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The in vivo metabolism of Althesin (alphaxalone + alphadolone acetate) in man

J. M. P. HOLLY*, D. J. H. TRAFFORD**, J. W. SEAR† AND H. L. J. MAKIN**

* *Medical Unit and ** Department of Chemical Pathology, London Hospital Medical College, Turner Street, London E1 2AD, and †Department of Anaesthetics, University of Bristol, Bristol Royal Infirmary, Bristol BS2 8HW, U.K.*

Gas chromatography-mass spectrometry has been used to study the metabolism of Althesin (alphaxalone and alphadolone acetate) in man. Two metabolites, 20α-reduced alphaxalone and alphadolone, as well as the two parent steroids, have been detected in the plasma during and after an infusion of Althesin. The main urinary metabolites were alphaxalone and 20α-reduced alphaxalone, with smaller amounts of alphadolone, all of which were excreted mainly as the glucuronide conjugates. No alphadolone acetate was detected in the urine. In 3 patients in whom bile was collected over the 1st 24 h post-operation from an indwelling T-tube, it has not been possible using the methods described to detect in bile the presence of the parent steroids, or any of their probable metabolites.

Althesin (alphaxalone (3α-hydroxy-5α-pregnane-11, 20-dione) and alphadolone acetate (3α-hydroxy-5α-pregnane-11,20-dione-21-acetate) (Glaxo Operations Ltd) may be used as a supplement to nitrous oxide for the maintenance of anaesthesia (Savege et al 1975; Sear & Prys-Roberts 1979a). In healthy man and in animals both repeated incremental doses and infusions of Althesin have been found to have a non-cumulative effect. Thus, complete recovery occurs rapidly on cessation of the infusion (Savege et al 1975; Jago & Restall 1977; Dechêne 1977; Dunn et al 1978).

Previous studies on the kinetics of the elimination of alphaxalone, the major active component of Althesin, have been carried out following a single induction dose of the drug (Dubois et al 1975; Simpson 1978). The relationship between the rate of infusion of Althesin and the plasma alphaxalone concentration has been described, but in those studies, the plasma alphaxalone and alphadolone acetate concentrations were not measured during the decay phase after a continuous infusion, nor were blood, urine or bile examined for the presence of metabolites of the two steroids (Sear & Prys-Roberts, 1979a, b). We now report studies where the plasma, urine and bile of patients who received an infusion of Althesin, has been examined by gas chromatography and gas chromatography–mass spectrometry (g.c.–m.s.) for the presence of metabolites of the two steroids. Plasma from a single patient has also been analysed by mass fragmentography for the presence of the components of Althesin and two metabolites during and after an infusion. On the basis of the results obtained a scheme for the metabolism of the steroids present in Althesin is outlined.

† Correspondence.

METHODS

Patients and materials

Patients (Table 1) undergoing peripheral vascular or abdominal surgery gave their consent to the studies which were approved by the Ethics Committee of the Bristol Royal Infirmary.

After premedication with morphine (10 mg intramuscularly), sleep was induced by the intravenous

Table 1. Patients studied.

Sex	Age (yr)	Wt (kg)	Operation	Total dose Althesin (mg)
Metabolites of Althesin in plasma:				
F	51	64	Mesocaval Anast.	166
F	36	71	Oesophageal Transection	272
F	63	65	Mastectomy	216
F	56	56	Laparotomy	144
M	58	68	Hernia Repair	204
F	63	60	Hemicolectomy	144
F	31	71	Cholecystectomy	181
F	27	58	Mesocaval Anast.	744
Metabolites of Althesin in urine:				
F	45	84	Paracetamol overdose	1320
M	44	77	Aorto-iliac graft	216
M	76	75	Aorto-iliac graft	228
M	57	65	Ilioprofundoplasty	122
F	64	64	Laparotomy for jaundice	340
F	51	66	Mesocaval Anast.	286
M	49	72	Ilio-femoral graft	268
M	61	72	Ilioprofundoplasty	113
M	57	75	Aorto-iliac graft	146
Metabolites of Althesin in bile:				
F	64	64	Laparotomy for jaundice	340
M	73	87	Cholecystectomy	240
F	48	68	Cholecystectomy	180

