CHROM. 5469

Analytical chemical studies on steroids

Part L. Determination of urinary metabolites of 16α-chloroestrone methyl ether by gas chromatography

In the previous paper of this series[1], the authors reported the isolation and characterization of the urinary metabolites in the rabbit with a large dosage of 16α-chloroestrone methyl ether which is widely used as a lipid-shifting drug[2]. During the continuation of the biochemical studies on the modified steroids[3-6], it has become necessary to determine the excreted amount of these biotransformation products. The present paper describes a method which employs thin-layer chromatography (TLC) as a preliminary purification procedure, followed by trifluoroacetylation of the metabolites derived from 16α-chloroestrone methyl ether and their determination by gas chromatography (GC).

Experimental

Materials. Almost all the samples employed in this work were prepared by the methods established in this laboratory[1].

Preparation of derivatives. Trifluoroacetates (TFA) were prepared by treatment with trifluoroacetic anhydride in tetrahydrofuran at room temperature and trimethylsilyl derivatives (TMS) with hexamethyldisilazane and trimethylchlorosilane in pyridine in the manner described by SWEELEY *et al.*[7].

Gas chromatography. The apparatus used was a Shimadzu Model GC-1C gas chromatograph equipped with a hydrogen flame ionization detector and a U-shaped stainless-steel column (3 mm I.D.). The column was packed with 3% SE-30 on a support of Chromosorb W (60–80 mesh), 5% SE-52 on Shimalite W (60–80 mesh) or 2% OV-17 on Shimalite W (60–80 mesh). The temperatures of column, flash heater and detector were kept at 240°, 250° and 260°, respectively. Nitrogen was used as the carrier gas at a flow rate of 40 ml/min with 4 kg/cm^2 pressure at the inlet.

Administration of 16α-chloroestrone methyl ether and separation of metabolites. A single dose of a suspension of 16α-chloroestrone methyl ether (200 mg) in Tween 80 was given orally to an adult male rabbit (body weight 2.1 kg) through a catheter. Urine was collected in a bottle containing a few drops of toluene as the preservative at 24, 48, 72 and 96 h after administration and was stored in the refrigerator until required for analysis. One-tenth aliquot of the pooled urine was adjusted to pH 5 with diluted H_2SO_4 and then to pH 4.5 with 0.1 *M* acetate buffer (3 ml per 10 ml of urine) and incubated with beef-liver β-glucuronidase (Tokyo Zokikagaku Co.) (300 Fishman units/ml) at 37° for 5 days. The incubated urine specimen was brought to pH 1 with 50% H_2SO_4, saturated with NaCl (2 g/10 ml) and extracted with ethyl acetate (3 × 50 ml). The organic phase was separated and allowed to stand at 37° for 24 h. The extract was washed with 5% $NaHCO_3$, H_2O and dried over anhydrous Na_2SO_4. After evaporation of solvent *in vacuo*, the gummy substance obtained was submitted to TLC for purification.

Purification by thin-layer chromatography. The gummy residue was applied to a Silica Gel HF (E. Merck A.G.) plate (20 × 20 cm) and developed with benzene. Of the expected metabolites, the most polar one, 16,17-epiestriol, remained at the

origin and 16α-chloro-17α-estradiol was most non-polar (R_F 0.30) on the thin-layer plate. The adsorbent of the zone (R_F 0–0.5) corresponding to the metabolites was eluted with acetone (3 × 50 ml). The eluate was again submitted to TLC using ethyl acetate as developing solvent. 16,17-Epiestriol and 16α-chloro-17α-estradiol gave R_F values of 0.60 and 0.94, respectively. The adsorbent of the zone (R_F 0.5–1.0) was eluted with acetone (3 × 50 ml) and the eluate thus obtained was transferred into a test-tube with tetrahydrofuran.

Determination by gas chromatography. To a solution of the metabolites in tetrahydrofuran (0.5 ml) was added trifluoroacetic anhydride (0.1 ml) and the mixture was allowed to stand at room temperature for 20 min. The reaction mixture was evaporated to dryness with the aid of a nitrogen gas stream. To this residue was added a tetrahydrofuran solution (0.5 ml) containing a known amount of 3-deoxyestrone (*ca.* 0.2–0.3 mg), and 1–2 μl of this solution was injected into the gas chromatograph.

Results and discussion

An initial attempt was made to establish the appropriate column conditions for the separation of five kinds of metabolites and unchanged 16α-chloroestrone methyl ether employing the synthetic samples. The retention values of these compounds under a variety of conditions are listed in Table I. When the specimens were transformed into the trimethylsilyl derivatives, satisfactory separation could not be attained on either the SE-30 or the OV-17 column. Therefore trifluoroacetylation was undertaken as pretreatment for GC. All the compounds, except estrone and 16β-

TABLE I

RELATIVE RETENTION TIMES OF STANDARD STEROIDS

Conditions: stainless-steel column (3 mm I.D.); N_2 flow rate, 40 ml/min; column temperature, 240°; flash heater temperature, 250°; detector temperature, 260°.

