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RECENT ADVANCES IN DOPING ANALYSIS (3)

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Trenbolone: Screening and Confirmation

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Introduction

The anabolic steroid trenbolone (β-trenbolone, 17β-hydroxyestra-4,9,11-trien-3-one, Figure 1) was originally developed for veterinary use as acetate or cyclohexylmethylcarbonate (Parabolan). Like several other veterinary anabolic agents, trenbolone is thought to be abused by athletes in power sports. Due to the unique chemical structure of this steroid, i.e. a chain of conjugated double bonds, trenbolone is difficult to derivatize for gas chromatographic analysis, and consequently is difficult to detect within the common steroid GC/MS screening procedure.

Screening by enzymatic immunoassay (1) and HPLC (2) are proposed. However, confirmation still requires GC/MS, which has not been reliably effective. Comprehensive study of trenbolone derivatization problems (3) demonstrates the advantage of methoxime (MO) shielding of the carbonyl group prior to trimethylsilylation (TMS). The use of MO-TMS derivatives is promising for GC/MS confirmation, but it does not seem to be practical for screening of just one rarely observed steroid.

Several metabolites of β-trenbolone are found (4), with α-trenbolone (epi-trenbolone) being a major human urinary metabolite. The purpose of this investigation was to find a practical analytical approach for detection of trenbolone using human excretion urine.

Material and Methods

β-Trenbolone was purchased from Sigma.

Excretion studies were made with two adult male volunteers. Non esterified β-trenbolone was administered orally in single doses of 15 mg. Urine was collected prior to and after drug ingestion for up to five days.

Assay: Routine screening procedure for total steroids was applied as follows:

Sample preparation

4 mL of urine and 1 mL of acetate buffer/β-glucuronidase/ISTD mixture are incubated for 3 hours at 52°C in a glass tube. Then 1mL of 1M sodium bicarbonate buffer is added and the tube is vortexed.

The mixture is applied to C18 solid phase extraction column prewashed with 3 mL methanol and 3mL water. The column is washed with 2 mL 20% acetonitril in water and steroids eluted with 3 ml methanol. The methanolic eluate is evaporated to dryness.

Derivatization

The dry residue is derivatized with one of the follows:

- 75 µL of MSTFA/NH4I/Dithioerythritol 1000:2:3 and heated for 15 minutes at 70°C.

- 75 µL of MSTFA and heated for 15 minutes at 70°C.

- 50 µL of 4% methoxyamine hydrochloride in pyridine for 30 minutes at 70° and after pyridine evaporation with 100 µL of TSIM at 70°C for 15 minutes.

GC/MS parameters

GC/MS HP 5890/5970

column:	HP-1 fused silica, crosslinked methylsilicon, 17 m, 0.2 mm i.D., 0.11 µm film thickness
temperature program:	180°C; 3°C/min - 231°C; 300°C

Results and Discussion

Derivatization of parent β-trenbolone was performed in three different ways: TMS without catalyst, TMS with enolization catalyst NH4I, and MO-TMS derivatization. Direct trimethylsilylation of this

steroid with or without enolization catalyst yields multiple derivatization products, as shown in Figure 2.

Trenbolone easily enolizes with plain MSTFA yielding several tautomeric di-TMS derivatives with molecular mass 414 and different positions of double bonds (Figure 2A). The "correct" mono-TMS derivative with molecular mass 342 is abundant, and does not have tautomers.

The application of a regular enol-TMS reagent results in a severe transformation of trenbolone into a large number of derivatives, shown in Figure 2B. Expected peaks with 414 molecular mass are weak. Interesting products of this enol-TMS derivatization are compounds with molecular mass 412, i.e. trenbolone di-TMS minus two hydrogens and 498, which are apparently linked one to another. Numerous peaks with 500 molecular mass evidently derive from tautomeric di-TMS β-trenbolones with 414 masses.

MO-TMS derivatization of β-trenbolone yields a single product shown in Figure 3B. Evidently, this derivatization is preferable for trenbolone confirmation, as has been previously suggested (2). Figure 3A shows trenbolone confirmation performed in urine as MO-TMS derivative in full SCAN mode. We are able to identify and confirm only one metabolite, epi-trenbolone, with a negligible amount of parent compound. Mass spectrum of epi-trenbolone MO-TMS is presented in Figure 4.

Routine anabolic steroid screening procedure with enol-TMS derivatization was applied to trenbolone excretion urine. We detect the abundant peak of epi-trenbolone derivative with molecular mass 412, as shown in Figure 5. Mass spectrum is presented in Figure 6. In contrast to the parent compound, expected derivatives with molecular masses 414 do not appear in measurable amounts, Figure 7. Apparently α- and β-trenbolone exhibit different behavior at enol-TMS derivatization, which could in part be due to the influence of the urine matrix. 43 Hours after ingestion detector signal remains strong in SIM mode for this derivative in our routine anabolic steroid screen, Figure 8.

In conclusion, a standard anabolic steroid screening procedure can be successfully applied to screening of trenbolone as well as other anabolic agents by monitoring its metabolized epimer as

a derivative with molecular mass 412. Trenbolone detection in a regular steroid screen should not present a problem. Trenbolone, when taken orally, is detectable for two to four days by using this procedure. Of course, injectable forms will be detected for longer time periods. For trenbolone confirmation, MO-TMS derivatization is preferable, especially when quantification is required.

