

## QUANTITATION OF OXANDROLONE(A SYNTHETIC ANABOLIC STEROID) IN HUMAN URINE BY GC / MS

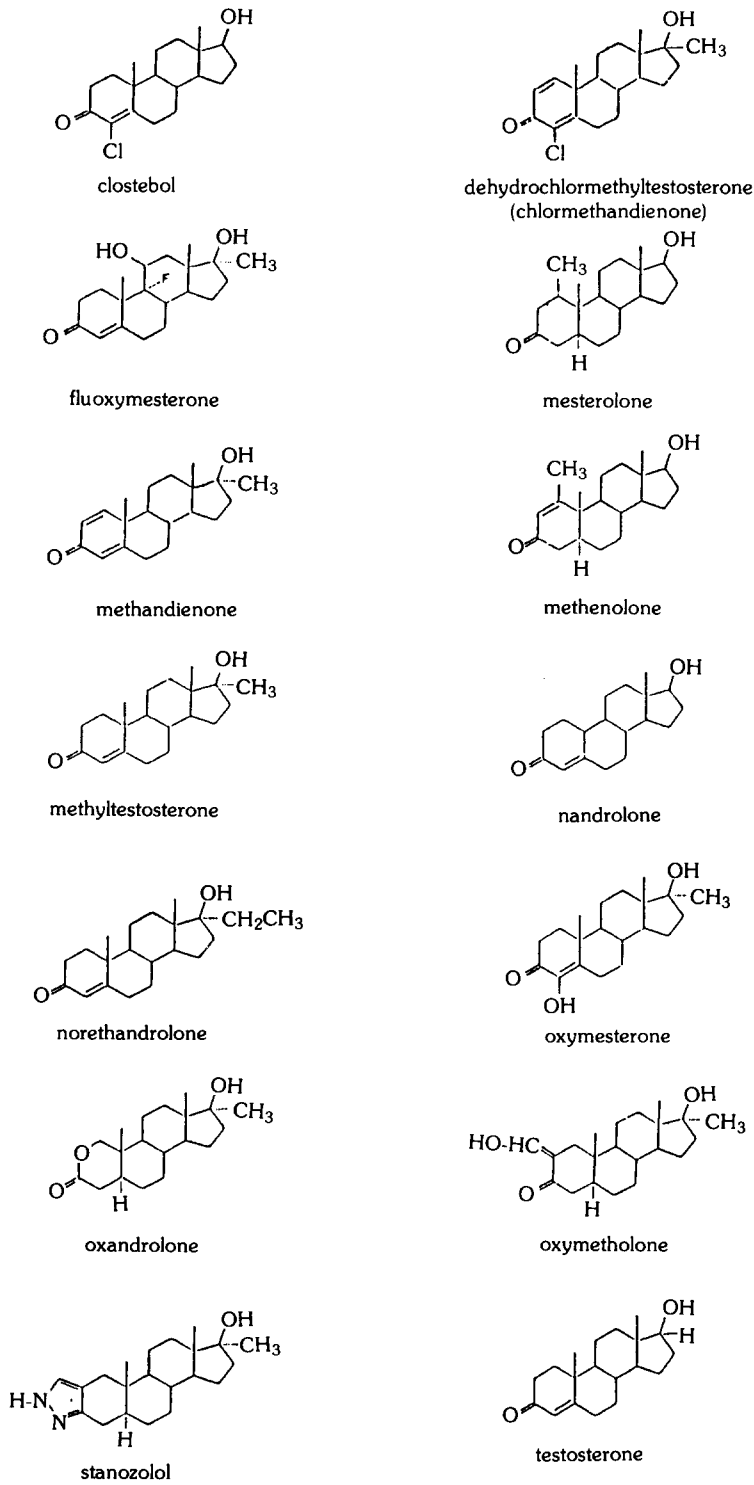
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**ABSTRACT:** A sensitive method for the quantitation of oxandrolone in urine was developed using GC/MS. After oral administration of 10mg oxandrolone, oxandrolone excreted in urine as unchanged form was extracted in ether and derivatized to its O-TMS. Oxandrolone excreted in urine as glucuronide conjugated form was extracted after enzymatic hydrolysis and derivatized to its O-TMS. The amount of oxandrolone-O-TMS was measured in GC/MS with selected ion monitoring. Calusterone, a structurally similar anabolic steroid, was employed as internal standard. The oxandrolone concentration in urine was 50-900 ng/ml for 26 hrs and the excretion half life was 10.4 hrs. The relative standard deviation was 1.0-8.5%.

### INTRODUCTION

Testosterone, a male sex hormone, has both androgenic effect and anabolic effect. There were many attempts to prepare anabolic steroid with minimum androgenic effects but with maximum anabolic effects. However, every anabolic steroid in the market at present time has some androgenic effects. Therapeutically anabolic steroids are used to enhance the weight gain, to reverse the negative nitrogen balance associated with post operative periods and glucocorticoid therapy (1-3). They are also successfully employed in treating the bone decalcification associated with osteoporosis and the aging process(4). In addition to these therapeutic purposes, anabolic steroids are widely abused by athletes in hopes to gain muscle strength and body weight. However, administration of anabolic steroids for long period is known to cause disorders of reproductive system and liver(5). Therefore, International Olympic Committee(IOC) has banned the use of such anabolic steroids on ethical grounds as well as for the health of athletes(6). Following anabolic steroids shown in Fig. 1 are banned.