administration of Althesin (24–36 mg total steroid). Intubation was achieved after neuromuscular paralysis with pancuronium 0·1 mg kg^{-1}, and anaesthesia maintained with N_2O (66%) in oxygen supplemented by an infusion of Althesin (12 mg ml^{-1} total steroid) administered into a peripheral vein by an Injectomat automatic syringe pump (Barclay, UK). The rate of administration varied between 1 and 2 × the *minimum infusion rate* (Sear & Prys-Roberts 1979a); that is between 13·5 and 27·0 μg kg^{-1} min^{-1} alphaxalone. The patients were artificially ventilated using the Oxford Penlon ventilator, with an inspired nitrous oxide concentration of 66% in oxygen, and a tidal volume of 10 ml kg^{-1}. The fresh gas flow to the ventilator was sufficient to maintain normocapnia (P_{CO_2} 4·8–5·7 kPa). Additional analgesia was provided by the synthetic narcotic, fentanyl (5–10 μg kg^{-1}) when clinically indicated.

Peripheral, central venous (antecubital) and arterial (brachial) cannulae were inserted using local anaesthesia (lignocaine 2%) before induction of anaesthesia. The blood pressure, heart rate and electrocardiogram were continuously recorded throughout surgery.

Blood samples were taken from the arterial cannula for estimation of the plasma concentrations of alphaxalone, alphadolone acetate and their metabolites. The samples were collected at approximately 30 min intervals during the infusion, and at 2, 5, 10, 15, 30, 60 and 120 min after its cessation. Plasma was separated by centrifugation and stored at −20 °C until analysed.

In 3 patients, the biliary excretion of Althesin was studied. These patients received an infusion of Althesin to supplement nitrous oxide/oxygen anaesthesia for surgery of the biliary tract. A 24 h collection of bile was made from an indwelling T-tube placed in the common bile duct at the time of surgery. The bile was stored at 4 °C until analysed.

In eight patients undergoing surgery, and in one patient receiving an infusion of Althesin to provide sedation in the Intensive Therapy Unit, a 24 h collection of urine was made, starting at the time the infusion began. The urine samples were stored at −20 °C. No additives or preservatives were used.

All reagents used, and details of mass spectrometer settings, unless otherwise specified, were as described by Sear et al (1980).

Measurement of alphaxalone, alphadolone acetate and their metabolites in plasma

Plasma (1 ml) was acidified with 200 μl of 5% (v/v) hydrochloric acid (BDH), and extracted with 4 ml of redistilled ether after the addition of accurate amounts of pregnenolone (pregn-5-en-3β-ol-20-one) as an internal standard (ca 1 μg). The extracts were evaporated to dryness at 40 °C under a gentle stream of nitrogen. Trimethylsilyl ethers were formed by heating with 100 μl *N,O*-bis-trimethylsilyltrifluroacetamide (BSTFA) for 15 min at 50 °C. Each extract was analysed by g.c.–m.s. monitoring of the molecular ion of each compound. The mass spectrometer was focussed on the molecular ion of pregnenolone-3-TMSi (*m/z*: 388) and by varying the accelerating voltage, molecular ion intensities of the other steroids were sequentially monitored (*m/z*: 404 for alphaxalone-3-TMSi; *m/z*: 478 for alphadolone-3,21-di TMSi and reduced alphaxalone-3,20-di TMSi; and *m/z*: 462 for alphadolone acetate-3-TMSi. Dwell time on each mass was 60 ms). Alphadolone acetate-3-TMSi has the same retention time on OV-1 as cholesterol-3-TMSi ether, and it was therefore necessary to use a more polar stationary phase, OV-17, to analyse the extracts for alphadolone acetate-3-TMSi (Sear et al 1980). Standard curves were prepared, using fixed amounts of pregnenolone and increasing quantities of standard steroids when available. Trimethylsilyl ether derivatives were formed and g.c.–m.s. was carried out. The amounts of steroids present in the plasma samples were obtained by comparison with the standard curves. Only alphaxalone and alphadolone acetate could be quantified in this way as pure standards for the other steroids were not available.

