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RECENT ADVANCES IN DOPING ANALYSIS (6)

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One Year Use of AUTOSPEC-TOF: Experience of Antidoping Lab in Rome

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INTRODUCTION

The use of high resolution instruments for routine GC/MS analyses is not popular: in Chemical Abstracts, General Subject indexes, during the last twenty years, combining HRMS with GC, there are not more that thirty entries. About ninety per cent of these reports are devoted to the use of GC/HRMS in searching for chlorinated substances of environmental interest, i.e. polychlorinated dioxins and polychloro biphenyles. Obviously, the mass defect of chlorine and the presence of several chlorine atoms make high resolution measuremets extremely useful in these fields.

The use of high resolution instruments in doping control laboratories started several years ago (1993) and it is apparent now that there are some advantages in the screening of anabolics performed with these instruments (1). However, there is no general agreement on the use of these instruments: for instance as far as the working resolution is concerned, the Cologne laboratory works with a standard resolution of 3000 (m/Δm) whereas Madrid and Athens laboratories work at 15000 resolution. As a matter of fact, the European Community gave criteria (2) for identification of an analyte by GC-HRMS: among them, any experiment to be classified as high resolution must be carried out at a resolution higher than 9500.

We started to use the AUTOSPEC-TOF early in 1997 in routine work. We wondered what resolution was appropriate for our analyses.

AUTOSPEC has a peculiar configuration, E-B-E. We suppose that this unusual configuration was chosen with the idea to have both advantages typical of instruments having «direct», or E-B (high trasmission at high resolution) and «reverse» or B-E (possibility of running MIKES spectra) configurations. However, whatever the configuration, it is well known that there is not only an inverse proportionality between trasmission (= sensitivity) and resolution: the relationship is more complicated and actually the transmission drops very rapidly at a certain value of resolution. Since in our laboratories the sensitivity is an extremely important parameter, it is imperative to find a compromise between sensitivity and

resolution. Moreover, ultimate resolution and performances of instruments are given without the GC coupling, i.e. without a gas flowing into the source.

Another question arises about the employment of an HR instrument in routine work: in screening or in confirmation? In screening, with reference to a lab engaged in a massive work (ten thousand samples/year as in our case) the instrument must process a high number of samples, if possible by running overnight. The reliability of these measurements depends on a multiplicity of factors, mainly the long term stability of resolution and calibration. Apparently, there were not many chances to process all samples on one HR instrument!

The AUTOSPEC-TOF offers the possibility of MS/MS experiments. It is a tandem Magnetic Sector/Time of Flight Analyser (3): TOF axis is placed orthogonally to the ion beam emerging from the sector stage. This configuration, as well as other characteristics like the collision cell float potential, were first suggested by Clayton e Bateman in 1992 (4); this design allows high energy fragmentation.

However, there was no experience in GC coupling, the instrument in this configuration being devoted mainly to the study of peptides and proteins, by using ESI sources. In this respect for this instrument an accessory is available: it is a by-pass for the magnet, in order to use the TOF as the lone mass analyser (mass limit being eight thousand amu)

In principle the TOF as MS2 has the advantage of a high sensitivity, since the mass analysis is not obtained through a scan: the drawback is the necessity of discontinuous sampling and the limitate trasmission of the system collision/acceleration chamber. Moreover, the TOF analyser as MS2 does not allow experiments which differ from the «daughter scan» since it is impossible to focus single or multiple ions with a definite mass.

In order to be useful for our work, the sensitivity should be sufficent to record spectra of analytes present at concentrations of 1 ng/ml of urine, without complicated enrichment of samples.

On the basis of the considerations presented above we started with some experiments which are described in the present paper.

EXPERIMENTAL

Samples preparation: Spiked urine samples were prepared according to the screening IV, by adding at the beginning the appropriate amount of standard dissolved in methanol. Clenbuterol, norandrosterone, 17β- methyl-5β- androst-1- ene-3α,17α-diol, 17α-methyl-5α-androstan-3α,17-β-diol, 3'OH stanozolol were chosen for these experiments, since these

substances are considered present in «small concentration» in urine samples.

Samples prepared for an evaluation of the most suitable resolution were prepared by adding 2 ng/ml (3 ng/ml for 3'OH stanozolol) of metabolites. Samples for MS/MS experiments were prepared by adding different amounts of substances (equivalent 1 or 2 ng/ml), but derivatizing with a reduced amount of reagent mixture (10 or 20 μl) in order to have the possibility to inject into the column an amount of analyte corresponding to 300-500 pg.

GC/MS: Gas-Chromatograph: FISONS GC8000; column HP-5 (5% phenyl-methylpolysiloxane cross-linked) 18 m x 0.22 i.d.. film thickness, 0.33 μm. Injector temp.: 280° C; Injected volume 1μl, split 1: 10 or splitless. Temperature program: 200° C, 2 min, rate 10° C/min, final T = 300° C. Transfer line: 300° C. Constant flow (helium) 0.8 ml/min.

