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# ANALYTICAL CHEMICAL STUDIES ON STEROIDS

## LIV. STUDIES ON METABOLISM OF 3-DEOXYSTEROIDS. IX. DETERMINATION OF URINARY METABOLITES OF ANDROST-5-EN-17-ONE BY GAS CHROMATOGRAPHY

TOSHIO NAMBARA* AND YOUNG HO BAE**
*Pharmaceutical Institute, Tohoku University, Sendai (Japan)*

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SUMMARY

A gas chromatographic method has been devised for determination of the metabolites formed from androst-5-en-17-one, a $C_{19}$-steroid lacking an oxygen function at C-3. Standard samples of the metabolites could be separated on an OV-17 column after conversion to the trimethylsilyl derivatives with the exception of androst-5-ene-3$\beta$,17$\alpha$-diol and 5$\alpha$-androstane-3$\beta$,17$\alpha$-diol. Resolution of these two compounds was achieved when the mixture was subjected to epoxidation with *m*-chloroperbenzoic acid followed by trimethylsilylation. The procedure was applied to quantitation of the metabolites excreted in rabbit urine after oral administration of androst-5-en-17-one. The cumulative excretion curve of the main metabolites is illustrated in Fig. 3. The steroid number contribution of the 5$\alpha$,6$\alpha$-epoxy group has also been determined.

## INTRODUCTION

In the previous paper of this series the authors reported the isolation and characterization of the urinary metabolites of androst-5-en-17-one (3-deoxydehydroepiandrosterone) administered to the rabbit[1]. During the continuation of biochemical studies on the modified steroids it has become necessary to determine the excreted amount of these biotransformation products. The present paper describes a method which employs thin-layer chromatography as a preliminary purification procedure, followed by preparation of the appropriate derivatives and their determination by gas chromatography (GC).

## EXPERIMENTAL

### *Materials*

Almost all the samples used in this work were prepared in this laboratory[2].

* To whom any inquiries should be addressed.
** On leave of absence from Chung Buk College of Pharmacy, Korea.

*Preparation of derivatives*

*Trimethylsilyl (TMS) derivatives.* TMS derivatives were prepared by treatment with hexamethyldisilazane and trimethylchlorosilane in pyridine according to the procedure of SWEELEY *et al.*[3].

*Epoxy-TMS derivatives. m*-Chloroperbenzoic acid (0.5 mg) was added to a solution of the sample in chloroform (0.2 ml) and the mixture was allowed to stand at room temperature for 1 h. After evaporation of solvent with the aid of a nitrogen gas stream the residue was submitted to trimethylsilylation.

*Gas chromatography*

The apparatus used for this work was a Shimadzu Model GC-5AIFE 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 or 5% SE-52 on a support of Chromosorb K (60–80 mesh), or 2% OV-17, 1.5% OV-1 or 1.5% NGS on Shimalite W (60–80 mesh). The detector and flash heater were kept at 250°, while the column temperature was 230°. The pressure and flow rate of nitrogen carrier gas were 1.4 kg/cm$^2$ and 54 ml/min, respectively.

*Administration of androst-5-en-17-one and separation of metabolites*

A single dose of a suspension of androst-5-en-17-one (100 mg) in Tween 80 was given orally to an adult female rabbit (body weight 2.2 kg) through a catheter. Urine was collected in a bottle, containing a few drops of toluene as the preservative, at 24, 48, 72, 96 and 120 h after administration and was stored in a refrigerator until required for analysis. One-tenth aliquot of the pooled urine was adjusted to pH 5 with diluted sulfuric acid and then to pH 4.5 with 0.1 *M* acetate buffer (3 ml per 10 ml of urine) and incubated with beef-liver $\beta$-glucuronidase (300 Fishman units per ml) (Tokyo Zōkikagaku Co.) at 37° for five 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% $NaHCO_3$ then water and dried over anhydrous $Na_2SO_4$. After evaporation of solvent *in vacuo* the gummy substance obtained was purified by thin-layer chromatography (TLC).

