魚尿中のステロイドホルモン代謝産物I

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Steroid Hormone Metabolites in Fish Urine-I

Identification of Androsterone, Etiocholanolone, and Dehydroepiandrosterone Isolated from the Urine of Carp

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The analysis of human urinary steroid metabolites has been widely used to diagnose the functional diseases of adrenal cortex, testis, ovary, and pituitary. This method may be applicable to elucidate some physiological effects of agricultural chemicals and toxicants in industrial wastes on fish endocrine organs. The present paper reports on the detection and identification of 17-ketosteroids in the urine of carp, *Cyprinus carpio*.

The urine collected by inserting a cannula into the urinary bladder of carp was percholated through an Amberlite XAD-2 column in order to eliminate β-glucuronidase inhibitors. The steroids were hydrolyzed with β-glucuronidase and by solvolysis, extracted with ethylacetate, converted to methoxime-trimethylsilyl derivatives, and subjected to gas liquid chromatographic (GLC) and mass spectrometric (MS) analyses.

The GLC separations were carried out on 1% OV-1 and 1% OV-17 columns. The three peaks observed in the 17-ketosteroid region of the chromatograms were identified as those of androsterone, etiocholanolone, and dehydroepiandrosterone by methylene unit value determination. Their structures were also confirmed by a combined GLC-Mass spectrometer.

Many studies have been made on the steroid hormones in fish, and a variety of steroids similar to those in mammals have been shown to be present in the blood and tissues of different fish species.[1–10] There are few studies, however, on their terminal metabolites and the mechanism of their excretion through kidney and/or gills.

In the present investigation, 17-ketosteroids (17-KS) in fish urine were detected and identified by use of gas liquid chromatography (GLC) and mass spectrometry (MS) developed by HORNING *et al.*[11–14]

Materials and Methods

Purification of Materials

Methanol, acetone and ethylacetate (analytical reagent grade) were distilled prior to use. Pyridine was refluxed over pellets of KOH, distilled on fresh pellets and stored in a dark bottle. Amberlite XAD-2 resin (Rohm and Haas Co.) was washed with distilled water untill all the fines were removed. Packed into a column, the resin was washed successively with methanol, acetone and water. Standard samples of androsterone (3α-hydroxy-5α-androstan-17-one), etiocholanolone (3α-hydroxy-5β-androstan-17-one) and dehydroepiandrosterone (DHEA, 3β-hydroxyandrost-5-en-17-one) were purchased from Sigma Chemical Co. and used without further purification because their homogeneities were confirmed both by thin layer chromatography and by GLC.

Test Animal

Twelve carps (body weight, 255–280 g) were purchased from a fish farm on March 5, 1977. All the carps were fed on commercial baits for about one month in six aquaria (29.5×60×36 cm) at 17±1°C.

Collection of Urine Samples

Five male and three female carps were selected for urine collection. The fish was anaesthetized with 30 ppm quinaldine solution and inserted a silicone catheter into the urinary bladder.[15–18] The catheter was made by attaching a silicone cannula (Fuji-kobunshi, Silascone, 30 cm×1 mm i. d.) to a polyethylene tube (Hibiki No 5, 1 cm×1 mm i. d.) which was tipped with a small silicone sleeve (*ca.* 4 mm×1 mm i. d.). The carp was then placed in a urine-collecting apparatus (Fig. 1),

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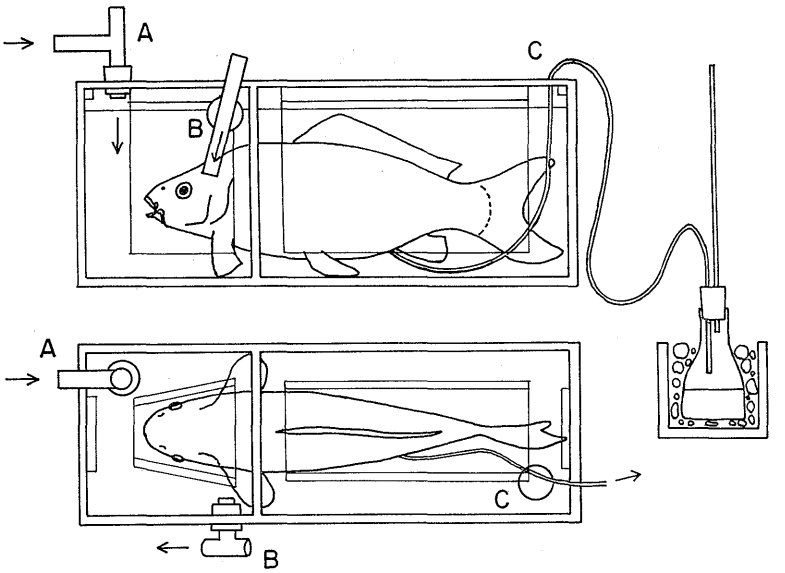

