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# Analysis of Urinary Metabolites of Mibolerone in Man by Gas Chromatography/Tandem Mass Spectrometry

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**Abstract**

This paper presents a study on the urinary metabolites of mibolerone in man. After enzymatic hydrolysis of the conjugates the metabolites of mibolerone were detected by GC-MS and GC-MS/MS. The GC-MS/MS features of mibolerone metabolites are presented and discussed. With GC-MS/MS in parent mode the source ions of most main fragments of the proposed metabolites were investigated. With GC-MS/MS in daughter mode the daughter mass spectra of all the major fragments showed hints of the structures of the ions. Based on the analytical data and the general knowledge about metabolism of anabolic androgenic steroids the metabolites of mibolerone in human urine were proposed as 5β-reduced mibolerone, tetrahydromibolerone, dihydroxymibolerone and mibolerone.

## 1. INTRODUCTION

Mibolerone with a CAS 3704-09-4, 17β-Hydroxy-7α, 17α-dimethylestr-4-ene-3-one, has synonyms U.10997, CDB 904, Matenon, Chequs® etc and is primarily used to prevent estrous in some animals. Though we have not seen any positive report for mibolerone it is banned by IOC. To our best knowledge, we have found only few publications about it, one is published by Dr. Schaenzer in Clinical Chemistry[1] and another is presented by Dr. Bowers in the Work Shop Cologne in 1995[2]. This fostered us to try to study the metabolites of mibolerone in human urine.

Our purpose of the study is focused on trying to find and confirm the possible metabolites of mibolerone in human urine. We used GC-MS to obtain the normal EI mass spectra of the possible metabolites of mibolerone and further on used GC/ tandem MS/MS to study the

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fragments of the proposed metabolites.

Through a comparison of ion chromatograms between blank urine and the mibolerone positive urine the possible metabolites of mibolerone were found according to the estimated molecular weights and the knowledge about the metabolism of steroids in general. All of the possible metabolites of mibolerone were studied by GC-MS/MS in daughter or in parent mode to see the relationship between the proposed structures and the mass spectra found in this experiment.

## 2. EXPERIMENTAL

### *2.1 Subject and Sample collection*

A 35 year old volunteer, healthy Chinese male with 73 kg body weight and 172 cm height, has taken orally a single dose of 14.5 mg mibolerone. 0~120 hr urine samples were collected and stored at -20°C till analysis.

### *2.2 GC/MS and GC-MS/MS analysis*

A gas chromatograph model 5890 series II plus (Hewlett-Packard) was connected to the TSQ-7000 (Finnigan) for GC-MS and GC-MS/MS determination. The separation was carried out with a HP 1 column (17m, 0.2 mm I.D., 0.11 μm film thickness). The injector operated in split mode (1:10 split ratio) and the interface were both maintained at 280°C. The temperature program was: initial 180°C, rate 1: 3.3°C/min to 231°C, rate 2: 30°C/min to 310°C and maintained for 2min at 310°C. Helium was used as carrier gas at flow rate 0.8ml/min (at 180°C). During the running the pressure was kept constant automatically. The mass spectra were obtained in full scan mode from m/z 50 to m/z 650 in 1 second. The transferline was set at 280°C. For GC-MS/MS the first MS worked in EI mode, (electron energy 70 eV), manifold was set at 70°C, ion source at 180 °C, conversion dynode at 13 kV, scan rate of the daughter mass spectra at 500 a.m.u./s, dwell time in RIM mode at 100 ms/per ion, emission current was 400 μA, electron multiplier voltage in the range of 1100~1300. PFTBA was used as the calibrate for tuning. Collision gas was argon gas at 1.8~2 mTorr. Collision energy depended on substances during -15 ~ -25 V. The correct factor (MSMSC) was 0.7.

*2.3 Extraction and Derivatization*

5 ml of urine sample were added to an Amberlite XAD-2 column. After the column was washed with 5 ml distilled water, 50 µl methyltestosterone in methanol (1 ng/µl) as an internal standard was added to the column. The absorbed fraction was eluted with 2 ml methanol. The methanolic eluate was evaporated to dryness. The residue was dissolved in 1 ml of 0.2 M phosphate buffer pH 6.8 with 100 µl β-glucuronidase from E.coli (125000 unit/3 ml phosphate buffer). After hydrolysis at 55°C for 3 h about 100 mg of solid carbonate buffer were added to alkalize the hydrolyzed solution. The mibolerone and its metabolites were extracted with 5 ml ether. After centrifugation the ethereal layer was transferred and evaporated to dryness. The residues were derivatized with 50 µl of MSTFA/TMSI/dithio-erythritol 1000:3:1 (v/v/w) and heated at 70°C for 30 min. 1 µl of the derivatized solution was injected into the GC-MS or GC-MS/MS.

## 3. RESULTS AND DISCUSSION

Unchanged mibolerone was found in the positive urine as a main metabolite of mibolerone. After derivatization with MSTFA the ion chromatogram and EI mass spectrum of mibolerone was showed in Fig.1. The major ions of mibolerone-di-*O*-TMS observed are m/z 446 (molecular ion), m/z 431, m/z 341 and m/z 301. Under our experiment conditions the daughter mass spectrum of ion m/z 446 gave the same major mass fragments as that EI mass spectrum. The only difference is the ratio of these fragments. The ion m/z 356 [(2)] could not be detected by GC-MS or by GC-MS/MS in our experiment conditions. The mass spectrum of m/z 446 in daughter mode showed in Fig.2 confirmed that the EI spectrum in Fig.1 was quite well without any interference from biomatrix. The ion m/z 431 may be due to the loss of methyl group from the molecular ion. The relationship between ions m/z 431 and 341 was showed in Fig.2 and Fig.3. It was clearly seen in Fig.3 (upper) that the ion m/z 341 could be raised by the loss of TMS-OH group from the ion m/z 431. On the other hand, the daughter mass spectrum of ion m/z 431 (see Fig. 2 lower) could produce ion m/z 341. Between the daughter mass spectra of ion m/z 431(Fig.2 lower) and ion m/z 341 (Fig.3 lower) there were a lot of common ions, such as m/z 272, m/z 251, m/z 211 and m/z 195 etc. This suggested that the major structures of ion m/z 431 and m/z 341 were quite similar. Fig.4, the mass spectrum in parent mode of the base peak with ion m/z 301, showed that ion m/z 301 came, mainly, directly from the molecular ion.

Its mechanism is not clear. The daughter mass spectrum of ion m/z 301 showed that the ion m/z 301 contained the structure of TMS-OH group and the structure of the characteristic ion m/z 143. The daughter mass spectrum of ion m/z 431 showed us that it produced a small part of ion m/z 301 (see Fig.3). Based on the above findings the possible fragmentation pathway was suggested in Fig.5.

One possible metabolite of mibolerone (Met.-I) is dihydromibolerone introduced by 5α- or 5β-reduction. Fig.6 showed the gas chromatogram and the EI mass spectrum of the 5- reduced metabolite of mibolerone. The fragmentation pattern of this proposed 5β-reduced metabolite-di-*O*-TMS looked like that of its precursor. The ion m/z 448 is the molecular ion. The ion m/z 433 and m/z 343 may be produced by the same mechanism as the ions m/z 431 and m/z 341 of mibolerone-di-*O*-TMS. Additional peak m/z 358 could be produced by loss of TMS-OH group from the molecular ion. The parent mass spectrum of ion m/z 304 (See Fig.7) showed that it came almost directly from the molecular ion and a small part of it from ion m/z 358. As the ion m/z 301 in the mibolerone spectrum, the ion 304 is not clear. Near this peak in the chromatogram we could not find any other peak with similar mass spectra; so it is supposed to be a 5β-reduced metabolite of mibolerone[1].

