Gas chromatographic and mass spectrometric analysis of conjugated steroids in urine

JONG MAN YOON* and KYUNG HO LEE†

Department of Marine Biomedical Science, College of Ocean Science and Technology, Kunsan National University, Kunsan City, Jeollabuk-do, 573-702, Korea

†*Department of Environmental Engineering, Kyongju University, Kyongju City, Kyongsangbuk-do, 780-210, Korea*

**Corresponding author (Fax, 82-63-463-9493; Email, jmyoon@kunsan.ac.kr).*

This study was carried out qualitatively and quantitatively to investigate the presence and the concentrations of anabolic steroids in urine collected from orally administered humans. Microanalysis of conjugated steroids by gas chromatography and mass spectrometry (GC/MS) has been carried out. Following oral administration three major metabolites of anabolic steroid drugs have been detected and partially characterized. The six steroids can be analysed at the same time in 17 min. The lower detection limit was 10 ng/ml in 5 ml of urine. The conjugated steroids from urine were centrifuged to 2,430 *g* for 10 min, the supernatant solution passed through Amberlite XAD-2 column and the steroids eluted fraction esterified by using MSTFA and TMSI. The rate of metabolism and urinary excretion seem to be reasonably fast.

1. Introduction

It is well established that the stimulating anabolic effect of steroid drugs is always accompanied by undesirable androgenic side effects, depending on structural differences of the various synthetic steroids (Ward *et al* 1977; Cartoni *et al* 1983). Anabolic steroids can be used not only for therapeutic purposes, but also as a doping agent in sports. Additionally, exposure to organic chemicals has been correlated with a reduction of reproductive performance in several fish species (Petit *et al* 1999). To prevent this abuse and the side effects of these drugs in various countries, the urine and fluids are analysed to detect the process of traces of doping compounds. In recent years, some sensitive and generally applicable methods for the determination of anabolic steroids and their metabolites have been developed (Axelson *et al* 1974; Verbeke 1979; Stan and Abraham 1980). Several methods are available for the analysis of anabolic steroids by gas chromatography (GC) and mass spectrometry (MS) for the analysis of residues in livestock or fish meat (Dürbeck *et al* 1978; Verbeke 1979; Stan and Abraham 1980; Cartoni *et al* 1983). In addition, methods to determine the precise origin and function of steroid has become dependent upon the availability of a sensitive and specific method for its measurement in small volumes of samples in fishes and animals (Wright and Hunt 1982; Young *et al* 1983; Kagawa *et al* 1984; McFarilane *et al* 1991; Swanson *et al* 1991; Yeoman *et al* 1991). In additional, the chemical composition of leaf oils obtained from plant species was investigated by capillary GC, GC/MS or ^{13}C NMR (Dunlop *et al* 2000; Lota *et al* 2000). Not much Information is available on the determination and metabolism of anabolic steroids in man or animal, although a knowledge of metabolism is necessary in order to understand the rationale of drug toxicity. This study of steroids was carried out qualitatively and quantitatively to determine the anabolic steroids in urine.

2. Materials and methods

2.1 *Materials*

Before administrating of the drug, urine samples were collected as control specimens (n = 30 men). Because of

Keywords. Conjugated steroids; gas chromatography; mass spectrometry; metabolite

Abbreviations used: GC, gas chromatography; MS, mass spectrometry; SIM, selected ion monitoring; T/E, testosterone/epitestosterone.

their particular significance as frequently used anabolic steroids, clostebol, oxymesterone and oxymetholone were selected as the compounds to be investigated. After the oral application of a single dose of clostebol (dose, 20 mg), oxymesterone (dose, 10 mg) and oxymetholone (dose, 20 mg), respectively, urine samples were collected following at least 8 h.

2.2 *Analytical apparatus*

All chromatograms were recorded with a Hewlett-Packard gas chromatography (Model 5890/5970B, USA), connected to a HP mass spectrometer (Model 5890/5970B, USA), and equipped with a HP data system. Vacuum evaporators were purchased from Heidolph, vortex mixer from Scientific Industries, centrifuge from Beckman and freezer from Eyela. The use of capillary columns facilitated at the analysis of the anabolic steroids, as it permits elution at lower temperatures in a short time and with a high resolving power.

2.3 *Procedure*

The reagents used in these experiments were purchased from Sigma (USA) and are of analytical grade. For the detection of the free steroids, 5 ml of urine were directly extracted with distilled diethylether. The solvent was dried over sodium sulphate, evaporated at room temperature under nitrogen and the residues dissolved in 30–50 µl of methanol and injected.

Table 1. Screening ions of steroids.