Fig. 1. Apparatus for holding carp during the period of urine collection. Water flows in the box at A and out at B. The free end of the cannula is passed out through a hole at C and attached to a ice-cooled receiver flask.

and the free end of the cannula was passed out through a hole at the rear of the chamber and attached to a ice-cooled receiver flask. The fish was then supplied with running dechlorinated tap water at a flow rate of 100 m*l*/min and with air by pump. The temperature was kept at 17±1°C throughout the experiment.

The urine collected for the first 24 hours was discarded in order to exclude the influence of diuretic stress[19] imposed on the fish, and the urine excreted for the following 48 hours was pooled and used for the analyses.

Enzymatic Hydrolysis and Solvolysis of Conjugated Steroids

The carp urine was passed through an Amberlite XAD-2 column in order to eliminate β-glucuronidase inhibitors, and then hydrolyzed with β-glucuronidase and by solvolysis according to the method of SAKAUCHI.[14]

Six urine samples (73–105 m*l*) collected from four male and two female carps were filtered and percholated through Amberlite XAD-2 columns (each *ca.* 2.5 g, 15 cm×0.5 cm i. d.).[20] After the column was washed with 10 m*l* of distilled water, the steroids were eluted with 12.5 m*l* of methanol at a flow rate of 0.5 m*l*/min and the eluate was evaporated to dryness *in vacuo* on a flash evaporator below 60°C. The residue was taken up in 25 m*l* of water and transferred to a 100 m*l* conical flask. Then 5 m*l* of 0.5 M phosphate buffer (pH 6.0), 1000 units of β-glucuronidase (*ca.* 16 mg, Sigma type I) and a few drops of chloroform were added, and the mixture was incubated for 24 hours at 37°C with continuous shaking. After incubation, 25 m*l* of ethylacetate, 0.5 m*l* of 2% formalin solution, 1.5 m*l* of 50% H_2SO_4 solution and 6.25 g NaCl were added to the flask and the mixture was shaken vigorously for 5 min and gently for 43 hours at 33°C. The reaction mixture was then transferred to a 100 m*l* separating funnel. The top ethylacetate layer was reserved, whereas the residual aqueous layer was re-extracted with 25 m*l* of ethylacetate. The combined ethylacetate extracts were washed with five 7.5 m*l* portions of 8% NaOH–10% NaCl solution and with three 7.5 m*l* portions of 10% NaCl solution, then dried over anhydrous Na_2SO_4, and concentrated to *ca.* 1 m*l* under reduced pressure below 60°C. The concentrate was transferred to a small sample tube (5 cm×5 mm i. d.) and the solvent was removed *in vacuo*.

Free Steroids

Two urine samples (65 m*l*, 81 m*l*) collected from one male and one female carp were filtered and diluted to 100 m*l* with distilled water. After adding 25 g of NaCl, the solution was transferred to a 300 m*l* separating funnel and extracted twice with 100 m*l* of ethylacetate. The combined extracts were washed with five 30 m*l* portions of 8% NaOH–10% NaCl solution and with three 30 m*l* portions of 10% NaCl solution, then dried over

anhydrous Na_2SO_4, and the solvent was removed under reduced pressure below 60°C.

Preparation of Methoxime-trimethylsilyl (MO–TMSi) Derivatives

MO–TMSi derivatives of carp urinary steroids and reference steroids were prepared according to the method of HORNING *et al.*[11,12]

The carp urinary steroid sample (or a mixture of reference steroids, each approx. 1–2 μg) was dissolved in 0.2 m*l* of dry pyridine containing 2 mg of methoxyamine hydrochloride.[21] The mixture was allowed to stand overnight at room temperature (or heated for 4 hours at 60°C), and then pyridine was removed under reduced pressure. The residue was extracted with 3 m*l* of ethylacetate, and the extract was washed with three 2 m*l* portions of 5% Na_2CO_3–10% NaCl solution, dried with anhydrous Na_2SO_4, and concentrated to 0.1 to 0.2 m*l*. The concentrate was transferred to a small sample tube (5 cm×5 mm i. d.) with a Teflon cap, and the solvent was removed *in vacuo*. Finally the residual contents were allowed to react with 20 μl of bis-trimethylsilylacetamide (BSA, Tokyo Chem. Product Co.) for 1 hour and the reaction mixture was subjected to GLC and GLC–MS analyses.

