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ANDROGEN METABOLISM IN THE BEAGLE: ENDOGENOUS $C_{19}O_2$ METABOLITES IN BILE AND FAECES AND THE EFFECT OF AMPICILLIN ADMINISTRATION

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SUMMARY

In gall bladder bile from 6 male beagles the total $C_{19}O_2$ steroid concentration, as measured by mass fragmentography, varied from 3.1 to 5.4 mg/l. More than 95% of the metabolites were present as glucuronide conjugates with the remainder as monosulphates. In the glucuronide fraction, androsterone, aetiocholanolone epiandrosterone, dehydroepiandrosterone, 5β-androstane-3α,17β-diol, 5α-androstane-3β,17α-diol, 5α-androstane-3β,17β-diol and 5-androstene-3β,17β-diol were identified. Quantitatively, the 3α,5β and 3β,5α isomers predominated. In the monosulphate fraction the unsaturated metabolites, 5-androstene-3α,17β-diol, 5-androstene-3β,17α-diol and 5-androstene-3β,17β-diol were relatively more abundant and androsterone and aetiocholanolone were the predominant oxosteroids.

The faecal excretion of these steroids in a male beagle, measured over two consecutive 24 h periods, was 157.3 and 194.2 μg/24 h. More than 92% of the steroids were unconjugated. Both the qualitative and quantitative pattern of the $C_{19}O_2$ steroids in the unconjugated and glucuronide fractions from faeces were very similar to those found in the biliary glucuronides. All the steroids found as monosulphates in bile were also detected in the monosulphate fraction of faeces. On the second day of a 3 day course of oral ampicillin administration the faecal excretion of $C_{19}O_2$ glucuronides was increased from 10.5, under control conditions, to 313.7 μg/24 h. The concentration of unconjugated steroids was unchanged. On the following day the concentration of steroid glucuronides returned to normal but the amounts of unconjugated $C_{19}O_2$ metabolites were significantly increased. Ampicillin had no major effect on the relative distribution of steroids measured in either the glucuronide or unconjugated steroid fraction.

These results suggest that the biliary–faecal axis is a major excretory route for androgen metabolites in the male beagle. In addition, the effects of ampicillin administration suggest that intestinal microflora play a significant role in the hydrolysis of the biliary steroid glucuronides.

INTRODUCTION

When testosterone was administered as a single intravenous injection or by intraduodenal instillation to dogs androgenic material could be detected in fistula bile for up to 72 h [1]. However, no systematic study of the androgen metabolites in body fluids and excreta of dogs has been carried out. Colorimetric assay of 17-oxosteroids in dog urine gave values of about 1 mg/24 h [2–4]. The plasma concentrations of testosterone, androstenedione and 5α-dihydrotestosterone (mean values: 115, 66 and 34 ng/100 ml, respectively) are lower in male dogs than in men. These findings suggest that testosterone secretion or metabolism and excretion are different in male dogs and men. In the present study we have determined $C_{19}O_2$ oxosteroids and androstane- and androstenediols in bile and faeces from male beagles using a mass fragmentographic method. The effect of oral ampicillin administration on the faecal steroids was also studied. The results, along with preliminary indications that urinary androgen excretion in the beagle is very low, suggest that the biliary–faecal axis is an important route of endogenous $C_{19}O_2$ steroid excretion in the beagle and that considerable intestinal reabsorption of biliary androgen metabolites takes place.

MATERIALS AND METHODS

Chemicals. All chemicals were reagent grade. Solvents were redistilled in all glass apparatus before use. Lipidex ™5000 was obtained from Packard Instrument International S.A., Zurich, Switzerland, Sephadex LH-20 from Pharmacia, Uppsala, Sweden and Adsorbocil from Applied Science Laboratories Inc., P.O. Box 440/State College, PA, U.S.A. Amberlite XAD-2 was obtained from B.D.H., Poole, England and used as previously described [6].

Steroids. Most of the reference steroids were purchased from Ikapharm, Ramat-Gan, Israel. Dr. D. N. Kirk, Steroid Reference Collection, London, England kindly provided samples of 5α-androstane-3β,17α-diol and 5-androstene-3β,17α-diol. [1,2,6,7(*n*)-^{3}H]-Testosterone (S.A., 80-110Ci/mmol) was purchased from the Radiochemical Centre, Amer-

sham, England and [1,2,-^{3}H]-testosterone glucuronide (S.A., 25 Ci/mmol) from New England Nuclear, Boston, MA, U.S.A. [1,2,6,7(n)-^{3}H]-Testosterone sulphate was synthesized by the procedure of Levitz[7], and purified by repeated chromatography on 4 g columns of Sephadex LH-20 using chloroform–methanol (1:1, v/v), containing 0.01 mol/l NaCl [8].