Oxandrolone(17 $\beta$ -hydroxy-17-methyl-2-oxa-5 $\alpha$ -androstan-3-one) is one of the most widely abused anabolic steroids since it was synthesized in 1962(7). It is a heterocyclic analog of 17 $\alpha$ -methyl testosterone and is orally active. The disposition and metabolic fate of oxandrolone in man was studied using <sup>14</sup>C-oxandrolone(8). However, the quantitation of oxandrolone in plasma or urine employing GC, HPLC or MS has not been reported. In this paper the concentration of oxandrolone in urine is determined



**Fig. 1.** Structure of anabolic steroids.

by using GC/MS quantitation with selected ion monitoring mode. Calusterone, a structurally similar 17 $\alpha$ -methyl anabolic steroid, is employed as the internal standard.

## EXPERIMENTAL

### Materials

Oxandrolone was supplied by Searle Co. (Seoul, Korea) and calusterone was purchased from Up John Co. (Kalamanzoo, MI, USA). Diethylether and methanol which were distilled over calcium hydride were used. Amberlite XAD-2 resin (particle size 0.15-0.2mm) was supplied by Serva Co. (Heidelberg, FRG) and was washed with acetone, methanol and distilled water before use.  $\beta$ -Glucuronidase (activity 25 U/ml) was purchased from Boehringer Co. (Indianapolis, USA). The silylating agents, MSTFA (N-methyl-N-trimethylsilyl trifluoroacetamide), TMS-Cl (trimethyl chlorosilane) and TMS-imidazole (trimethylsilyl imidazole) was obtained from Sigma Co. (St. Louis, USA). A methanolic solution of 10  $\mu$ g/ml calusterone was prepared and stored at 4°C.

### Instruments

A Hewlett-Packard Gas Chromatograph/Mass Selective Detector (5890/5970B) was used. A cross-linked 5% phenyl methylsilica capillary column (length 16m, ID 0.2 mm, film thickness 0.33 $\mu$ m) was directly connected to the ion source. Helium, at a flow rate of 0.85 ml/min, was employed as carrier gas. Samples were injected in splitless mode. Temperatures of injector and transfer line were set at 300°C. Oven temperature was 180°C and increased by 25°C to 300°C and stayed for 2.5 min.

### Drug Administration

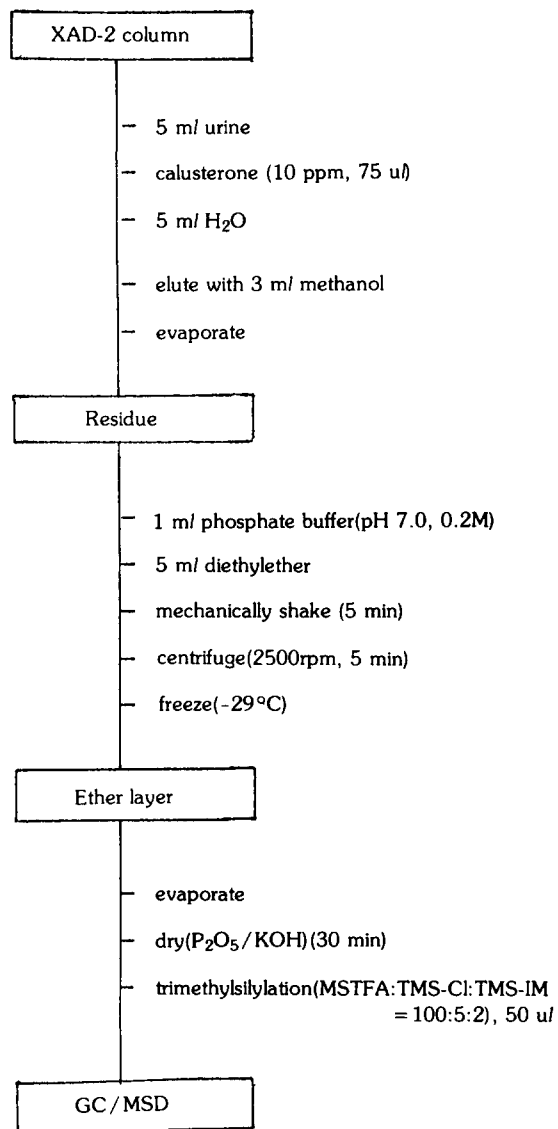
Oxandrolone (10mg) was administered to a healthy man (70kg) orally. Urine samples were collected at 0, 2, 5, 10, 17, 20 and 26 hrs post-dose. The urine samples were stored at 4°C.