References:

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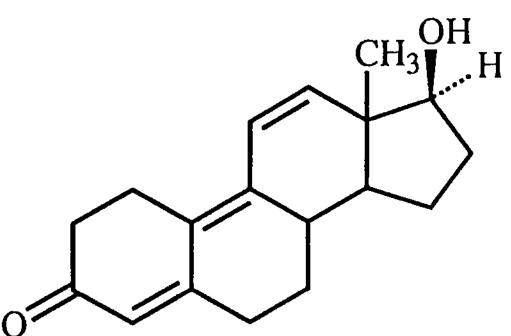
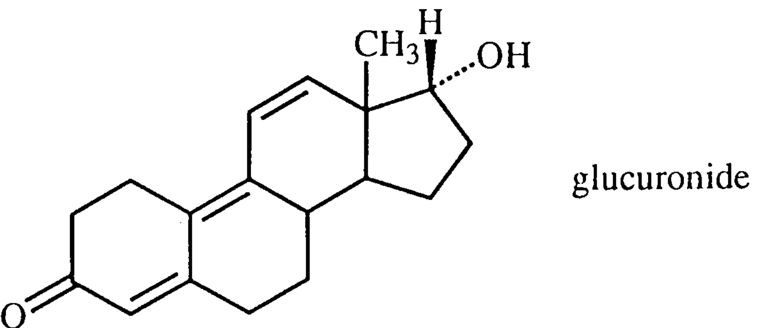
Trenbolone

17β–hydroxyestra-4,9,11-triene-3-one



β–Trenbolone
Parernt



α–Trenbolone (epi-Trenbolone)
Human urinary metabolite

glucuronide

M.W. 270

Derivatives:

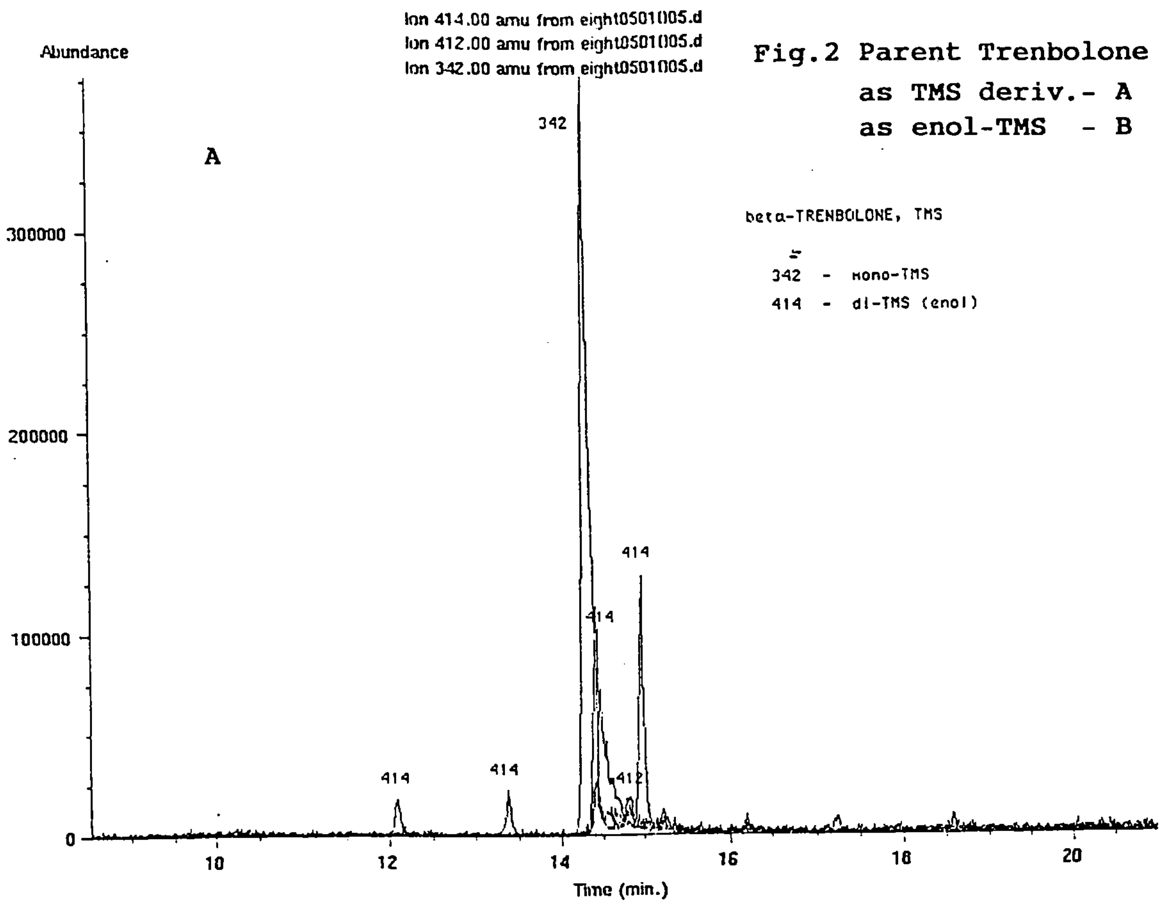
TMS 342

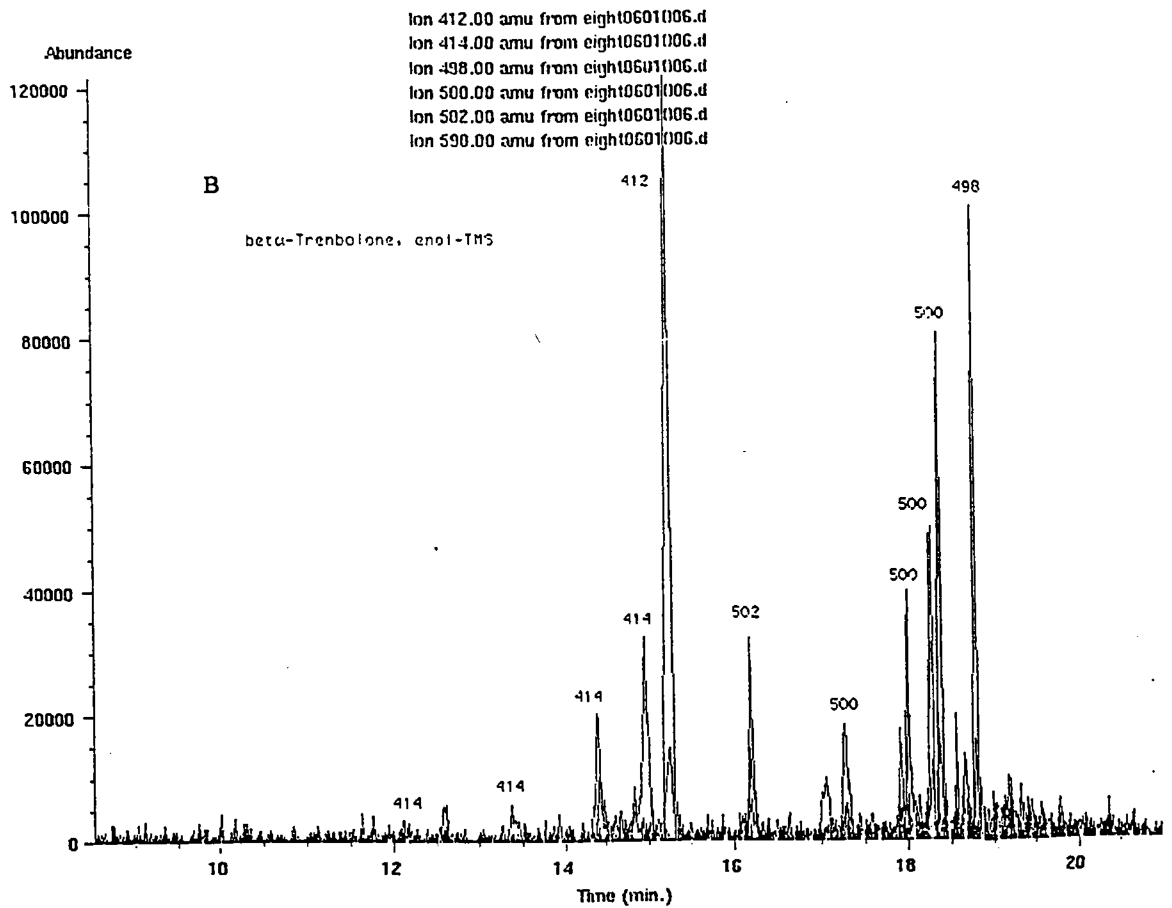
enol-TMS 414

MO-TMS 371

Fig. 1