Steroids	Molecular weight of screening ions		
Nandrolone	405	422	420
Mesterolone	432	433	480
D_2-testosterone	–	434	–
Testosterone	–	432	–
Clostebol	466	451	468
Oxymesterone	534	519	389
Oxymetholone	550	495	143

Table 2. Composition of STD-K mixtures.

Administered drug	Excreted time (h)	Administered drug	Excreted time (h)
Nandrolone	8·0	Clostebol	8·0
Mestersterone	8·0	Oxymetholone	7·0
Testosterone	13·5	Oxymesterone	8·0
Boldenone	7·0		

2.4 *Urine collection, extraction, hydrolysis and derivatization*

Following collection of a 24 h control urine specimen, the anabolic steroid under investigation was administered orally to a human. For the analysis of conjugated steroids, 5 ml of urine were passed through a XAD-2 column, which was washed with 10 ml of distilled water and eluted with 0·9 ml methanol. The methanol was evaporated under reduced pressure, the residue was dissolved in 1 ml of 0·2 M phosphate buffer at pH 7·0 and 0·1 ml of enzyme was added. The human urine specimens were initially passed through Amberlite XAD-2 columns in order to concentrate the steroids prior to hydrolysis. The conjugated steroids were enzymatically hydrolyzed for

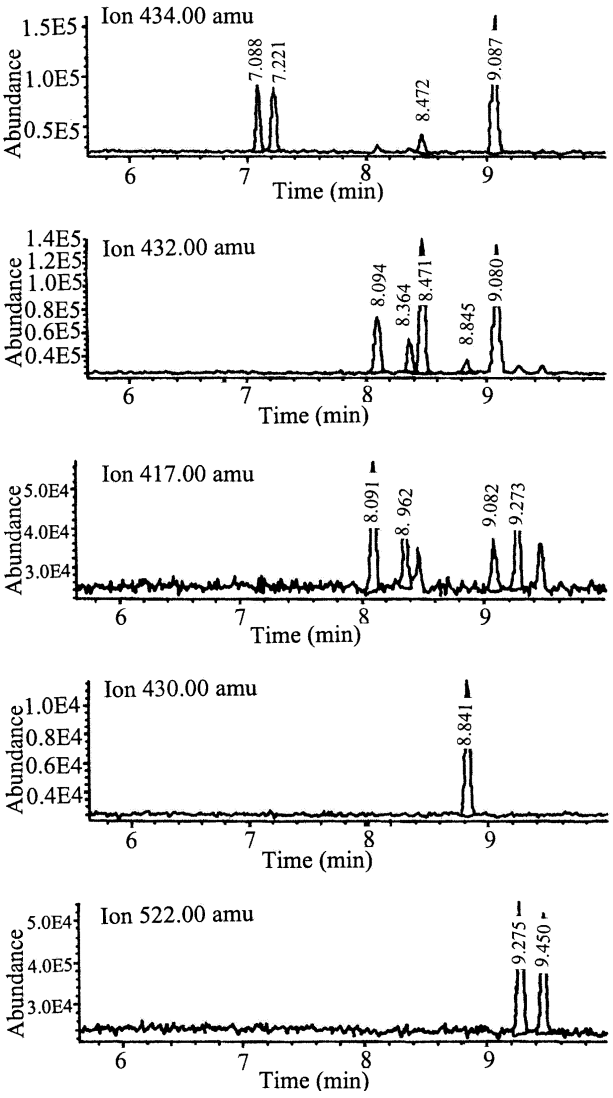

Figure 1. Total ion chromatogram of STD-13 mixture (E means 10 × and next number of E latter means exponential).

12–24 h at 40°C. This solution was brought to pH 8·5 with potassium bicarbonate and extracted with 5 ml of diethylether. The residue, after evaporation of the diethylether, was derivatized with 50 µl of a solution of N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA), N-trimethylsilylimidazole (TMSI) and dithioerythritol, according to the ratio 1,000 : 2 : 1, at 60°C for 15 min. The solution was then ready for gas chromatography. After 48 h incubation at 37°C, the free steroids were extracted on Amberlite XAD-2 columns.