### Extraction of steroid

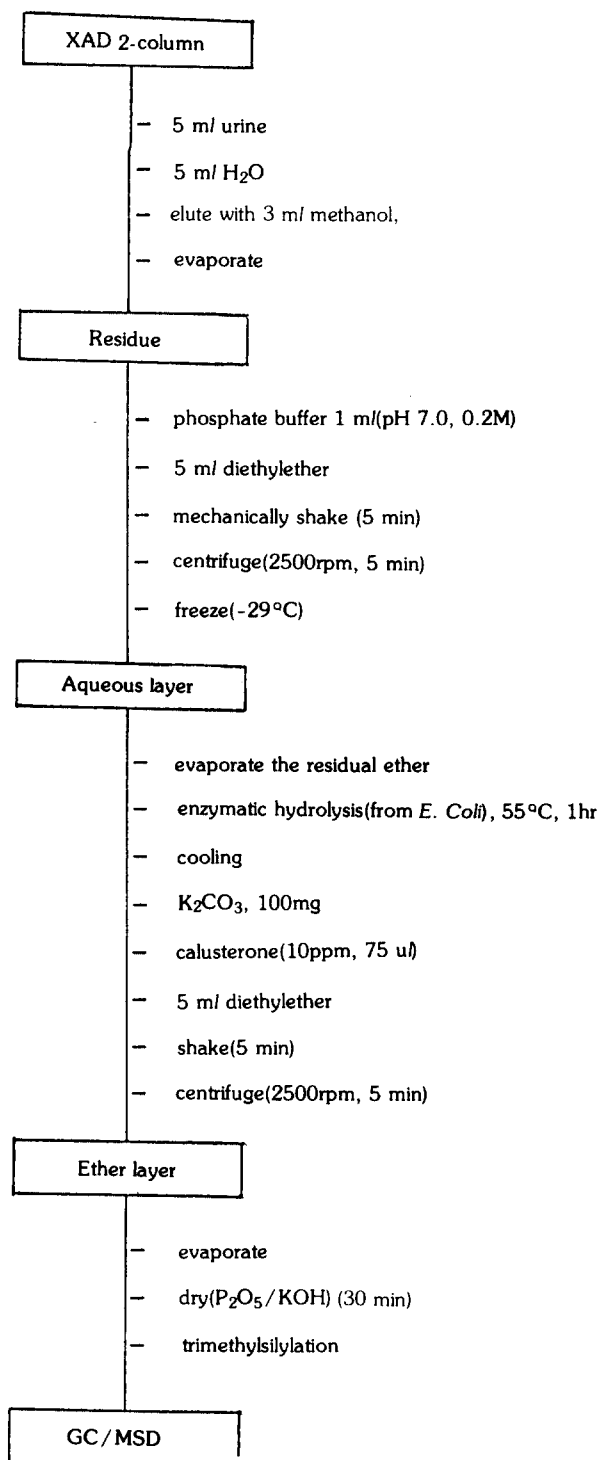
#### a) Extraction of the free steroid fraction

An aqueous XAD-2 slurry was filled into a pasteur pipet (I.D 0.5cm) until a bed of 3cm of height is achieved. Urine (5 ml) and internal standard solution (10 ppm, 75  $\mu$ l) were applied to the column. The XAD-2 column was washed with an equal volume of water. The absorbed steroids were eluted with 3 ml of methanol. The methanol solution was evaporated to dryness by vacuum rotary evaporator. Phosphate buffer (0.2M, pH 7.0) (1 ml) and ether (5 ml) were added to the residue. After mechanical shaking (5 min) and centrifugation (2500rpm, 5 min), the organic layer was transferred to another test tube. The ether was removed by a rotating evaporator. The residue was dried in vacuum desiccator over P<sub>2</sub>O<sub>5</sub>/KOH for at least 30 minutes.

#### b) Extraction of the conjugated steroid fraction



**Scheme 1.** Extraction and derivatization procedure for oxan drolone of free form



**Scheme 2.** Extraction and derivatization procedure for oxandrolone of conjugated form.

To the XAD-2 column, 5 ml urine was applied. The column was washed with water, then eluted with methanol. The methanolic solution was evaporated. Phosphate buffer (0.2M, pH 7.0) (1 ml) and ether (5 ml) were added to the residue. After mechanical shaking (5 min) and centrifugation (2500 rpm, 5 min), the ethereal layer was removed. The phosphate buffer was rinsed with 2 ml of diethylether. The ethereal layer was removed and discarded. To the phosphate buffer layer 10  $\mu$ l of the  $\beta$ -glucuronidase (25 U/ml) were added and heated to 55°C for 1 hr. The solution was cooled to room temperature and 100 mg of potassium carbonate, calusterone internal standard solution (100ppm, 75  $\mu$ l) and 5 ml of diethylether were added. The mixture was mechanically shaken (5 min) and centrifuged (2500 rpm, 5 min) and the organic phase was transferred to another test tube. The ether was evaporated to dryness in vacuum rotary evaporator. The residue was dried in vacuum desiccator over  $P_2O_5$ /KOH.

### Derivatization

The residue was trimethylsilylated (9-10) with 50  $\mu$ l of the reagent mixture, MSTFA/TMS-Cl/TMS-imidazole (100:5:2, v:v:v) and heated to 80°C for 15 minutes.

### Quantitation

Selected ion chromatograms were obtained at ion  $m/z$  143 and ion  $m/z$  315 for the analysis of oxandrolone and calusterone, respectively. Peak area ratio was calculated by dividing the area of the  $m/z$  143 peak of oxandrolone-OTMS by the  $m/z$  315 peak of calusterone-OTMS-enol-OTMS in Chemstation.