*Purification by thin-layer chromatography*

The gummy residue was applied to a Silica Gel G (E.Merck AG) plate (20 × 20 cm) and developed with benzene. Of the metabolites the most polar one, 5$\beta$-androstane-3$\alpha$,17$\alpha$-diol, remained at the origin and androst-5-en-17$\beta$-ol was the most non-polar ($R_F$ 0.12). The adsorbent of the zone ($R_F$ 0–0.30) corresponding to the metabolites was eluted with acetone (2 × 50 ml). The eluate was again submitted to TLC using ethyl acetate as developing solvent. 5$\beta$-Androstane-3$\alpha$,17$\alpha$-diol and androst-5-en-17$\beta$-ol gave $R_F$ values of 0.88 and 0.75, respectively. The adsorbent of the zone ($R_F$ 0.5–1.0) was eluted with acetone (2 × 50 ml) and the eluate thus obtained was transferred into a test-tube with tetrahydrofuran.

## RESULTS AND DISCUSSION

An initial attempt was made to establish the appropriate column conditions

TABLE I

RELATIVE RETENTION TIMES OF STANDARD STEROIDS

Conditions: stainless-steel column (2.250 × 3 mm I.D.); nitrogen flow rate, 54 ml/min; column temperature, 230°; flash heater temperature, 250°; detector temperature, 250°.

| *Compound* | *Column* | | | | |
|---|---|---|---|---|---|
| | *3% SE-30 (2.250 m)* | *2% OV-17 (3.375 m)* | *3% SE-30 (2.250 m)* | *2% OV-17 (6.750 m)* | *1.5% NGS (2.250 m)* |
| | | | *TMS derivative* | | *Epoxy-TMS derivative* |
| Androst-5-en-17-one (1) | 0.27 | 0.23 | 0.27 | 0.31 | 1.39 |
| Androst-5-en-17β-ol (2) | 0.22 | 0.32 | 0.27 | 0.23 | 1.39 |
| Androst-5-ene-3β,17α-diol (3) | 0.46 | 0.80 | 0.50 | 0.41 | 1.00 |
| 5α-Androstane-3β,17α-diol (4) | 0.44 | 0.80 | 0.50 | 0.41 | 0.48 |
| 5β-Androstane-3α,17β-diol (5) | 0.42 | 0.72 | 0.52 | 0.36 | 0.38 |
| Androst-5-ene-2α,17α-diol (6) | 0.45 | 0.80 | 0.55 | 0.34 | 1.16 |
| 5β-Androstane-3α,17α-diol (7) | 0.41 | 0.70 | 0.50 | 0.27 | 0.33 |
| Aldrin (IS) | — | — | — | 0.12 | — |
| 4-Methyl-3-deoxyestrone (IS) | — | — | — | — | 1.28 |
| Cholestane | 1.00 (13.00 min) | 1.00 (15.48 min) | 1.00 (13.00 min) | 1.00 (42.50 min) | 1.00 (6.24 min) |

