Reprint from

10th Cologne Workshop On Dope Analysis 7th to 12th June 1992 - Proceedings -

M. Donike
H. Geyer
A. Gotzmann
U. Mareck-Engelke
S. Rauth
(Editors)

Sport und Buch Strauß, Köln, 1993

V. VLADIMIROVA, A. TSOUTSOULOVA-DRAGANOVA, M. ANGELOVA:
Detection, Identification and Excretion Profile of Oxymetholone Metabolites in Urine
In: M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.) 10th Cologne Workshop On Dope Analysis 7th to 12th June 1992. Sport und Buch Strauß, Köln, (1993) 101-120

V. Vladimirova, A. Tsoutsoulova-Draganova and M. Angelova

Detection, Identification and Excretion Profile of Oxymetholone Metabolites in Urine

Doping Control Laboratory, Sofia, Bulgaria

Summary

The metabolites of oxymetholone have been investigated in urine samples taken from three healthy volunteers after oral administration of Plenastril tablet. Extraction, derivatization and gas chromatography/mass spectrometry analyses of metabolites have been performed by routine procedure used in our laboratory for free and conjugated anabolic steroids.
Two metabolites were isolated from the free fraction under acidic conditions (pH 1). 19 metabolites of oxymetholone have been detected and identified by three derivatization techniques. The fragmentation of major metabolites has been presented and discussed. The kinetic excretion of 6 main oxymetholone metabolites has been studied. The behaviour of oxymetholone (Plenastril tablet) isolated from water solutions at pH 5 and 9,6 has been discussed.

Introduction

The oxymetholone (17ß-hydroxy,17α-methyl-2-hydroxy methylene, 5α-androstan-3-on) is a synthetic anabolic steroid known for a long time. Its chemical structure presume the formation of many metabolites in human organism. Honggang BI, Robert Masse and Robert Dugal (1) have investigated the metabolic pathway of oxymetholone. They have established the major transformation route by the reduction of A-ring functional groups with concomitant dehydration of the C-2 hydroxymethyl group. The oxidation and decarboxylation of C-2 hydroxymethylene group they accept as the minor in the steroid metabolism. L.M.Harrison, D.Martin, R.W.Gotlin and P.V.Fennessey (2) have identified 3,17-dihydroxy-17-methylandrostane, 2,17-dihydroxy-17-methyl-3-oxo-2-androstene and 2,3,17-trihydroxyandrostane as major excreted compounds of oxymetholone after administration.

The gas chromatography/mass spectrometry (GC/MS) screening analysis for detection of conjugated anabolic steroids includes many compounds and the presence of oxymetholone is estimated usually by metabolite 3ϵ,6ϵ,17ß-hydroxy,2-hydroxymethylene, 17α-methyl-5α-androstane. Ji Zhang, Chun Sheng Liu, Yi Zhong Zhang, Chang Jiu ZHang, Li Ye and Tong Hiu Zhou (3) propose for screening of anabolic steroids two metabolites of oxymetholone corresponding to metabolites with M^+ 550 and M^+ 640. The authors have established full reduction of parent compound.

The aim of this paper was to investigate the excretion kinetic of oxymetholone metabolites in urines of three volunteers (first-woman, 48 years, collected up to 96 hours; second-man, 24 years, collected up to 96 hours; third man, 27-years, collected up to 47 hours) after oral administration.

Experimental

Chemicals

All the chemicals used were of analytical reagent grade.
Plenastril^R was purchased from Grünenthal GmbH.
ß-Glucuronidase/ arylsulfatase from Helix Pomatia, Serva.
1,4-Dithioerythritol, trimethyliodsilane (TMSI), N-methyl,N-trimethylsilyl-trifluoracetamide (MSTFA), 1-trimethylsilyl-imidazol (TMSIm), methoxyamine and pyridine (MOX) by Fluka HG.
N-methyl,N-trimethylsilyl heptafluorbutyramide (MSHFBA) from Macherey Nagel.
Trimethylchlorsilane (TMSCl) from Pierce.
Inorganic salts were of analytical grade.
Anhydrous diethyl ether, methyl alcohol were redistilled before use.

Chemical Procedure

Routine chemical procedure for screening of anabolic steroids (shown on Figure 1) has been used for treatment of the urine samples.

The extraction of acidic metabolites was performed as follow: To 3ml urine add 0.2 M H_3PO_4 up to pH 1 and extract two times with 6ml diethyl ether. Shake for 20 minutes. Then centrifuge at 2500 rpm for 5 minutes. The ethereal phase is transferred to another tube and evaporated to dryness under nitrogen or vacuum rotary evaporator. Dry in a desiccator over P_2O_5/KOH for at least 30 minutes before derivatization (as shown on Fig.1).

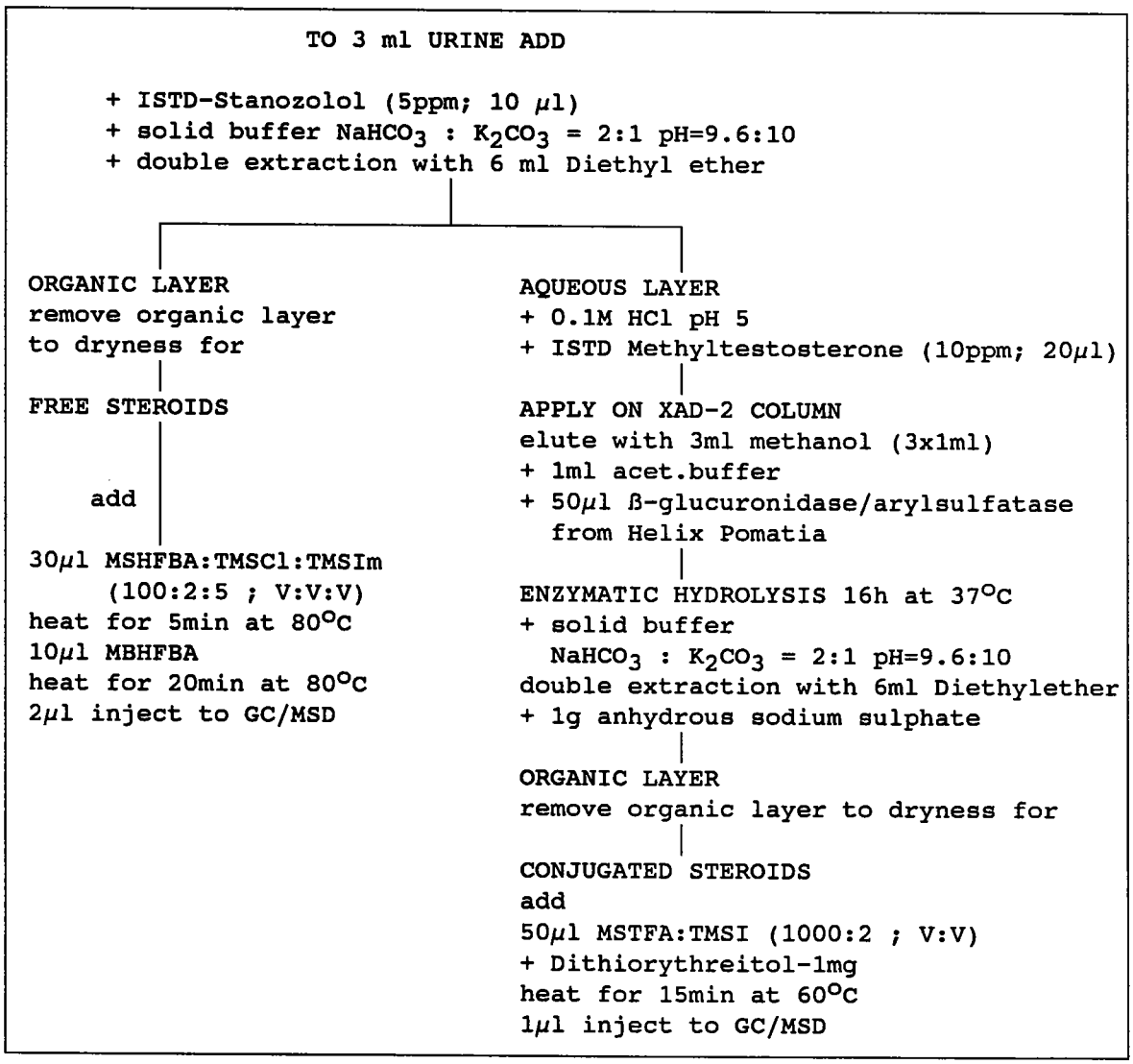

Fig.1: Chemical treatment procedure of urine for anabolic steroids

Gas chromatography-mass spectrometry

The mass spectra were obtained on a Hewlett-Packard 5890A/5970B apparatus. HP 59970C MS Chem Station was used for treating the data. The analytical conditions are shown on Fig. 2.