Experimental animals. Gall bladder bile was collected from 6 adult male beagles (23–29 months old; 11.4–14.2 kg body weight) maintained at the Department of Experimental Toxicology, Schering AG., Berlin. The dogs were fasted for 20 h and then killed by the administration of Epivan®, intravenously. The bile (12.0–20.7 ml) was collected directly by cutting the gall bladder. The samples were frozen and transported on dry ice to Helsinki, where they were stored at −18° until analysed. Complete 24 h collections of faeces were made from a young adult male beagle over a seven day period. On the third, fourth and fifth day he was given 2 × 500 mg doses of ampicillin (Penbritin Powder, Beechams, Dublin) orally in water. The daily faeces collections were frozen and stored at −18° until analyzed.

PROCEDURE

Extraction, separation of conjugates and steroid conjugate hydrolysis. Gall bladder bile samples, 2.5 ml, were treated overnight with 10 vol. of acetone–ethanol (1:1, v/v) at 39° [9]. The daily faecal samples for the two control days and the second and third days of ampicillin administration were extracted exactly as described by Laatikainen and Vihko[10]. The dry residue obtained was suspended in methanol–water (7:3, v/v) and the suspension left at −18° overnight. The sample was then centrifuged at 1000 *g* for 30 min at −20°, and the resultant supernatant evaporated to dryness.

To monitor the efficiency of the extraction procedures small amounts (50–100,000 c.p.m.) of [^{3}H]-testosterone, [^{3}H]-testosterone glucuronide and [^{3}H]-testosterone sulphate were added to the bile samples before processing and to the faecal extracts before Sephadex LH-20 chromatography. The recovery of added radioactivity was determined prior to silicic acid (or Lipidex) chromatography, by counting an aliquot of each fraction in a Wallac-LKB liquid scintillation counter, with external standardisation correction.

The entire bile extracts and 5% of each faecal extract were applied to 20 g columns of Sephadex LH-20 in 3- and 2-ml portions of chloroform–methanol (1:1, v/v, 0.01 mol/l NaCl) which was also used as eluting solvent [9]. Fractions of unconjugated + glucuronide, monosulphate and disulphated steroids were collected [11]. The unconjugated steroids and steroid glucuronides were then separated by solvent partition between ethyl acetate and 8.4% sodium bicarbonate [12]. The steroid glucuronides were hydrolysed using Ketodase [11] and the mono- and disulphates solvolyzed [8].

Batch separation of steroids. All steroid extracts were then subjected to chromatography on 200 mg columns of silicic acid (adsorbocil) [13] or on columns (100 × 3 mm) of Lipidex ™5000 and fractions containing the 17-oxosteroids and androstene- and androstanediols collected. The Lipidex ™5000 was prepared for use and the columns packed as described in [14]. The extracts were applied in petroleum ether (b.p. 40–60): chloroform (95:5, v/v) and the columns eluted step-wise with 4 ml of the same solvent, 4 ml of petroleum ether–chloroform (90:10, v/v) (elutes oxosteroids) and 4 ml of petroleum ether–chloroform (60:40, v/v) (elutes androstane- and androstenediols). These fractions were then evaporated to dryness, the appropriate amount of internal standard(s) for mass fragmentography added (see below) and trimethylsilyl (TMSi) ethers prepared.

Mass fragmentography was carried out on an LKB 9000 gas chromatograph–mass spectrometer fitted with an accelerating voltage alternator; the signals from two of the three available fragment ion monitoring channels were fed to separate single pen electrical recorders [15]. The gas chromatographic column contained 3% OV-210 on Gas-Chrom Q (column length: 2.7 m, column oven temperature: 220° (isothermal; for oxosteroid determinations) and 189 or 200° (isothermal, for androstane- and androstenediol determinations), flash heater: 248° and separator: 250°). The energy of the bombarding electrons was 22.5 eV.

In order to *identify* the steroids in the androstane- and androstenediol fractions four m/e values which were characteristic and abundant in their mass spectra were monitored during GC–MS analysis. Routinely, the molecular ion (M^+) and fragments M-15, M-90 and M-(90 + 15) were monitored in these identification studies. An established mass fragmentographic method [15, 16] was used for the quantitative analysis of the oxosteroids, thus no further identification studies were made.