Compound	*Column*					
	3% SE-30 (1.875 m)		*2% OV-17 (3.375 m)*		*5% SE-52 (4.125 m)*	*5% SE-52 (3.375 m) + 2% OV-17 (4.500 m)*
	TMS	*TFA*	*TMS*	*TFA*	*TFA*	*TFA*
16α-Chloroestrone methyl ether (1)[a]	—	—	—	—	—	1.12
16α-Chloro-17α-estradiol (2)	0.98	0.45	1.01	0.50	0.47	0.47
Estrone (3)	0.66	0.33	0.91	0.50	0.37	0.40
16β-Chloro-17α-estradiol (4)	0.94	0.31	0.81	0.38	0.36	0.35
17α-Estradiol (5)	0.59	0.18	0.62	0.10	0.19	0.20
16,17-Epiestriol (6)	1.08	0.25	0.91	0.10	0.24	0.22
3-Deoxyestrone (I.S.)	—	—	—	—	—	0.31
Cholestane	1.00 (7.36 min)	1.00 (25.12 min)	1.00 (11.24 min)	1.00 (10.36 min)	1.00 (55.48 min)	1.00 (65.24 min)

[a] Numbers in brackets refer to peaks and curves in Figs. 1 and 2.

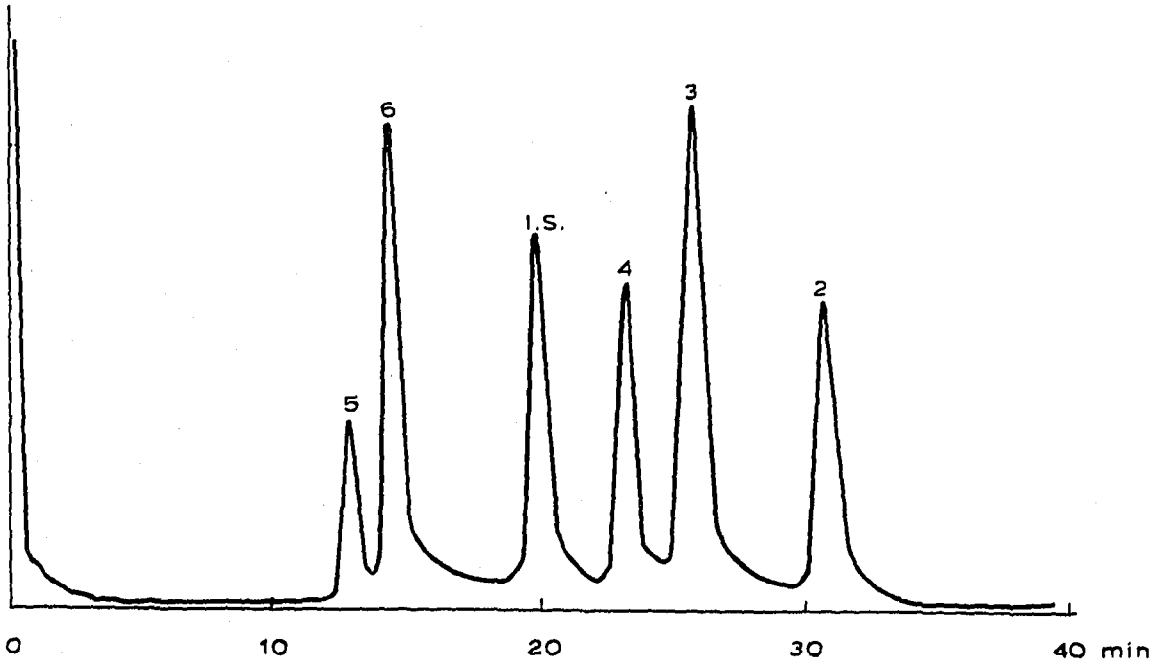

Fig. 1. Gas chromatogram of a mixture of standard steroids. Conditions: stainless-steel column (3 mm I.D.) packed with 5% SE-52 (3.375 m) + 2% OV-17 (4.500 m). Other conditions were the same as in Table I. To identify numbers on peaks, see Table I.