Column: HP Ultra 1 Capillary column
internal diameter : 0.2mm
length : 12.5m
film thickness : 0.32μm

Injector temperature : 280°C
Transfer line temperature : 280°C

Injection mode : split 1:15
Carrier gas : Helium at 0.85ml/min

TEMPERATURE PROGRAM

OF FREE STEROIDS		**OF CONJUGATED STEROIDS**	
Initial temperature	: 180°C	Initial temperature	: 203°C
Initial time	:	Initial time	: 0min
Rate	: 25°C/min	Rate	: 2°C/min
Final temperature	: 300°C	Final temperature 1	: 225°C
Final time	: 5.2min	Final time	: 2.3min
Total run time	: 10min	Rate	: 15°C/min
		Final temperature 2	: 295°C
		Final time	: 6min
		Total run time	: 24min

Fig.2: Chromatographic conditions for analysis of anabolic steroids

For excretion kinetic of oxymetholone free and conjugated metabolites the mass spectrometer was operated in the selected ions monitoring (SIM) mode using m/z 143, 218,231,358,360, 370, 448, 450, 458, 460, 462, 536, 538, 548, 550, 552, 638 and 640 with dwell time 70. For the free steroids m/z 596, m/z 581 (ISTD-stanozolol) were added and for the conjugated - m/z 301, m/z 446 (ISTD-methyltestosterone). The analyses in SCAN mode was performed in range 100:700 amu.

Results and discussion

The isolated oxymetholone from Plenastril tablet was analysed as TMS- and enol-TMS-ethers at GC/MS analytical conditions given on Fig.2. MOX-TMS derivatives were received adding 200 μl 2 % methoxyamine in pyridine and heating for 15 min at 60^{o}C. The pyridine was removed under nitrogen stream and was added 50μl of mixture MSHFBA:TMSIm:TMSCl (100:2:5). After heating for 15min at 60^{o}C the sample was analysed at GC/MS analytical conditions given on Fig.2.

The functional groups on C-2 and C-3 in A-ring determine the behaviour of oxymetholone in acidic and basic medium. In presence of hydrogen cations the hydroxy form of methylene group in C-2 predominates. In basic medium the methylene group of C-2 is reduced and the equilibrium is moved to keto form of oxymetholone (Fig.3).

By TMS-ether derivatization we found two di-TMS isomers of oxymetholone with M^+ 476 and retention times (RT) 18.69 min and 19.24 min (see Table 1). The enol-TMS-ether quantity was about 13% when extracted at pH 5 and 33% at pH 9,6. The mass spectra of TMS-derivatives of oxymetholone standard are illustrated on Fig. 4.

By enol-TMS-derivatization we obtained peaks with the same retention times and M^+ as by the TMS-derivatization but in different ratio. The peak with M^+ 548 was 100 % at pH 9,6 and 78% at pH 5, while the peak with M^+ 476 was found only at pH 5 (14%) (Table 1).

After MOX-TMS derivatization of oxymetholone extracted at pH 5 we have obtained four peaks with M^+ 462 corresponding to di-MOX derivatives. Two of them were very intensive (33% with RT=18.23 min, 62% with RT=18.44 min) and two small ones. That confirms the presence of two oxym derivatives (both in sin- and anti forms specified for oxyms). At the same pH value we obtained a peak with M^+ 505 which proved the presence of mono-MOX-TMS-derivative (3%, RT=18.76 min). The mass spectra are shown on Fig.5. The spectra of di-MOX-TMS derivatives show the basic ion m/z 143 and specific fragmentation for oxym (M^+, intensive M^+ -CH_3O and M^+-CH_3). In the spectrum of mono-MOX-TMS derivative the M^+ is more intensive then M^+ - CH_3O.

Table 1: Percent of derivatization of oxymetholone standard and retention times* of different derivatives

pH	Molecular ion M^+	RT min	TMS-ether %	RT min	enol-TMS-ether %	RT min	MOX-TMS-ether %
5	568	17.43	4.1	17.43	7.8	17.43	2.3
	548	18.18	13.1	18.18	78.0		
	462					18.23	33.2
	462					18.44	61.7
	505					18.76	2.8
	476	18.69	56.4	18.69	14.2		
	476	19.24	26.3				
9,6	548	18.18	32.6	18.18	100.0		
	462					18.23	47.6
	462					18.44	52.4
	476	18.69	43.5				
	476	19.24	23.9				

*Remark: GC conditions for conjugated anabolic were used (fig.2)

When the medium is basic only di-MOX derivatives (RT=18.23min, 48%, RT=18.44min, 52%) are present. That underlines the influence of the pH value for shifting the equilibrium of the oxymetholone tautomeric forms towards the keto form in C-2 (Fig. 3).

By all three types of derivatization used a peak with M^+ 568 (about 5%,RT=17.43min) was registered only at pH 5. It corresponds to opened A-ring with carboxylic group in C-2. Its mass spectrum is given on Fig.5.

The results obtained from the analyses of the oxymetholone standard (pill) were used in our further investigation of oxymetholone metabolites in urine.

The hydroxy- and carbonyl- groups in oxymetholone parent compound having in mind C-2, C-3 and C-17 could be transformed to many metabolites after administration. Oxidation, reduction, decarboxylation of the C-2 -hydroxymethyl group, reduction of carbonyl group at C-3, oxidation of C-6 and C-16 give many different metabolites (1).

It was very interesting to observe the excretion kinetic of all of possible oxymetholone metabolites in urine samples from different volunteers after administration of a single therapeutic dose. At the beginning of the investigation the samples were analysed by GC/MS in SIM mode described above. In collected urine samples between 3 or 8 hours from the three volunteers we had possibility to obtain the retention times and mass spectra of most metabolites. They were confirmed by performing three different types of

derivatization of the same urine samples. Figure 3 shows 19 metabolites detected in the free and conjugated fractions of anabolic steroids. In all urine samples oxymetholone parent compound was not found. That means a full reduction after its administration. Three metabolites with molecular weight (Mwt) 304, 306 and 334 were detected in the free fraction from basic extract and two metabolites with Mw 350 and 352 from acidic extract. These metabolites were found in the first ten hours of collecting the urines. The metabolite with Mwt 304 has a carbonyl group in C-3 and shows unspecific MOX-TMS fragmentation. It has intensive M^+-15 (M^+-CH_3), but M^+-31 (M^+-OCH_3) is absent (Fig.6a).
Acidic metabolite with Mwt 350 is four time more intensive than metabolite with Mwt 352 (see Fig.6b). The first one has an opened A-ring at C-2 and C-3. Its mass spectrum is formed by basic ion at m/z 143, clear M^+-15 (M^+-CH_3) and specific ions at 215 and 347 according to R.Masse (4) (see Fig.6c). The second acidic metabolite is illustrated on Fig.6d.
The intensity of six of the metabolites detected in conjugated fraction have given us the possibility to study their excretion kinetic. These metabolites (Met-*) are shown on Fig.7 as follows:

Met-1 3α,17ß-dihydroxy,17α-methyl-androstan
Met-2 3ϵ,17ß-dihydroxy,17α-methyl,2ϵ-hydroxymethylene-androstan
Met-3 17ß-hydroxy,17α-methyl,2ϵ-hydroxymethylene-androstan-3-on
Met-4 3ϵ,6ϵ,17ß-3hydroxy,17α-methyl,2ϵ-hydroxymethylene-androstan
Met-5 6ϵ,17ß-dihydroxy,17α-methyl,2ϵ-hydroxymethylene-androstan-3-on
Met-6 16ϵ,17ß-dihydroxy,17α-methyl,2ϵ-hydroxymethylene-androstan-3-on

Figure 7 illustrates their presence in the total ion chromatogram of urine sample by ion chromatograms of m/z 143 and m/z 218. The retention times (RT) of different derivatives and relative retention times (RRT) as enol-TMS-ethers to ISTD-methyltestosterone with RT=16.00 are presented on Table 2.