For the *quantitative* estimation of the various steroids identified, it was first established that the groups of analytical samples did not have significant peaks at the t_r* values of the internal standards used under normal analysis conditions. For the quantitation of the oxosteroids two internal standards were added: 5α-dihydrotestosterone (m/e value monitored: 347) and testosterone (m/e value monitored: 360). For the androstene- and androstanediols, a single standard: 5α-androstane-3β,11β,17β-triol was used (m/e values monitored: 421 and 434).

Peak areas were measured and the amount of steroid calculated by comparing the ratio of peak areas of analytical:internal standard to the peak areas

* The abbreviation t_r is used to denote relative retention time. All t_r values are expressed relative to 5α-cholestane (t_r, 1.00).

Table 1. Androstene- and androstanediol metabolites in bile and faeces of the male beagle

Steroids	t_r values			Steroid conjugate fraction†	
	Bile	Faeces	Reference standard	Bile	Faeces
5-androstene-3α,17β-diol	0.44	0.43	(0.43)*	MoS‡	Mos§
5-androstene-3β,17α-diol	0.49	0.50	0.49(0.48)	MoS	MoS§
5-androstene-3β,17β-diol	0.59	0.58	0.59(0.57)	G,MoS	U§,G§,MoS§
5α-androstane-3α,17β-diol**		0.45	(0.45)		U¶,G¶
5β-androstane-3α,17β-diol	0.48	0.47	0.49(0.47)	G	U¶,G¶
5α-androstane-3β,17α-diol	0.54	0.53	0.51(0.51)	G,MoS	U‖,G¶,MoS¶
5α-androstane-3β,17β-diol	0.64	0.62	0.64(0.62)	G,MoS	U¶,G¶,MoS¶

* The t_r values given in parenthesis are those reported by Thompson Jr. *et al.* [39] for a QF-I Column. † The abbreviations used are: U, unconjugated; G, glucuronide and MoS, monosulphate. ‡ In the case of the bile samples the presence of the steroid peak was monitored at four appropriate *m/e* values: the molecular ion (M^+) and fragments M-15, M-90 and M-(90 + 15). § Steroid peaks detected at one *m/e* value only: M^+. ‖ Steroid peaks detected at two *m/e* values only: M^+, M-90. ¶ Steroid peaks detected at three *m/e* values: M^+, M-90, M-(90 + 15). ** 5β-androstane-3β,17β-diol has the same retention time as this compound on QF-I liquid phase [18] and is thus not separated from it under the conditions used here.

of appropriate standard:internal standard. In the case of the steroids for which it was not possible to prepare a standard solution quantitation was based on a closely related standard.

RESULTS

The recovery of [^{3}H]-testosterone glucuronide and [^{3}H]-testosterone sulphate added to the bile and faeces samples was 67–93 and 42–55% (bile) and 34–67 and about 16% (faeces), respectively. No attempt was made to quantitate the faecal steroid sulphates. The recovery of [^{3}H]-testosterone added to the faeces samples was 87–96%. The results, however, have not been corrected for recovery on the basis of these radioactivity measurements.

The androstene- and androstanediol metabolites identified in the various fractions of beagle bile and faeces are listed in Table 1 together with the gas chromatographic t_r data and the conjugate fractions in which they were found. Identification is based on the criteria outlined in the Materials and Methods section. However, because of the small amounts of androstenediols in the faeces samples, identification studies were not attempted; in addition, the presence of large interfering peaks hampered some of the faecal steroid identification studies at the lower *m/e* values (Table 1). No steroid could be identified in the unconjugated or disulphate fractions of beagle bile. The disulphate fractions from the faeces samples were not analysed. The fragment ion chromatograms on which the identification of three androstanediols in the glucuronide fraction of beagle bile is based are shown in Fig. 1.

There was little qualitative difference between the $C_{19}O_2$ steroids found in the bile and faeces. Androsterone, aetiocholanolone, epiandrosterone and dehydroepiandrosterone were found in the glucuronide and monosulphate fractions from bile and in