Fig.2 Parent Trenbolone as TMS deriv.- A as enol-TMS - B





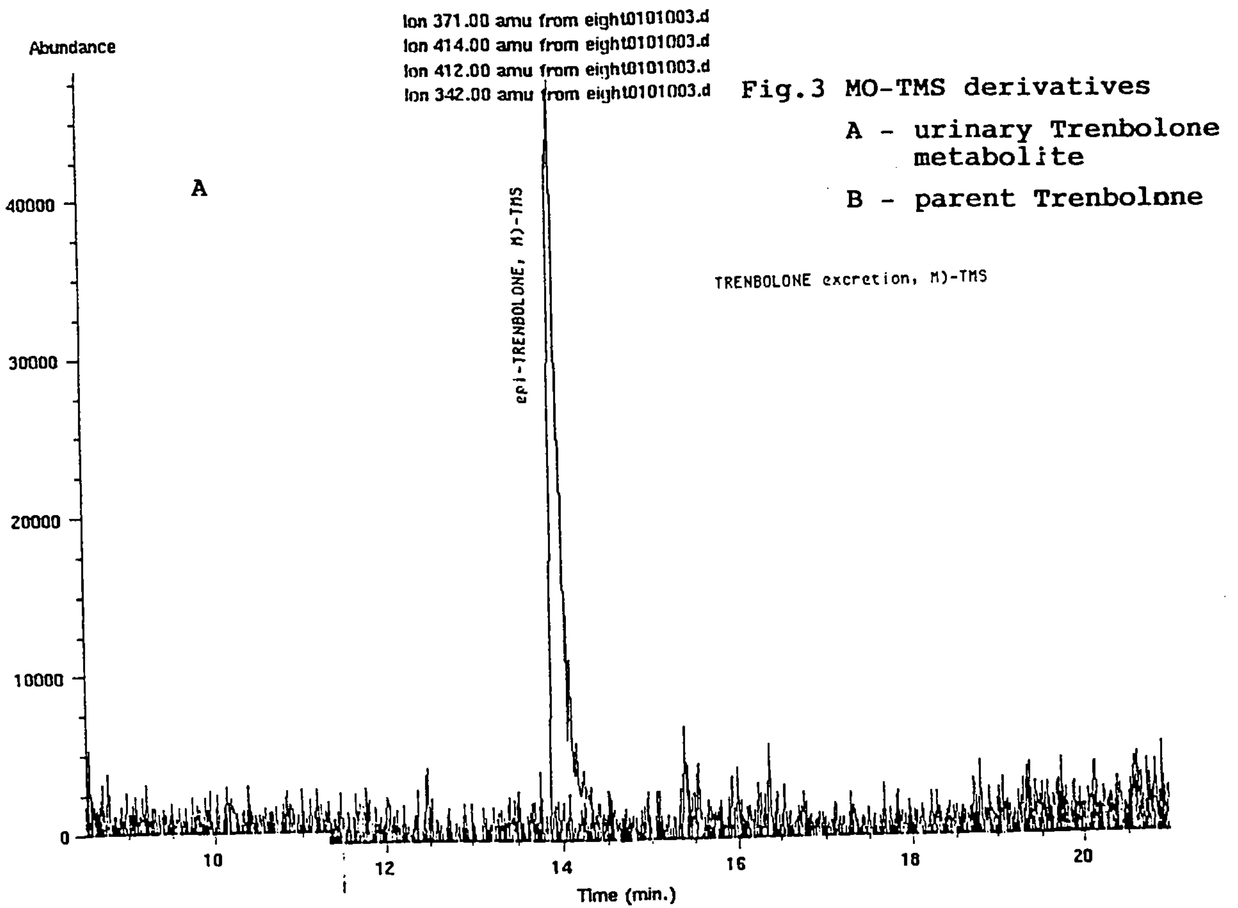
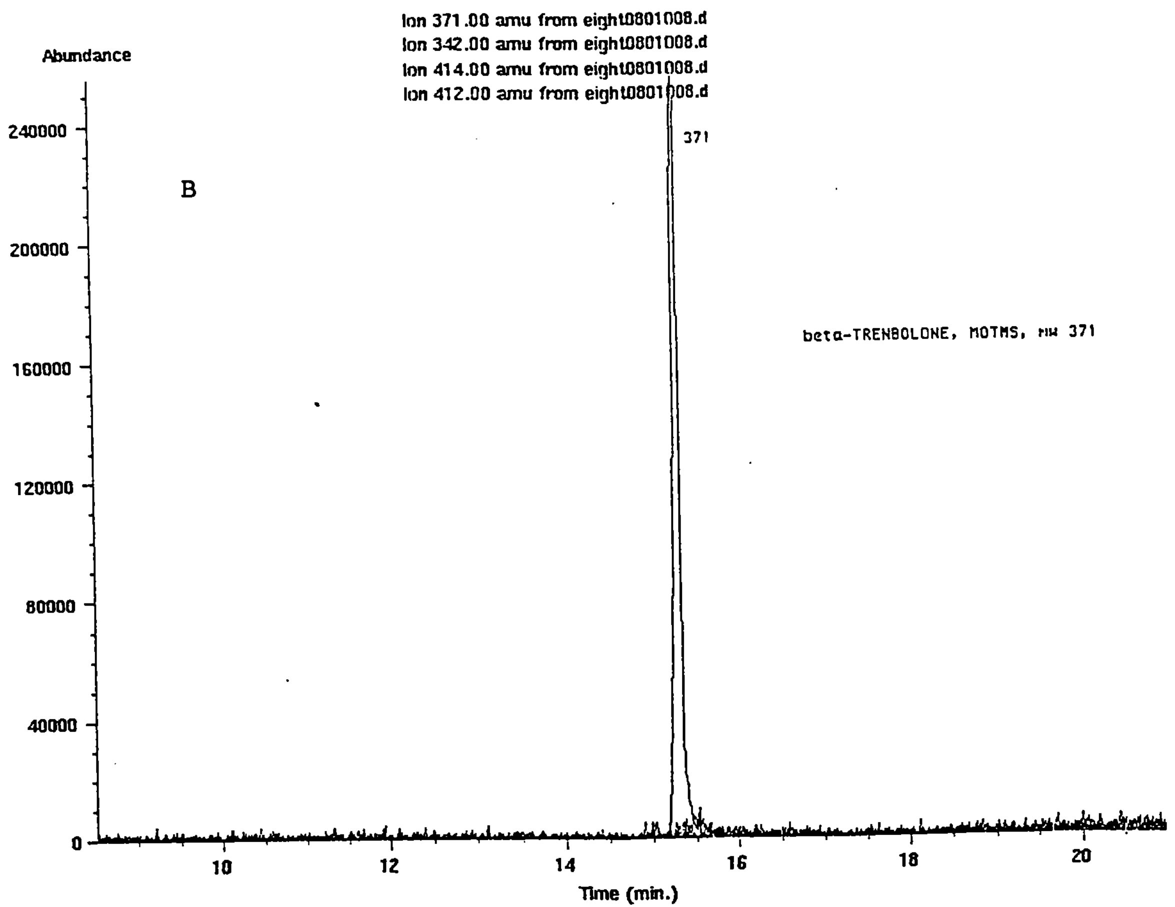