Table 2: Retention and relative retention times of 6 oxymetholone metabolites as different derivatives

Metabo-lites	Mwt	Derivatives			
		Enol-TMS-ether		TMS-ether	MOX-TMS-ether
		RT min	RRT	RT min	RT min
Met-1	304	13.62	0.85	13.62	13.62
Met-2	336	17.33	1.08	17.33	17.33
Met-3	334	18.00	1.12	17.80	17.96
Met-4	352	18.30	1.14	18.30	18.30
Met-5	350	18.94	1.18	18.80	18.91
Met-6	350	19.92	1.23	19.78	19.60

The derivatives of metabolite 1 obtained by the three different derivatization methods show the same retention times. This phenomenon is observed for derivatives of the metabolites 2 and 4 as well (see Table 2) This proves the presence of only hydroxy groups in the molecules of metabolites 1, 2 and 4. The mass spectra are illustrated on Fig.8. The metabolites 3, 5 and 6 have hydroxy- and carbonyl groups and they have different RT for their three derivatives. That was proved by mass spectra shown on Fig.9, 10 and 11. TMS- and enol-TMS- derivatives have well known fragmentation for 17-hydroxy-17-methyl- and 16, 17-hydroxy-17-methyl anabolic steroids with basic ion at m/z 143 or m/z 218. They have clear M^+, M^+-90 (M^+-HOTMS), M^+-180 (M^+-2HOTMS), M^+-CH_3. The metabolites 3 and 5 have specific fragmentation for MOX-TMS-ethers and basic ion at m/z 143.

Metabolite 6 gives more intensive M^+ than M^+-31 (M^+-OCH_3).

We have investigated the excretion kinetic of the sixth metabolites in collected urine samples of the three volunteers. Their intensities between the 3rd and 8th hours are the highest (Fig.12). After that they decrease and about the 45 hour there is a little increase in intensity. The metabolites 2 and 4 remain the major ones till the last hours of excretion by all three volunteers. Metabolite-2 is more intensive in men urines than Met-4 while in woman urine their ratio varies during studied excretion (Fig.13). That is why in the screening analysis of conjugated anabolic steroids it is necessary both Met-2 and Met-4 to be included. This would ensure higher reliability in detecting oxymetholone in urine samples. During all the duration of the analysis we recommend the observation of the ion at m/z 143. It increases the reliability of detecting anabolic steroids.

Literature:

1. Hoggang Bi, Robert Masse, Robert Dugal, 7th Cologne Workshop in Dope analysis, Cologne 1989.
2. L.M.Harrison, D.Martin, R.W.Gotlin, P.V.Fennessey, J.Chromatogr.,489, (1989) 127.
3. Ji ZHANG, Chun Sheng LIU, Yi Zhong ZHANG, Chang Jiu ZHANG, Li YE, Tong Hui ZHOU, Chinese Chemical Letters, 1,2, (1990) 143.
4. Robert Masse, Honggang Bi, Christiane Ayotte, Robert Dugal, Biomedical and Environment Mass Spectrometry, 18, (1989) 429.

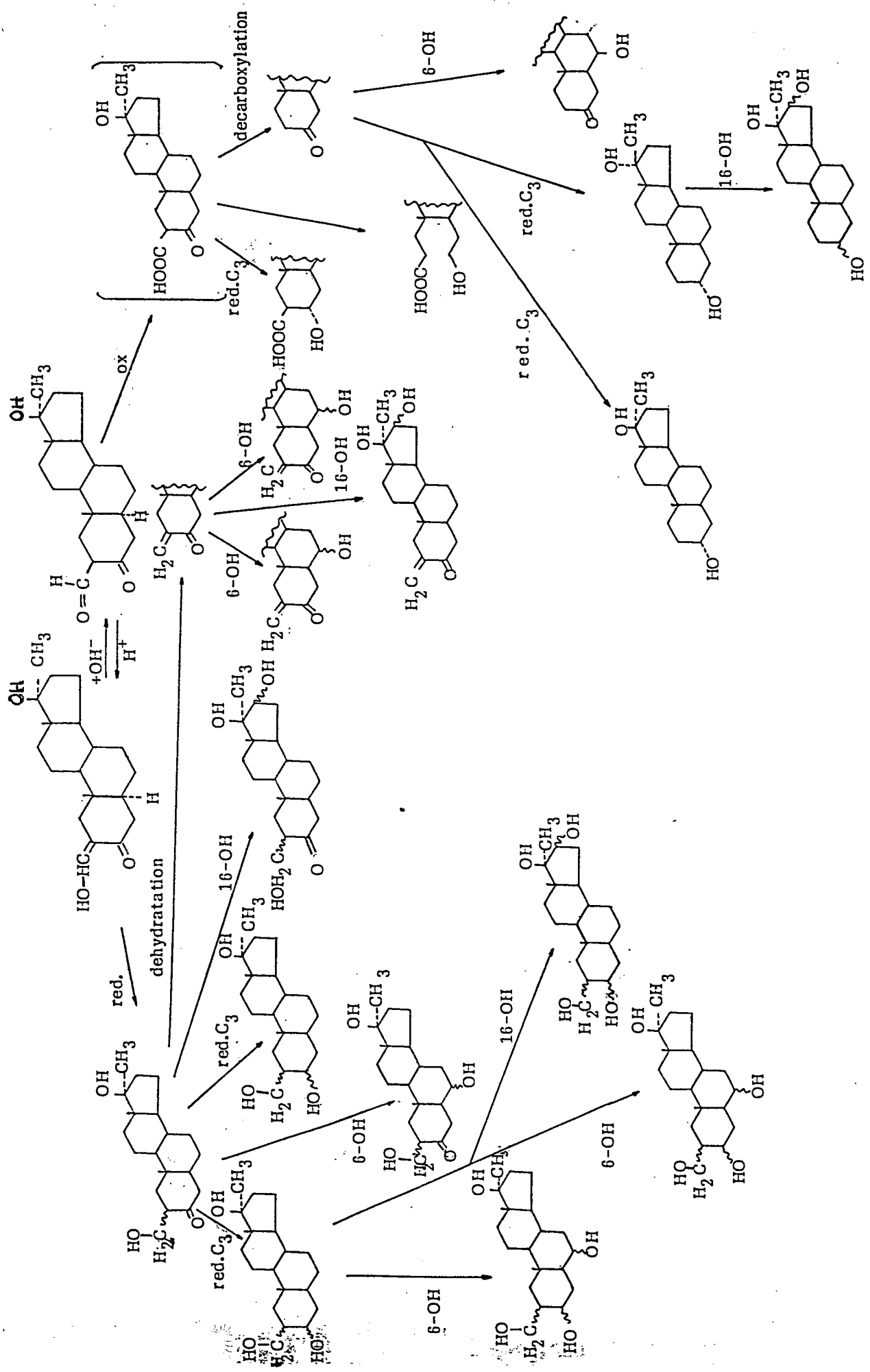

Fig.3. Oxymetholone and confirmed metabolites in urine samples from the volunteers.