2.5 *Gas chromatography/mass spectrometry*

The samples were then analysed by selected ion monitoring (SIM). The detection was carried out by selectively monitoring the same molecular ion and other characteristic

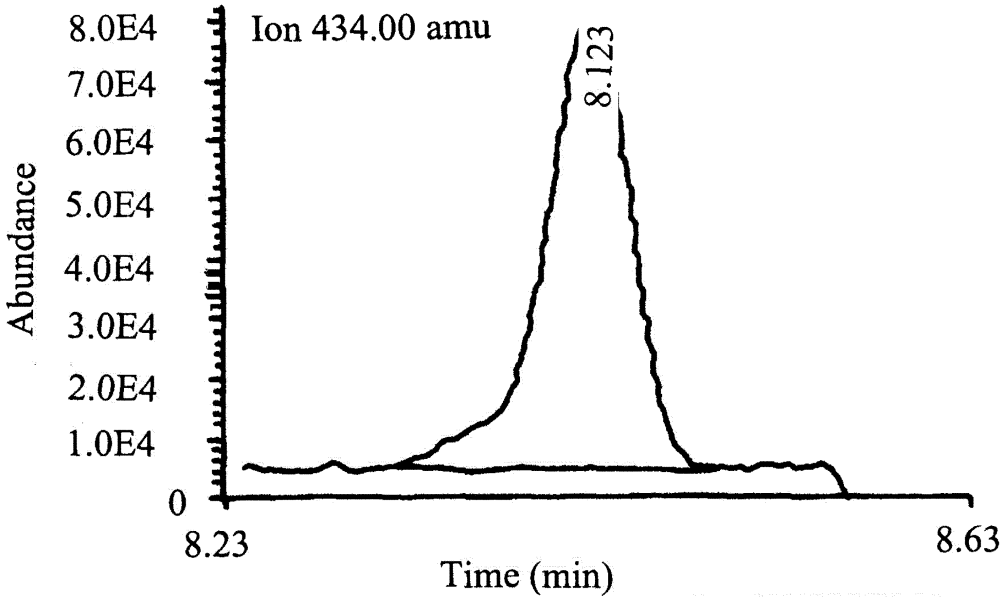

Figure 2. Chromatogram of testosterone passed through SE-30 column of 17 m length.

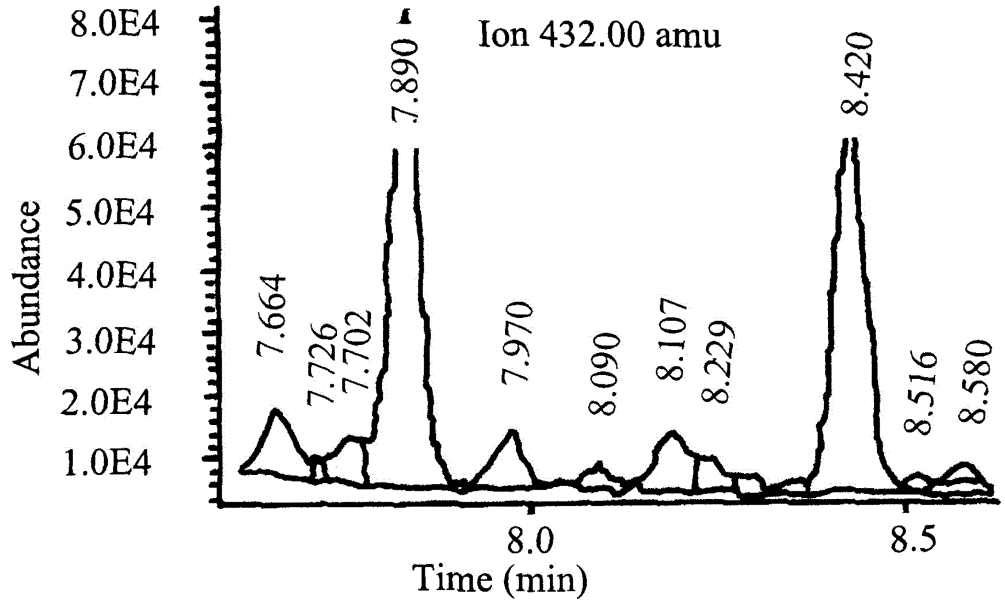

Figure 3. Chromatograms of testosterone and epitestosterone.

fragments of metabolites of the drug. The values of the retention time and of the ion intensity of various metabolites clearly verify the presence of anabolic steroids. Some of the more frequently used anabolic steroids with their molecular weights of screening ions are shown in table 1.

The capillary column used was a 17 m × 2 mm SE-30 glass column. The carrier gas used was helium (He) at a flow-rate of 1·2 ml/min at 300°C and a split ratio of 1 : 10. The temperature programmes were (i) 6 min isothermal at 180°C, then programmed at 4°C/min to 224°C, and (ii) at 15°C/min to 300°C. The column was interfaced to the mass spectrometer by direct insertion of the capillary in the ion source. Spectra were obtained by electron impact (70 eV) and in some cases by chemical ionization with methane, for a better identification of metabolites. A 1 µl aliquot of the purified sample solution was injected at 280°C in the split/splitless system or 3 µl aliquot with the splitter opened. Wherever possible, full spectra were recorded, and for samples where small amounts of drugs or metabolites were present, multiple ion detection was used with at least three or four selected masses.