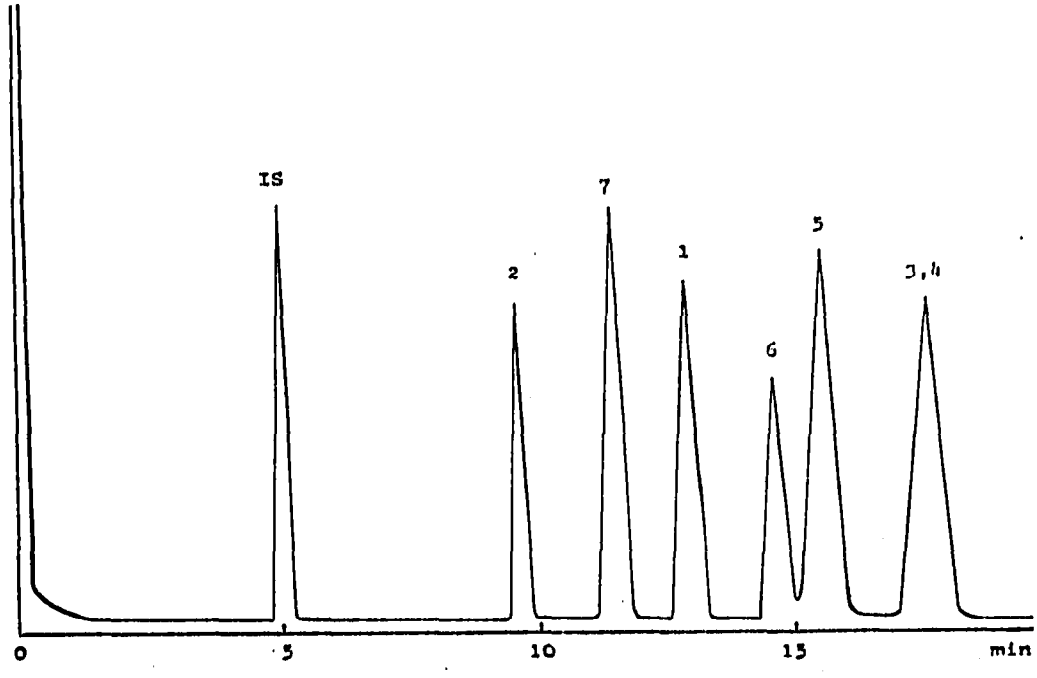


Fig. 1. Gas chromatogram of TMS derivatives of standard steroids. Column: stainless-steel tube (6.750 m × 3 mm I.D.) packed with 2% OV-17 (Condition I). Other conditions were the same as in Table I.

for the separation of six kinds of the metabolites (2–7) and the unchanged androst-5-en-17-one (1), employing the synthetic samples. The retention times of these compounds relative to cholestane under a variety of conditions are tabulated in Table I. When the free steroids were applied, a satisfactory separation was not achieved on either an SE-30 or an OV-17 column. Accordingly, trimethylsilylation was carried out by the method of SWEELEY *et al.*[3]. Hereupon the compounds, except androst-5-ene-3β,17α-diol (3) and 5α-androstane-3β,17α-diol (4), were resolved satisfactorily on the 2% OV-17 column (Condition I). A typical gas chromatogram of the TMS derivatives of standard steroids is illustrated in Fig. 1.

The aim of the next project was the separation of the two unresolved compounds, which differ only in their C-5 and C-6 structures. Transformation of the unsaturated compound into a suitable derivative to accentuate the existing difference appeared to be promising for this purpose. It has been reported that the 3β-hydroxy-$\Delta^5$-steroid readily undergoes epoxidation with *m*-chloroperbenzoic acid yielding the

TABLE II

RELATIVE RETENTION TIMES OF TMS DERIVATIVES

Conditions: stainless-steel column (2.250 m × 3 mm I.D.); nitrogen flow rate, 54 ml/min; column temperature, 230°; flash heater temperature, 250°; detector temperature, 250°.

| *Compound* | *Column* | | | | |
|---|---|---|---|---|---|
| | *3% SE-30* | *5% SE-52* | *2% OV-17* | *1.5% OV-1* | *1.5% NGS* |
| Androst-5-ene-3β,17α-diol | 0.50 | 0.50 | 0.40 | 0.52 | 0.48 |
| 5α-Androstane-3β,17α-diol | 0.50 | 0.51 | 0.40 | 0.52 | 0.48 |
| 5α,6α-Epoxyandrostane-3β,17α-diol | 0.72 | 0.72 | 0.67 | 0.73 | 1.00 |
| 3β-Hydroxyandrost-5-en-17-one | 0.48 | 0.51 | 0.67 | 0.47 | 1.44 |
| 3β-Hydroxy-5α-androstan-17-one | 0.48 | 0.52 | 0.67 | 0.49 | 1.46 |
| 5α,6α-Epoxy-3β-hydroxyandrostan-17-one | 0.65 | 0.71 | 1.10 | 0.66 | 2.88 |
| Androst-5-ene-3β,17β-diol | 0.58 | 0.54 | 0.48 | 0.61 | 0.54 |
| 5α-Androstane-3β,17β-diol | 0.62 | 0.60 | 0.48 | 0.61 | 0.55 |
| 5α,6α-Epoxyandrostane-3β,17β-diol | 0.81 | 0.82 | 0.77 | 0.83 | 1.14 |
| Androst-5-ene-3β,16β-diol | 0.56 | 0.53 | 0.46 | 0.57 | 0.52 |
| 5α-Androstane-3β,16β-diol | 0.56 | 0.54 | 0.46 | 0.57 | 0.52 |
| 5α,6α-Epoxyandrostane-3β,16β-diol | 0.78 | 0.75 | 0.75 | 0.77 | 1.02 |
| 3β-Hydroxyandrost-5-en-16-one | 0.48 | 0.51 | 0.67 | 0.48 | 1.59 |
| 3β-Hydroxy-5α-androstan-16-one | 0.48 | 0.51 | 0.67 | 0.49 | 1.60 |
| 5α,6α-Epoxy-3β-hydroxy-androstan-16-one | 0.65 | 0.72 | 1.11 | 0.63 | 3.08 |
| Cholestane | 1.00 (13.00 min) | 1.00 (10.00 min) | 1.00 (11.00 min) | 1.00 (18.12 min) | 1.00 (6.24 min) |