### Calibration samples

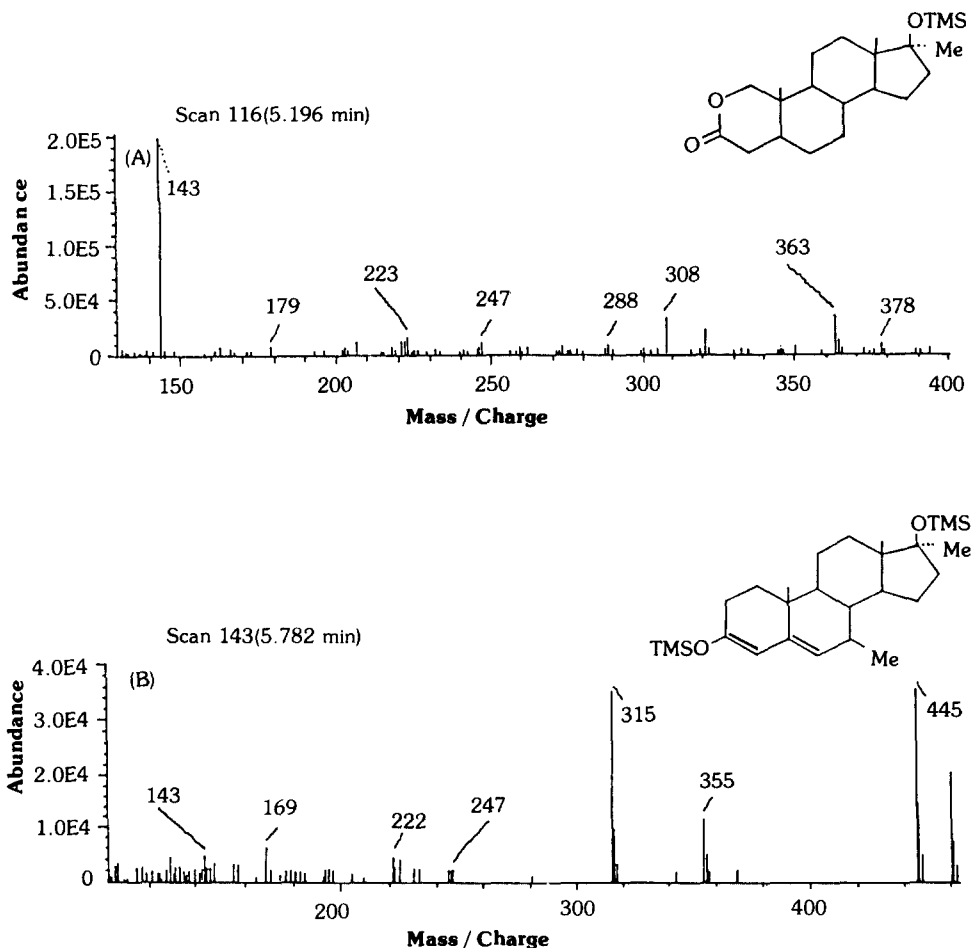
For the preparation of calibration samples, methanolic solutions of oxandrolone and internal standard were spiked to a blank urine. Sample concentrations were 10, 20, 50, 100, 150 and 250 ng/ml of oxandrolone and the internal standard calusterone concentration was 150 ng/ml. For estimating the concentration of conjugated oxandrolone fraction, the drug and internal standard solutions were spiked to the phosphate buffer layer after enzyme hydrolysis.

## RESULTS AND DISCUSSION

### Analytical considerations

The mass spectra of the TMS derivatives of oxandrolone and calusterone are shown in Fig. 2. The structures of the major ion fragments are also shown in Fig. 2. Oxandrolone-OTMS gave the ions  $m/z$  143(D-ring), 308, 321, 363 ( $M^+$ -CH<sub>3</sub>) and 378 ( $M^+$ ). The ion  $m/z$  143 was formed by D-ring cleavage and it is the characteristic ion for TMS derivatives of 17-methyl-17-hydroxy anabolic steroids. Calusterone-OTMS-enol-OTMS gave ions  $m/z$  143 (D-ring cleavage) but  $m/z$  315( $M^+$ -D-ring) was shown as the base peak.

Therefore,  $m/z$  143 and  $m/z$  315 peaks were selected for the mass spectral quantitation of oxandrolone and calusterone, respectively, on the basis of maximum abun-



**Fig. 2.** Full spectrums of oxandrolone-OTMS(A) and calusterone-bis-OTMS (B).

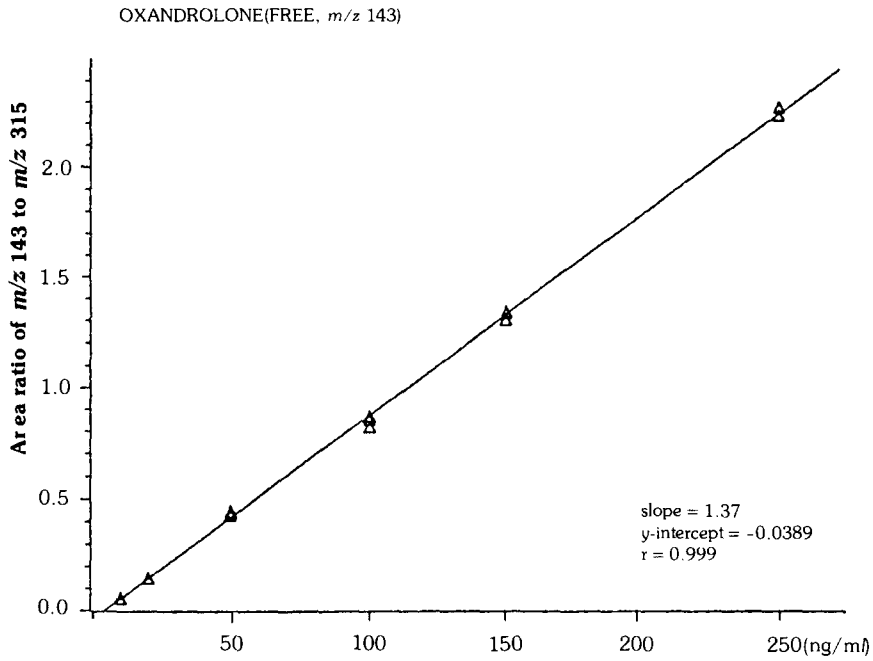
dance without interference at its retention time.