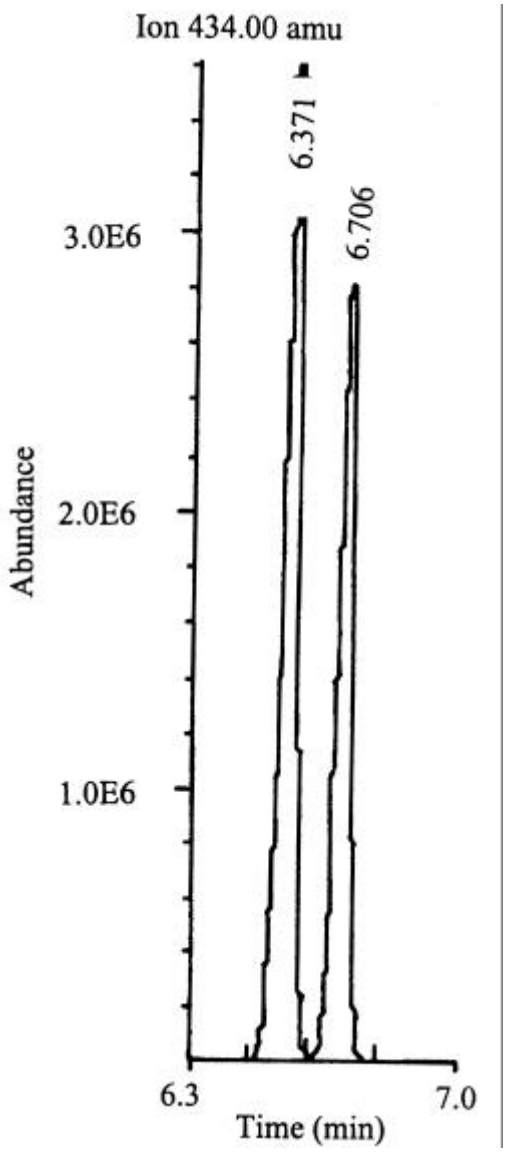

Figure 4. Chromatograms of *cis*-androsterone and etiocholanolone for determination of abundance.

The compositions of STD-K mixtures of anabolic steroids, were obtained from the total ion monitor of the mass spectrometer with the fused-silica capillary column is shown in table 2. The silylating reagent was evaporated, the residue was dissolved in cyclohexane and injected into the gas chromatograph. The chromatogram of untreated urine (blank) and of hydrolyzed urine collected and pretreated 8 h after the ingestions of single therapeutic dose of 4 ng/µl of *cis*-androsterone, etiocholanolone, dehydroepiandrosterone, androstanedione, epitestosterone, testosterone, androstendione, 11*b*-OH androsterone and 11*b*-OH etiocholanolone, respectively, was shown.

The detection of the administration of testosterone depends on the values of testosterone/epitestosterone (T/E) ratio (more than 6).

3. Results

Comparing various chromatograms, it appears that there were many interfering compounds in urine, but from the mass spectra recorded the presence of anabolic steroid and of its by the corresponding peaks, metabolite was clearly identified (figures 5–7). On the basis of computer-aided multiple-ion mass fragmentography and by comparison of the relative retention data, five peaks were identified for *cis*-androsterone and its epimer, etiocholanolone (m/z = 434), 11*b*-OH androsterone and its epimer, 11*b*-OH etiocholanolone (m/z = 522), and D_2-testosterone, respectively, is shown in figure 1. The chromatograms of STD-K mixtures of anabolic steroids, obtained from the total ion monitor of the mass spectrometer, with the fused-silica capillary column are shown in figure 2. Figures 3 and 4 show the chromatograms of the same mixture after derivatization. The silylated testosterone and its metabolite had retention times different from the underivatized steroid.

Their therapeutic dosages and main excretion forms of some of the more frequently used conjugated and endogenous steroids are given in table 3. The relative retention times of metabolites were shown in urine samples that were collected within 7 h of the administrating the conjugated steroids, respectively. The compounds and closely related metabolites were detectable in urine samples.