Gas Liquid Chromatography

Analytical separations were performed with Shimazu model 4BMPF instruments equipped with hydrogen flame ionization detectors. The columns were 3 m×4 mm glass coils and the phases employed were 1% OV–1 (a methylsiloxane polymer) and 1% OV–17 (a phenylmethylsiloxane polymer) on Shimalite W (acid washed, silanized, 80–100 mesh). The column temperatures were programmed at 2°C/min from 170°C for 1% OV–1 and from 200°C for 1% OV–17. The injection zone and detector were kept at 280°C. The flow rate of nitrogen was 60 m*l*/min.

Aliquots (1–2 μl) of sample were injected into the column with a microsyringe. The GLC retention data of carp urinary steroids were compared with those of reference steroids by use of methylene unit (MU) values: MU values[11] were measured with respect to a qualitative hydrocarbon mixture (Applied Science Laboratories Inc.) co-injected with the steroid derivatives.

Gas Liquid Chromatography-Mass Spectrometry

A Hitachi model RM–50GC gas liquid chromatograph-mass spectrometer with a 2 m×4 mm i. d. glass column packed with 1% OV–1 on Shimalite W (acid washed, silanized, 80–100 mesh) was used. The column temperature was programmed at 1°C/min from 130°C to 230°C. The injection zone and interface were kept at 250°C. The flow rate of herium gas was *ca.* 30 m*l*/min. The ionizing potential and current were 50 eV and 80 μA, respectively.

Results

Volumn of Urine Samples

The flow rate of carp urine was close to 25 m*l*/kg/h shortly after cannulation, probably due to laboratory diuresis.[19] But this flow rate felt to 5.1–8.2 m*l*/kg/h within 24 hours, and remained fairly constant thereafter. The urine excreted for the first 24 hours was discarded and that collected volumn the following 48 hours was examined. The for of urine samples ranged from 65 to 105 m*l* per individual.

Identification of the Carp Urinary Steroids by GLC

Figs. 2 and 3 show chromatograms of carp urinary steroids on 1% OV–1 and 1% OV–17 column. MU values of compounds I, II and III in the 17-ketosteroid (17–KS) region were compared with those of reference steroids (Table 1).

Table 1. Methylene unit (MU) values for MO–TMSi derivatives of carp urinary steroids and reference steroids determined by temperature programming with OV-1 and OV-17 liquid phases.

Steroid	Liquid phase	
	OV-1*1	OV-17*2
Compound I	25.02	27.14
Compound II	25.23	27.33
Compound III	25.63	28.28
Androsterone*3	25.02	27.15
Etiocholanolone*4	25.22	27.33
Dehydroepiandrosterone*5 (DHEA)	25.65	28.19

*1 1% OV–1 column, temperature programmed at 2°C/min, starting at 170°C.
*2 1% OV–17 column, temperature programmed at 2°C/min, starting at 200°C.
*3 3α-hydroxy-5α-androstan-17-one.
*4 3α-hydroxy-5β-androstan-17-one.
*5 3β-hydroxyandrost-5-en-17-one.
*6 presumably overlapped with pregnanediol peak (28.32).

As shown in Table 1, compound I corresponds in MU value to authentic androsterone, compound II to etiocholanolone, and compound III to DHEA, respectively. In Fig. 3, the peak of compound III seems to be overlapped with that of presumably pregnanediol at 28.32.

The peaks of androsterone and etiocholanolone were relatively high and constant in all the samples,

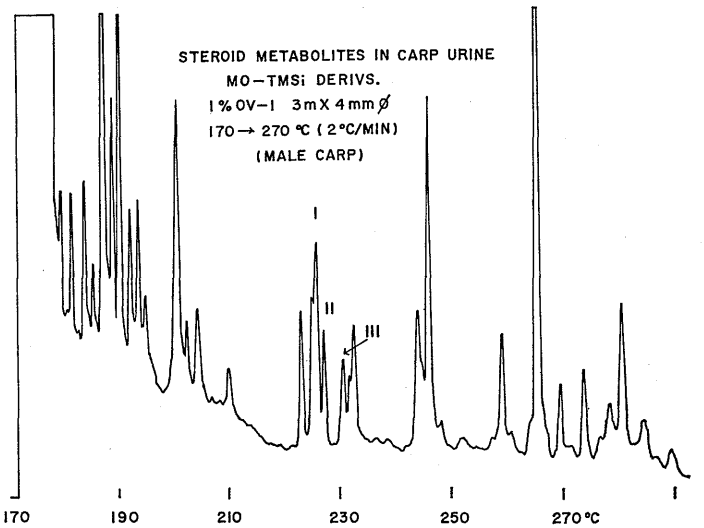

Fig. 2. Gas liquid chromatogram of carp urinary steroids as MO-TMSi derivatives. Column conditions: 3 m×4 mm i. d. glass column, 1% OV-1 on Shimalite W (80–100 mesh), temperature programmed at 2°C/min from 170°C. Peaks I, II and III correspond in MU value to androsterone, etiocholanolone and DHEA, respectively.