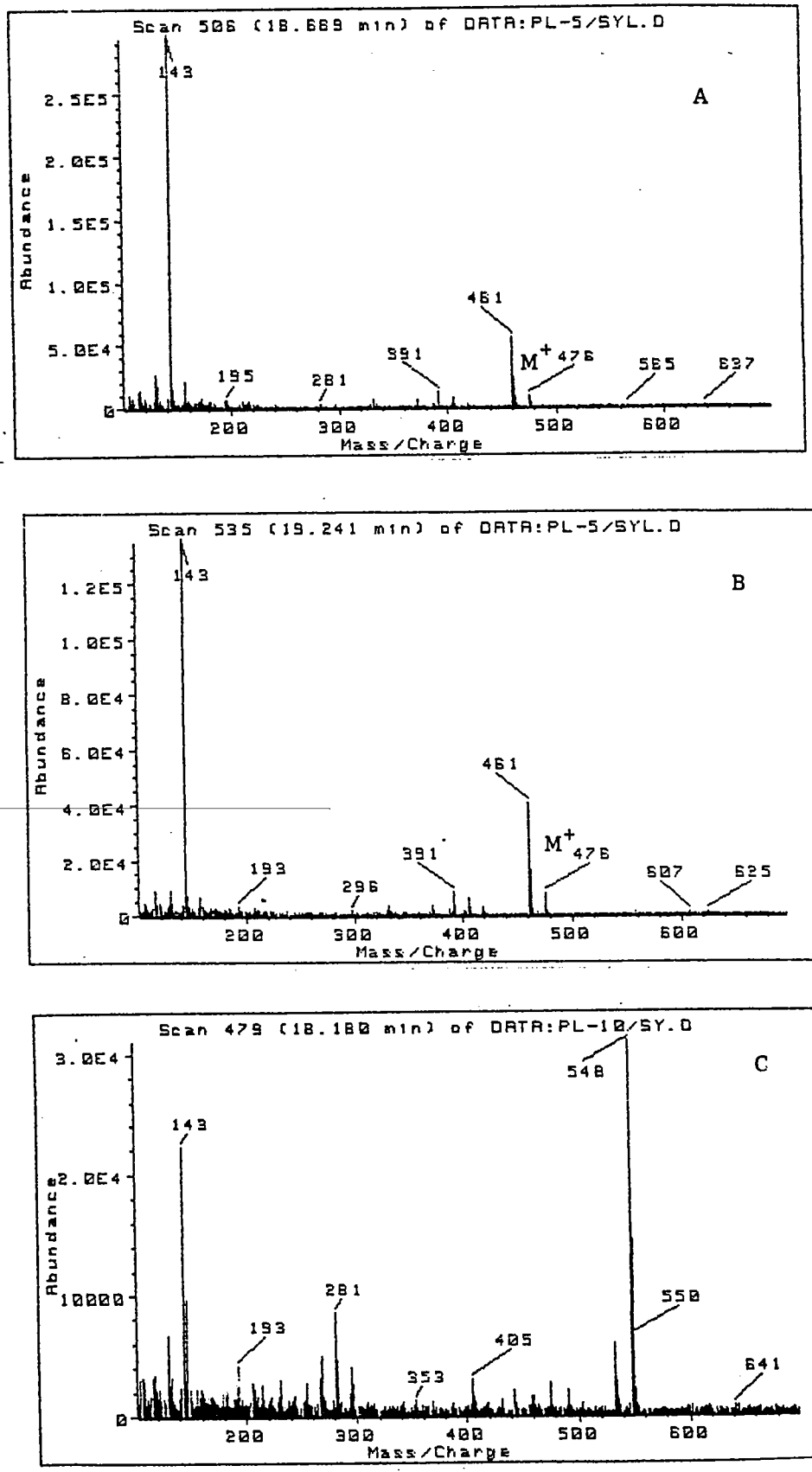

Fig.4. Mass spectra of two TMS-ethers of Oxymetholone standard (A,B); Mass spectrum of enol-TMS-ether of Oxymetholone standard (C).

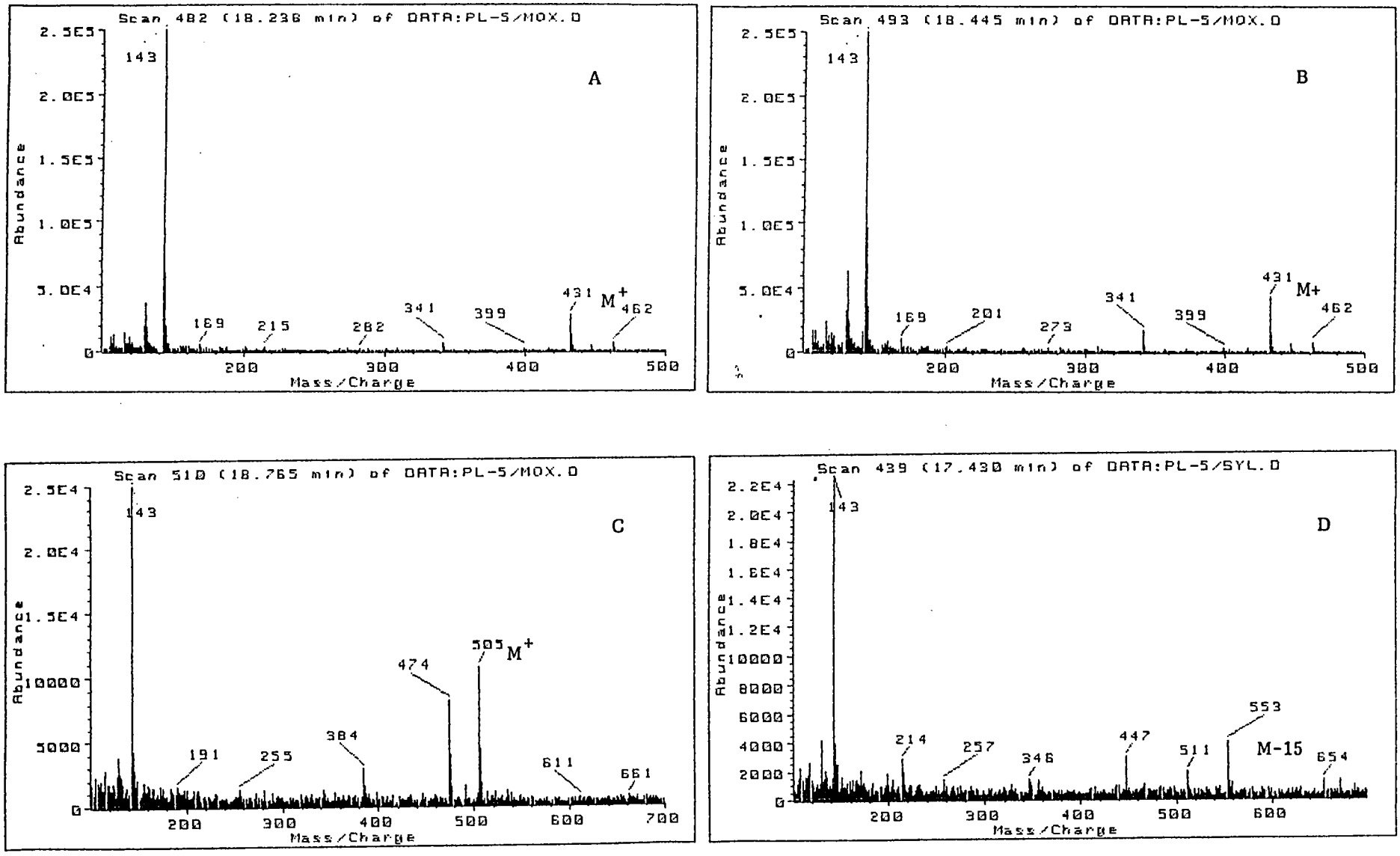

Fig.5. Mass spectra of diMOX-TMS-ethers of Oxymetholone standard (A,B); Mass spectrum of mono-MOX-TMS-ether of Oxymetholone standard (C); Mass spectrum of Oxymetholone standard-acid (D).