Identification of metabolites in plasma, urine and bile

Pooled plasma (15 ml) from several patients who had received Althesin by infusion was extracted with diethyl ether as described above. Urine and bile (25 ml) from individual patients were extracted as described by Edwards et al (1953) and the extract was separated into three fractions; free (unconjugated), glucuronide and sulphate as described by Edwards (1969). Steroid glucuronides were hydrolysed by overnight incubation at 37 °C with β-glucuronidase (EC 3.2.1.31-*Helix pomatia*; Baylove Chemicals Ltd., Musselburgh, Scotland), and steroid sulphates were solvolysed at pH2 in ethyl acetate (Edwards 1969). Trimethylsilyl ethers (TMSi) were formed with BSTFA (50 °C for 15 min) and each extract was analysed by g.c.–m.s. using both OV-1 and OV-17 columns. A total ion current trace for each of the three fractions was obtained, and the separate peaks scanned at 70 eV. The spectra were compared with the mass spectra obtained from standards run in the same g.c. system.

Reference standards. Pure alphaxalone and alphadolone acetate were available by courtesy of Glaxo Laboratories, Greenford. Alphadolone was prepared in an unpure state by hydrolysis of alphadolone acetate with a cholesterol esterase (EC 3.1.1.13; Sigma Chemicals Co., Poole, Dorset). Alkaline hydrolysis of alphadolone acetate with bicarbonate, commonly used for steroid ester hydrolysis (Edwards 1969), could not be used as isomerization at C17 of the steroid side chain took place.

RESULTS

Normalized mass spectra of the three standard trimethylsilyl ethers (alphaxalone, alphadolone and alphadolone acetate) are: alphaxalone m/z 404 (100), 389 (65), 361 (18), 346 (30), 324 (75), 299 (28), 256 (29); alphadolone m/z 462 (46), 447 (27), 402 (15), 390 (55), 372 (38), 270 (100); alphadolone acetate 492 (3), 477 (52), 387 (33), 361 (15), 271 (100), 256 (33). Since no authentic standards of the possible metabolites of the two steroid components of Althesin were available to us, it was necessary to use ΔRm analysis (Edwards 1969) to predict the likely retention times of these metabolites in the g.l.c. systems used. Table 2 gives an example of the use of ΔRm analysis in the tentative identification of 20α-reduced alphaxalone. The actual retention time of the isolated metabolite is also shown. The only evidence for characterization of the alphaxalone metabolites as the 20α-reduced isomer rather than 20β-reduced isomer is based on ΔRm analysis (Table 2), where the actual retention time is closer to that predicted for 20α-reduced rather than 20β-reduced alphaxalone.

Only the free (or unconjugated) steroids present in plasma were examined since diethyl ether extraction of acidified plasma does not extract steroid glucuronides or sulphates to any significant extent. Fig. 1 shows the results of single ion monitoring (SIM) of an extract of pooled plasma. Three peaks were identified on the basis of their retention times and their mass spectra. The changes in the levels of alphaxalone, alphadolone acetate and their metabolites, alphadolone and 20α-reduced alphaxalone measured during and after infusion in a single patient are illustrated in Fig. 2.

Characterization of metabolite of alphaxalone

To further characterize the 20-reduced alphaxalone found in plasma, a portion of the extracts from pooled plasma was acetylated by treatment with acetic anhydride–pyridine (1 : 1 v/v) at 50 °C for 1 h, and re-chromatographed on OV-1. The acetylated

Table 2 (a). Example of the use of ΔRm analysis in the identification of 20α-reduced alphaxalone as a metabolite of alphaxalone. Predicted Rt values can be calculated by multiplying the Rt of alphaxalone by the appropriate separation factor or by adding the ΔRm factor to the Rm ($\log_{10}$ retention time) of alphaxalone, giving the new Rm (which can then be converted to retention time by taking the antilog).