### Calibration curve

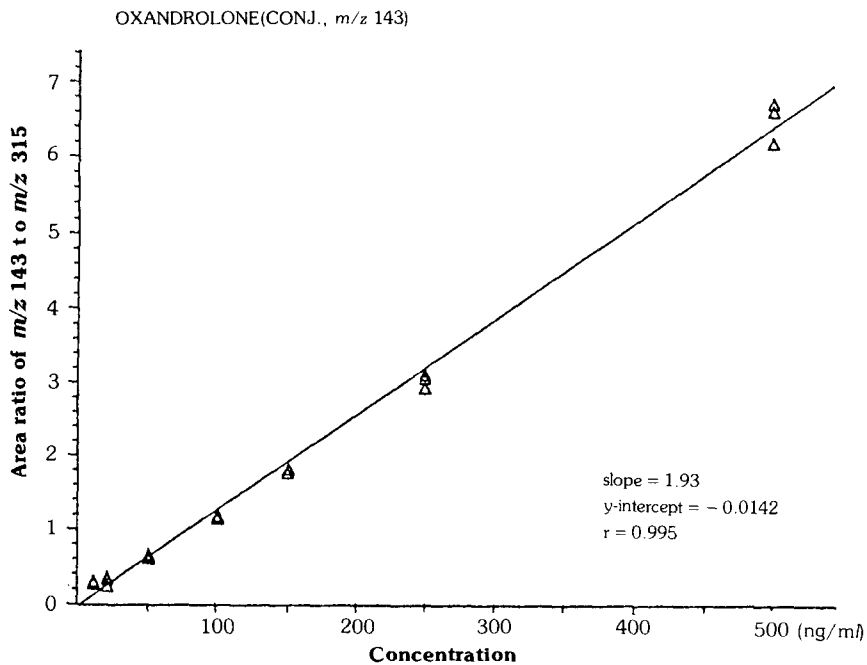
Calibration curves were linear from 10 to 250 ng/ml. The calibration curve for free steroid fraction is illustrated in Fig. 3 ( $r=0.984$ ). Quality control sample with 120 ng/ml has resulted in  $122.7 \pm 1.76$  ng/ml (RSD = 1.4%,  $n = 3$ ). Fig. 4 shows the calibration curve for conjugated steroid for fraction 0.995. Quality control samples with 120 ng/ml showed  $115.9 \pm 11.2$  ppm (RSD  $\pm 9.7\%$ ,  $n = 3$ ).

### Determination of oxandrolone in urine

The free fraction of oxandrolone post-dose urine showed  $m/z$  143, 308, 321, 363 peaks at retention time of 5.720 min and 5.314 min as illustrated in Fig. 5. The peaks at 5.720 min was identical to authentic oxandrolone-OTMS. The peaks at 5.314 min was considered as a metabolite of oxandrolone (epioxandrolone-OTMS). As in Fig. 5,