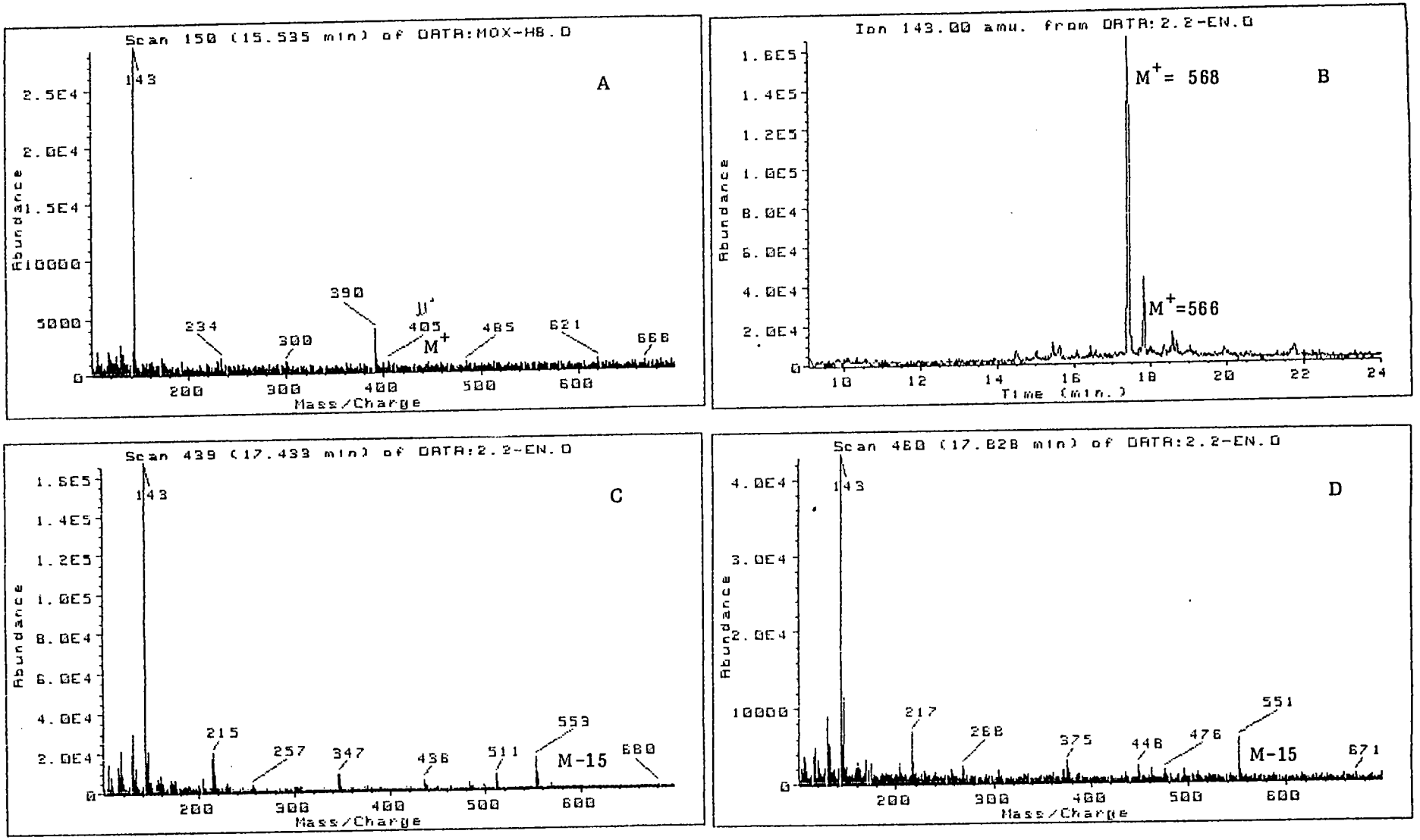

Fig.6. Mass spectrum of MOX-TMS-ether of Oxymetholone metabolite with M_{wt}= 304(A). Ion chromatogram of m/z143 of acidic metabolites of Oxymetholone (B). Mass spectra of acidic metabolites with M_{wt} 352(C) and M_{wt} 350(D).

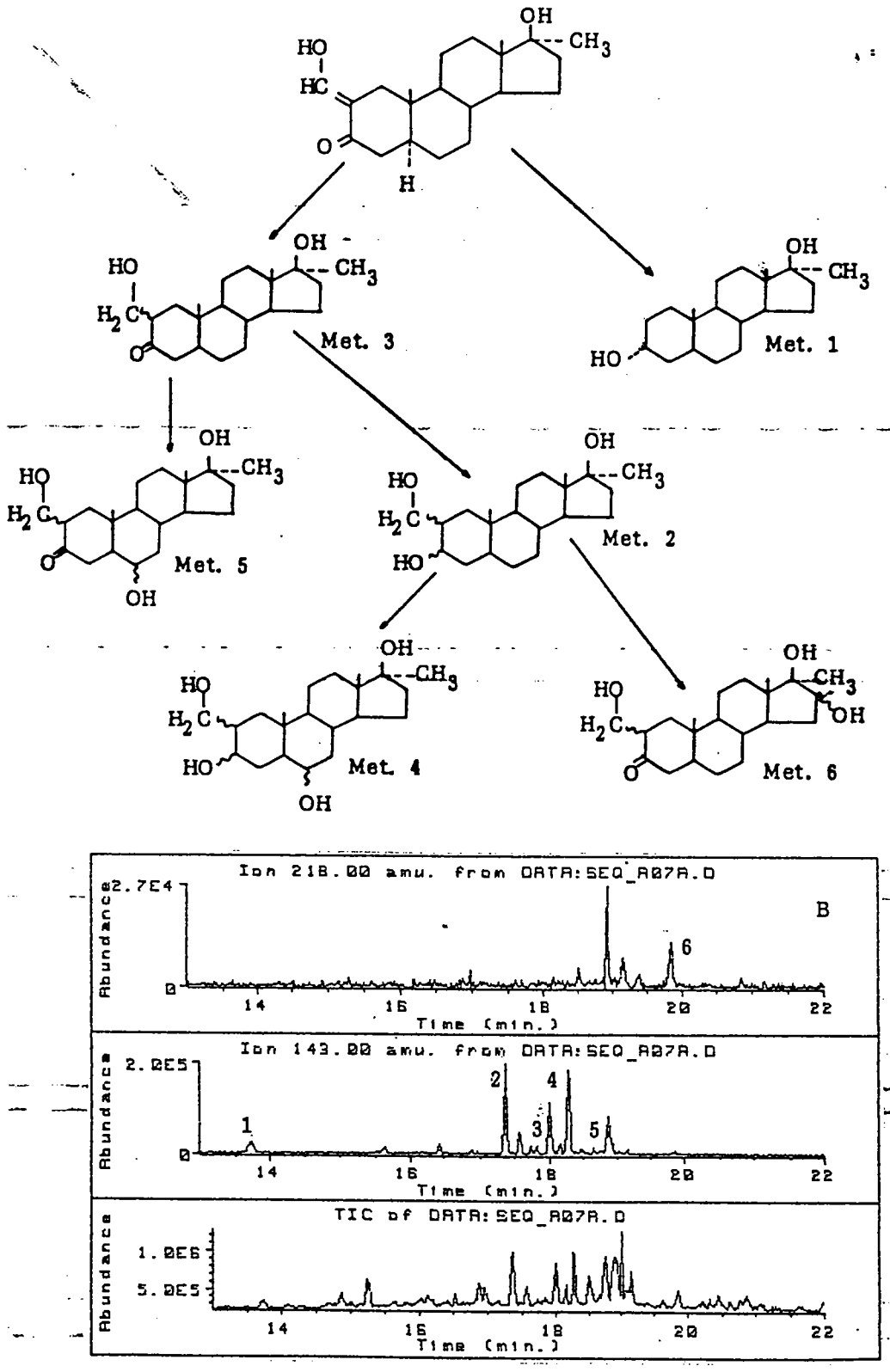

Fig.7. Chemical structures of six Oxymetholone metabolites (A).
Position of six Oxymetholone metabolites in TIC and Ion Chromatograms of m/z 218 and m/z 143 (B) of urine sample