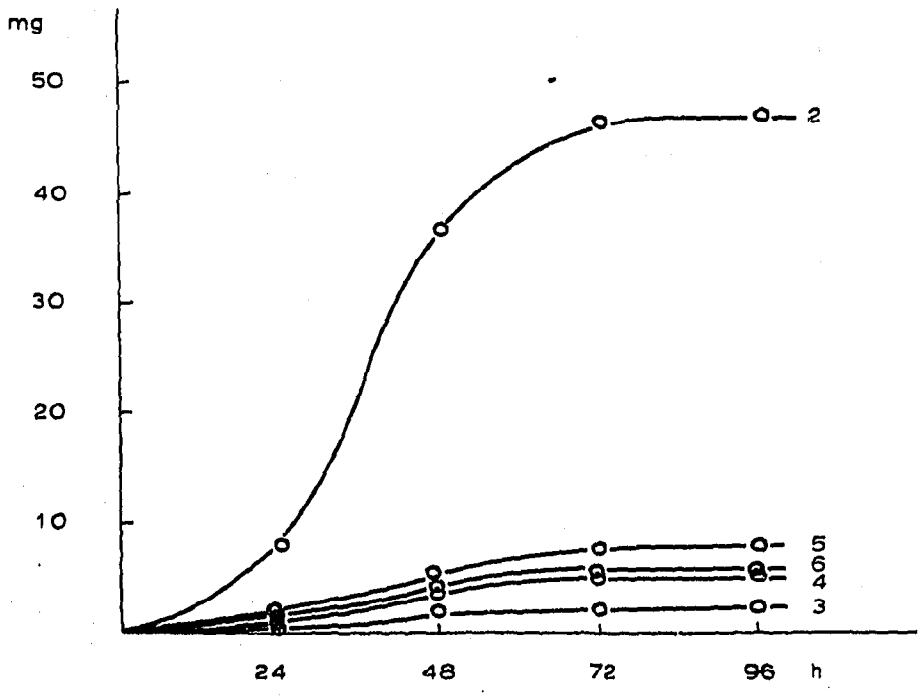

Fig. 2. Cumulative excretion curves of urinary metabolites formed from 16α-chloroestrone methyl ether in the rabbit. To identify numbers on curves, see Table I.

chloro-17α-estradiol, then showed excellent separation on SE-52. With respect to these two, however, distinct resolution was attained on the OV-17 column. Thus, the use of a connected column of SE-52 and OV-17 seemed to be promising. In practice, complete separation of all the main metabolites was accomplished with success.

Then several compounds were tested for use as an internal standard, and in consequence 3-deoxyestrone was chosen as the most suitable one in every respect. A typical gas chromatogram of a mixture of the standard steroids is illustrated in Fig. 1. The calibration curves for determination were constructed by plotting the ratio of peak area of each sample to the standard against the weight ratio of these two, whereby good linearity was observed.

In order to apply the present method to quantitation of the urinary metabolites, it was necessary to remove the interference due to the undesirable substances in the rabbit urine. Examinations were made on the preliminary purification by means or TLC using two kinds of solvent systems. This procedure proved to be effective fof elimination of the substances which are less and more polar than the expected metabolites. A known amount of each steroid was recovered in a yield of *ca.* 92% by this treatment.

The metabolites excreted in the rabbit urine were determined by the method thus established after oral administration of 16α-chloroestrone methyl ether. The cumulative excretion curve of the main metabolites is shown in Fig. 2. The result demonstrated that 33% of the administered steroid was excreted in 96 h and 16α-chloro-17α-estradiol formed 70% of the excreted amount. It should be noted that the presence of chlorine at C-16 appears to prevent the oxidative biotransformation leading to the polar metabolites. It is hoped that the present method will serve to clarify the effects of the dose and mode of administration upon the metabolic pattern.

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1 T. NAMBARA, M. NOKUBO AND Y. H. BAE, *Chem. Pharm. Bull. (Tokyo)*, in press.
2 G. P. MUELLER, W. F. JOHNS, D. L. COOK AND R. A. EDGREN, *J. Amer. Chem. Soc.*, 80 (1958) 1769.
3 T. NAMBARA AND M. NUMAZAWA, *Chem. Pharm. Bull. (Tokyo)*, 17 (1969) 1200.
4 T. NAMBARA AND H. TAKAHASHI, *Chem. Pharm. Bull. (Tokyo)*, 18 (1970) 2309.
5 T. NAMBARA AND Y. H. BAE, *Chem. Pharm. Bull. (Tokyo)*, 18 (1970) 2119.
6 T. NAMBARA AND M. NUMAZAWA, *Chem. Pharm. Bull. (Tokyo)*, 19 (1971) 990.
7 C. C. SWEELEY, R. BENTLEY, M. MAKITA AND W. W. WELLS, *J. Amer. Chem. Soc.*, 85 (1963) 2497.

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