Fig.3 MO-TMS derivatives

A - urinary Trenbolone metabolite

B - parent Trenbolone



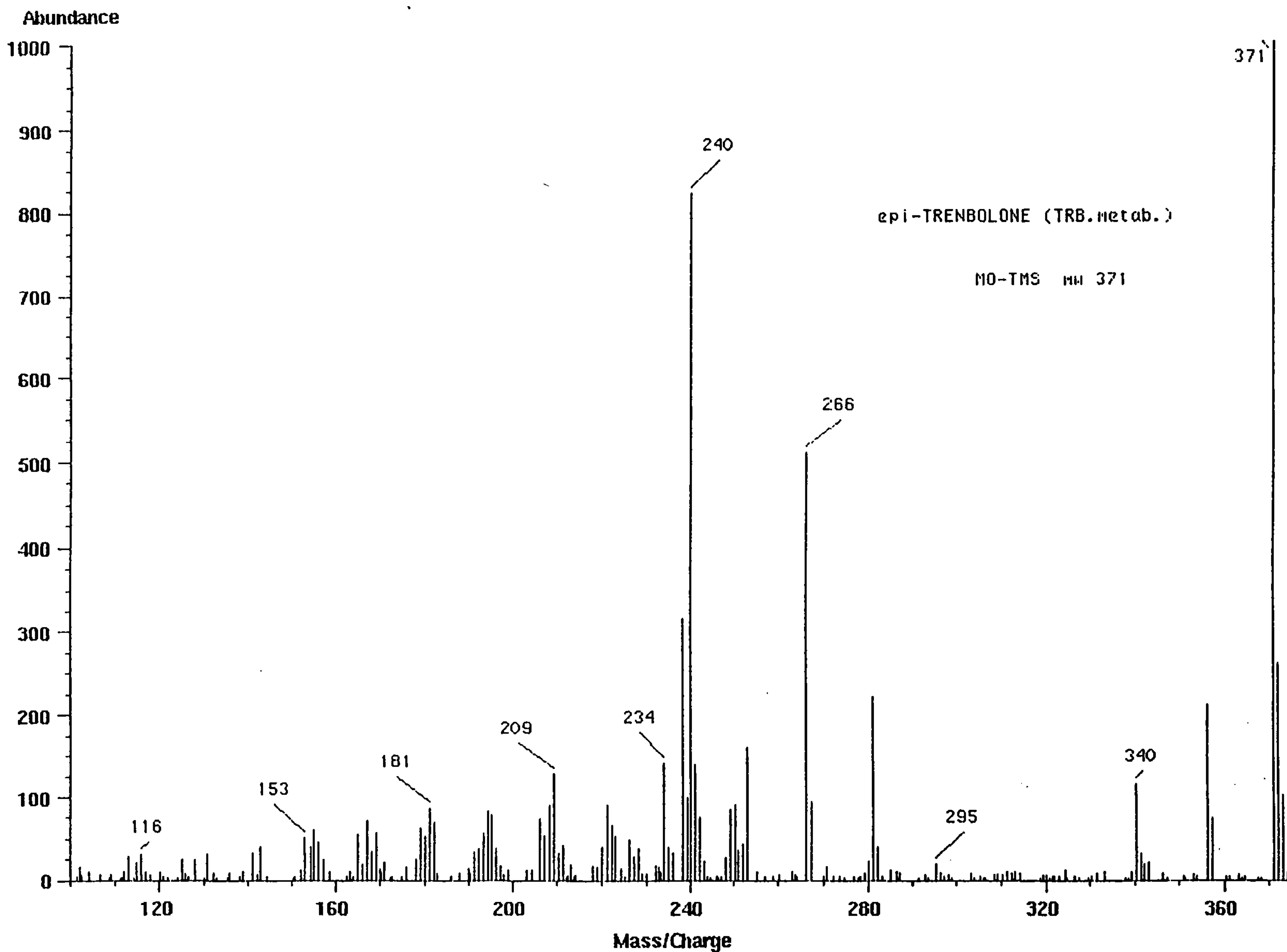


Fig. 4 EI mass spectrum of epi-Trenbolone MO-TMS derivative