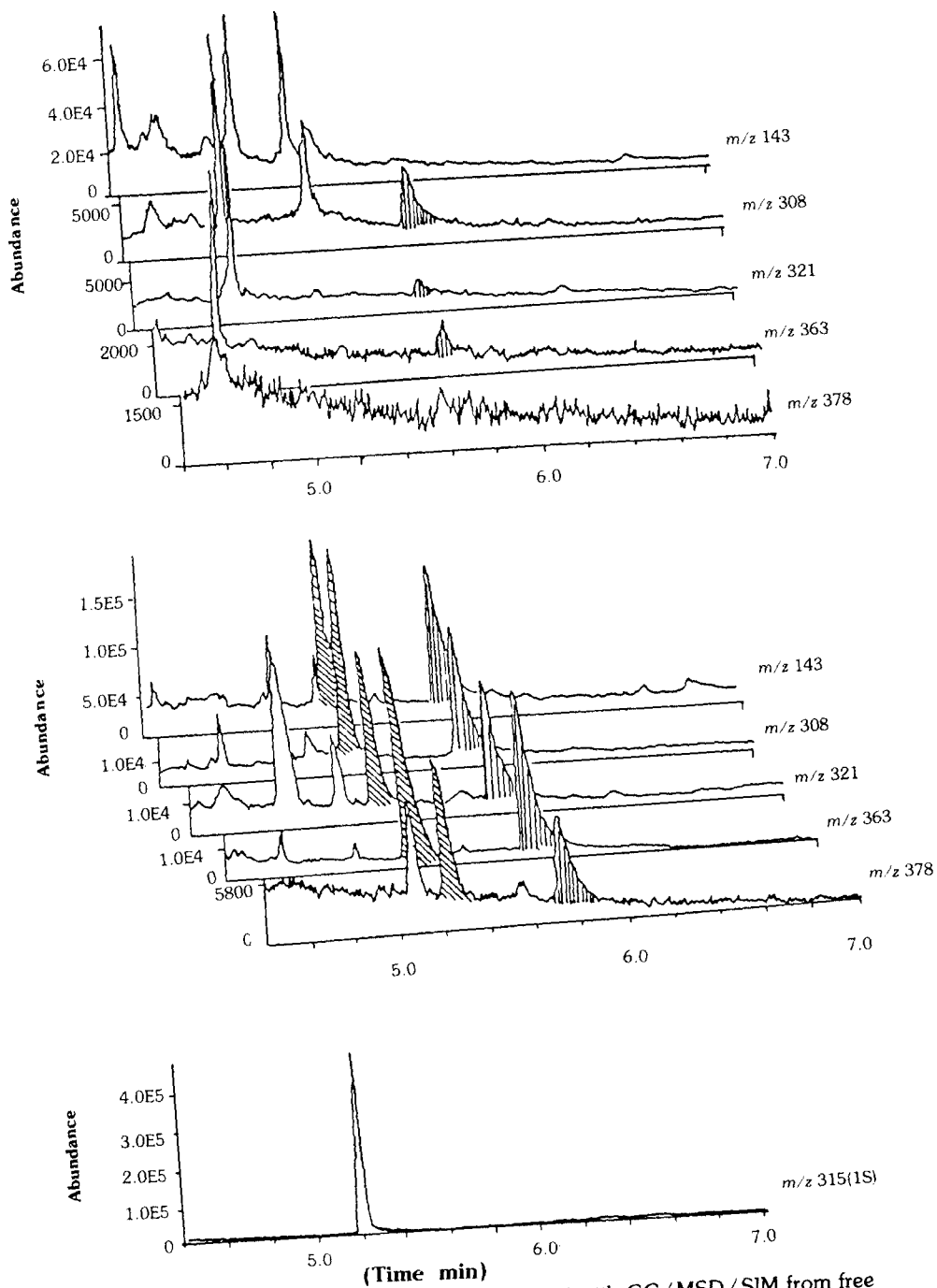


**Fig. 3.** Calibration curve of oxandrolone from free fraction ( $n = 3$ ).

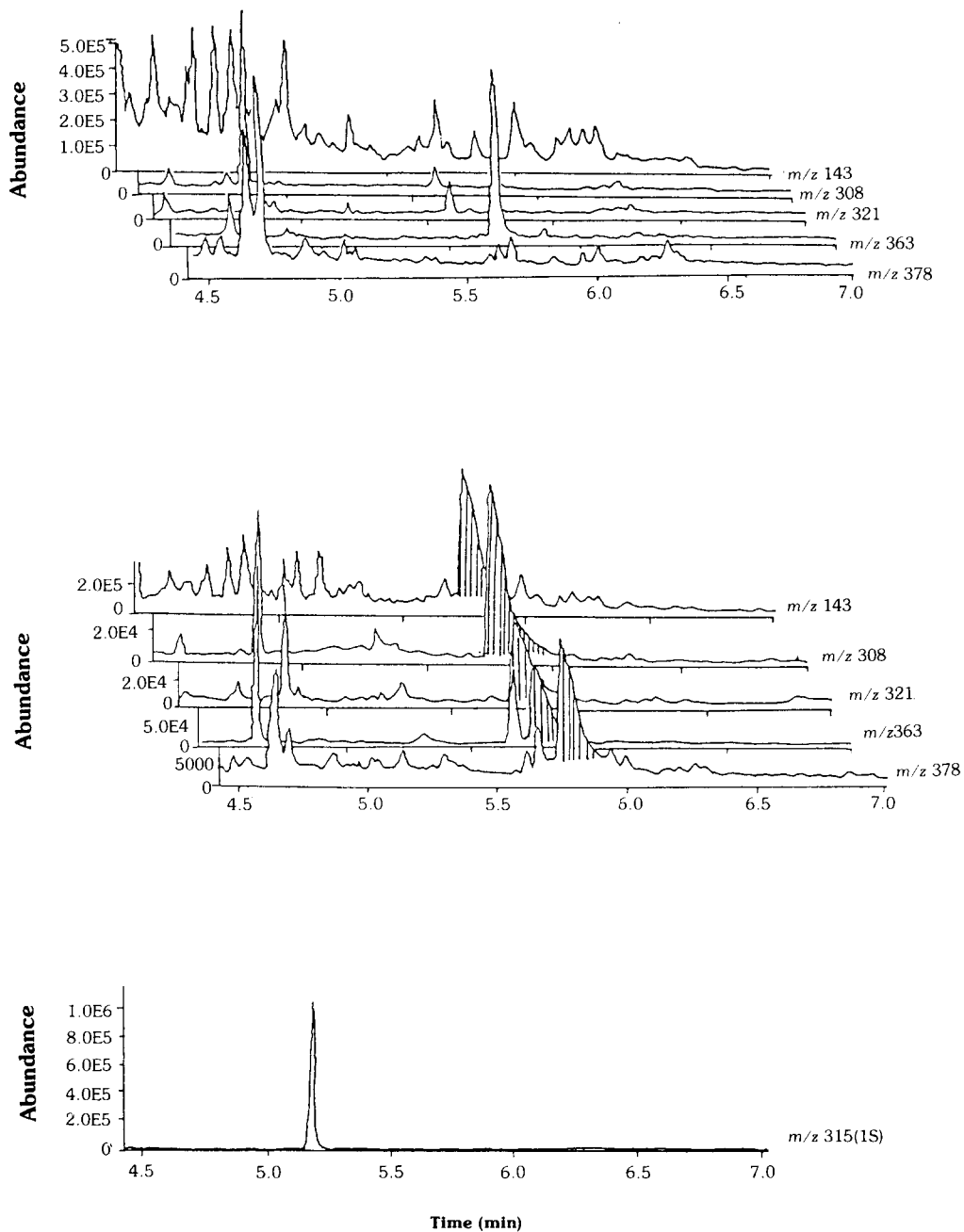


**Fig. 4.** Calibration curve of oxandrolone from conjugated fraction ( $n = 3$ ).





**Fig. 5.** Chromatograms of each ion obtained with GC/MSD/SIM from free fraction;  
 (A) Blank urine  
 (B) Urine at 34 hr after oral administration of oxandrolone (10mg)  
 (C) Calusterone (15 ppm) in urine



**Fig. 6.** Chromatograms of each ion obtained with GC/MSD/SIM from conjugated fraction;  
 (A) Blank urine  
 (B) Urine at 7.5 hr after oral administration of 10mg oxandrolone  
 (C) Calusterone (15 ppm) in urine

the free fraction of blank urine showed no interference peak for  $m/z$  143 ion at 5.720 min. Therefore,  $m/z$  143 ion peak was employed for the quantitation of oxandrolone in urine. The detection limit was 4 ng/ml.

The conjugated fraction of blank urine showed more peaks for ion  $m/z$  143 as illustrate in Fig. 6. The detection limit of 20 ng/ml was considerably higher than the free fraction. This may be due to the small interference peak appearing at 5.6 min.

The oxandrolone concentration present in the urine after oral administration of 10 ng oxandrolone at different time interval was shown in Table 1. The oxandrolone concentration in the free fraction was about two times higher than that of conjugated fraction. The mean urinary excretion half-life of the steroid derived from the semilogarithmic plot (Fig. 