The second possible metabolite of mibolerone (Met.-II) is tetrahydro-mibolerone. After 5β-reduction the 5β-dihydromibolerone could be metabolized to tetrahydromibolerone. Fig. 8 showed the gas chromatogram and EI mass spectrum of the possible tetrahydromibolerone-di-*O*-TMS. Its molecular ion is m/z 450. Loss of methyl from m/z 450 produced the ion m/z 435. The loss of m/z TMS-OH from the molecular ion m/z 450 and the ion m/z 435 resulted in the ion m/z 360 and m/z 345 respectively. Continuously the loss of m/z 90 from the ion m/z 360, and m/z 345 produced ion m/z 270 and m/z 255. Fig.9 showed that the ion m/z 318 contained the fragment of ion m/z 228. This may be also due to the loss of TMS-OH from the ion m/z 318. The differences between m/z 360 and 318, between 270 and 228, between 255 and 213 are equal to 42, which may be due to the loss of $C_3H_6$. The mass spectrum of ion 213 in parent mode (see Fig.10) showed the relationship between ion m/z 213 and other parent ions. The base peak is m/z 143 in Fig 8 for tetrahydromibolerone-di-*O*-TMS.

The third possible metabolite of mibolerone we have found is dihydroxy-mibolerone (Met.-III).

The gas chromatogram and EI mass spectrum were showed in Fig.11. The molecular ion of dihydroxymibolerone-tetra-*O*-TMS is m/z 624. The ion m/z 534 came from its molecular ion m/z 624 due to the loss of TMS-OH. The loss of TMS-OH from ion m/z 534 and from ion m/z 444 produced ion m/z 444 and m/z 354 respectively. The presence of m/z 231 and m/z 218 suggested the presence of 16β- or 16α-OH structure in the metabolite. The mass spectra of ion m/z 218 and ion m/z 231 in daughter mode (see Fig.12) showed the fragmentation of 16-OH metabolites of mibolerone, supporting the hypothesis of 16-OH structure. The possible fragmentation pathway of ions m/z 218 and 231 was showed in Fig.13. It was showed by the mass spectrum of ion m/z 494 in parent mode that the loss of ion m/z 130 from the molecular ion 624 resulted in ion m/z 494. The presence of ion m/z 534, which came from the molecular ion m/z 624, and the presence of the ions m/z 444 and m/z 354 in Fig.11 suggested the presence of dihydroxymibolerone.

In our results, we could not find any isomers of these proposed metabolites. This is in agreement with the publications in which only 5β-isomers were proposed. So our proposed metabolites of mibolerone are: 5β-dihydromibolerone, tetrahydromibolerone, dihydroxy-mibolerone and unchanged mibolerone. Fig.14 showed our proposal for the possible chemical structures of the metabolites of mibolerone. In our experiment the exact structures of mibolerone metabolites are still open.

In summary table 1 listed the retention data of the possible metabolites of mibolerone.

Tab.1 The retention times and relative retention times of mibolerone and its possible metabolites

| Substances | Retention time (min) | Relative Rt |
|---|---|---|
| Mibolerone (Di-*O*-TMS) | 14.55 | 0.964 |
| Met I (Di-*O*-TMS) | 12.90 | 0.854 |
| Met II (Di-*O*-TMS) | 13.20 | 0.874 |
| Met III (Tetra-*O*-TMS) | 16.13 | 1.068 |
| IS (Methyl-T-di-*O*-TMS | 15.10 | 1.000 |

Fig. 15 shows the excretion variation of mibolerone metabolites in the urine. The excretion study showed that mibolerone in urine was excreted mainly in unchanged form and tetrahydromibolerone in conjugation. In 28 hr. after oral administration with a single dose of 14.5 mg only tetrahydro-mibolerone could be detected in urine. According to our experiment most of the metabolites of mibolerone were conjugated in urine. With screening procedure IV the unchanged mibolerone and its proposed metabolites were detected by GC-MSD (HP 5973).

**References:**


1. W. Schaenzer, Metabolism of Anabolic Androgenic Steroids, Clin. Chem. 1996 42:7 pp 1001-1020
2. L. D. Bowers, Metabolic Pattern of Mibolerone, Recent Advances in Doping Analysis (3), SPORT und BUCH Strauss, 1996, pp 81-82

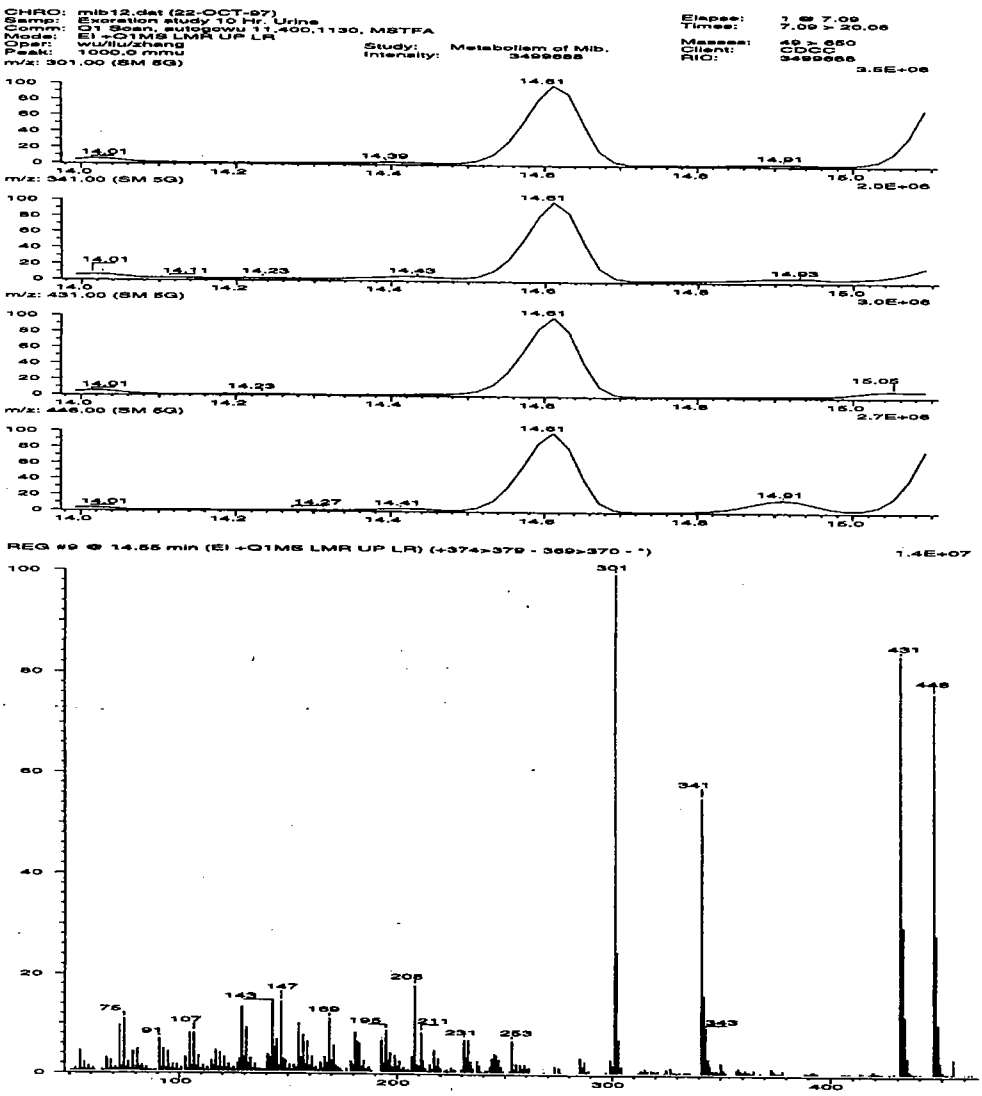
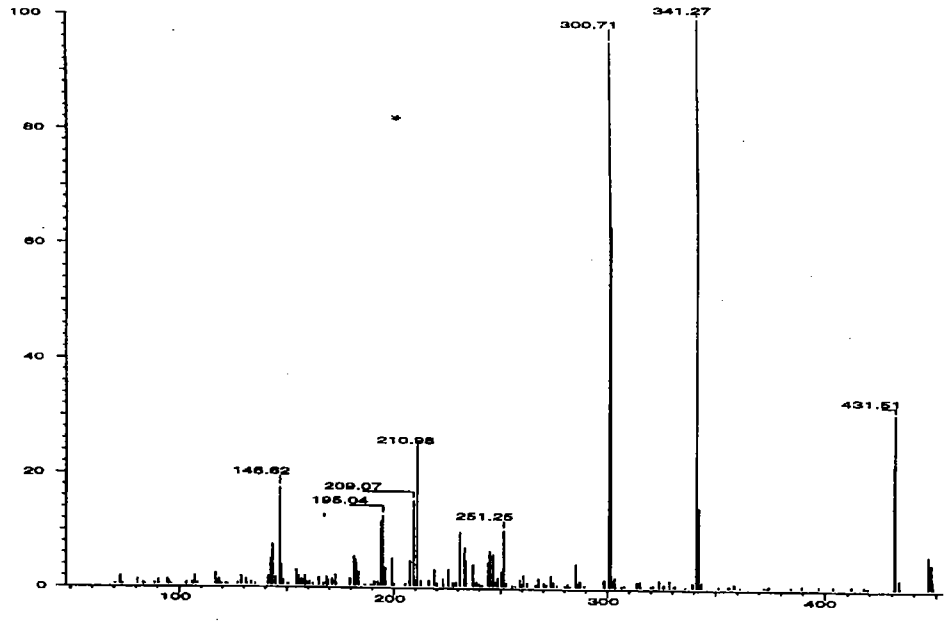