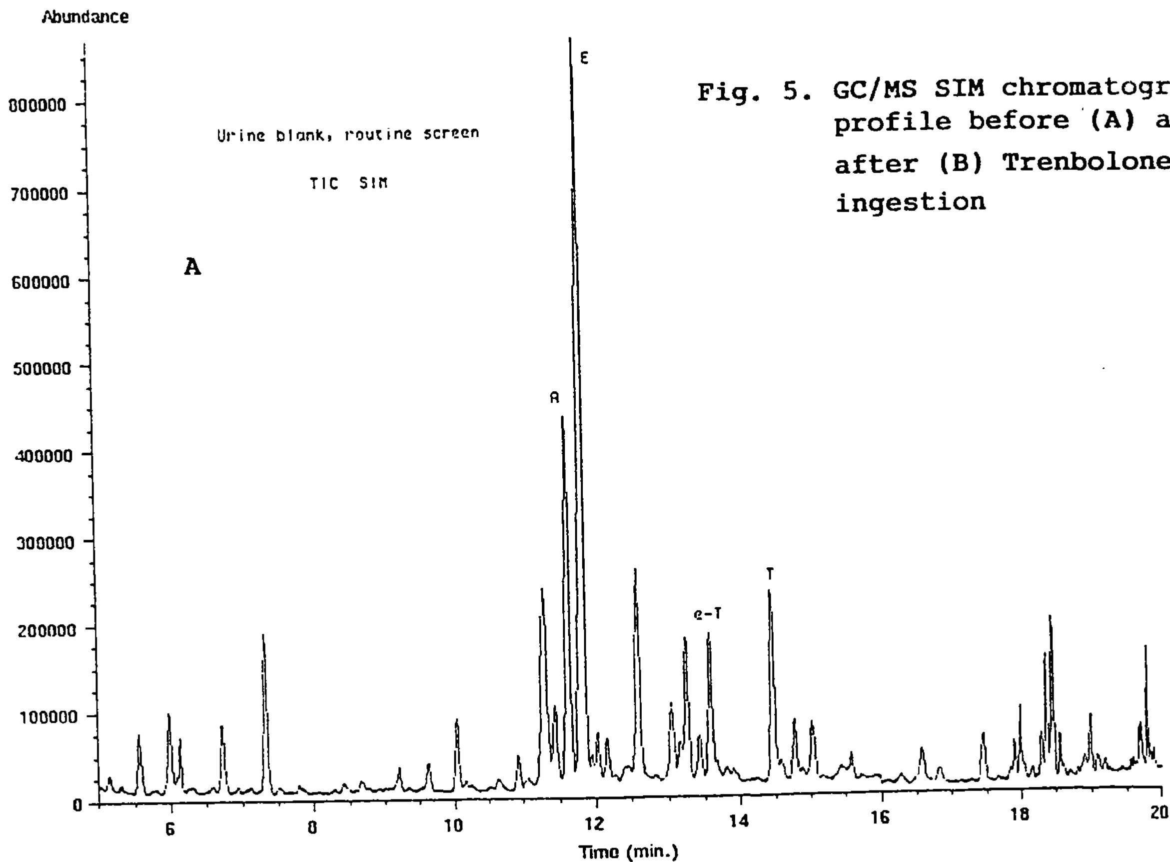
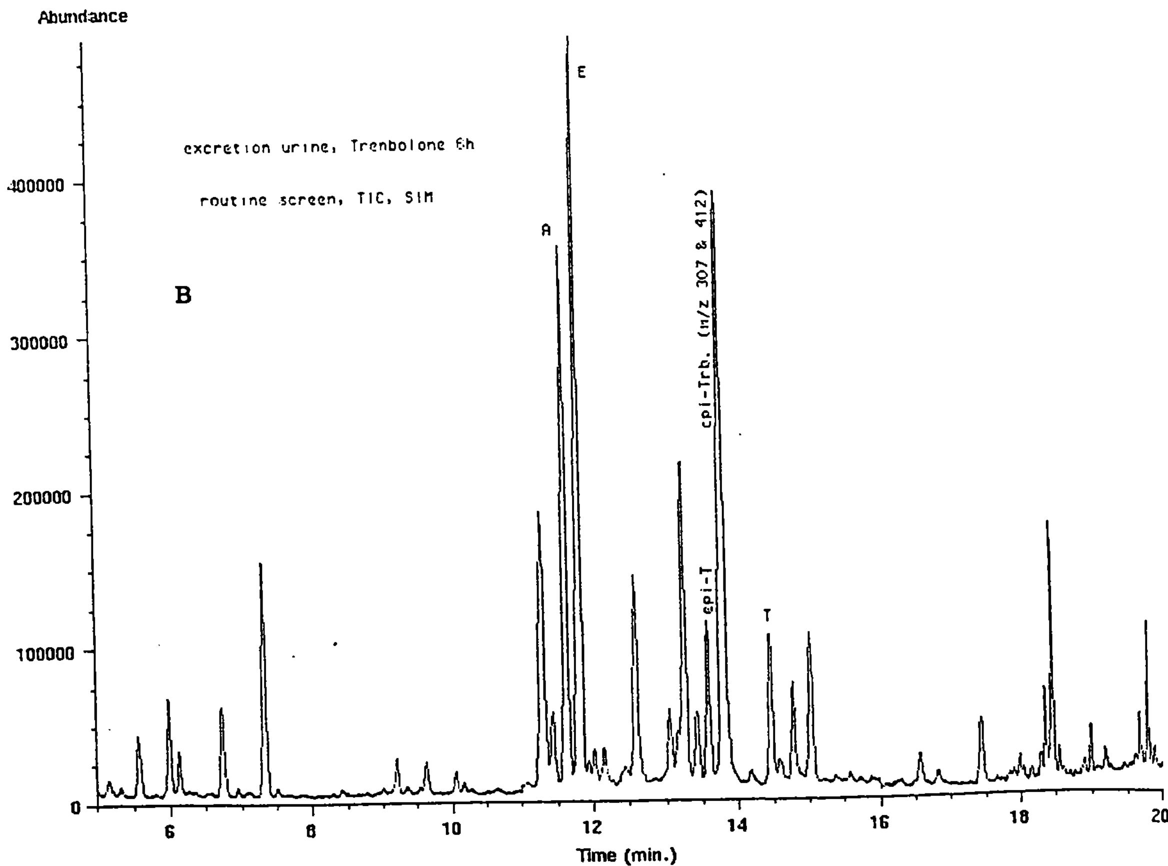


Fig. 5. GC/MS SIM chromatographic profile before (A) and after (B) Trenbolone ingestion