5α, 6α-oxido derivative as the sole product[4]. Indeed, on brief treatment with the peracid, followed by trimethylsilylation, androst-5-ene-3β,17α-diol showed a single peak of the theoretical shape, indicating the satisfactory GC properties of the reaction product. As was expected, this derivatized steroid exhibited an altered retention value, which was obviously different from that of the parent compound. The applicability of the present method was examined with five pairs of the $\Delta^5$- and 5α-steroids. It is evident from the data listed in Table II that satisfactory resolution was obtained on all the columns used. In order to estimate the effect of the structural alteration on the retention value, the steroid number (SN)[5] contribution of the 5α,6α-epoxy group was determined. The SN value was obtained graphically from the relative retention time on an SE-30 column, and the SN difference between the 5α,6α-epoxide and the parent 5α-steroid was calculated. As can be seen in Table III the SN contribution of the oxido function is almost constant with a mean of 1.0. The

TABLE III

RELATIVE RETENTION TIMES AND STEROID NUMBERS OF TMS DERIVATIVES

Conditions: stainless-steel column (2.250 m × 3 mm I.D.) packed with 3% SE-30; nitrogen flow rate, 54 ml/min; column temperature, 230°; flash heater temperature, 250°; detector temperature, 250°.

| *Compound* | *RRT* | *SN* | *SN contribution of 5α,6α-epoxy group* |
|---|---|---|---|
| 5α-Androstane-3β,17α-diol | 0.50 | 24.80 | |
| 5α,6α-Epoxyandrostane-3β,17α-diol | 0.72 | 25.80 | 1.00 |
| 3β-Hydroxy-5α-androstan-17-one | 0.48 | 24.55 | |
| 5α,6α-Epoxy-3β-hydroxyandrostan-17-one | 0.65 | 25.55 | 1.00 |
| 5α-Androstane-3β,17β-diol | 0.62 | 25.30 | |
| 5α,6α-Epoxyandrostane-3β,17β-diol | 0.81 | 26.30 | 1.00 |
| 5α-Androstane-3β,16β-diol | 0.56 | 25.10 | |
| 5α,6α-Epoxyandrostane-3β,16β-diol | 0.78 | 26.17 | 1.07 |
| 3β-Hydroxy-5α-androstan-16-one | 0.48 | 24.55 | |
| 5α,6α-Epoxy-3β-hydroxyandrostan-16-one | 0.65 | 25.55 | 1.00 |
| Androstane | 0.09 | | |
| Cholestane | 1.00 (13.00 min) | | |

constancy of this value may be useful for distinguishing the $\Delta^5$- and 5α-steroids by GC techniques. Upon conversion to the epoxy-TMS derivative the complete separation of androst-5-ene-3β,17α-diol and 5α-androstane-3β,17α-diol without interference from other metabolites, could be attained on a 1.5% NGS column (Condition II) as shown in Fig. 2 (see also Table I). These results led to the conclusion that separatory determination of all the metabolites is possible when the duplicate samples are transformed into the TMS and epoxy-TMS derivatives and then subjected to GC employing two different columns (Condition I and II), respectively.

Then the selection of the internal standards suitable for the established conditions was examined. Of several substances tested aldrin and 4-methyl-3-deoxyestrone were chosen as the most suitable standards for Condition I and II, respectively. The calibration curves for determinations were constructed by plotting the ratio of peak area of each sample to the internal standard against the weight ratio of these two, whereby good linearity was observed.