(b) Separation factors[1] (ΔRm)[2] of several possible metabolic transformations[3] (A → B) of alphaxalone.

(a)	Predicted Rt (min)	Actual Rt (min)
Alphaxalone		5·21
20α-reduced alphaxalone	7·76	7·75
20β-reduced alphaxalone	7·24	—
11-reduced alphaxalone (free)	7·07	—
(TMSi)	7·86	—
11,20α-direduced alphaxalone	11·72	11·69**

(b) A	B	Separation factors[1] (ΔRm)[2]
11-oxo	11β-hydroxyl	1·51 (+0.179)
11-oxo	11β-trimethylsilyl ether	1·36 (+0·133)
20-oxo	20α-trimethylsilyl ether	1·49 (+0·173)
20-oxo	20β-trimethylsilyl ether	1·39 (+0·143)

** This peak had a mass spectrum consistent with the structure of alphadolone.

[1] Separation factor $= \frac{\text{Retention time of Peak B}}{\text{Retention time of Peak A}}$

[2] ΔRm = Rm of Peak B — Rm of Peak A (where Rm is $\log_{10}$ (retention time), if $Rt_A > Rt_B$ then Rm is +ve, if $Rt_A < Rt_B$ then Rm is −ve).

[3] Values given were calculated from duplicate retention times of various steroids run on OV1 at 215 °C.

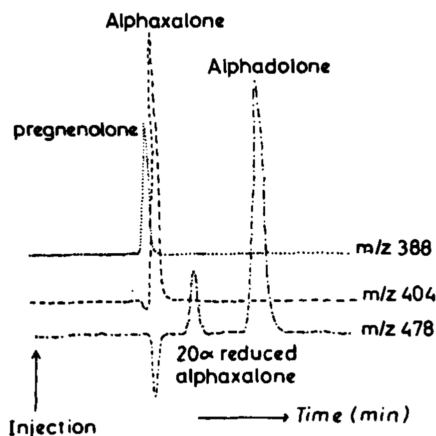

FIG. 1. Mass fragmentogram obtained after extraction of pooled plasma as described in the text. The mass spectrometer was focussed on the molecular ion of pregnenolone-3-trimethylsilyl ether and the ions monitored are indicated in the figure. Trimethylsilyl ethers were prepared and run on OV-1 at 250 °C.