Fig.1 The Gas Chromatogram and EI Mass Spectrum of Mibolerone-di-*O*-TMS




*(Fig.2 continueing)*

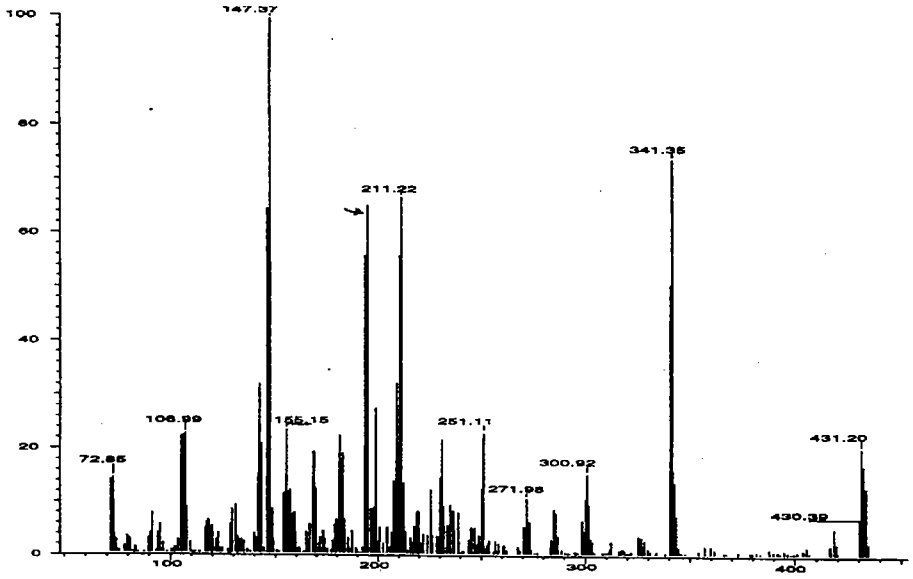



Fig.2 The Daughter Mass Spectra of Ions m/z 446 (in the previous page) and m/z 431

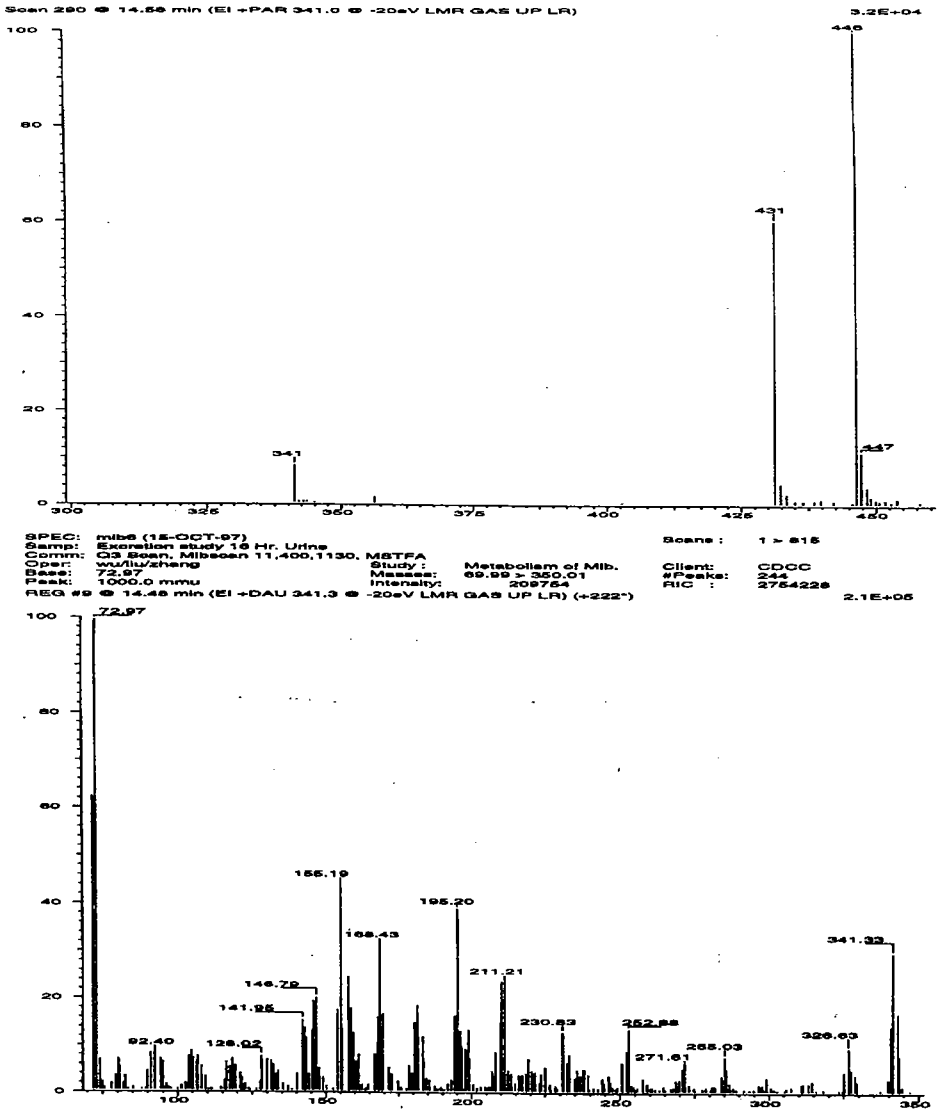



Fig.3 The Mass Spectra of Ion m/z 341 in Parent Mode (upper) and in Daughter Mode (lower)