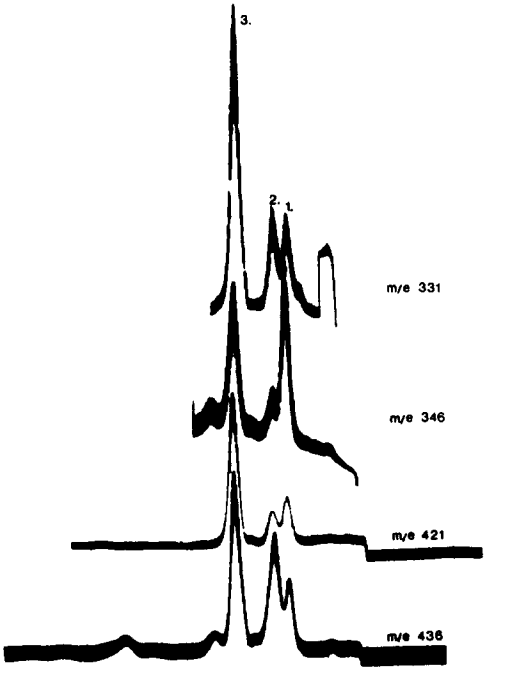

Fig. 1. The mass fragmentograms (fragment ion chromatograms) on which the identification of three androstanediols in the glucuronide fraction of beagle bile is based. The androstane- and androstenediol fraction from beagle bile, animal 1 (glucuronides) (Table 2) after TMSi ether formation (see Procedure) was subjected to mass fragmentography and the ions *m/e* 436, 421, 346 and 331 monitored; these ions correspond to the molecular ion (M^+), M-15, M-90 and M-(90 + 15) of an androstanediol-*bis* TMSi derivative. The three compounds identified are: 1. 5β-androstane-3α,17β-diol-*bis* TMSi ether (t_r, 0.48), 2. 5α-androstane-3β,17α-diol-*bis* TMSi ether (t_r, 0.54) and 3. 5α-androstane-3β,17β-diol-*bis* TMSi ether (t_r, 0.64). The column used contained 3% OV-210. The column oven temperature was 200°, flash heater, 248°, separator 250° and the energy of the bombarding electrons 22.5 eV. The t_r values are expressed relative to 5α-cholestane (t_r, 1.00). The chromatograms run from right to left.

the unconjugated and glucuronide conjugated fractions from faeces. Epiandrosterone was the only oxosteroid that was consistently detected in the monosulphate fraction from faeces. The androstanediols and 5-androstene-3β,17β-diol identified as glucuronides in bile were all found in the unconjugated and glucuronide fractions of faeces. 5α-Androstane-3α,17β-diol was the only metabolite detected in faeces which was not also identified in bile. It is possible that the 5α-androstane-3α,17β-diol peak also contains some 5β-androstane-3β,17β-diol since these compounds are not separated on QF-1 [9]. All five androstene- and androstanediols identified in the monosulphate fraction of bile were also detected as monosulphates in faeces (Table 1).

Examples of typical single ion chromatograms at *m/e* values 347, 360, 421 and 434 on which the quantitative analyses are based are shown in Figs. 2 and 3. The concentrations of $C_{19}O_2$ steroids found in the glucuronide and monosulphate fractions of bile from