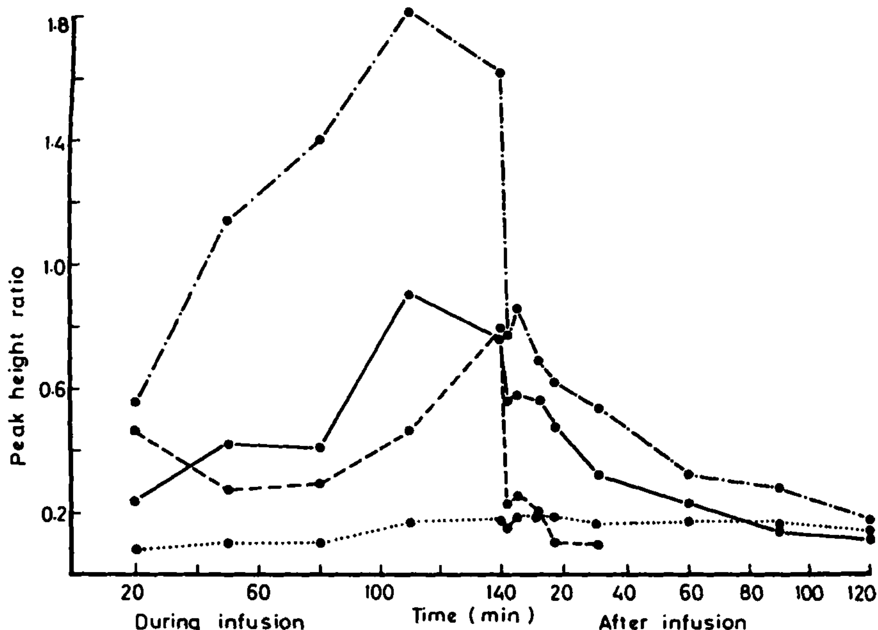

FIG. 2. Concentrations of alphaxalone (●- · - · -●), alphadolone acetate (●—●), alphadolone (●- - -●), and 20-reduced alphaxalone (●·····●) in plasma during and after a constant infusion of Althesin to a single patient. The peak height ratios (peak height of steroid: peak height of internal standard) are recorded. To convert to $\mu g\ ml^{-1}$, the peak height ratio should be multiplied by 0·4 (for alphaxalone) and 0·44 (for alphadolone acetate).

metabolite peak was scanned and the mass spectrum [(m/z) gave: 418 (3), 358 (100), 316 (84), 298 (34)] was consistent with the structure alphaxalone-3,20-diacetate. Urine extracts treated similarly gave a mass spectrum which was exactly the same as that obtained from plasma.

Portions of the extracts from urine and plasma were also treated with methoxyamine hydrochloride (forming an *O*-methyl-oxime derivative) before the formation of trimethylsilyl ethers as described by Sear et al (1980). The relative retention times were again as predicted and mass spectra were obtained during g.c. on OV-1 and OV-17. The mass spectrum of each peak was the same as that obtained from authentic material. The 20-reduced alphaxalone did not form an *O*-methyl oxime derivative, and thus its retention time was unchanged by reaction with methoxyamine hydrochloride.

Urine analysis

24 hr urine collections were made in nine patients. 3 urine extracts (free, glucuronide and sulphate fractions obtained as described above) were analysed by g.c.–m.s., and Fig. 3(a) shows the results from the glucuronide fraction; similar traces were obtained from the free and sulphate fractions, but the concentrations of steroids present were much less than in the glucuronide fraction. It is estimated that the glucuronide fraction in urine accounts for most of the total urinary metabolites of the steroid components of Althesin. Fig. 3(b) shows the same fraction as that analysed in Fig. 3(a), the total ion current (TIC) being monitored. Examination of the TIC establishes that the major metabolite in the urine glucuronide fraction is 20α-reduced alphaxalone.

In some of the other patients, there were greater amounts of alphaxalone and less of its metabolite, 20-reduced alphaxalone—indicating variability in the degree of biotransformation within individuals.

No alphadolone acetate could be detected in any urine samples studied using SIM.

No 11-reduced compounds (derived from alphaxalone or alphadolone acetate) nor 20-reduced alphadolone were detected in any samples analysed. The possibility of metabolites with additional hydroxyl groups was also considered, and the likely mass of the molecular ions calculated. However, no evidence for the presence of these compounds in urine, plasma or bile was found, although our extraction procedure would have extracted them quantitatively from urine and bile, and would have probably detected significant amounts present in plasma.