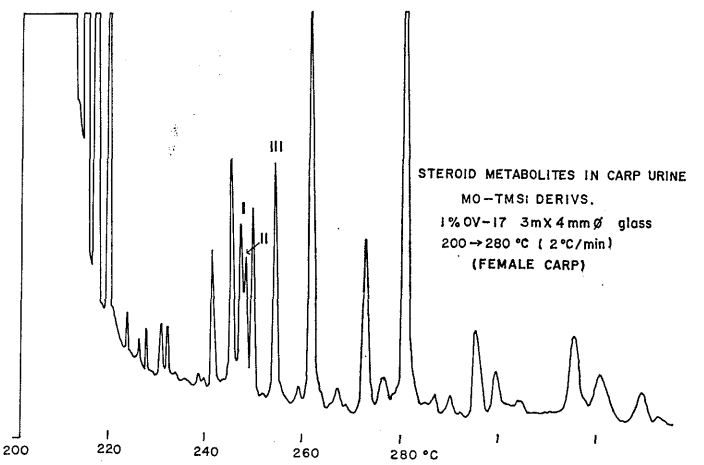

Fig. 3. Gas liquid chromatogram of carp urinary steroids as MO-TMSi derivatives. Column conditions: 3 m×4 mm i. d. glass column, 1% OV-17 on Shimalite W (80–100 mesh), temperature programmed at 2°C/min from 200°C. Peaks I, II and III correspond in MU value to androsterone, etiocholanolone and DHEA, respectively. Peak III seems to be overlapped with a peak of presumably pregnanediol.

whereas that of DHEA was low and varied greatly, some sample contained only trace amount of this steroid. On the other hand, no significant difference was observed in the chromatographic patterns between male and female carps. The GLC analyses of free steroids showed no peak in the region of 17-KS on both 1% OV-1 and 1% OV-17 columns, indicating that mostly all 17-KS excreted in carp urine are present in conjugated forms as in human urine.[22,23]

Identification of the Carp Urinary Steroids by GLC–MS

Fig. 4 shows the mass spectrum of compound I, which fragmentation pattern coincided with that of authentic androsterone MO-TMSi derivative, giving molecular ion peak at m/e 391. The loss of 31 amu, due to cleavage of the methoxy group, results in a strong peak at m/e 360 (M–31). An intense peak is also seen at m/e 270 (M–31–90), due to subsequent cleavage of the trimethylsilanol.

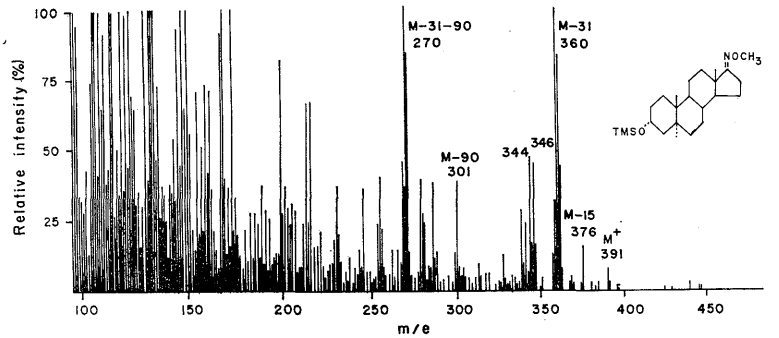

Fig. 4. Mass spectrum of compound I. The fragmentation pattern was identical with that of authentic androsterone MO-TMSi derivative. The GLC column was a 2 m×4 mm i. d. glass coil packed with 1% OV-1 on Shimalite W. The column temperature was programmed at 1°C/min from 130°C to 230°C.