7.) was 10.24 hours. This urinary half-life of oxandrolone is well correlated with the result of A. Kalim *et al*(8). However the total excreted amount of oxandrolone was only 7.5% of the total dose administered. This recovery is far below than the recovery of 60% of radioactivity which were reported by A. Kalim *et al*(8). However, Dubeck *et al.* recovered only 5% of methandienone as two major metabolites when they analysed the urine with GC/MS (11). And they suggested that the rest of steroids might be stored in fats or muscles.

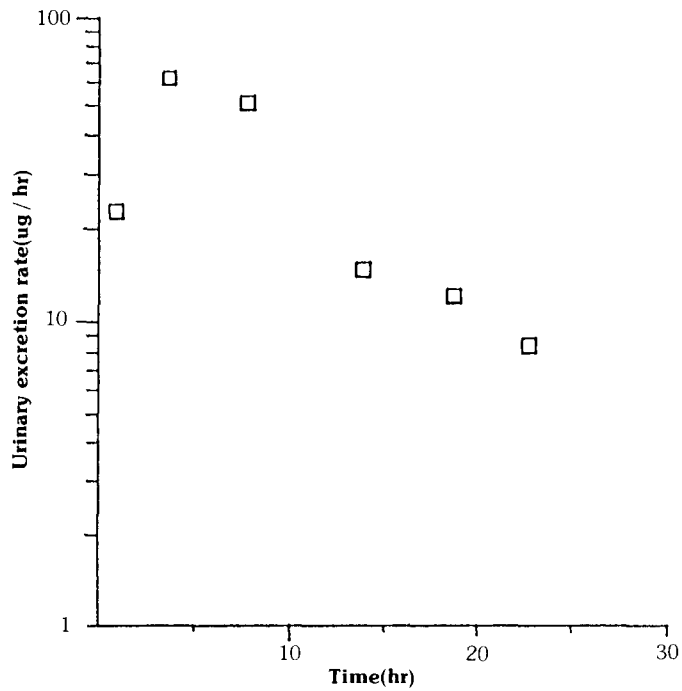
## Urinary metabolites

It is known that oxandrolone is relatively resistant to metabolic transformation due to a presence of lactone structure in the A-ring. However, two metabolite peaks were detected. The one found in free fraction showed same mass spectrum as the oxandrolone-OTMS and it was assigned as 17-epi-oxandrolone-OTMS. Methandienone, which has 17-methyl and 17-hydroxyl group in its structure, is known to be metabolized to its epimer (12). The other peak found in the conjugated fraction showed mass spectrum  $m/z$  218, 231 (13) and 466 at retention time of 6.93 min. Since the 16-hydroxylated metabolite of stanozolol showed  $m/z$  218 and 231 after derivatization, this compound could be 16-hydroxyoxandrolone-bis-OTMS. In addition, 16-hydroxyoxandrolone is known as the metabolite of oxandrolone produced in rabbit (14). Two more metabolite peaks were detected, but the structure couldn't be elucidated.