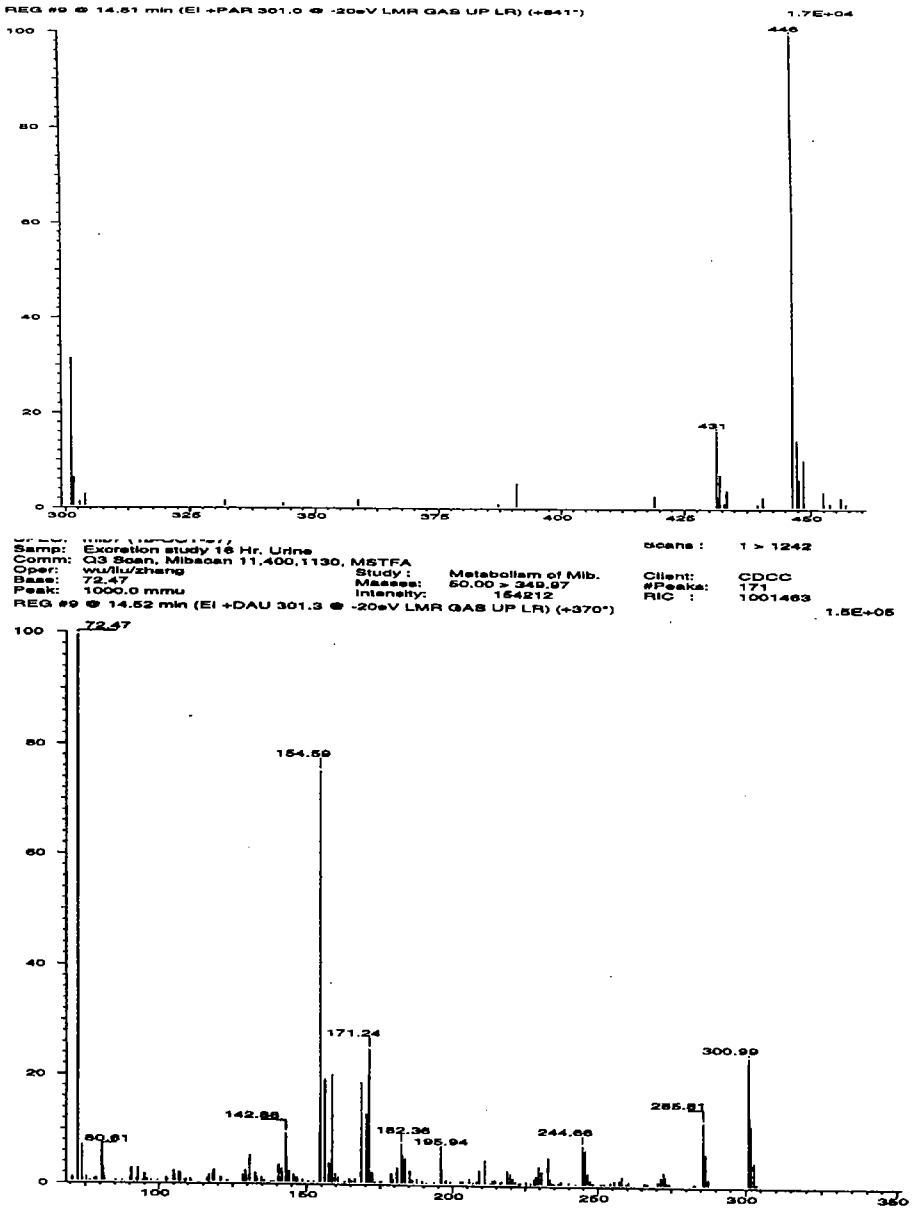
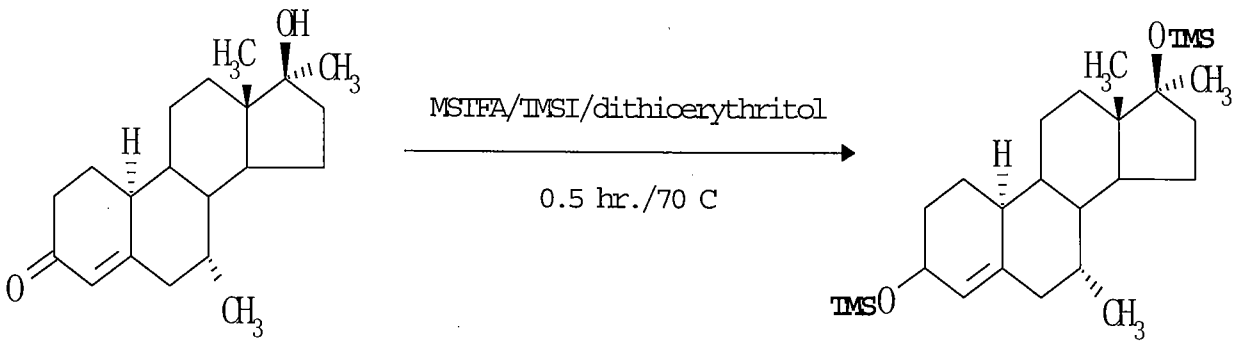



Fig.4 The Mass Spectra of Ion m/z 301 in Parent Mode (upper) and in Daughter Mode (lower)