HRMS: PFK was used as reference. Any resolution (5%valley definition) was obtained by tuning with 404.97 or 430.97 ions. Aquisition in SIR voltage mode: the magnet is parked 5 amu below the lowest mass to be monitored and the voltage scanned down from the lowest mass (starting from full accelerating voltage). To ensure that the top of mass peak is monitored at high resolution, a lock mass correction is used to compensate the possible magnet drift. The calibrant is continuously bled into the source: a constant correction can be applied to the calibration. Windows of aquisition were the following:

- time 3.00-4.30 min (lock mass 330.979) - clenbuterol, masses 335.069; 336.059; 337.067;
- time 6.15-8.00 (lock mas 404.976) - norandrosterone, 17β- methyl-5β -androst-1-ene-3α,17α-diol , masses 405.264, 420.288 and 358.269, 448.319, respectively
- time 8.00-9.30 (lock mass 430.973) - 17α-methyl-5α-androstan-3α,17-β-diol, masses 435.311, 450.335
- time 9.30-11.00 (lock mass 442.973) - 17-methyl-testosterone (internal standard), mass 446.304
- time 13.30-15.00 (lock mass 554.966) - 3'OH stanozolol, masses 545.341, 560.365

In those cases in which the span considered was larger than 100 amu (mass to be monitored being in the interval 400-500 amu) the tuning was made not at 8 kV but at an intermediate value of acceleration potential. A tipical case, when mass 315 had to be monitored for norandrosterone.

Tandem MS: GC conditions Injector temp. 260° C; injected volume 1 μl (splitless: purge off time=0.8 min). Initial temp 120° C (1 min) rate: 40° C/min to 220° C, after rate: 10°C/min, final temp= 300° C (5 min); transfer line 300° C.

TOF: High mass: 600; low mass 50; minimum resolution 1500 (higher values can be obtained by tuning MS1); accelerating voltage 8 kV. Magnet control: field. Collision gas: Argon, collision energy: 800 eV. Experiments have been performed with samples containing norandrosterone, 17β- methyl-5β- androst-1-ene-3α,17α-diol, and stanozolol.

RESULTS AND DISCUSSION

As already observed (5), it is not possible to anticipate a value of resolution appropriate to separate and distinguish each analyte from the background. On considering that the background - made by interfering substances present in the urine - is often similar in all samples, a reasonable possibility is to run several analyses at different resolutions and to observe the results Therefore we analyzed same samples (spiked urines) at four different resolutions: 1000, 3000, 5000, 10000.

The most significant results are presented in figures 1-5: clenbuterol ions do not merge from the traces at 1000 res., whereas signals are apparent at 3000 res. (fig 1) Traces are clean at 5000 and at 10000, but in the latter case the signal is approx. one tenth of that at 3000.

Norandrosterone is perfecly detectable at 1000 res: in the window the trace is clean. (fig 3). Stanozolol can be observed at any resolution: but the trace is clean only at 10000 res. The same behaviour is presented by the two other analytes.

On the basis of these experiments, we decided to use for routine work - first confirmation of anabolics, see later - a standard resolution of 5000 which seems to be a reasonable compromise between selectivity and sensitivity. In our opinion, the advantage in the use of GC/HRMS does not lie simply in the resolution - which lowers the «chemical noise» - but also in an improved stability of electronics, which reduces the overall noise, which is the sum of chemical and electrical noise.

To understand what the practical possibilities of the instrument were, we started to repeat samples processed in the usual screenings with quadrupolar GC/MS. When we had doubtful «positivities», on repeating the same analyses on HR instrument we always obtained a clear-cut answer. In other words, doubtful cases became positive or negative (fig 6). In this way we found the best employment of HRMS for our laboratory: to have a «first confirmation» about the presence of a prohibited substance.

When we start with tandem MS experiments, we had some difficulties, given by the old software (OPUS 3.1) which did not allow a change in mass during the chromatographic run. In this way, only a single substance could be detected. This difficulty was overcome with the

updated software, i.e. when the OPUS 5.1 was installed.

Trial experiments showed that an amount of 500 pg of a substance showing in the spectrum a reasonably intense ion, for instance, norandrosterone, injected into the column give - by selecting the ion 420 - a perfectly readable collision spectrum. Argon as colliding gas is not the best, probably Xenon could give better results (6), but owing to the difficulties (and cost) related to the availability of Xenon, we used Argon: actually, fragmentation is scarce, and it is necessary to amplify the region of fragments, in order to have significant patterns.

Collision spectra of standard substances (10 ng injected into the column) compared with spectra obtained with 500 pg injected are reported in fig.7. Reproducibility is excellent.

The last example is striking: a sample (D8, lab's code) was found to contain stanazolol metabolites. In a single run we confirmed the presence of three metabolites, by comparing collision spectra with those of a standard urine, containing stanazolol metabolites. Spectra are reported in fig 8.

Now we use the Tandem MS routinely as a tool to confirm analytes. The procedure is very fast and even the same sample of screening can be suitable for these measurements.