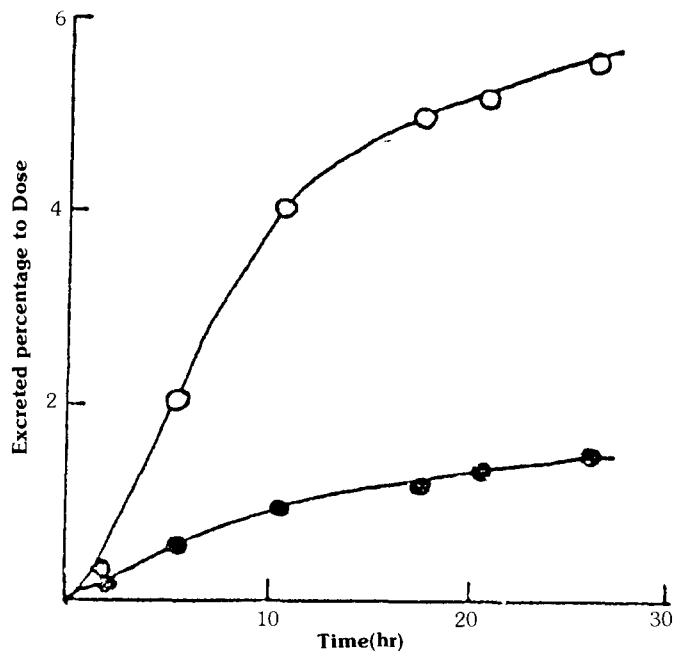
**Table 1.** Concentration of oxandrolone in human urine after oral administration.

Time(hr)	Free	Concentration(ng/ml)		RSD(%)
		RSD(%)	Conjugated	
0 -2.0	102.9	1.0	70.4	1.8
2.0-5.3	858.2	1.3	179.3	1.9
5.3-10.4	891.4	3.6	153.1	1.6
10.4-17.3	215.2	8.5	641.2	4.4
17.3-20.1	48.7	2.9	476.2	5.7
20.1-26.0	143.2	5.1	62.4	1.4

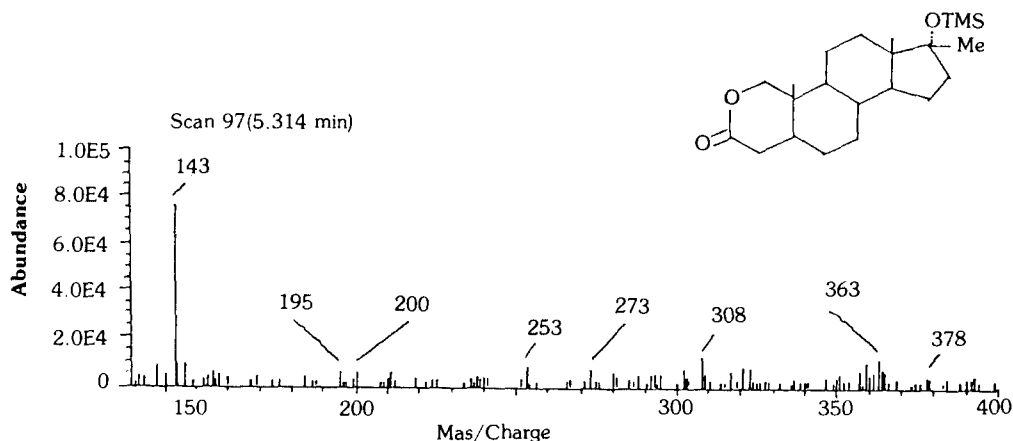
Each represents mean of 3 experiments.



**Fig. 7.** Urinary excretion rate versus midpoint time plot of oxandrolone after oral dosing of 10 mg to volunteer.



**Fig. 8.** Urinary accumulative curve after oral administration of oxandrolone (10 mg);  
- ○ - : from free fraction  
- ● - : from conjugated fraction



**Fig. 9.** Full spectrum of 17-epioxandrolone as a metabolite of oxandrolone.

## Conclusion

The amounts of oxandrolone excreted in urine were determined by GC/MS. The detection limit was 4 ng/ml and the half-life was 10.24 hours.

This study showed good specificity and sensitivity and therefore, GC/MS could be employed in metabolic studies as well as nanogram range quantitation without using radiolabelled compounds. Also the present method employed for the oxandrolone study could be used in the detection of other anabolic steroids.

## REFERENCES

- Pacetti, W.A. (1964); *Curr. Ther. Res.*, 6: 261.
- Renzi, A.A. and Chart, J. J. (1972); *Proc. Soc. Exp. Biol. Med.*, 110: 259.
- Albancese, A.A. (1965); *J. New Drugs*, 5: 208.
- Kopera H., in *Anabolic-Androgenic Steroids*, C.D. Kochakian, Ed., Springer-Verlag, New York, 1976, p. 573.
- Humplt, H.A., Rovere, G.D. (1984); *Am. J. Sports. Med.*, 12: 469.
- "Games of XXI Olympiad Montreal" (IOC Medical Commission) 1976.
- Fox, M., Minot, A.S. and Liddle, G.W. (1962); *J. Clin. Endocrinol Metab.*, 22: 921.
- Kalim, A., Ranney, R.E., Zagarella, J. and Maibach, H.I. (1973); *Clin. Pharm. Therapeutics*, 14: 862.
- Donike, M. (1969); *J. Chromatogr.*, 42: 103.
- Donike, M. (1975); *J. Chromatogr.*, 103: 91.
- Dubeck, H. W., Buker, I., Scheulen, B. and Telin, B. (1978); *J. Chromatogr.*, 167: 117.
- MacDonald, B.S., Sykes, P.J., Adhikary P.M. and Hackness R.A. (1971); *Biochem. J.*, 122: 26.
- Schanzer, W., Opfermann, G. and Donike, M. *in press*.
- Watabe, T., Yagishita, S. and Hara, S. (1970); *Biochem. Pharmacol.*, 19: 1485.