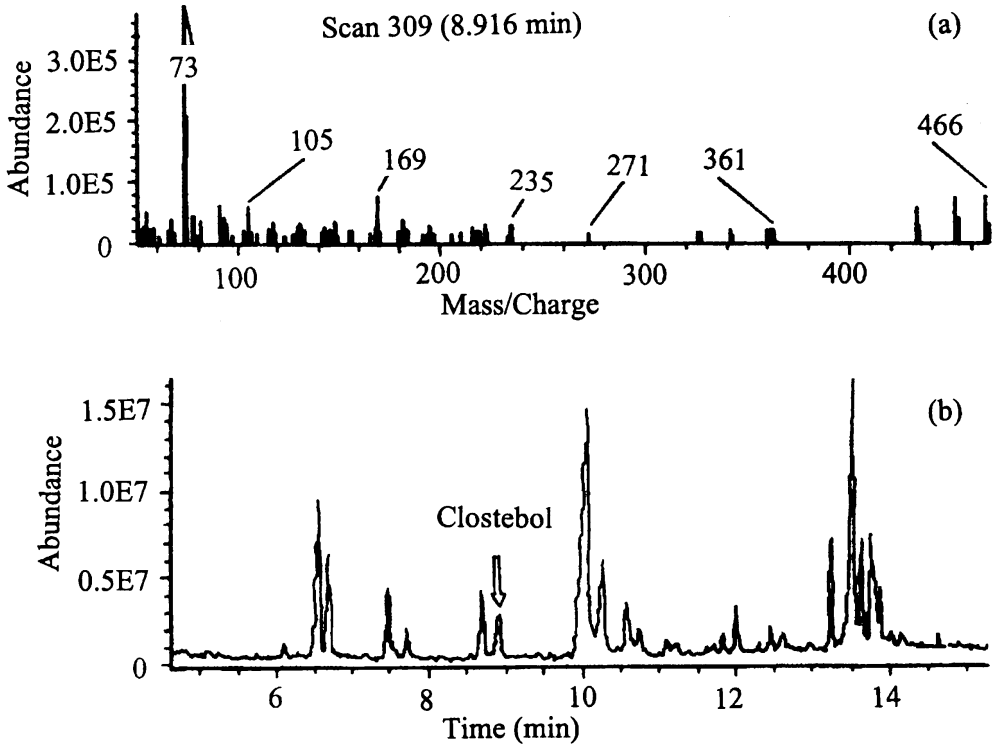

Figure 5. Mass spectrum (**a**) and total ion chromatogram (**b**) of clostebol.

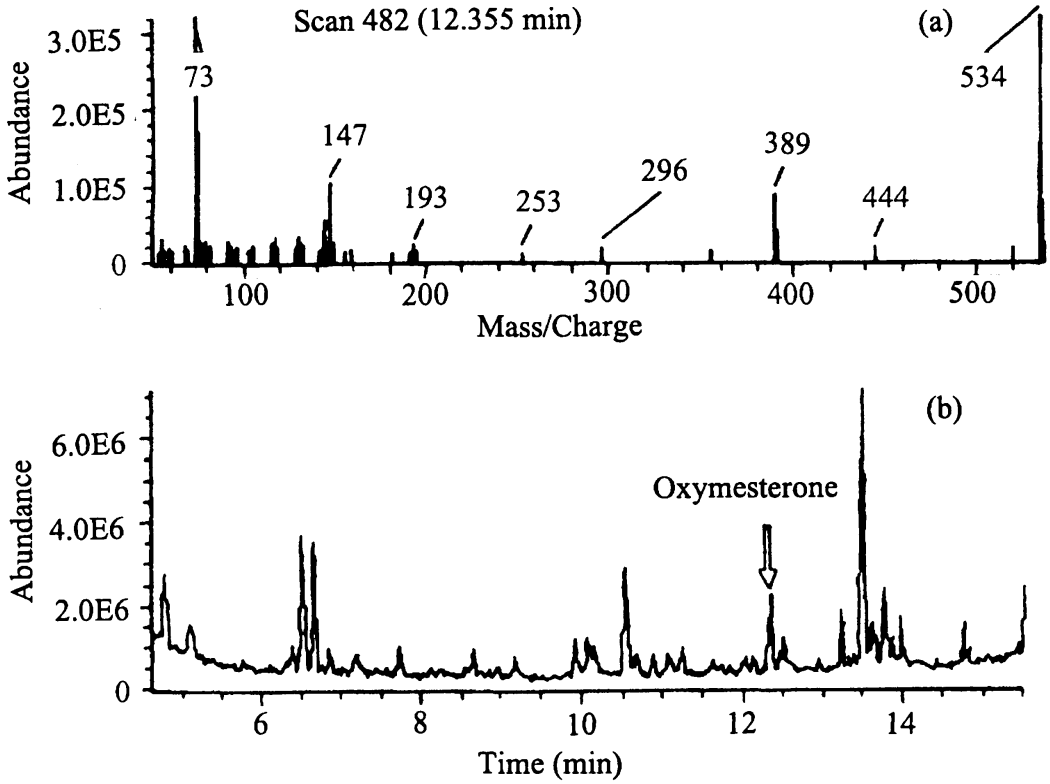

Figure 6. Mass spectrum (**a**) and total ion chromatogram (**b**) of oxymesterone.