m/z 302

Mibolerone

m/z 446

Di-O-TMS-Mibolerone

*(Fig.5 continueing)*

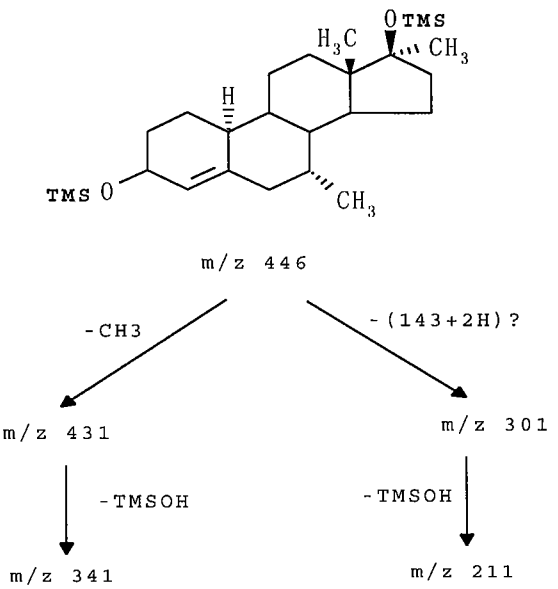



Fig.5 The Structure and Fragmentation Pathwey of Mibolerone-di-O-TMS

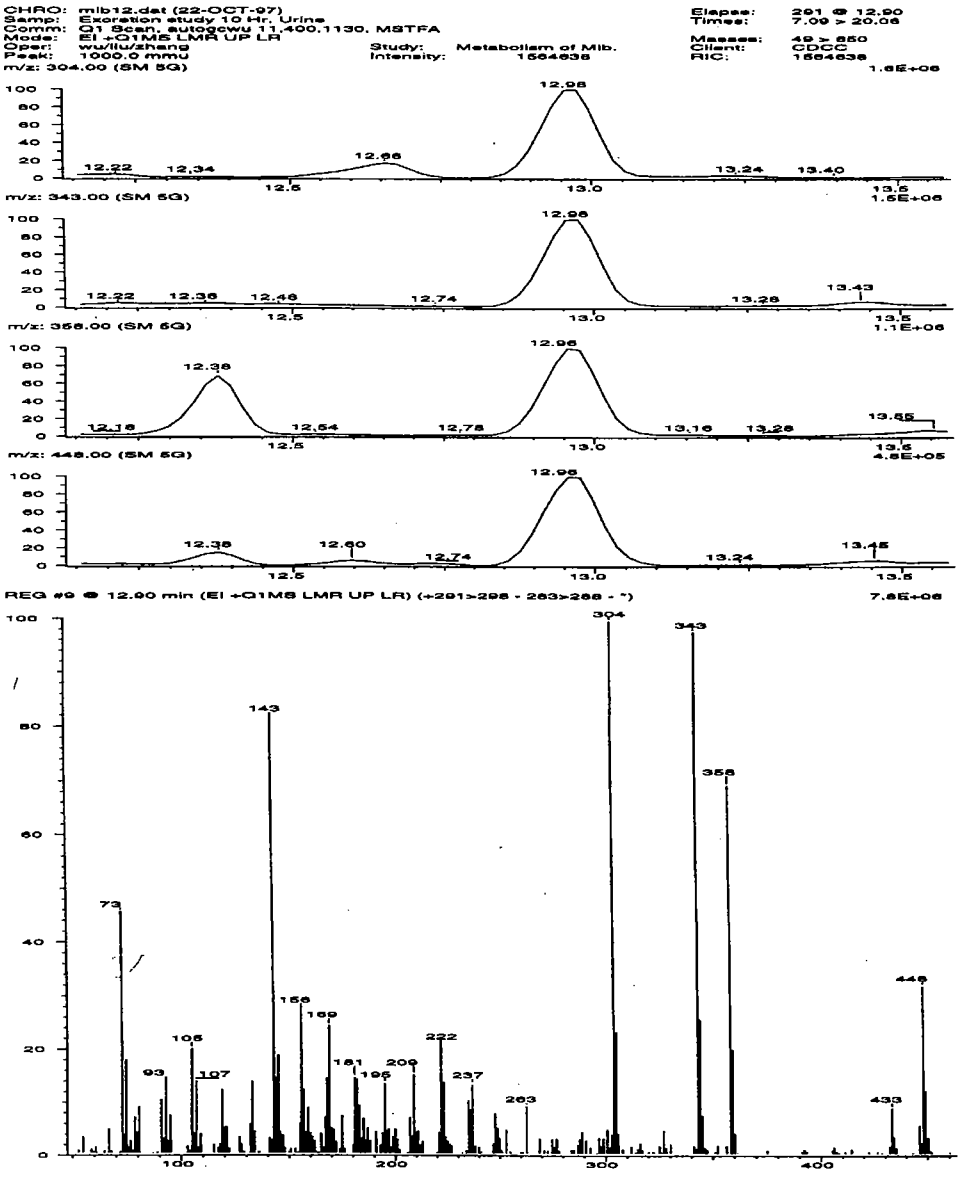



Fig.6 The Chromatogram and EI Mass Spectrum of Met.-I-di-*O*-TMS

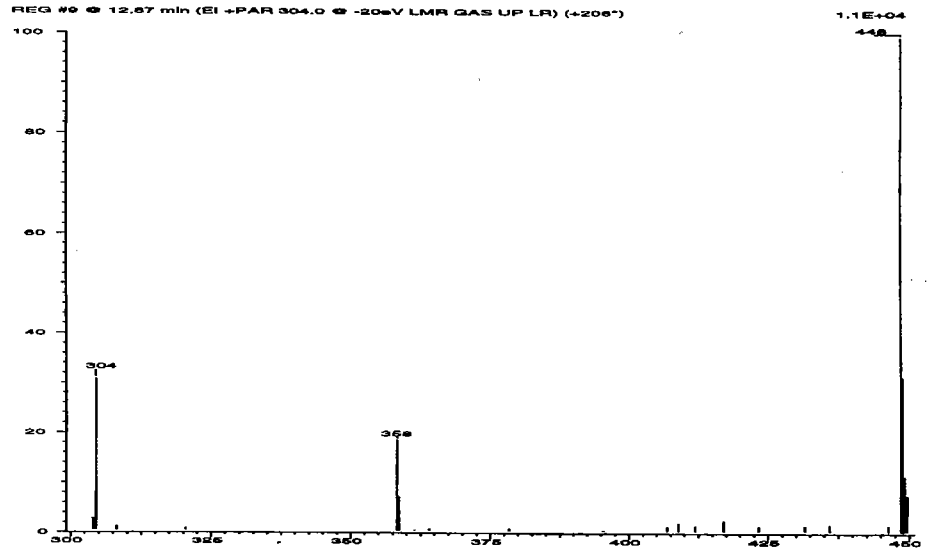



Fig.7 The Mass Spectrum of Ion m/z 304 in Parent Mode