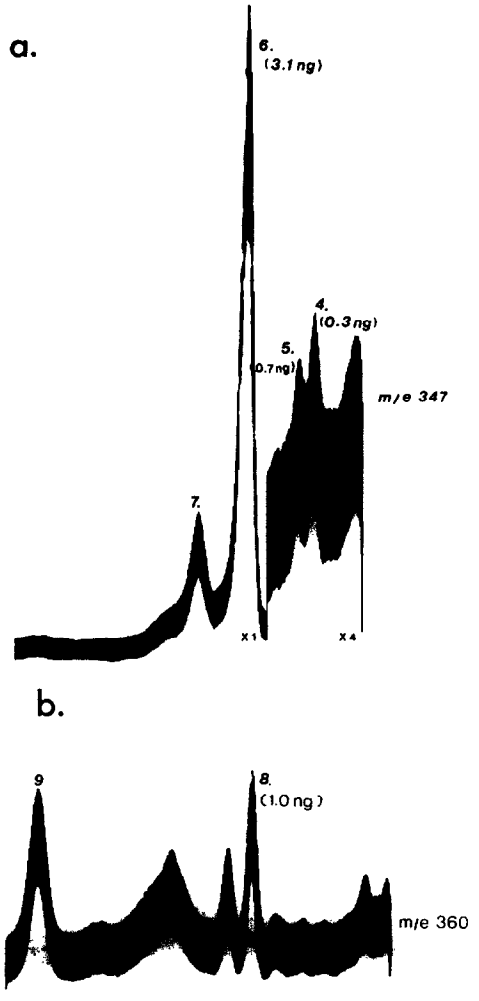

Fig. 2. Typical mass fragmentograms of oxosteroid fractions on which quantitation is based. (a) Ring A and B saturated oxosteroids from faeces, Day 2 (unconjugated steroids) (Table 3); derivative, TMSi ether; ion monitored, *m/e* 347 (M-15); steroids: 4, androsterone-TMSi ether, 5, aetiocholanolone-TMSi ether, 6, epiandrosterone-TMSi ether and 7, 5α-dihydrotestosterone (internal standard). (b) Ring A or B unsaturated oxosteroids from bile (glucuronides), beagle 2 (Table 2); derivative, TMSi ether; ion monitored, *m/e* 360 (M^+); steroids: 8, dehydroepiandrosterone and 9, testosterone (internal standard). Gas chromatography–mass spectrometry conditions were as in Fig. 1 except that the column oven temperature was 220°. The quantities of steroid in each analytical peak is shown. Where recorder gain has been changed during a run, the relative sensitivity is indicated for each section at the bottom of the chromatogram.

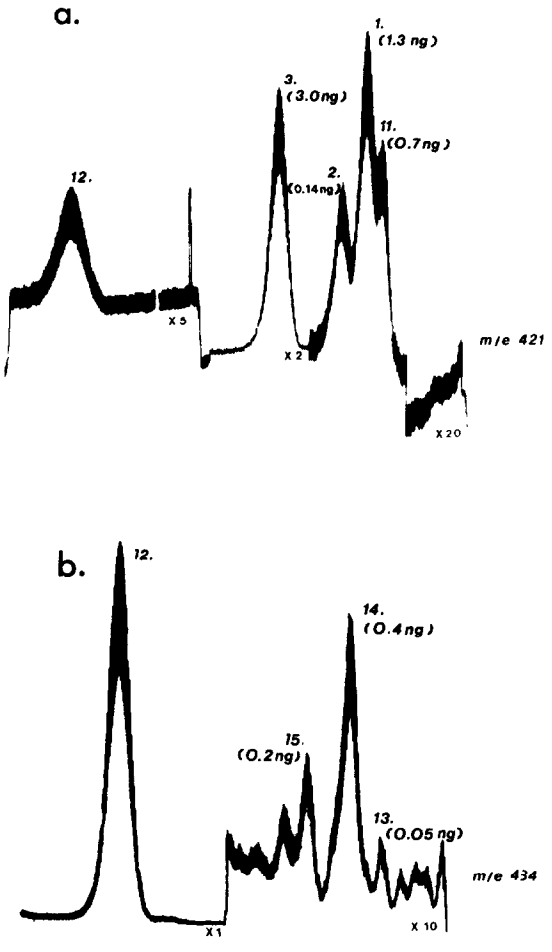

Fig. 3. Typical mass fragmentograms of androstane- and androstenediol fractions on which quantitation is based. (a) Androstanediols from faeces, Day 2 (unconjugated steroids) (Table 3); derivative, *bis*TMSi ether; ion monitored, *m/e* 421 (M-15); steroids: 11, 5α-androstane-3α,17β-diol-*bis*TMSi, 1–3, see Fig. 1. 12, 5α-androstane-3β,11β,17β-triol-*bis*TMSi (internal standard). (b) Androstenediols from bile (monosulphates), beagle 2 (Table 2); derivative, *bis*TMSi ether; ion monitored, *m/e* 434(M^+); steroids: 13, 5-androstene-3α,17β-diol-*bis*TMSi ether, 14, 5-androstene-3β,17α-diol-*bis*TMSi ether, 15, 5-androstene-3β,17β-diol-*bis*TMSi ether, 12, see (a). Gas chromatography–mass spectrometry conditions were as in Fig. 1 except that the column temperature was 189° (a) and 200° (b). The quantities of steroid in each analytical peak is shown. Where recorder gain has been changed during a run, the relative sensitivity is indicated for each section at the bottom of the chromatogram.

Table 2. Concentration of $C_{19}O_2$ steroids (μg/l) in the glucuronide and monosulphate fraction of beagle bile

	Animal					
	1	2	3	4	5	6
Glucuronides						
Androsterone	125	137	87	45	34	49
Aetiocholanolone	529	659	696	145	297	503
Epiandrosterone	583	1430	837	778	599	895
Dehydroepiandrosterone	91	263	198	272	80	316
5β-androstane-3α,17β-diol	1283	1203	1626	2214	1061	1066
5α-androstane-3β,17α-diol*	184	90	129	136	50	162
5α-androstane-3β,17β-diol	1040	1334	1068	1318	561	773
5-androstene-3β,17β-diol	143	173	212	331	39	238
Monosulphates						
Androsterone	28	45	27	27	37	—
Aetiocholanolone	15	27	12	12	49	—
Epiandrosterone	13	23	7	7	10	—
Dehydroepiandrosterone	10	15	10	7	5	—
5α-androstane-3β,17α-diol*	9	4	3	4	4	22
5α-androstane-3β,17α-diol	2	4	1	2	3	2
5-androstene-3α,17β-diol†	0.7	0.5	0.3	0.4	0.4	—
5-androstene-3β,17α-diol†	3	4	2	3	2	—
5-androstene-3β,17β-diol	0.5	2	0.6	1	1	—
Total conjugates	3979	5441	4916	5304	3051	4026