Peak 16 was identified as the metabolite of clostebol, 4-chloro-3*a*-hydroxy-5*a*-androstan-17-one and its retention time was 8·916 min. Its compound was most suitable without interface (figure 5). There appeared the retention time, abundances and chromatogram of oxymesterone (figure 6, table 4). The metabolite was found as a minute quantity and its retention time was 12·355 min. In the mass spectrum (a) and total ion chromatogram (b) of oxymetholone (figure 7). 3*a*,6*b*,17*b*-trihydroxy-2-hydroxymethyl-17*a*-methylandrostan was identified as the

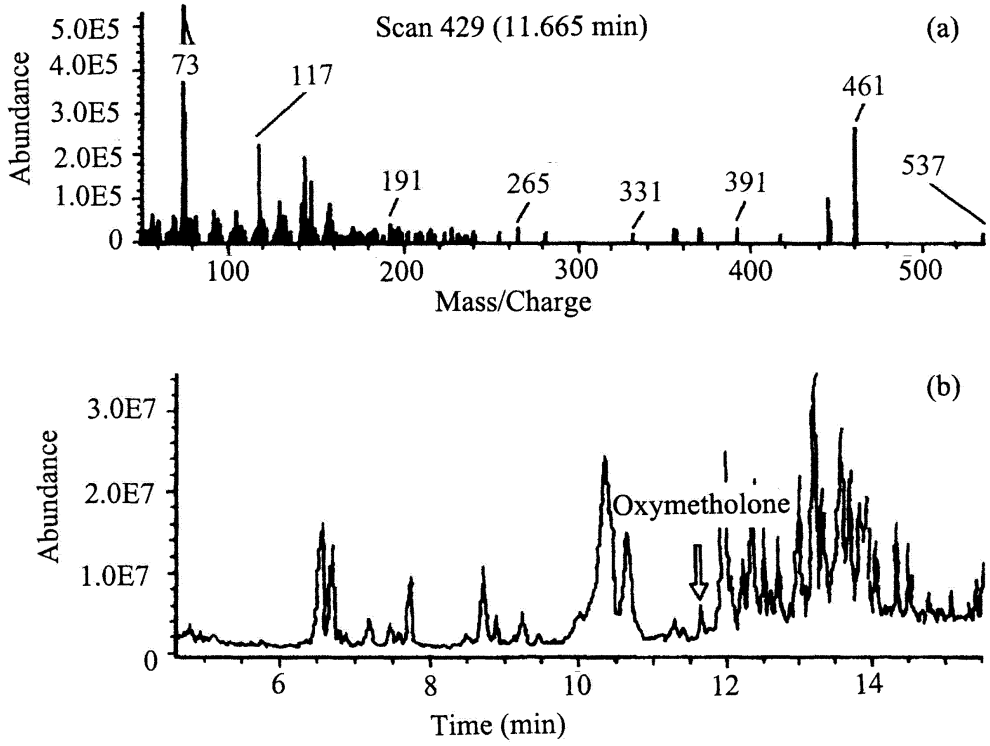

Figure 7. Mass spectrum (**a**) and total ion chromatogram (**b**) of oxymetholone.

Table 3. Relative retention time of conjugated and endogenous steroids.

Substances	Metabolites (TMS-derivatives)	Relative time (min)
Nandrolone	*cis*-norandrosterone	0·6626
	Noretiocholanolone	0·7417
cis-Androsterone		0·7776
Etiocholanolone		0·7936
DHEA		0·8792
Mesterolone	3 -hydroxy-1 -methyl-5 -androstan-17-one	0·9202
5 -androstandione		0·9203
Epitestosterone		0·9234
Mesterolone	3 -hydroxy-1 -methyl-5 -androstan-17-one	0·9728
Androstendione		0·9732
Boldenone	1-dehydrotestosterone	0·9810
D_2-testosterone		1·0000
Testosterone		1·0016
11 -OH androsterone		1·0319
11 -OH etiocholanolone		1·0539
Clostebol	4-chloro-3 -hydoxy-5 -androstan-17-one	1·0582
Oxymesterone		1·4283
Oxymetholone	3 ,6 ,17 -trihydroxy-2-hydroxymethyl-17 -methylandrostan	1·4706

Table 4. Relative abundance of oxymesterone.