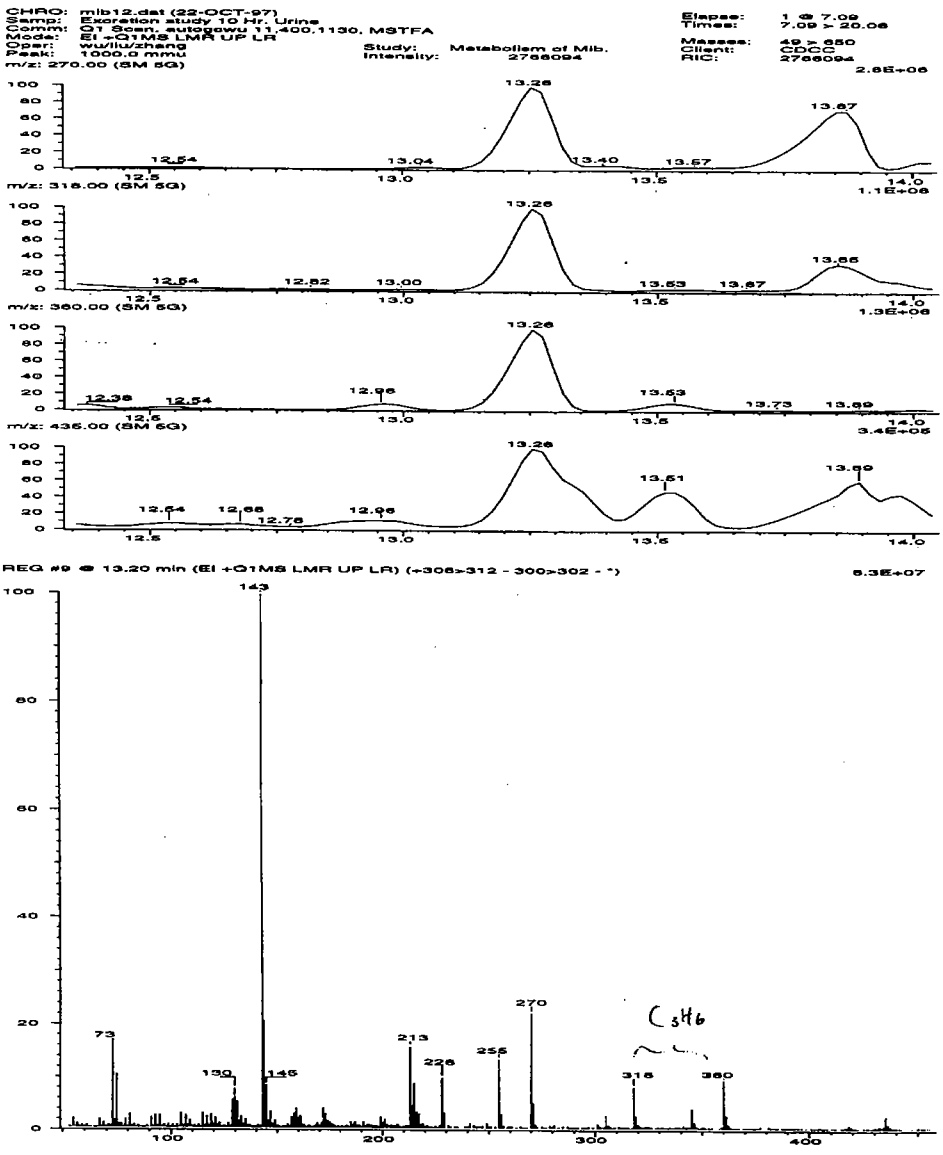



Fig.8 The Chromatogram and EI Mass Spectrum of Met.-II-di-*O*-TMS

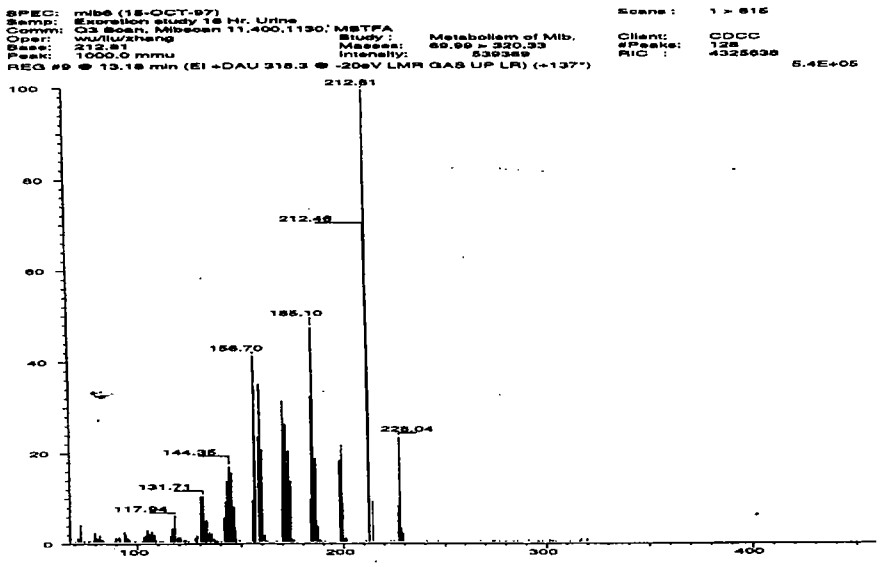



Fig.9 The Daughter Mass Spectrum of Ion m/z 318

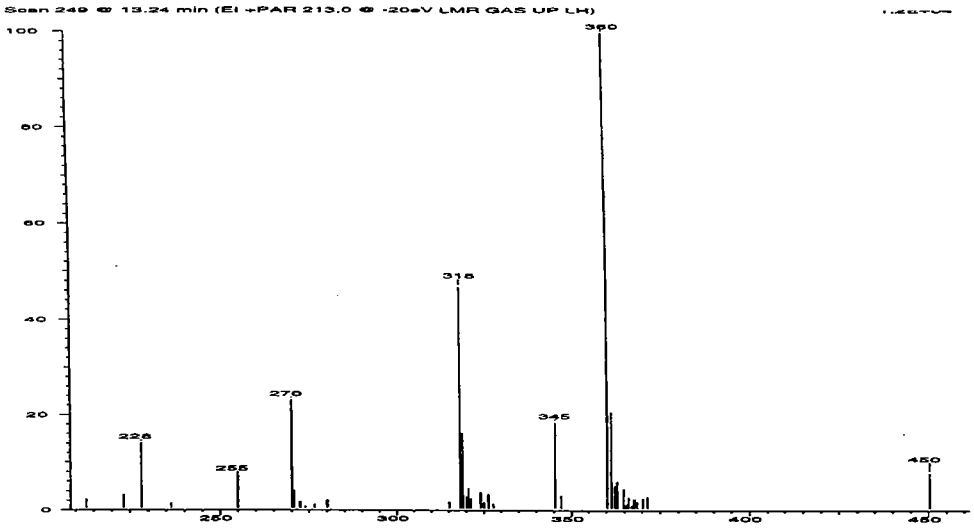
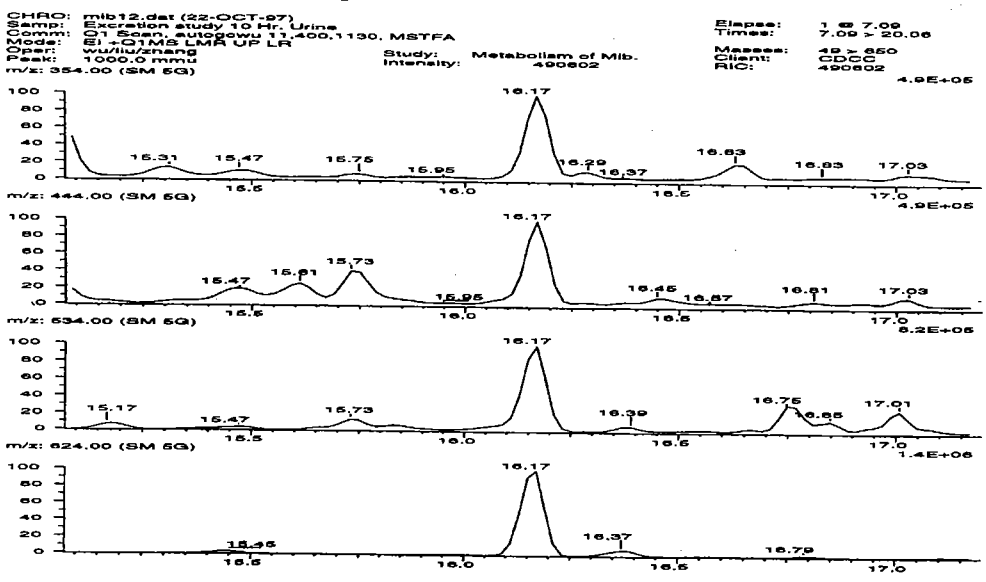



Fig.10 The Mass Spectrum of Ion m/z 213 in Parent Mode




(Fig.11 continueing)