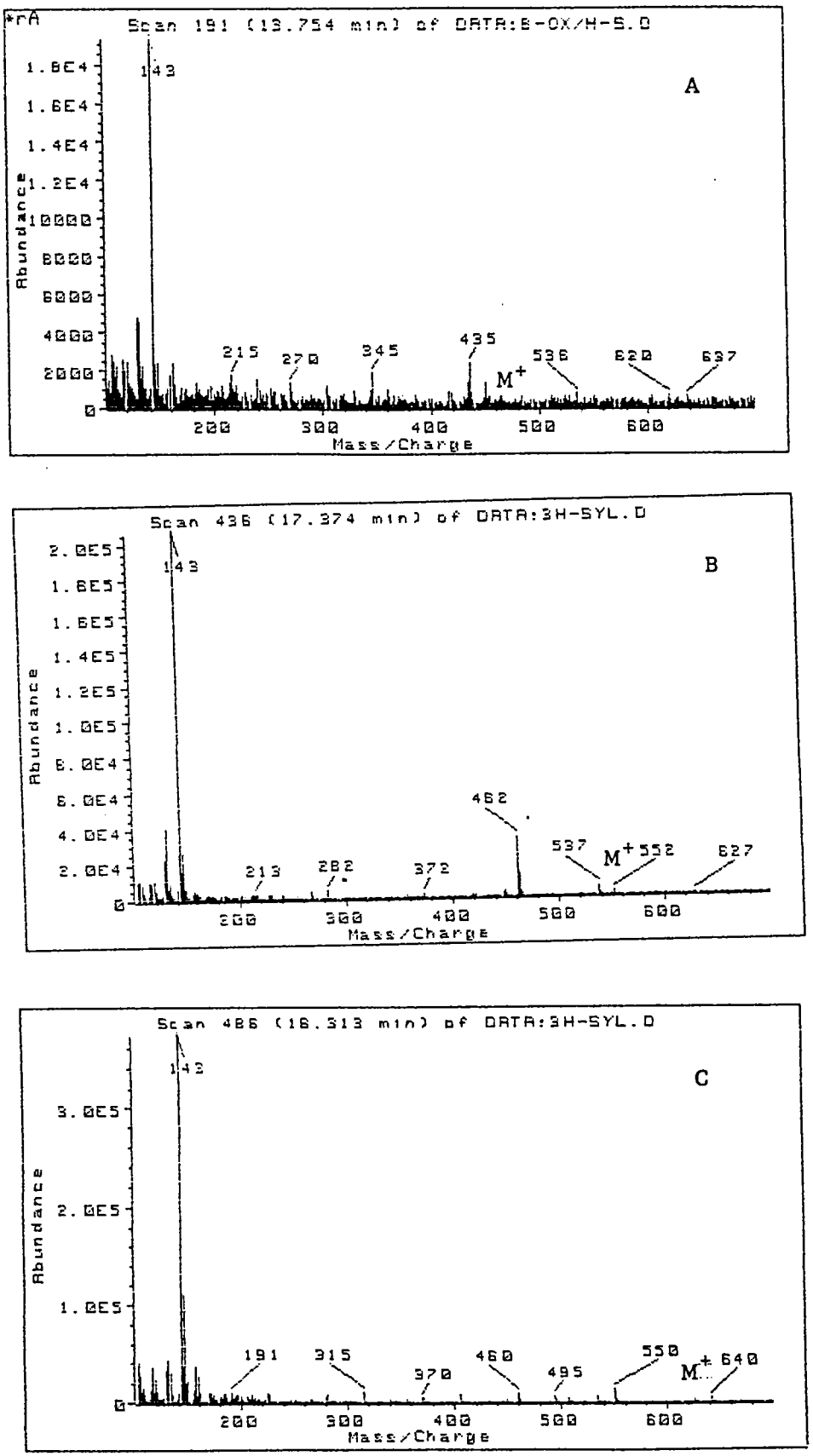

Fig.8. Mass spectra of TMS-ethers of Oxymetholone Metabolites 1(A), 2(B) and 4(C).

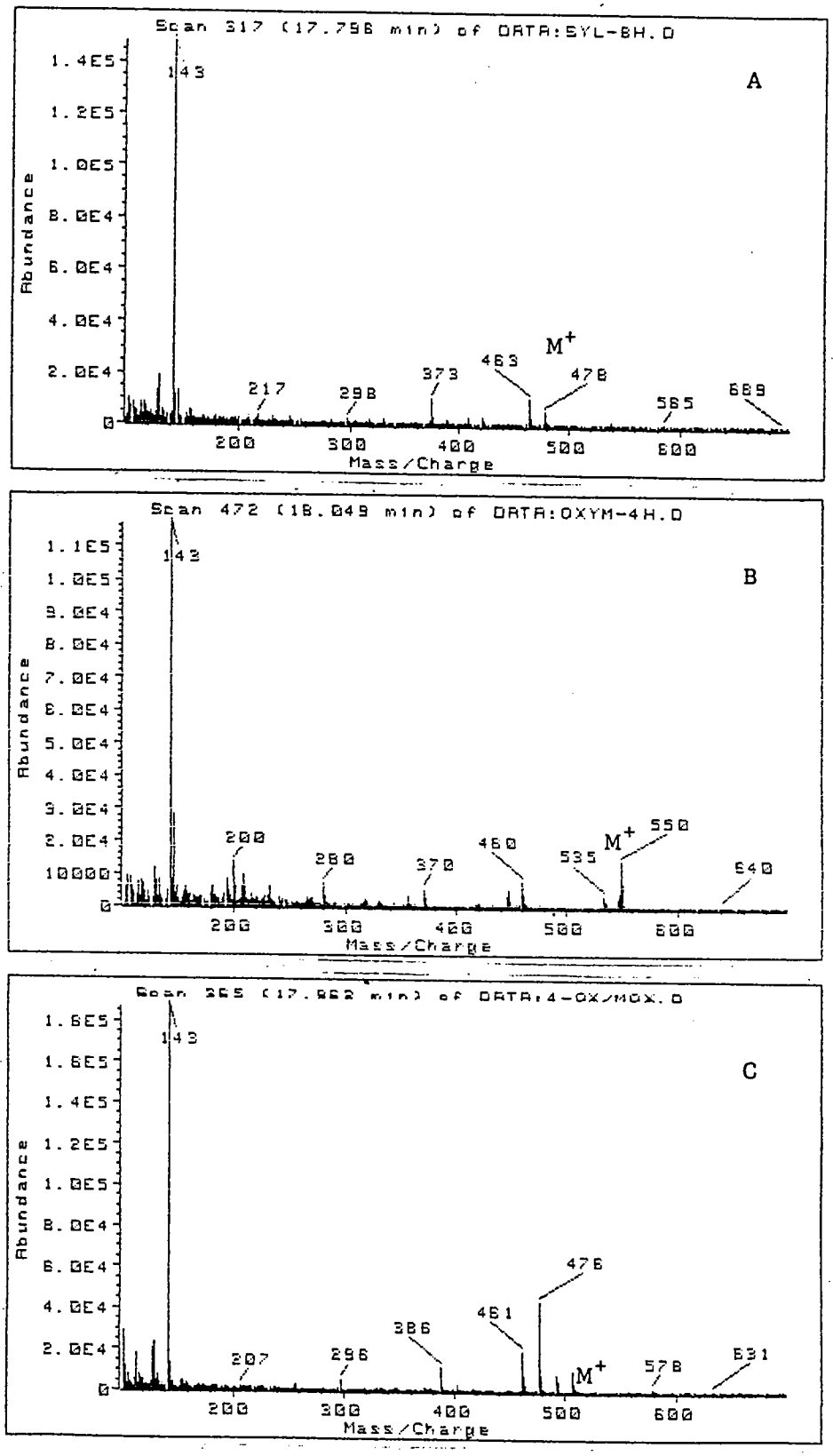

Fig.9. Mass spectra of Oxymetholone metabolite 3;. TMS-ether (A);. enol-TMS-ether (B) and MOX-TMS-ether (C).