m/z	Abundance	m/z	Abundance	m/z	Abundance	m/z	Abundance
51·15	407	78·15	402	128·15	538	191·20	433
53·15	507	79·05	900	129·15	1110	193·10	607
54·25	416	80·15	391	130·05	686	195·30	504
55·15	920	81·15	684	131·15	823	253·20	424
56·15	544	91·15	862	133·05	699	296·20	578
59·15	581	92·15	510	141·15	412	355·20	419
61·05	451	93·15	666	143·15	1794	389·30	2124
67·15	795	95·15	620	144·15	460	390·30	1156
69·05	518	102·95	470	147·05	3188	444·40	701
73·15	9733	105·15	629	148·15	697	519·40	653
74·15	1390	106·15	432	149·25	679	534·40	10000
75·15	5319	115·05	746	155·25	425	535·40	4709
76·05	597	117·15	1037	159·25	490	536·40	2157
77·15	862	119·15	634	181·25	439	537·40	790

m/z, Ratio of mass/charge.

metabolite of oxymetholone and its retention time was 11·665 min. The peak heights of testosterone and epitestosterone in the left window showed the ratio of integration (figure 8). Two peaks of cis-androsterone and etiocholanolone appeared in right window, the T/E values of which appeared more than 6. This shows that testosterone has been administered.

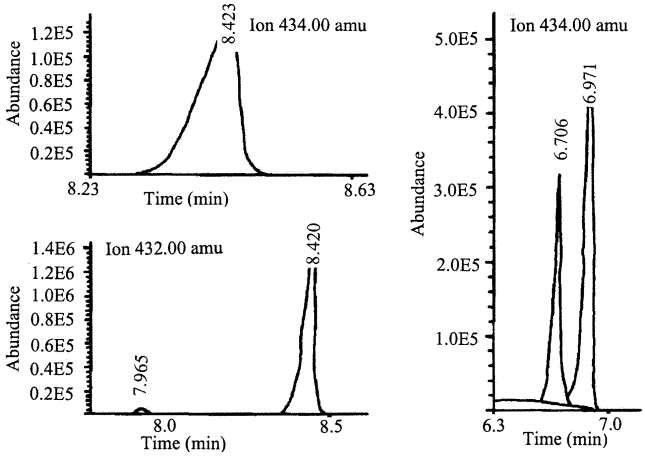

Figure 8. Determination of testosterone/epitestosterone ratio.

4. Discussion

The urinary metabolites of steroid hormones in men or mammals such as monkeys have been previously studied by Ward *et al* (1977), Dürbeck *et al* (1978), Cartoni *et al* (1983). Comparing the five chromatograms, they were capable of determining at least 10 mg more of either metabolite, with a standard deviation of ± 8%. The amount was equivalent to a concentration of 30 µg/l in urine and was similar to the results obtained following the administration of other steroid, such as methandienone (Cartoni *et al* 1983). Moreover, the result was in accordance with the detected level described by Dürbeck *et al* (1978). These results showed that on the basis of well-established GC/MS techniques, either with automated sampling or with data-processing devices, the administration of steroids could be detected with sufficient sensitivity.

Comparing the two chromatograms, it appears that the compounds were always eluted with symmetrical peaks, and the changes in the retention time between the free and the silylated steroids were very useful for the identification of the compounds in urine samples. Even though the silylated testosterone and its metabolite had retention times different from the underivatized steroid, in both cases they were easily detectable.

The results from the chromatograms obtained in this study showed that at least one major metabolite could be detected. The chromatograms presented exhibited various peaks (peak 1 ~18). By comparing of the relative retention data, the fastest peak (peak 1) was identified as the metabolite of nandrolone, *cis*-norandrosterone and its epimer, noretiocholanolone (peak 2). The slowest peak (peak 18) was identified to be the metabolite of oxymetholone, 3*a*,6*b*,17*b*-trihydroxy-2-hydroxymethyl-17*a*-methylandrostane (m/z = 550). Peaks 3 and 4 were identified as *cis*-androsterone and etiocholanolone, respectively. Peaks 14 and 15 were confirmed to be 11*b*-OH androsterone and 11*b*-OH etiocholanolone, respectively. Owing to the presence of the metabolite of oxymesterone, it was considered that this compound was administered. There was no any interface because the retention time of oxymesterone was slower than any other steroids and was therefore easily detectable. Generally, the values of the retention time and the ion intensity of these metabolites

clearly verify the presence of the anabolic steroids (Dürbeck *et al* 1978; Cartoni *et al* 1983). Peak 16 was identified as the metabolite of clostebol, 4-chloro-3*a*-hydroxy-5*a*-androstan-17-one and its retention time was 8·426 min. Its compound was relatively-easily detectable without interface. Peak 18 was identified as the metabolite of oxymetholone,3*a*,6*b*,17*b*-trihydroxy-2-hydroxymethyl-17*a*-methylandrostane. Two peaks of the metabolite were found at m/z = 550 and m/z = 495. These two fragment ions appear in the spectra of all oxymetholone metabolites.