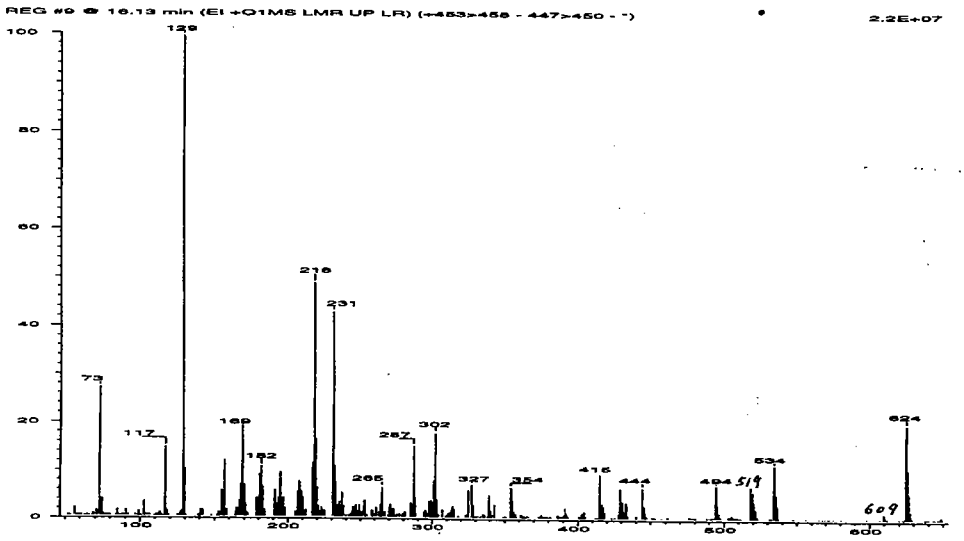



Fig.11 The Chromatogram and EI Mass Spectrum of Met.-III-tetra-*O*-TMS

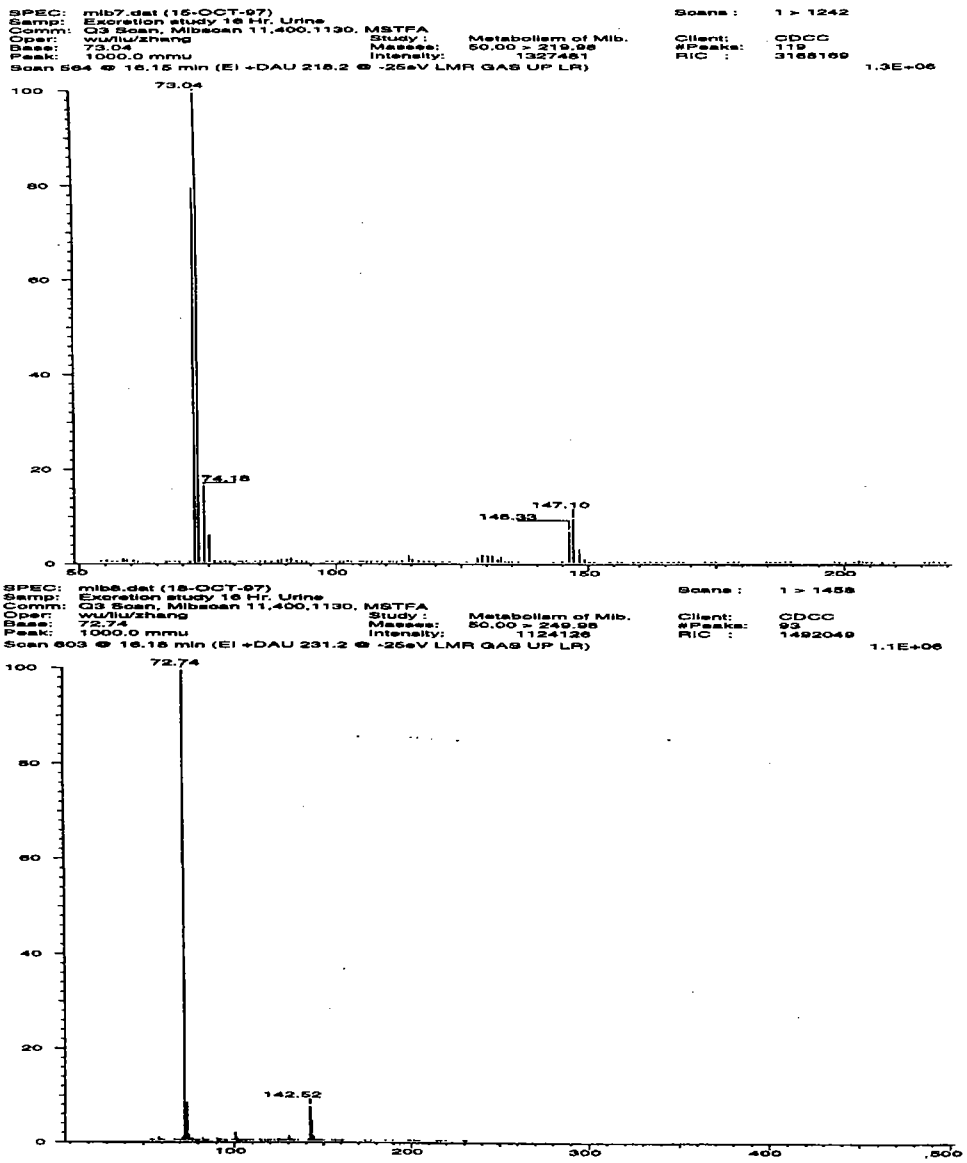



Fig.12 The Daughter Mass Spectra of Ion m/z 218 (upper) and 231 (lower)