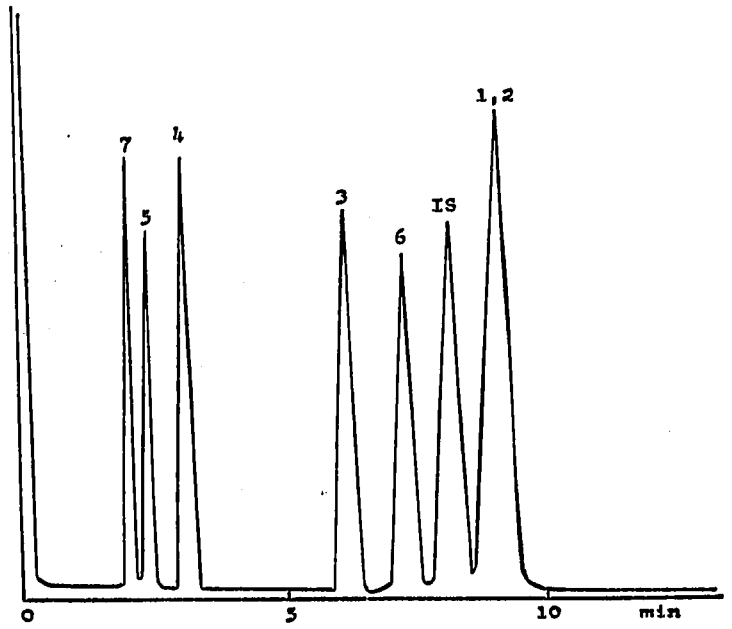


Fig. 2. Gas chromatogram of epoxy-TMS derivatives of standard steroids. Column: stainless-steel tube (2.250 m × 3 mm I.D.) packed with 1.5% NGS (Condition II). Other conditions were the same as in Table I.

In order to apply the present method to quantitation of the urinary metabolites it was necessary to remove the interferences due to undesirable substances in the urine. TLC purification employing two kinds of solvent systems proved to be effective for removal of substances less and more polar than the metabolites.

The recovery test for the mixture of standard steroids added to the rabbit urine was performed employing the procedure thus established. It is evident from the data listed in Table IV that each metabolite was recovered with satisfactory rate and reproducibility.

A single dose of androst-5-en-17-one was given orally to an adult female rabbit and the urine was collected for the following 120 h. An aliquot of the urine specimen was taken and hydrolysis with β-glucuronidase followed by solvolysis was carried out in the manner described in the preceding papers[6,7]. The free steroid fraction was purified by TLC. The eluate thus obtained was divided into two portions, which were

TABLE IV

RECOVERY TEST FOR STANDARD STEROIDS ADDED TO URINE[a]

| *Added amount (μg)* | *Metabolite* | | | | | | | *Mean* |
|---|---|---|---|---|---|---|---|---|
| | *1* | *2* | *3* | *4* | *5* | *6* | *7* | |
| 300 | 94.0 | 94.6 | 95.0 | — | 93.7 | 95.2 | — | |
| 500 | 95.0 | 96.3 | — | — | 93.6 | 94.5 | 94.1 | |
| | | | | | | | | 94.6 |

[a] Values were obtained by Condition I and are expressed in mean percentage for duplicate determinations.

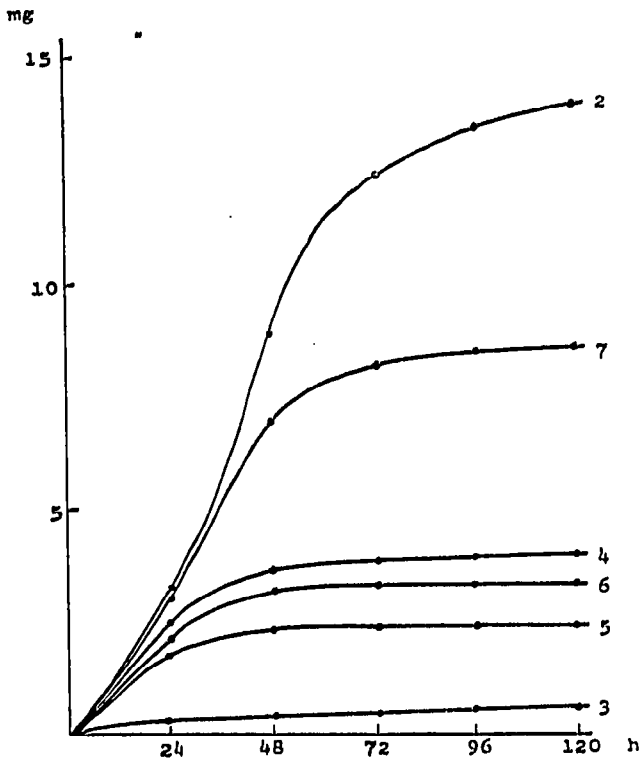


Fig. 3. Cumulative excretion curves of urinary metabolites formed from androst-5-en-17-one in the rabbit.

converted to the TMS derivatives with or without previous epoxidation, and then subjected to GC determination.

The cumulative urinary excretion of the metabolites is illustrated in Fig. 3. The results demonstrated that 33% of the administered steroid was excreted in 120 h and that androst-5-en-17$\beta$-ol and 5$\beta$-androstane-3$\alpha$,17$\alpha$-diol formed 70% of the excreted amount. It is hoped that the present method will serve to clarify the effects of dosage and mode of administration upon the metabolic pattern.

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