* The quantitation of this steroid is based on the 5α-androstane-3β,17β-diol standard. † The quantitation of this steroid is based on the 5-androstene-3β,17β-diol standard.

six beagles are given in Table 2. The total $C_{19}O_2$ steroid content measured, uncorrected for methodological losses, varied from 3.1 to 5.4 mg/l of which more than 95% was present in the glucuronide fraction. In the glucuronide fraction there were appreciably more androstene- and androstanediols than $C_{19}O_2$ 17-oxosteroids. Saturated 3β,5α and 3α,5β isomers were present in highest concentrations, with unsaturated metabolites accounting for less than 7.5% of the total steroids. In the monosulphate fraction unsaturated metabolites accounted for a greater proportion (~15%) of total metabolites and androsterone and aetiocholanolane were the predominant oxosteroids.

The concentration of $C_{19}O_2$ steroids in faeces from a male beagle before and during ampicillin administration are shown in Table 3. Metabolites were quantitated in the unconjugated and glucuronide fractions. The total excretion measured on the control days, uncorrected for methodological losses, was 157.3 and 194 μg/24 h. Of this more than 92% was present as unconjugated steroids. The relative quantitative distribution pattern of the various oxosteroid and androstene- and androstanediols was similar in both the unconjugated and glucuronide fractions (Table 3) and reflected quite closely that found in the biliary glucuronides (Table 2).

The faecal metabolites were also analysed on the second and third days of a three day course of oral ampicillin administration (days 4* and 5*, Table 3). These two days were chosen on the basis of our previous studies on the effect of this antibiotic on urinary and faecal steroid excretion in pregnant human subjects [11, 17].

On day 4, the second day of ampicillin administration total steroid excretion was increased more than two fold. The increase occurred entirely in the glucuronide fraction with the values rising from a mean of 10.5, on the control days, to 313.7 μg/24 h. The steroid concentration and the relative distribution pattern in the unconjugated fraction was similar to that found under control conditions (Table 3,2). The quantitative steroid pattern in the glucuronide fraction on day 4 was very similar to that found in the biliary glucuronides (Table 2) excepting the aetiocholanolone level which remained unexpectedly low (Table 3).

On day 5, the final day of ampicillin administration, total $C_{19}O_2$ steroid excretion was also increased (329 μg/24 h) but not as dramatically as on day 4 (Table 3). However, the increase was confined almost totally to the unconjugated steroid fraction. The concentration of glucuronides had almost returned to normal. The relative amounts of the various oxosteroids and androstane- and androstenediols in both the unconjugated and glucuronide fraction were similar to that seen under control conditions.

The quantitative data described above are based on single mass fragmentographic determinations of each analytical sample with the standard being determined every fifth or sixth injection. The coefficient of variation for the various standards (in 0.4–30 ng amounts) assayed 3–8 times during each batch of analyses (not necessarily on the same day) averaged

Table 3. Concentration of $C_{19}O_2$ steroids (μg/24 h) in the unconjugated and glucuronide fractions of beagle faeces before and during ampicillin administration. Days 1 and 2 are control days and days 4* and 5* the second and third days of ampicillin (1 g per day per os) administration

	Day			
	1	2	4*	5*
Unconjugated				
Androsterone	1.5	5.3	5.5	12.9
Aetiocholanolone	13.9	14.7	4.9	24.6
Epiandrosterone	30.9	61.4	77.4	110.4
Dehydroepiandrosterone†	—	—	—	—
5α-androstane-3α,17β-diol‡	14.3	14.8	12.8	24.1
5β-androstane-3α,17β-diol	29.7	25.9	27.6	38.9
5α-androstane-3β,17α-diol§	3.7	2.7	5.6	6.6
5α-androstane-3β,17β-diol	48.5	59.0	38.0	85.2
5-androstene-3β,17β-diol	2.1	2.3	2.4	3.2
Total	144.6	186.1	174.2	305.9
Glucuronides				
Androsterone	0.6	0.7	3.4	1.5
Aetiocholanolone	1.4	1.4	10.6	3.6
Epiandrosterone	3.9	2.4	137.5	6.6
Dehydroepiandrosterone	1.9	—	3.5	1.3
5α-androstane-3α,17β-diol‡	0.4	0.4	1.6	0.2
5β-androstane-3α,17β-diol	1.6	0.7	59.7	3.1
5α-androstane-3β,17α-diol§	0.2	0.1	7.4	0.5
5α-androstane-3β,17β-diol	2.7	2.4	86.8	6.1
5-androstene-3β,17β-diol	—	—	3.2	—
Total	12.7	8.1	313.7	22.9
Total steroids	157.3	194.2	487.9	328.8

