[Chem. Pharm. Bull. 18(10)2119—2122(1970)]

UDC 547.92.09 : 591.149.05 : 543.544.25

Studies on Metabolism of 3-Deoxysteroids. V. Determination of Urinary Metabolites of 3-Deoxyestrone by Gas Chromatography[1,2]

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(Received May 23, 1970)

Gas chromatographic method has been devised for determination of the metabolites of 3-deoxyestrone. Satisfactory separation of the standard steroids was achieved, when the mixture was trimethylsilylated as pretreatment and then was subjected to gas chromatography using a connected column of 3% SE-30 and 2% OV-17 as the stationary phase. The established procedure was applied to quantitation of the urinary metabolites of 3-deoxyestrone administered to a rabbit. The typical gas chromatogram and cumulative excretion curve of the principal metabolites are shown in Fig. 2 and 3, respectively.

In the preceding papers the authors reported the isolation and characterization of the urinary metabolites of 3-deoxysteroids administered to a rabbit.[4] During the continuation of biochemical studies on these modified steroids it has become requisite to determine the excreted amount of the biotransformation products. The present paper describes a method which employs thin-layer chromatography as a preliminary purification procedure, followed by trimethylsilylation of the metabolites derived from 3-deoxyestrone and their determination by gas chromatography.

Experimental

Materials——Almost all the samples were prepared by the methods described in the preceding papers[5] and the others by the known procedures. 3-Deoxyestrone was donated from Teikoku Hormone Manufacturing Co., Ltd.

Apparatus——Shimadzu Model GC-1C gas chromatograph equipped with a hydrogen flame ionization detector was used. The U-shaped stainless steel column (3 mm *i.d.*) was packed with either 1.5% (or 3%) SE-30 on a support of Chromosorb W (60—80 mesh) or 2% OV-17 on Shimalite W (60—80 mesh).

Administration of 3-Deoxyestrone and Separation of Metabolites——A single dose of a suspension of 3-deoxyestrone (100 mg) in Tween 80 was orally given to an adult male rabbit (body weight 2.3 kg) through a catheter. Urine was collected in a bottle containing a few drops of toluene as preservative at 24, 48, 72 and 120 hr after administration and was stored in refrigerator until required for analysis. One-tenth aliquot of the pooled urine was adjusted to pH 5 with dil. H_2SO_4 and then to pH 4.5 with 0.1M acetate buffer (3 ml/10 ml of urine) and incubated with beef-liver β-glucuronidase (Tokyo Zōkikagaku 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 AcOEt (70 ml×2, 60 ml×1). The organic phase was separated and allowed to stand at 37° for 24 hr. The extract was washed with 5% $NaHCO_3$, H_2O and dried over

1) This paper constitutes Part XL of the series entitled "Analytical Chemical Studies on Steroids"; Part XXXIX: N. Shinriki and T. Nambara, *Yakugaku Zasshi*, submitted.
2) In this paper the following trivial names are used. 3-Deoxyestrone=estra-1,3,5(10)-trien-17-one, 3-deoxyestradiol=estra-1,3,5(10)-trien-17β-ol, 3-deoxy-17α-estradiol=estra-1,3,5(10)-trien-17α-ol, estrone=3-hydroxyestra-1,3,5(10)-trien-17-one, estradiol=estra-1,3,5(10)-triene-3,17β-diol, 17α-estradiol=estra-1,3,5(10)-triene-3,17α-diol, 16,17-epiestriol=estra-1,3,5(10)-triene-3,16β,17α-triol.
3) Location: *Aobayama, Sendai*; *a*) On leave of absence from Chung Buk College of Pharmacy, Korea.
4) *a*) T. Nambara and M. Numazawa, *Chem. Pharm. Bull.* (Tokyo), **16**, 383 (1968); *b*) T. Nambara, M. Numazawa, and H. Takahashi, *ibid.*, **16**, 1148 (1968); *c*) T. Nambara and M. Numazawa, *ibid.*, **17**, 1200 (1969); *d*) T. Nambara, M. Numazawa, and S. Akiyama, *ibid.*, **17**, 2394 (1969); *e*) T. Nambara and H. Takahashi, *ibid.*, in press.
5) T. Nambara and M. Numazawa, *Chem. Pharm. Bull.* (Tokyo), **16**, 863 (1968); T. Nambara, M. Numazawa, and H. Takahashi, *ibid.*, **17**, 1725 (1969).

anhydrous Na_2SO_4. After evaporation of solvent *in vacuo* a gummy substance obtained was submitted to thin-layer chromatography.

Purification by Thin-Layer Chromatography——The residue was applied to silica gel HF (E. Merck AG) plate (20×20 cm) and developed with benzene. Of the expected metabolites the most polar one, 16,17-epiestriol, remained at origin and 3-deoxyestrone was most nonpolar (*Rf* 0.30) on the thin-layer plate. Adsorbent of the zone corresponding to these metabolites (*Rf* 0—0.5) was eluted with acetone (20 ml×3). Eluate was again submitted to thin-layer chromatography using AcOEt as developer. 16,17-Epiestriol and 3-deoxyestrone gave *Rf* values of 0.60 and 0.90, respectively. Adsorbent of the zone (*Rf* 0.5—1.0) was eluted with acetone (20 ml×3) and the eluate was dissolved in THF (5 ml). Two 2 ml aliquots were taken up, and one portion was submitted to alkaline treatment, while the other to trimethylsilylation without hydrolysis.

