, the director of the Laboratories, for their interest and encouragement. The facilities given by Dr. M. Hasegawa of Daiichi Pure Chemicals Co., Ltd. for work with radioisotopic materials are gratefully acknowledged.