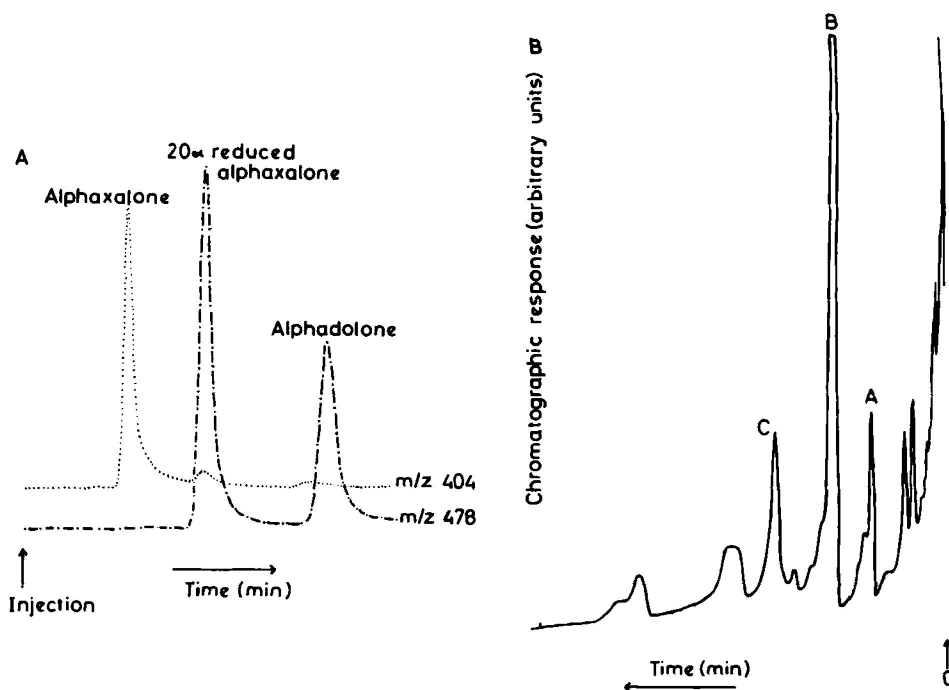

FIG. 3. Mass fragmentogram (a) and Total ion current trace (b) obtained after the extraction of urine as described in the text. The glucuronide fraction of the urine was derivatized to form the trimethylsilyl ethers and run on OV-1 at 250 °C. The peaks are identified as follows: A, alphaxalone. B, 20α-reduced alphaxalone. C, alphadolone.

Bile analysis

In these patients, bile was collected over the immediate 24 h post-operative period, and analysed in a similar fashion to that described for urine. Three fractions (free, glucuronide and sulphate) were examined, but the presence of alphaxalone, alphadolone acetate or their metabolites could not be detected in any of the three fractions. However, cholesterol-3-TMSi was present in significant amounts.

DISCUSSION

Alphaxalone and alphadolone acetate, and their metabolites, are not normal constituents of body fluids. Although Fukushima & Gallagher (1957) reported the isolation of the 5β-isomer of alphaxalone from human urine, this compound proved to be an artifact of their isolation procedure. It was produced from the urinary metabolite 5β-pregnane-3α, 17α, 20α-triol-11-one by the removal of the 17α-hydroxyl group. The presence of steroids identified as described above in plasma and urine may therefore be taken as evidence that they are derived from the administered Althesin steroids and not from de novo synthesis in vivo.

To date, little work has been published on the metabolism of the steroid components of Althesin. This study, while not comprehensive, confirms the previous unpublished findings of Glaxo Research Ltd that the plasma metabolite found in significant quantities after infusion of Althesin is 20α-reduced alphaxalone (presumably mainly derived from alphaxalone). As would be expected, alphaxalone. alphadolone and alphadolone acetate can also be detected in the plasma of patients receiving a continuous infusion of Althesin. However, 20α-reduction of alphadolone probably does not occur to any large degree, as 21-hydroxylated steroids are poor substrates for the 20α-hydroxysteroid dehydrogenase (Gower 1975). 11β-Hydroxysteroid dehydrogenase normally acts on substrates containing unsaturated A rings; for example in the interconversion of cortisone and cortisol (Gower 1975). Thus, significant amounts of 11-reduced metabolites of the steroid components of Althesin would not be expected and were not in fact detected by our procedures. This is perhaps surprising since Nicholas et al (1980) identified 11-reduced metabolites of alphaxalone incubated with rat lung in vitro.

After A ring reduction (Gower 1975; Samuels &

Eik-Nes 1968), most endogenous secreted steroids are conjugated to glucuronic or sulphuric acid and excreted in the urine.

Similarly the most quantitatively important urinary metabolite of alphaxalone following an infusion of Althesin appears to be 20α-reduced alphaxalone glucuronide. In plasma, the unconjugated 20α-reduced alphaxalone is present in low concentrations, and does not increase significantly despite changes in the plasma alphaxalone concentrations. The high metabolic clearance rate (MCR) of glucuronide conjugates, which approximates to the glomerular filtration rate (GFR) may offer an explanation for this.