† Large interfering peaks prevented quantitation of this steroid. ‡ The quantitation of this steroid is based on the 5α-androstane-3β,17β-diol standard. § The quantitation of this steroid is based on the 5α-androstane-3β,17β-diol standard.

14.5%. This is similar to the values found for oestrogens in this laboratory and would be difficult to improve on with our present instrumentation [18]. While the precision of the analytical determinations may have been inferior to this, it is felt that it was adequate for obtaining an estimate of the biliary and faecal concentrations of this group of compounds and of the quantitative relationships between the compounds within the group.

DISCUSSION

The pattern of $C_{19}O_2$ steroids found in male beagle bile (Tables 1, 2) has two very striking features: firstly, the predominance of glucuronide conjugates and secondly, the predominance of 5β,3α- and 5α,3β-isomers within the glucuronide fraction. Available data on other species suggest that this dominance of glucuronide conjugation in the male beagle may be unique: in the rabbit and cat, for instance, only half the metabolites found after [^{14}C]-testosterone administration were glucuronides [19, 20]. In bile from human males, androstane- and androstenediols are excreted mainly as disulphates with smaller but similar concentrations as monosulphates and glucuronides [21] while only 8–25% of administered [^{14}C]-corticosterone, [^{14}C]-pregnenolone and [^{14}C]-pregnenolone sulphate were found in the glucuronide fraction of rat bile [22].

Available evidence from studies in man and rat suggests that the distribution pattern of the various $C_{19}O_2$ steroids in bile and faeces is determined to a large extent by 1. the relative activity and specificity of the hepatic steroid reductases and hydroxysteroid dehydrogenases, 2. the relative activity and specificity of the hepatic uridine diphosphate glucuronyl (UDPG) transferase and sulphokinase systems, 3. the nature of the steroid conjugates secreted in the bile and 4. the activity of the intestinal bacterial hydroxysteroid dehydrogenases. For instance, Schriefers [23] deduced from liver perfusion studies in male rats, using testosterone as substrate, that equal amounts of 5β- and 5α-reduced metabolites would be formed with the 5β-compounds being exclusively metabolized to 5β,3α-products and the 5α-compounds being preferentially metabolized to 5α,3β-products. In contrast, female rats produced 5α,3α-metabolites almost exclusively. There is also a good deal of evidence to suggest that the UPDG transferase and sulphokinase systems show preference for conjugating steroids with different Ring A structures: In humans, 5β,3α-steroids such as 5β-pregnane-3α,20α-diol and aetiocholanolone (3α-hydroxy-5β-androstan-17-one) are found in bile [21] and urine [24–26] principally as glucuronides while 3β,5-ene-steroids, in bile [21] and urine [26, 27], and 5α,3α-steroids, in bile [21] are predominantly sulphated. Examination of the steroid content of pooled faeces from male and female germfree rats

showed a preponderance of sulphate conjugated 3α,5α-, 3β,5α- and 3β,5-ene steroids [28]; the steroids identified in faeces from conventional rats were unconjugated or sulphated and had the same structures [29, 30]. These findings led Gustafsson, Eriksson and Sjövall [28–30] to suggest that the lack of 5β steroids may be related to a very low level of steroid glucuronides in the faeces of these rats. Steroids with a 3β,5α-structure were much more abundant in faeces from conventional than from germfree rats [28–30]. This is indicative of 3α,5α → 3β,5α conversion by intestinal bacteria in the conventional rats. Our present results (Tables 2, 3) suggest that the conjugating systems in the male beagle may also show a preference for different isomers. Thus, the predominance of the *trans*-isomers (5β,3α and 5α,3β) seen in the glucuronide fraction in bile is not reflected in the sulphates. Again, the preference of the UDPG transferase system for 5β steroids would seem to extend to the beagle. However, unlike the rat (see above, 28–30) the formation of 3β,5α-steroids in the beagle (Tables 2, 3) would seem to take place in the liver rather than in the intestine.