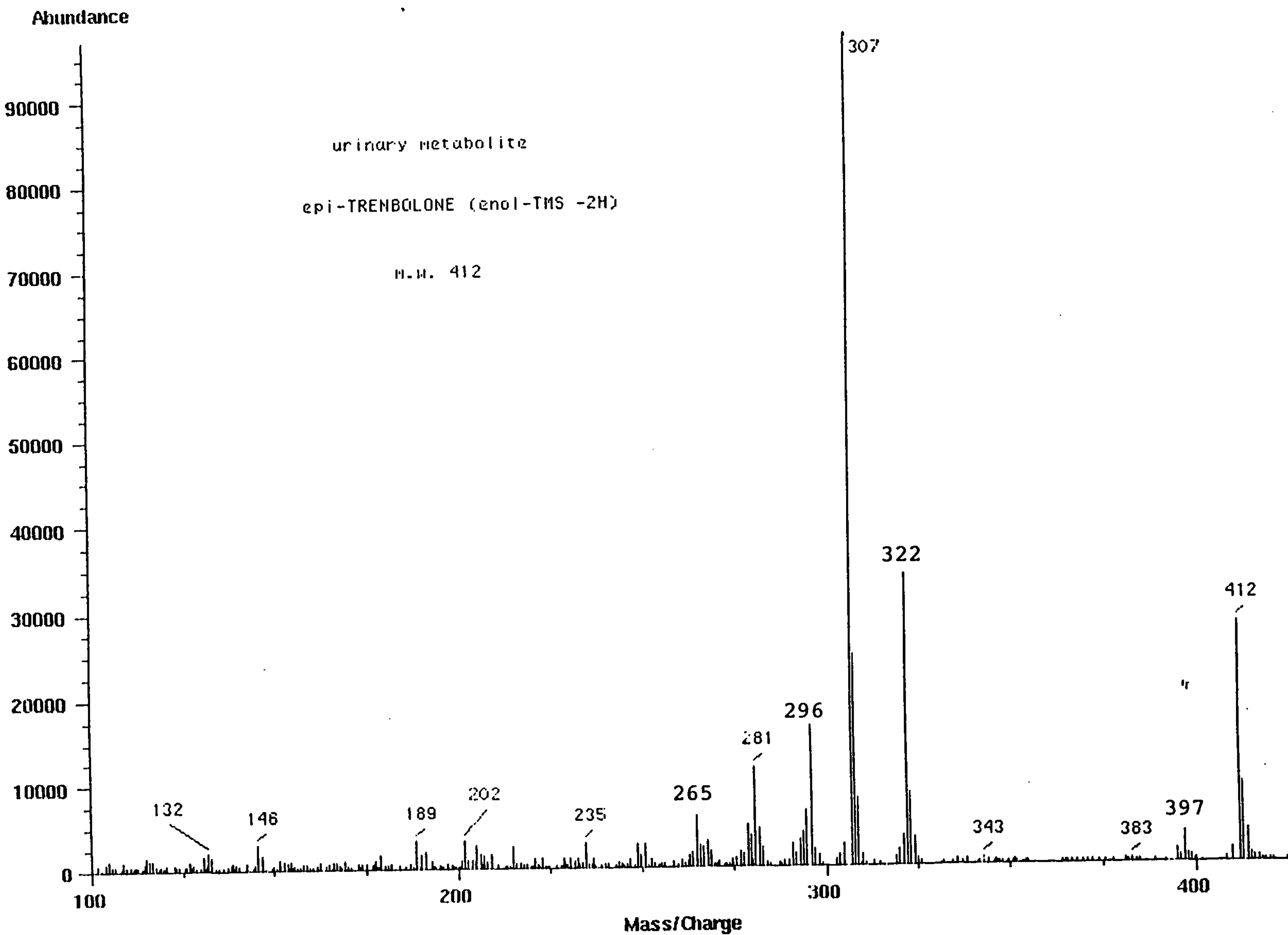


Fig. 6. EI mass spectrum of epi-Trenbolone enol-TMS (-2H) derivative

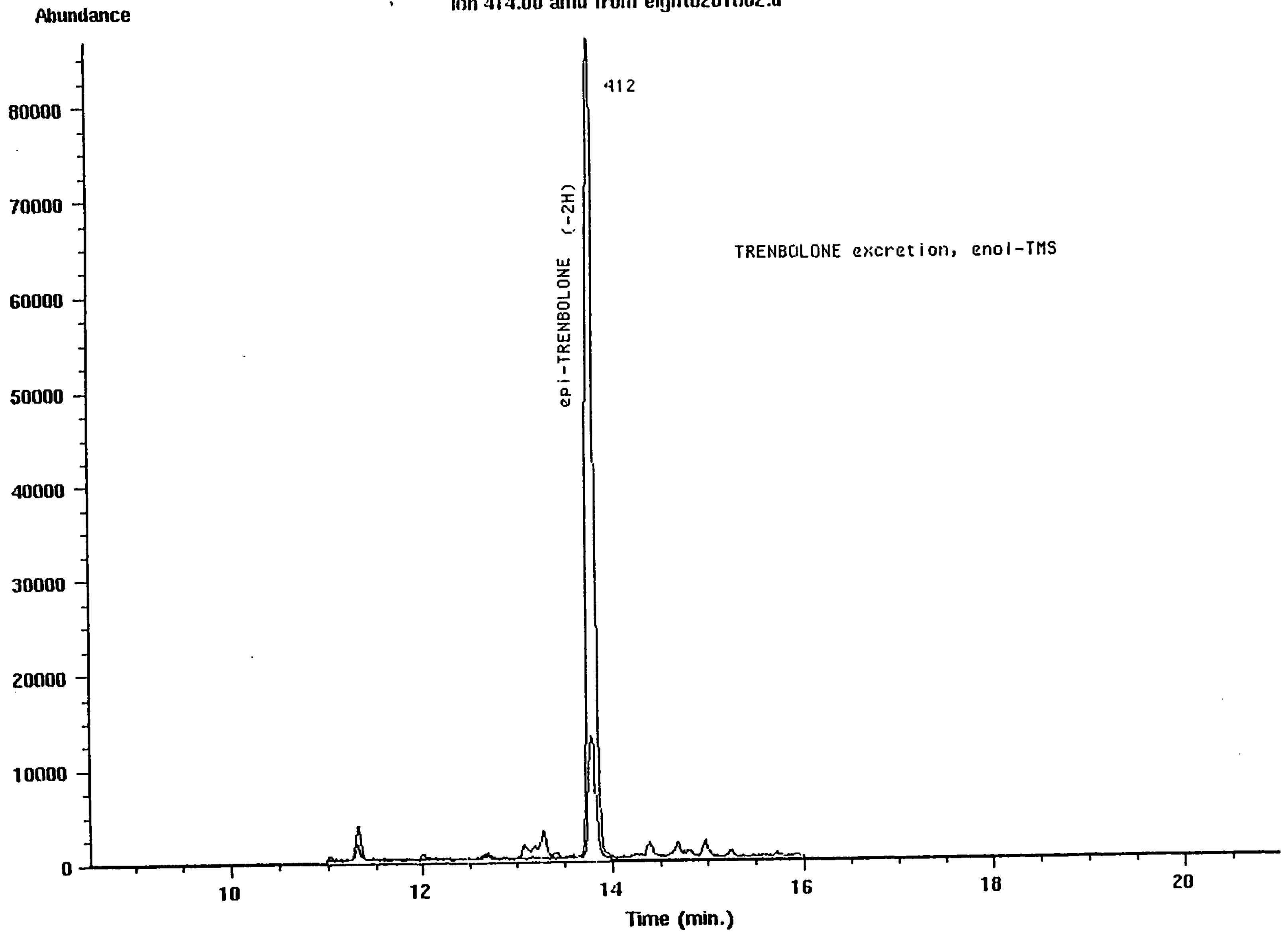


Fig. 7. SIM chromatogram of Trenbolone excretion urine