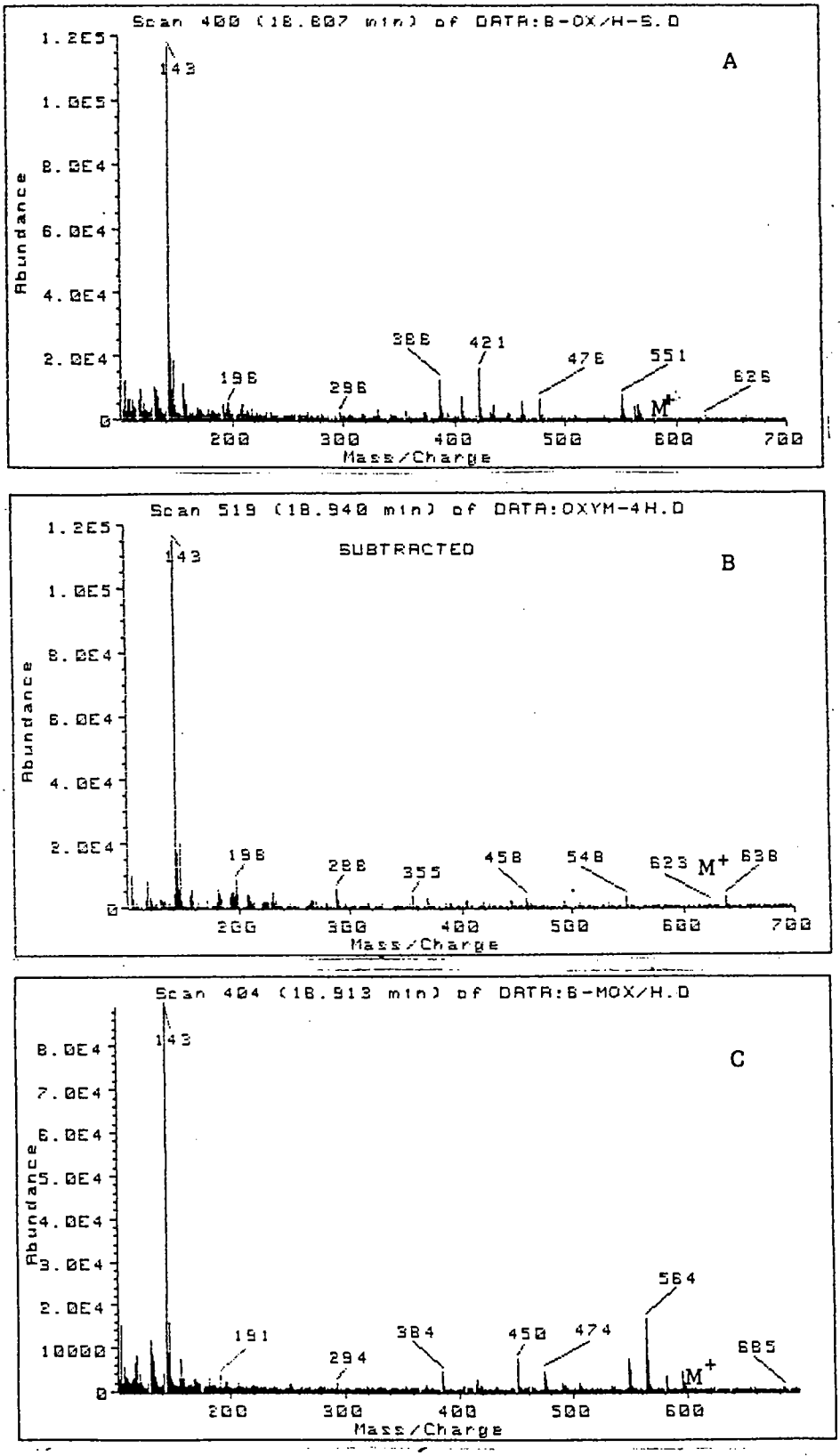

Fig.10. Mass spectra of Oxymetholone metabolite 5;. TMS-ether (A);. enol-TMS-ether (B) and MOX-TMS-ether (C)..

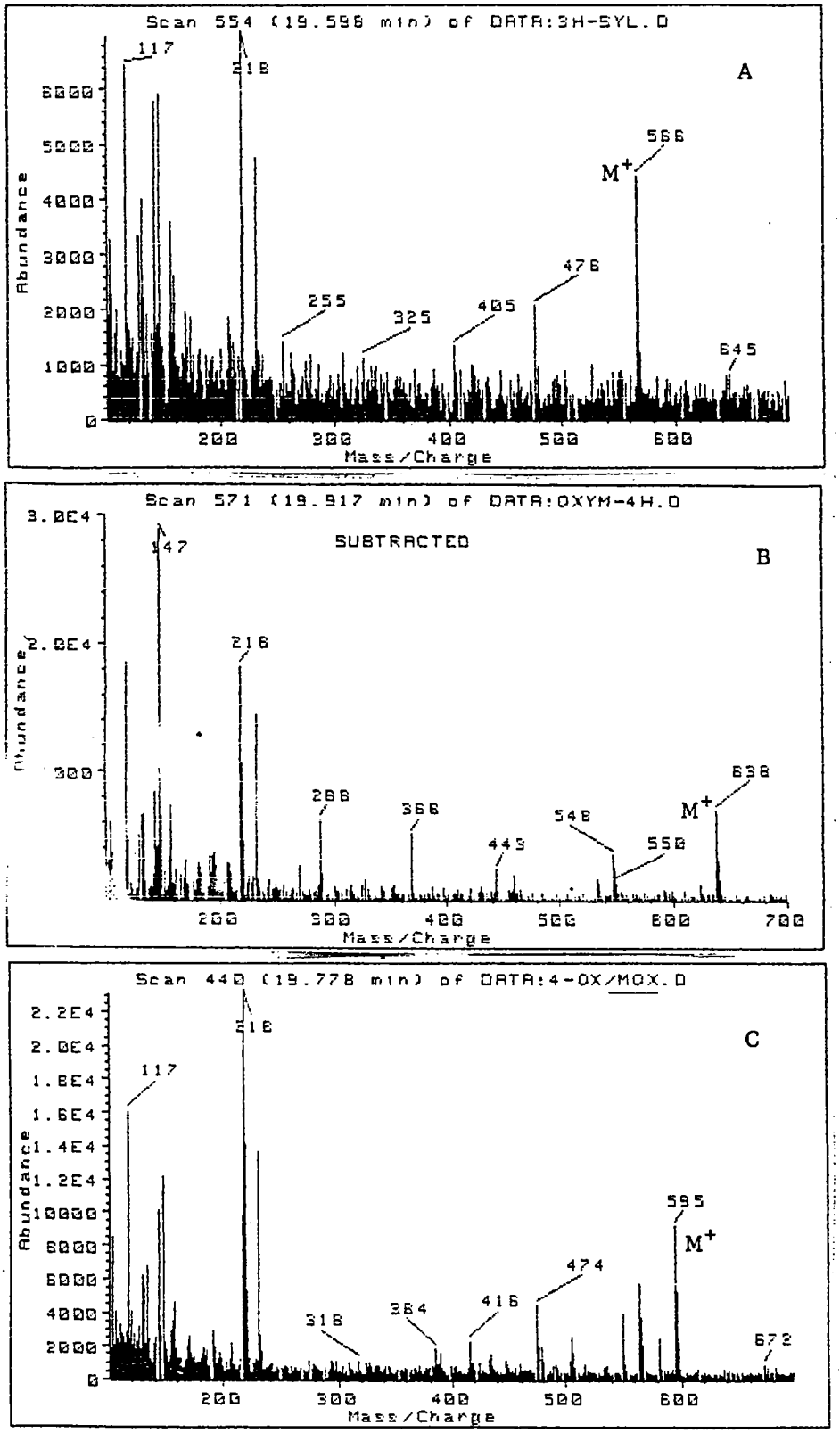

Fig.11. Mass spectra of Oxymetholone Metabolite 6; TMS-ether (A) ; enol-TMS-ether (B) and MOX-TMS-ether (C).