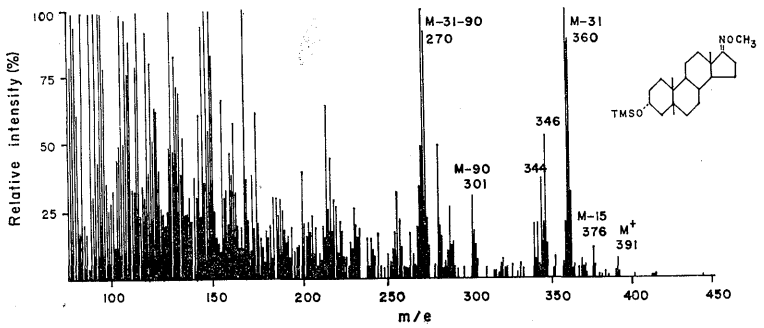

Fig. 5. Mass spectrum of compound II. The fragmentation pattern was identical with that of authentic etiocholanolone MO-TMSi derivative. GLC-MS conditions as in Fig. 4.

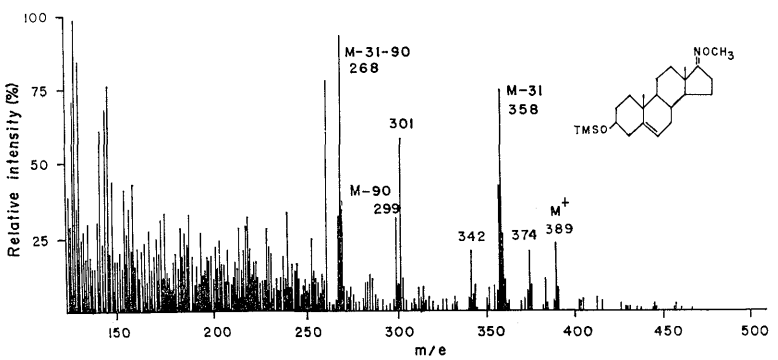

Fig. 6. Mass spectrum of compound III. The fragmentation pattern was identical with that of authentic DHEA MO-TMSi derivative. GLC-MS conditions as in Fig. 4.

In addition, peaks are also observed at m/e 376 (M–15) and 301 (M–90) corresponding to the loss of methyl group and trimethylsilanol.

Fig. 5 shows the mass spectrum of compound II, which fragmentation pattern coincided with that of authentic etiocholanolone MO–TMSi derivative, giving molecular peak at m/e 391. Peaks at m/e 376 and 301 corresponds to the loss of methyl group and trimethylsilanol. Intense peaks seen at m/e 360 (M–31) and m/e 270 (M–31–90) are due to cleavage of methoxy group and trimethylsilanol.

Fig. 6 shows the mass spectrum of compound III, which fragmentation pattern coincided with that of authentic DHEA MO–TMSi derivative, giving molecular peak at m/e 389. The peaks at m/e 374 (M–15), 358 (M–31), 299 (M–90) and 268 (M–31–90) are due to cleavage of the methyl group, methoxy group and trimethylsilanol.

Discussion

The present study revealed that androsterone, etiocholanolone and DHEA are present in carp urine as in human urine. The presence of these 17-ketosteroids (17–KS) suggests that the metabolic pathway which involves the following sequence exists in carp.

Testosterone(or DHEA)→androstenedione→androstanedione→androsterone (or etiocholanolone)

Other 17–KS such as 11-ketoandrosterone, 11-ketoetiocholanolone, 11-hydroxyandrosterone and 11-hydroxyetiocholanolone were not detected in carp urine probably because they were in minor quantities.

In human urine, all the 17–KS are known to be present as glucuronides or sulfates.[22,23] Of the major 17–KS of human urinary steroids, etiocholanolone is excreted predominantly as a glucuronide, DHEA mainly as a sulfate, and androsterone appears both as a glucuronide and as a sulfate.[24] In carp urine, on the contrary, androsterone and etiocholanolone were excreted as glucuronides, because they were completely liberated by hydrolysis with β-glucuronidase, whereas DHEA both as a glucuronide and as a sulfate, because its complete liberation was performed by solvolysis after enzymatic hydrolysis. This problem is, however, still inconclusive and further work will be conducted in a later report.

The most widely used methods for hydrolysis of human urinary steroids are acid hydrolysis and enzymatic hydrolysis combined with solvolysis. Although acid hydrolysis is convenient and speedy, it is not recommended since some urinary steroids undergo some structural alternations, forming unkown artifacts. In the present study, therefore, the carp urinary steroids were hydrolyzed by enzymatic hydrolysis and solvolysis according to SAKAUCHI.[14]

In the course of this work, it was also noted that the activity of β-glucuronidase was greatly reduced by some interfering substance present in carp urine. However, it was easily eliminated from urine by Amberlite XAD-2 treatment. This inhibitor may be a substance similar to 1, 4-glucuronolactone which is well known as a β-glucuronidase inhibitor in human urine.[25]

The identification of other peaks observed on GLC charts (Figs. 2 and 3) will be shown in a subsequent paper.

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