The absence of either alphaxalone, alphadolone acetate or their metabolites in the bile of the three patients studied was of interest, as Strunin et al (1977) had shown the presence of radioactivity in the bile within 10 min of a single dose of 10–20 μCi of [^{14}C]alphaxalone; although in one of their 5 patients the amount of radioactivity was low. There is no evidence that [^{14}C]alphaxalone labelled in the C21 position will undergo radiolysis or isotopic exchange in the body. However, there are a number of possible explanations for this discrepancy; (a) the radio-labelled alphaxalone used by Strunin et al was not radiochemically pure, (b) the levels of the steroid present in the bile were below the limits of detection by g.c.-m.s. or (c) the extraction procedure used in the present study failed to recover the steroid or its metabolites. In particular the possibility that bile contains hydrophilic metabolites should be considered. A further explanation may be that the patients studied here, all of whom had an elevated plasma alkaline phosphatase, had significant intra-hepatic cholestasis. This might prevent excretion (with the first 24 h post-operation) of all bile other than that already present within the major intra-hepatic and extrahepatic biliary apparatus. However, although significant obstructive jaundice was present in one of the patients, it was *not* seen in the others. Thus it is necessary to conclude that the radioactivity measured by Strunin et al was *not* due to the presence of alphaxalone or 20α-reduced alphaxalone in bile but may be due to other hydrophilic metabolites which we have not measured.

There are no previously published data on the metabolism of alphadolone acetate. The main urinary metabolite is alphadolone (21-hydroxy alphaxalone), and this has less anaesthetic activity than alphaxalone. It might be suggested that the absence of alphadolone acetate in urine could be an artifact produced by the extraction method used, but this is unlikely as alphadolone acetate added to plasma and urine could be recovered by our procedure with no detectable hydrolysis. It is suggested, therefore, that the hydrolysis of the acetate group occurs in vivo before excretion. 21-Dehydroxylation has shown to occur in some steroids excreted in the bile by the action of bacteria in the gastrointestinal tract (Bokkenheuser et al 1977; Dehazya et al 1978; Winter & Bokkenheuser 1978). It is therefore possible that, if alphadolone enters the enterohepatic circulation, it could be transformed into alphaxalone in the gastrointestinal tract. Ethical considerations prevented any studies on the metabolism of alphaxalone and alphadolone acetate separately. There is no evidence that 21-hydroxylation of steroids with saturated A rings occur, and thus there is little likelihood of the conversion of alphaxalone to alphadolone.

The study of the metabolism of alphaxalone and alphadolone acetate described here has shown that these steroids are metabolized by the same pathways as those already described for endogenous steroids (Gower 1975), and a possible scheme for Althesin metabolism is shown in Fig. 4.

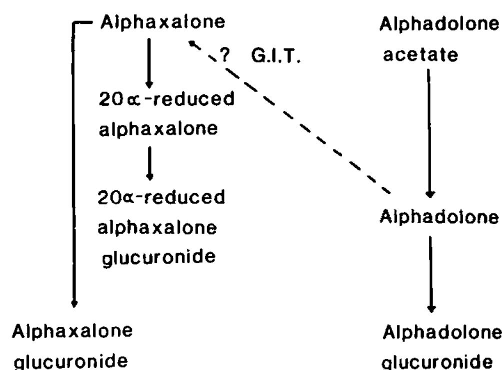

FIG. 4. Proposed scheme for the metabolic degradation of the Althesin steroids in man. Although not detected in the plasma, it is necessary for the glucuronide conjugates of alphaxalone, alphadolone and 20α-reduced alphaxalone to be transported in the blood from the liver to the kidney, from whence their excretion takes place.

A number of metabolic studies have been undertaken in the rat, where the main metabolites of alphaxalone are 2α-hydroxy, 16α-hydroxy and 2α,16-dihydroxy alphaxalone. These steroids have been detected in both the urine and bile following the administration of Althesin. Our studies indicate, therefore, a species difference in the metabolic

products of Althesin in man in comparison with those present in the rat, as well as a difference in the route of elimination of these metabolites.

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