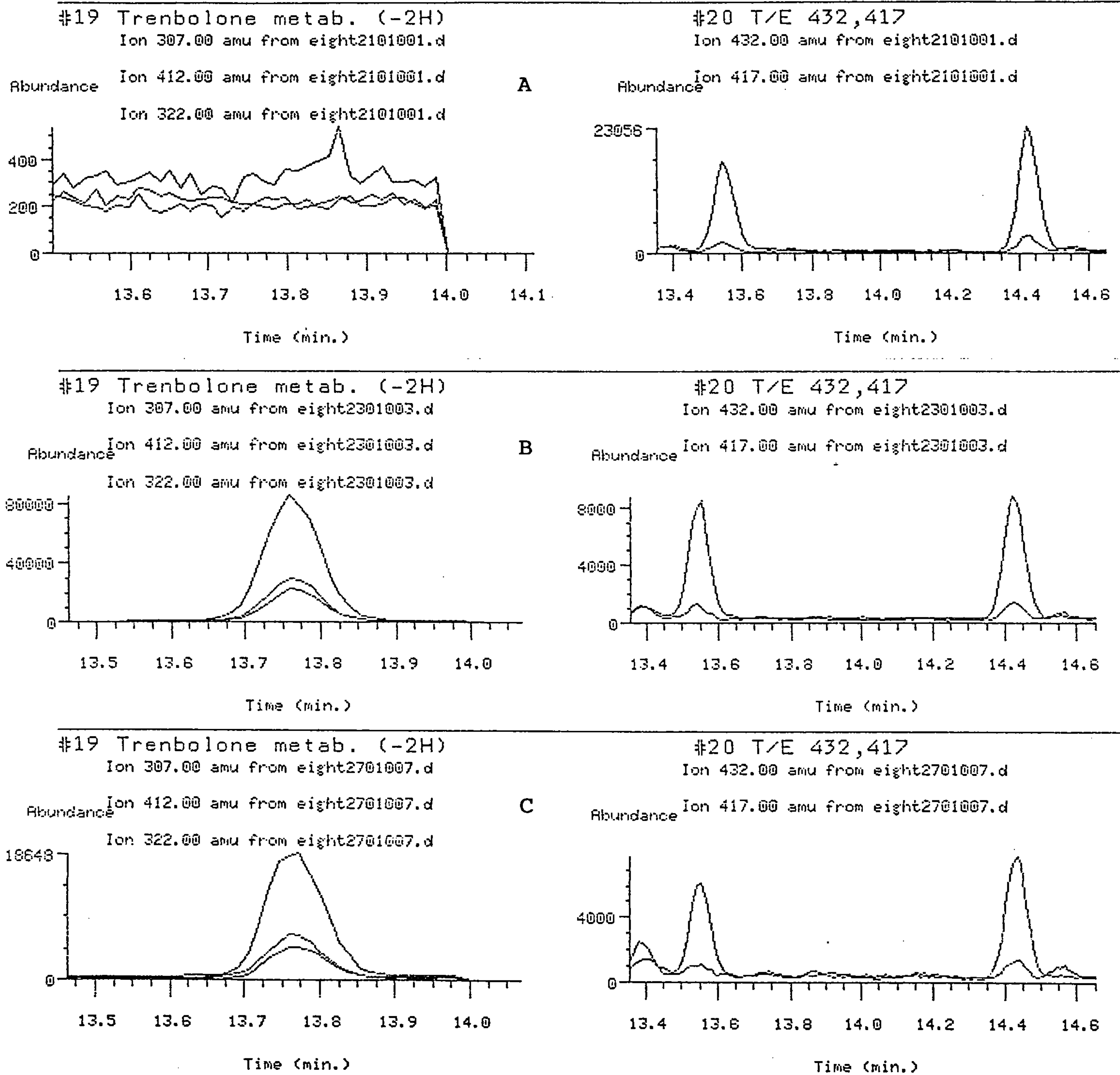


Fig. 8. SIM ion chromatograms for Trenbolone metabolite and T/E
A - before ingestion
B - 6 hours after ingestion
C - 43 hours after ingestion