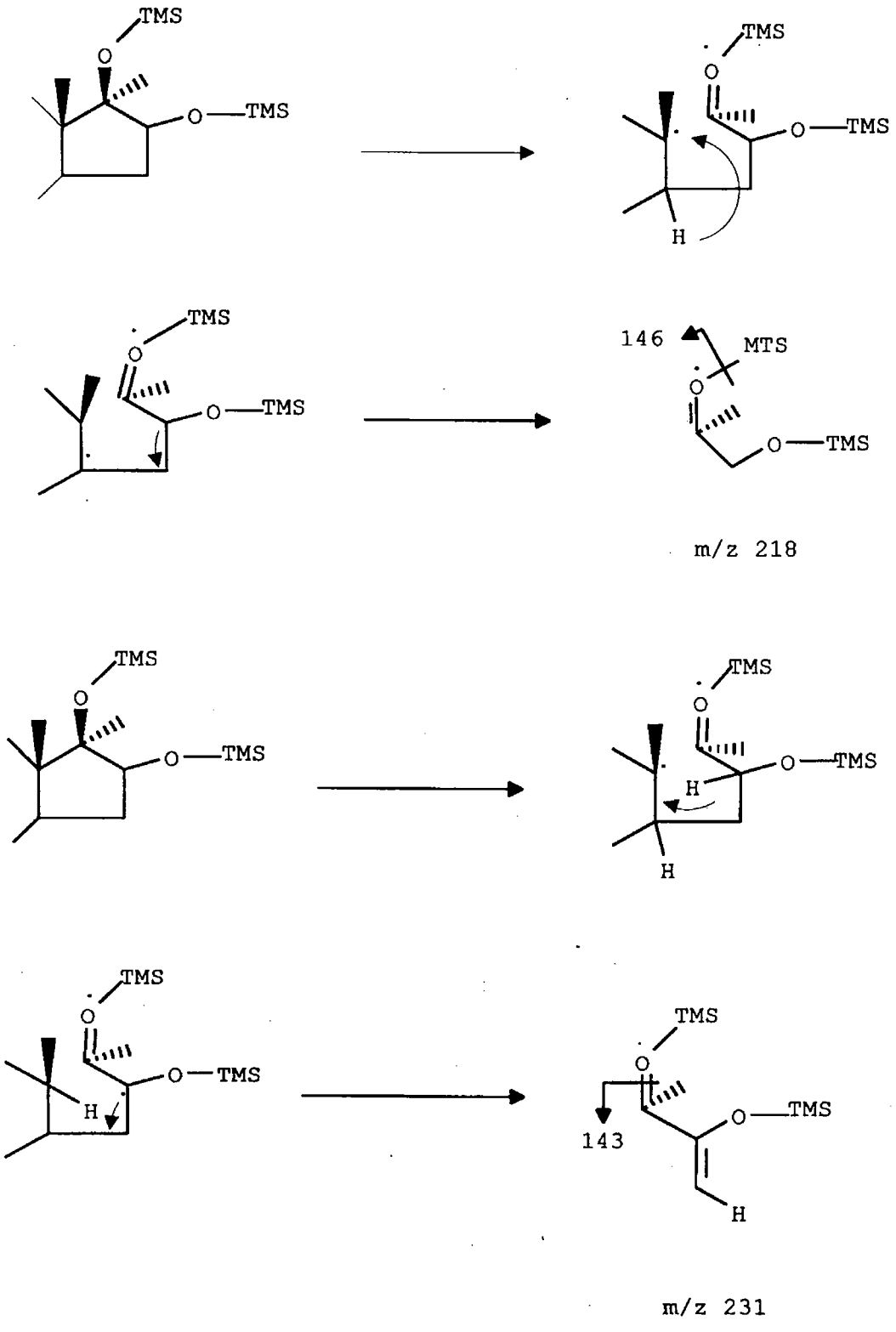



Fig.13 The Possible Mechanism of Ion m/z 218 and m/z 231

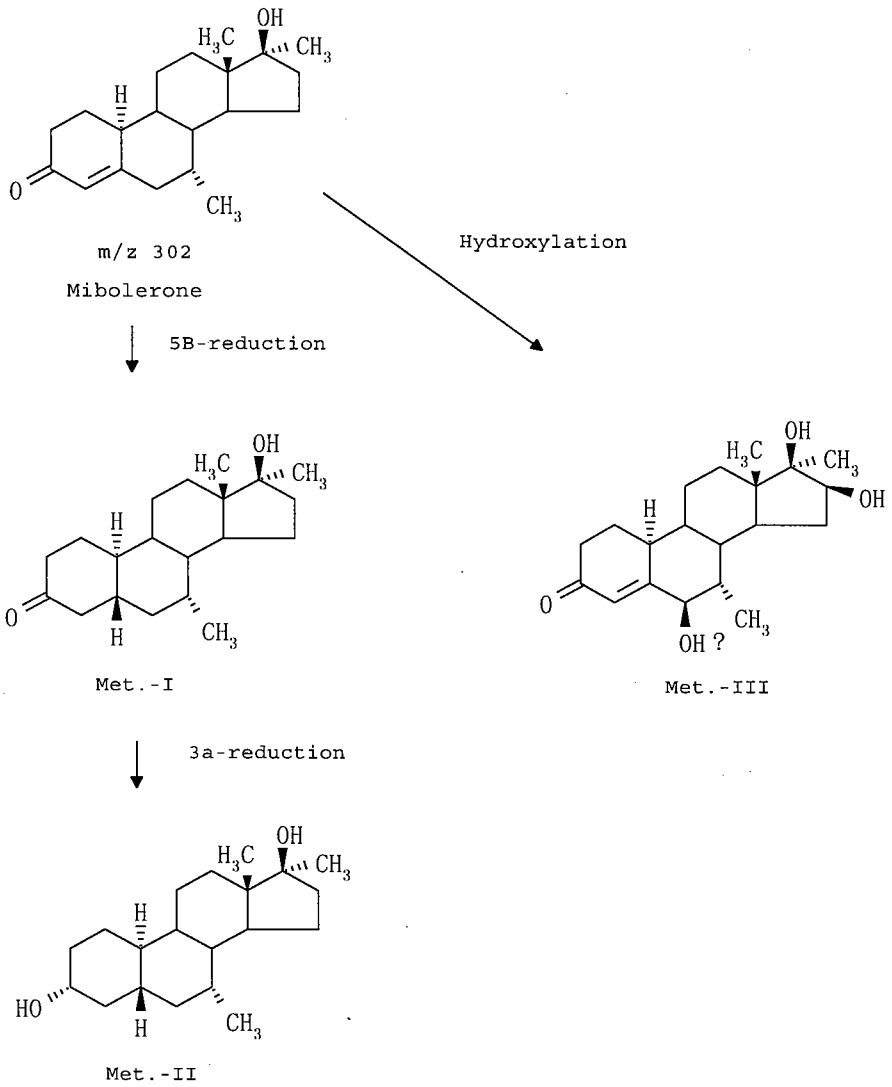



Fig.14 The Possible Structures of Mibolerone Metabolites

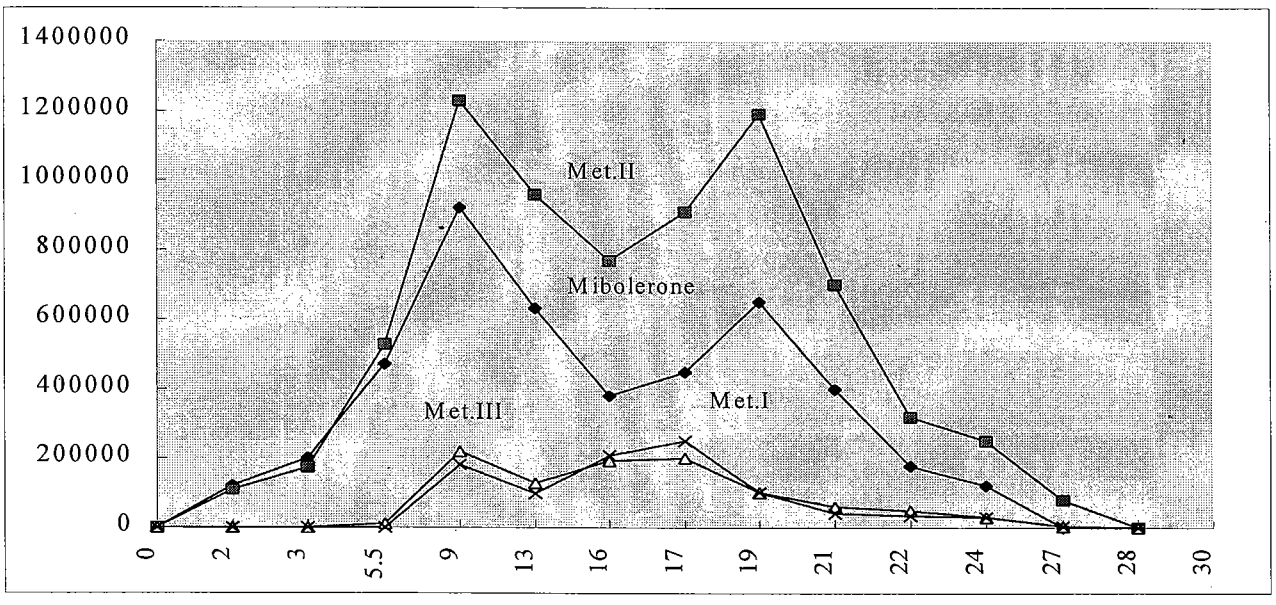



Fig.15 The Excretion Variations of Mibolerone Metabolites