Treatment of Alkaline Hydrolysis——To a solution of the purified metabolites in THF (2 ml) were added MeOH (2.5 ml) and 2.5% K_2CO_3 (0.5 ml) and refluxed for 4 hr. The resulting solution was transfered to a separatory funnel with AcOEt (10 ml×3) and washed with H_2O (2 ml×2, 1 ml×1). Evaporation of solvent gave a residue, which in turn was again transfered to a glass-stoppered test tube with THF for trimethylsilylation.

Gas Chromatography——After evaporation of THF the residue obtained was treated with hexamethyldisilazane (0.1 ml) and trimethylchlorosilane (0.1 ml) in pyridine (5 drops) according to the procedure of Sweeley, *et al.*[6] The reaction mixture was evaporated to dryness with the aid of a N_2 gas stream. To this residue was added THF solution (0.5 ml) containing a known amount of 4-methyl-3-deoxyestrone (*ca.* 0.2—0.3 mg), centrifuged and 1—2 μl of the supernatant was injected into the sample chamber.

The column, flash-heater and detector bath were maintained at 230°, 250° and 260°, respectively. N_2 gas flow rate was 45 ml/min with 4 kg/cm² at the inlet.

Result and Discussion

An initial attempt was made to establish the appropriate column conditions for separation of eight kinds of expected metabolites[4c] and unchanged 3-deoxyestrone employing the synthetic specimens. The retention values of these compounds under a variety of conditions are listed in Table I. Among these steroids 16,17-epiestriol itself did not give any peak on either SE-30 or OV-17 probably due to the thermal decomposition. Accordingly trimethylsilylation was carried out as pretreatment for gas chromatography in the manner described by Sweeley, *et al.*[6] Hereupon the compounds except estrone and 16,17-epiestriol showed satisfactory separation on OV-17 but not on SE-30. As for these two, however, an excellent

Table I. Relative Retention Times of Standard Steroids in Gas Chromatography[a]

Compounds	Column				
	1.5%SE-30 (3.375 m) Free	2%OV-17 (1.875 m) Free	1.5%SE-30 (1.875 m) TMS	2%OV-17 (3.375 m) TMS	3%SE-30(1.5m) +2%OV-17 (3.375m) TMS
3-Deoxy-17α-estradiol (1)	0.29	0.41	0.27	0.28	0.29
3-Deoxyestradiol (2)	0.32	0.43	0.30	0.33	0.32
3-Deoxyestrone (3)	0.27	0.44	0.32	0.46	0.39
6β-Hydroxy-3-deoxyestrone (4)	0.32	0.46	0.36	0.57	0.50
17α-Estradiol (5)	0.61	1.31	0.61	0.65	0.62
2-Hydroxy-3-deoxyestrone (6)	0.56	1.40	0.55	0.90	0.76
Estrone (7)	0.61	1.40	0.59	1.03	0.87
16,17-Epiestriol (8)	None	None	1.04	1.02	0.99
17α-Estradiol 17-Acetate (9)	0.75	1.71	0.77	1.20	1.05
4-Methyl-3-deoxyestrone (IS)	—	—	—	—	0.56
Cholestane	1.00 (8.12 min)	1.00 (9.36 min)	1.00 (4.24 min)	1.00 (19.12 min)	1.00 (21.48min)

a) temperature: column 230°, flash-heater 250°, detector 260°
carrier gas: N_2 flow rate 45 ml/min
sensitivity: 100 range: 0.8 V

6) C.C. Sweeley, R. Bentley, M. Makita, and W.W. Wells, *J. Am. Chem. Soc.*, **85**, 2497 (1963).

resolution was attained on SE-30 column. Thus the use of a connected column of SE-30 and OV-17 appeared to be promising. In fact it proved to be of advantage for complete separation of all the metabolites when trimethylsilylation was previously performed.

Unfortunately it was then found that a non-steroidal substance excreted in urine showed almost the same retention time as 17α-estradiol 17-acetate on this column. Therefore the quantity of this metabolite was indirectly determined with use of duplicate samples. Difference in 17α-estradiol value between the cases with and without hydrolysis was regarded as that of the acetate. Alkaline hydrolysis under mild conditions readily proceeded without any undesirable side-reaction. The recovery rate of the 17-acetate in the presence of 17α-estradiol determined by gas chromatography was found to be 98.5%.