In the ampicillin experiment, under control conditions almost all the androgen metabolites detected in the faeces of the male beagle were unconjugated. With the exception of the presence of 5α-androstane-3α,17β-diol, the pattern of metabolites in both the unconjugated and glucuronide fractions of faeces was very similar to that found in the glucuronide fraction of bile (Tables 2, 3). All the compounds found in the monosulphate fraction of faeces were also present as monosulphates in bile (Table 1). It would thus seem that the major metabolic occurrence in the passage of the biliary androgens through the intestinal tract is their deglucuronidation. Hydrolysis of steroid glucuronide and sulphate conjugates in the intestinal tract of both rats [31, 32] and humans [17, 33] has been amply demonstrated. One of the major probable consequences of steroid conjugate hydrolysis in the intestinal tract is the facilitation of the reabsorption of biliary steroids [34]. The changes in the pattern of faecal androgen excretion which were observed during oral administration of ampicillin (Table 3) (discussed below) suggest that a significant quantity of the biliary metabolites is reabsorbed from the intestinal tract and undergoes enterohepatic circulation.

Both the administration of antibiotics [17, 33, 35] and the comparison of faecal metabolites in conventional and germ-free strains of animals [31, 32] have been used to assess the contribution of the intestinal microflora to intestinal steroid metabolism. By comparing faecal steroid metabolites in germfree and conventional rats it was demonstrated that the intestinal microflora in the rat could hydrolyse steroid glucuronides and sulphates and catalyze oxido-reduction of 3α-, 3β-, 11α-, 11β- and 17β-hydroxysteroids and 16α- and 21-steroid dehydroxylation (reviewed in 32 and 36).

On the other hand, the administration of ampicillin to pregnant women has been shown to cause transient but extensive faecal loss of conjugated steroids [17, 33] accompanied by reduced plasma levels [37] and urinary excretion of selected steroid metabolites of intestinal origin [11, 38].

On the second day of ampicillin administration to a male beagle (day 4*, Table 3) there was a very significant increase in the total faecal androgens measured, as compared to that found under control conditions. The increase was confined to the steroid glucuronides with the level of unconjugated steroids remaining unchanged. The total faecal androgens measured on the second day of ampicillin administration was 488 μg/24 h which was more than twice the total excretion on either of the control days (157 and 194 μg/24 h, respectively (Table 3)). These results indicate that, under normal conditions, a considerable fraction of the biliary androgens may be reabsorbed during their passage through the intestine. The very large increase in faecal glucuronides observed during ampicillin administration would suggest that hydrolysis of these conjugates is a pre-requisite to their reabsorption and that the intestinal microflora play a significant role in this hydrolysis. From their studies of conventional and germfree rats Eriksson[32] concluded that glucuronidase activity in rat intestine originates from both intestinal micro-organisms and mucosal cells in the intestinal wall and contents [32].

On the third day of ampicillin administration (day 5) faecal androgen metabolite levels were also elevated but glucuronide levels had returned to near normal (Table 3). Previous studies on the effect of ampicillin administration on urinary and faecal steroid metabolites in pregnant women has shown the effect of the antibiotic to be transient and greatest on the second and third day of oral administration [11, 17]. The increase in faecal unconjugated steroids seen on day 5 may possibly be due to hydrolysis of biliary conjugates at a level of the intestine where reabsorption is no longer possible.

The pattern of androgen metabolites in both the unconjugated and glucuronide fractions on both ampicillin treatment days studied was quite similar to that found in faeces and bile under control conditions. This would suggest that no major metabolism of these biliary androgens other than deglucuronidation is catalyzed by the intestinal micro-organisms in the male beagle. Alternatively, the present experimental system is not sensitive enough to detect any other changes which might occur. No explanation can presently be offered for the one difference which was found, *vis.* the very great increase in the epiandrosterone: aetiocholanolone ratio seen in the glucuronide fraction on the second day of ampicillin administration (Table 3).

These studies demonstrate that considerable quantities of androgen metabolites are secreted in bile, as glucuronides, by the male beagle. Although, a considerable portion of these metabolites may be reab-

sorbed, after hydrolysis, faecal excretion is considerable. Preliminary investigation of these metabolites in the urine of male beagles using this technique showed the levels to be extremely low (Martin F., Bhargava A. and Adlercreutz H., unpublished). Thus, the biliary–faecal axis is probably the main route of androgen metabolite excretion in the male beagle.

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