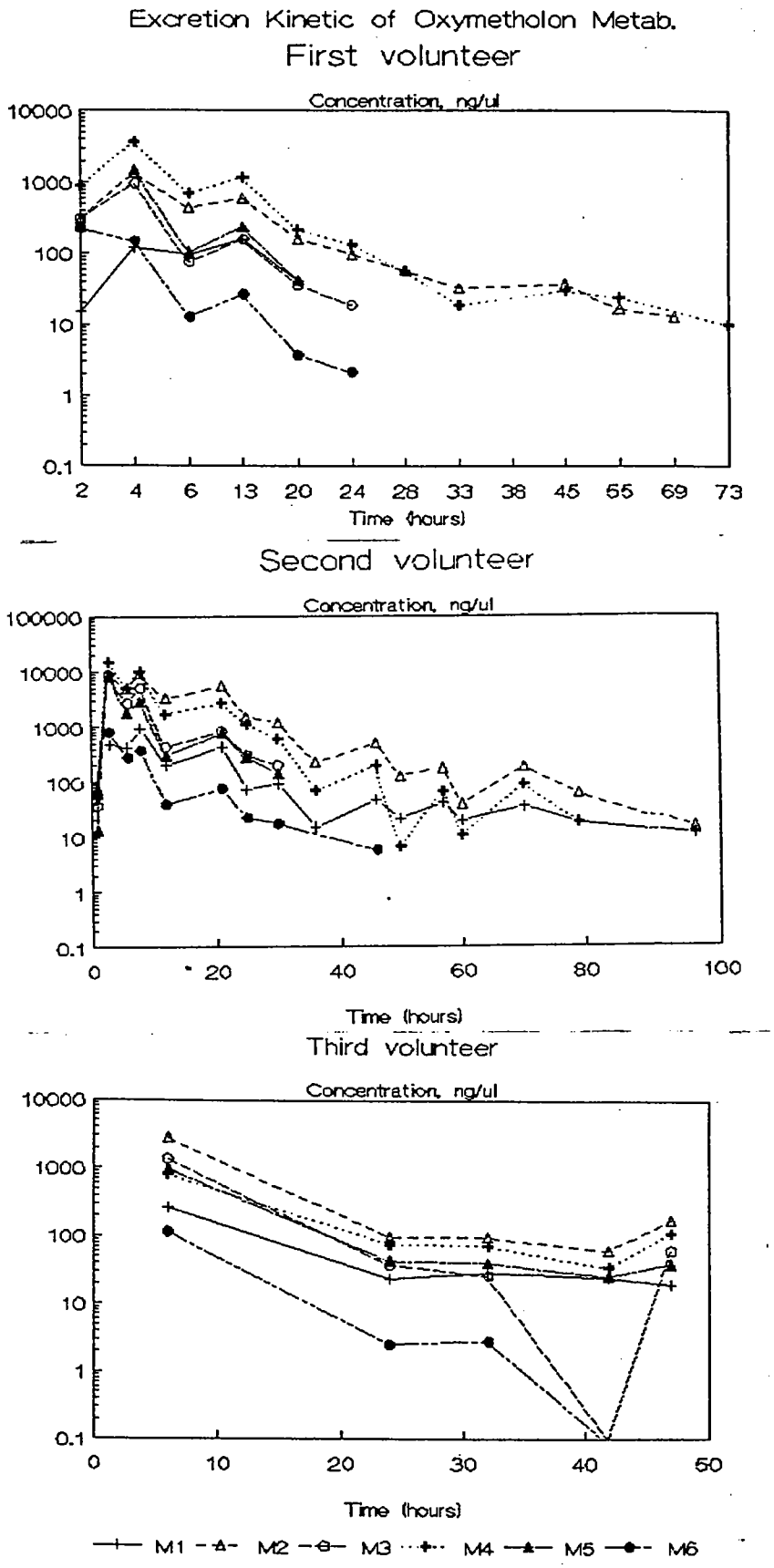

Fig.12. Excretion Kinetic of the sixth Oxymetholone Metabolites.

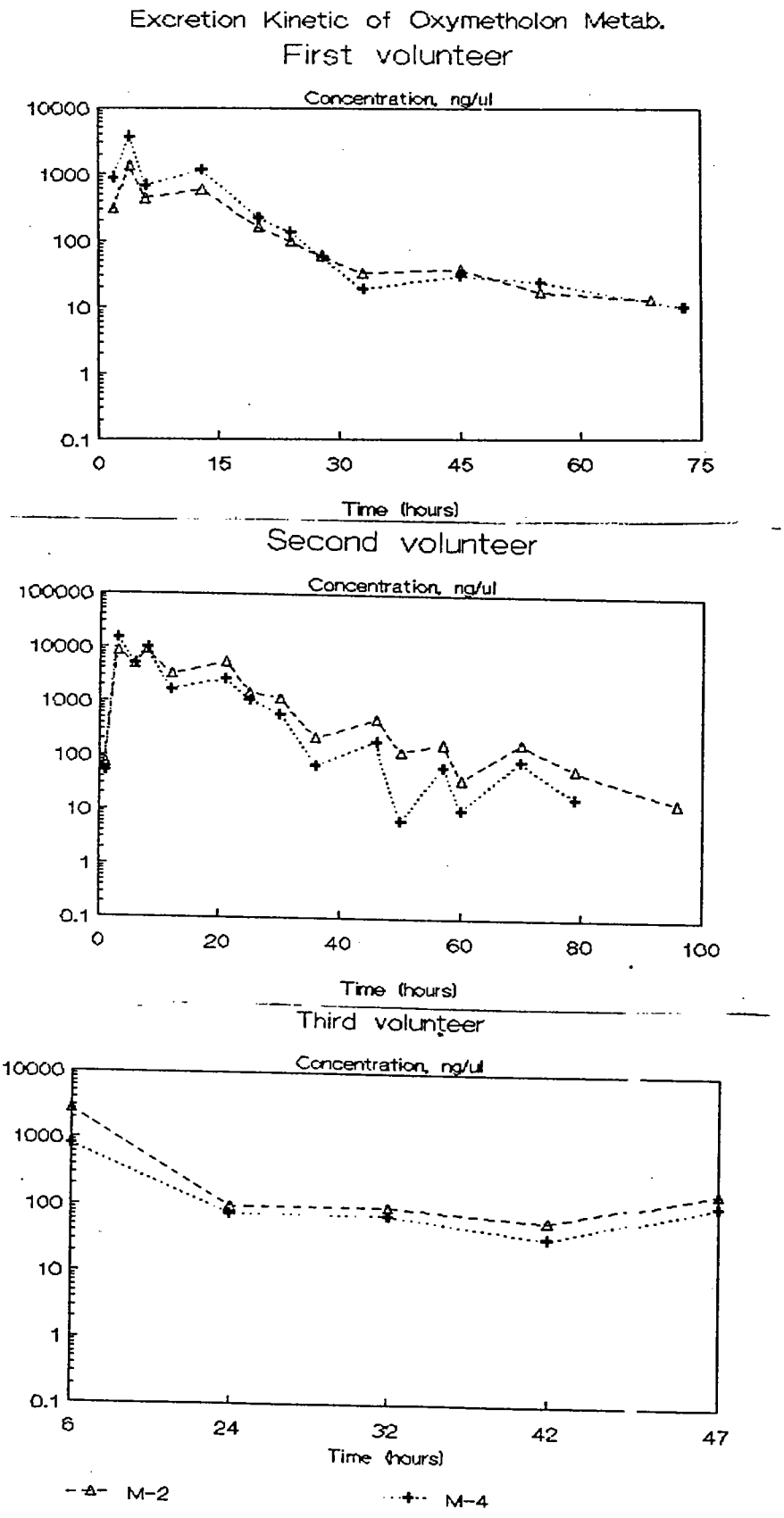

Fig.13. Excretion Kinetic of the Oxymetholone Metabolites 2 and 4.