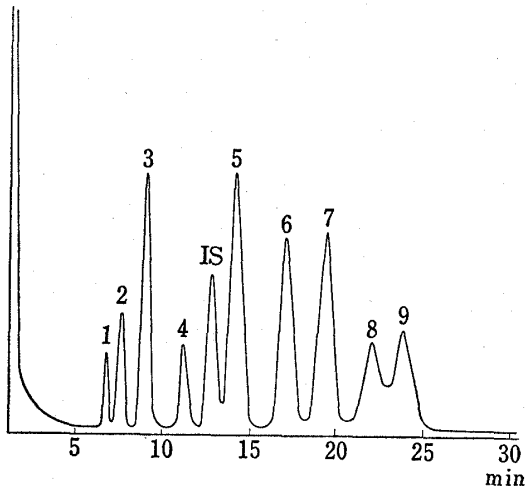

Fig. 1. Gas Chromatogram of a Mixture of Standard Steroids

column: 3% SE-30 on Chromosorb W (1.5 m) + 2% OV-17 on Shimalite W (3.375 m)
temperature: column 230°, flash-heater 250°, detector 260°
carrier gas: N_2 flow rate 45 ml/min
sensitivity: 100 range: 0.8V

Then selection of the internal standard suitable for the established conditions was examined. Of several compounds tested 4-methyl-3-deoxyestrone was chosen as the most pertinent standard in every respect. A typical gas chromatogram of the metabolites and internal reference is illustrated in Fig. 1. 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 respectively, 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 urine. Thin-layer chromatography was carried out for preliminary purification employing two kinds of solvent

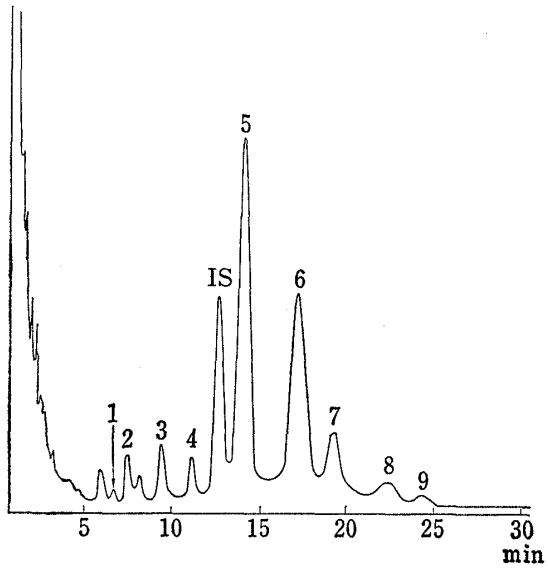

Fig. 2. Gas Chromatogram of Urinary Metabolites of 3-Deoxyestrone[a)]

a) Gas chromatographic conditions were the same as in Fig. 1.

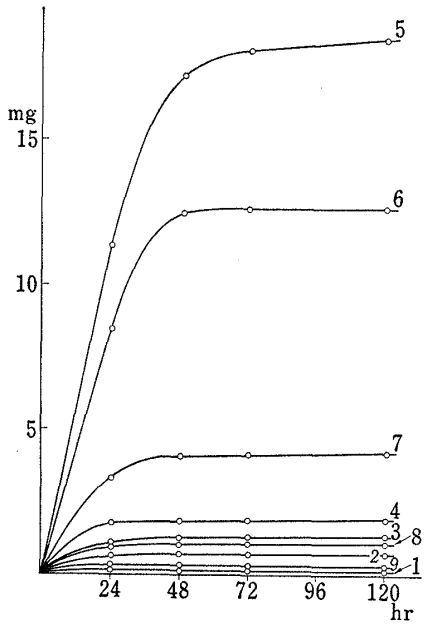

Fig. 3. Cumulative Excretion Curves of Urinary Metabolites of 3-Deoxyestrone

systems. This procedure proved to be effective for removal of the less and more polar substances than the 3-deoxyestrone metabolites. In addition a known amount of each steroid was recovered at the rate of *ca.* 92% on this treatment.

A single dose of 3-deoxyestrone was orally given to an adult male rabbit and the urine was collected for the following five days. A portion of the urine specimen was taken up and hydrolysis with β-glucuronidase followed by solvolysis was processed in the manner as described in the preceding papers.[4] According to the established procedure mentioned above, the free steroids fraction was subjected to preliminary purification, hydrolysis, if necessary, and then to gas chromatography. A typical gas chromatogram of the urinary metabolites and the internal standard is shown in Fig. 2. It is evident that 17α-estradiol and 2-hydroxy-3-deoxyestrone were excreted in urine together with minor metabolites, *i.e.* estrone, 6β-hydroxy-3-deoxyestrone, 16,17-epiestriol, 3-deoxyestradiol, 3-deoxy-17α-estradiol, and 17α-estradiol 17-acetate. The cumulative urinary excretion of these metabolites is illustrated in Fig. 3. The result demonstrated that 40% of the administered steroid was excreted in five days and the phenolic metabolites having a hydroxyl group at C-3 or C-2 formed 90% of the excreted amount. Aromatic hydroxylation, in particular at C-3, seems to be most important biotransformation in the metabolic pathway of this modified steroid. It is hoped that the present method may serve to clarify the effects of the dose and mode of administration upon the metabolites pattern.

Acknowledgement This work was supported in part by a grant from Yakurikenkyukai (Iatrochemical Research Foundation), which is gratefully acknowledged. Thanks are also due to Teikoku Hormone Manufacturing Co., Ltd. for generous gift of 3-deoxyestrone.