The results from the chromatograms of testosterone and epitestosterone indicated that at least two major metabolites could be detected. Two peaks were identified as *cis*-androsterone and epicholanolone, respectively. For the ratio of testosterone and epitestosterone (T/E ratio), the record was generated as;

$$\text{T/E} = \frac{\text{peak height of testosterone} - \text{peak height of } D_2\text{-testosterone} \times 0{\cdot}03}{\text{peak height of epitestosterone}}.$$

Administration of testosterone can be detected if the value of T/E ratio is more than 6. In a normal situation approximately 10 ng should be injected in the gas chromatograph to obtain a full spectrum, and 50–100 pg for SIM detection. These amounts, according to the procedure described, correspond to concentrations in the urine of from 0·1 ppm to 1 ppb. We have thus shown that capillary GC/MS can be utilized in the detection of anabolic steroids and in the study of their metabolism (Cartoni *et al* 1983).

Acknowledgments

The authors are grateful to the Fisheries Science Institute of Kunsan National University for equipment and funding in the programme year of 2001. Thanks are also due to the referees who assisted in the preparation of this paper.

References

Axelson M, Schumacher G and Sjövall J 1974 Analysis of tissue steroids by liquid-gel chromatography and computerized gas chromatography-mass spectrometry; *J. Chromatogr. Sci.* **12** 535–540

Cartoni G P, Ciardi M, Giarrusso A and Rosati F 1983 Capillary gas chromatographic-mass spectrometric detection of anabolic steroids; *J. Chromatogr.* **279** 515–522

Dunlop P J, Bignell C M and Hibbert D B 2000 Use of gas chromatograms of essential leaf oils to compare clones of *Eucalyptus camaldulensis*; *Biochem. Syst. Ecol.* **28** 383–391

Dürbeck H W, Büker I, Scheulen B and Telin B 1978 Gas chromatographic and capillary column gas chromatographic-mass spectrometric determination of synthetic anabolic steroids; *J. Chromatogr.* **167** 117–124

Kagawa H, Young G, and Nagahama Y 1984 *In vitro* estradiol-17*â* and testosterone production by ovarian follicles of the goldfish, *Carassius auratus*; *Gen. Comp. Endocrinol.* **54** 139–143

Lota M L, de Rocca Serra D, Tomi F and Casanova J 2000 Chemical variability of peel and leaf essential oils of mandarins from *Citrus reticulata* Blanco; *Biochem. Syst. Ecol.* **28** 61–78

McFarilane J R, Cabrera C M, Coulson S A and Papkoff H 1991 Partial purification and characterization of rhinoceros gonadotropins, growth hormone, and prolactin: Comparison with the horse and sheep; *Biol. Reprod.* **44** 94–101

Petit F, Goff P L, Crvedi J P, Kah O, Valotaire Y and Pakdel F 1999 Trout estrogen receptor sensitivity to xenobiotics as tested by different bioassays; *Aquaculture* **177** 353–365

Stan H J and Abraham B 1980 Determination of residues of anabolic drugs in meat by gas chromatography-mass spectrometry; *J. Chromatogr.* **195** 231–241

Swanson P, Suzuki K, Kawauchi H and Dickhoff W W 1991 Isolation and characterization of two coho salmon gonadotropins, GTH I and GTH II; *Biol. Reprod.* **44** 29–38

Verbeke R 1979 Sensitive multi-residue method for detection of anabolics in urine and in tissues of slaughtered animals; *J. Chromatogr.* **177** 69–84

Ward R J, Lawson A M and Shackleton C H L 1977 Metabolism of anabolic steroid drugs in man and the marmoset monkey (*Callithrix jacchus*)-1. Nilevar and orabolin; *J. Steroid Biochem.* **8** 1057–1063

Wright R S and Hunt S M 1982 A radioimmunoassay for 17*á*20*â*-dihydroxy-4-pregnen-3-one: Its use in measuring changes in serum levels at ovulation in Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*), and rainbow trout (*Salmo gairdneri*); *Gen. Comp. Endocrinol.* **47** 475–482

Yeoman R R, Williams L E, Aksel S and Abee C R 1991 Mating-related estradiol fluctuations during the estrous cycle of the Bolivian squirrel monkey (*Samimiri boliviensis*); *Biol. Reprod.* **44** 640–647

Young G, Ueda H and Nagahama Y 1983 Estradiol-17*â* and 17*á*,20*â*-dihydroxy-4-pregnen-3-one production by isolated ovarian follicles of amago salmon (*Oncorhynchus rhodurus*) in response to mammalian pituitary and placental hormones and salmon gonadotropin; *Gen. Comp. Endocrinol.* **52** 329–335

MS received 18 June 2001; accepted 12 September 2001

Corresponding editor: SAMIR BHATTACHARYA