NOTES

1) Horning S. and Schanzer W. - Steroid screening Using GC/HRMS - Proc. of 14th Cologne Workshop on Dope Analysis - page 261, Sport und Buch Strauss Edition Sport - Koln 1995

2) European Community Commission - 14th of July 1987 - Gazz. Uff. Comunita' Europee - 11 / 8 / 1987.

3) See for a short review: Burlingame A.L., Boyd R. K., Gaskell S.J. «Mass Spectrometry» Anal. Chem. 68, 608R-609R (1996)

4) Clayton E., Bateman R.H. - Rapid Comm. in Mass Spectrom. 6, 719 (1992)

5) Thieme D. et al. - Application of High-Resolution-MS and Tandem MS to the identification of anabolic agents - Proc. of 13th Cologne Workshop on Dope Analysis - page 285, Sport und Buch Strauss Edition Sport - Koln 1996

6) Clayton E., Bateman R.H. loc. cit.

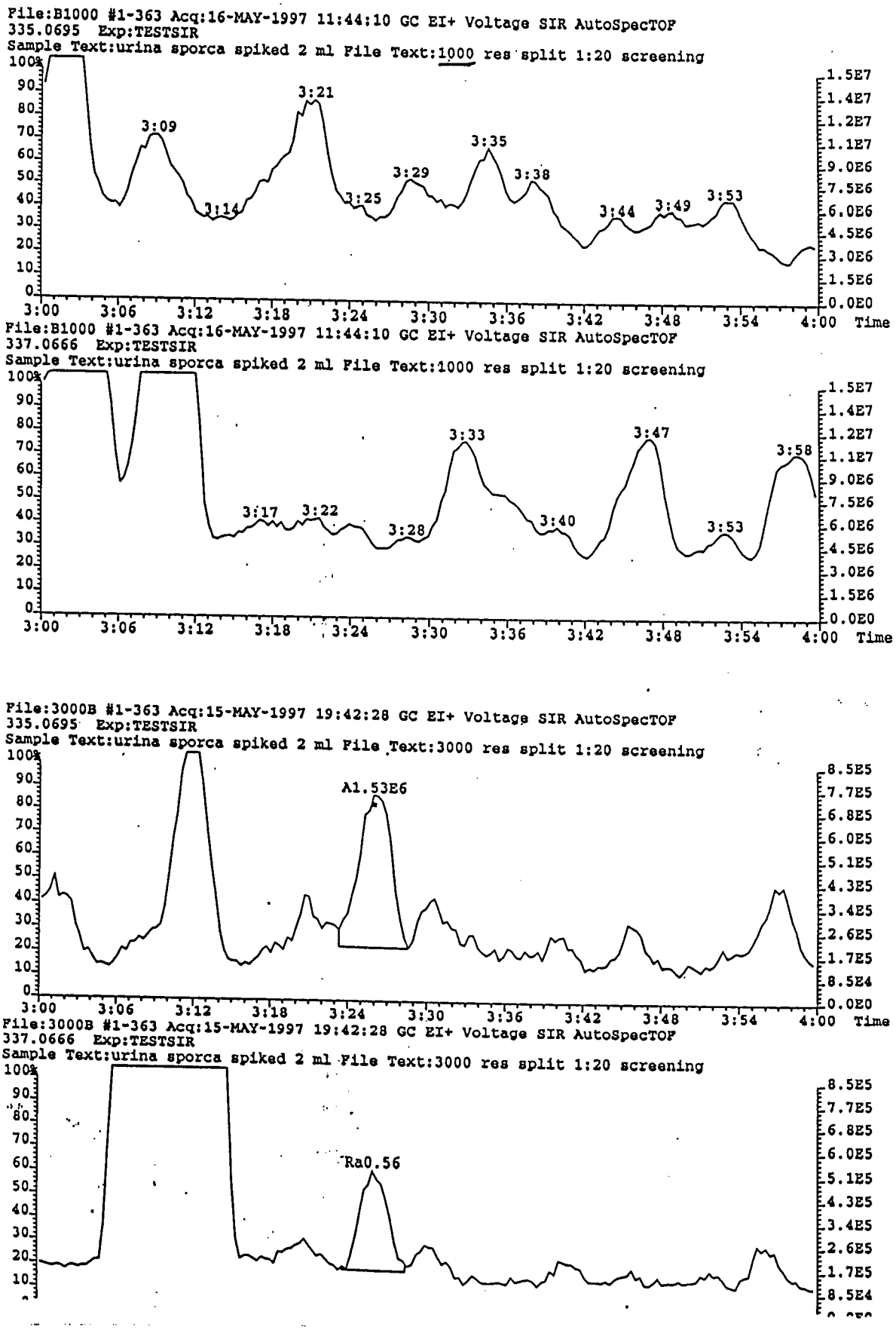

Fig. 1 spiked urine (2 ng/ml): clenbuterol: Rt 3.26 min. top: 1000 res, ions 335-337; bottom: 3000 res same ions.

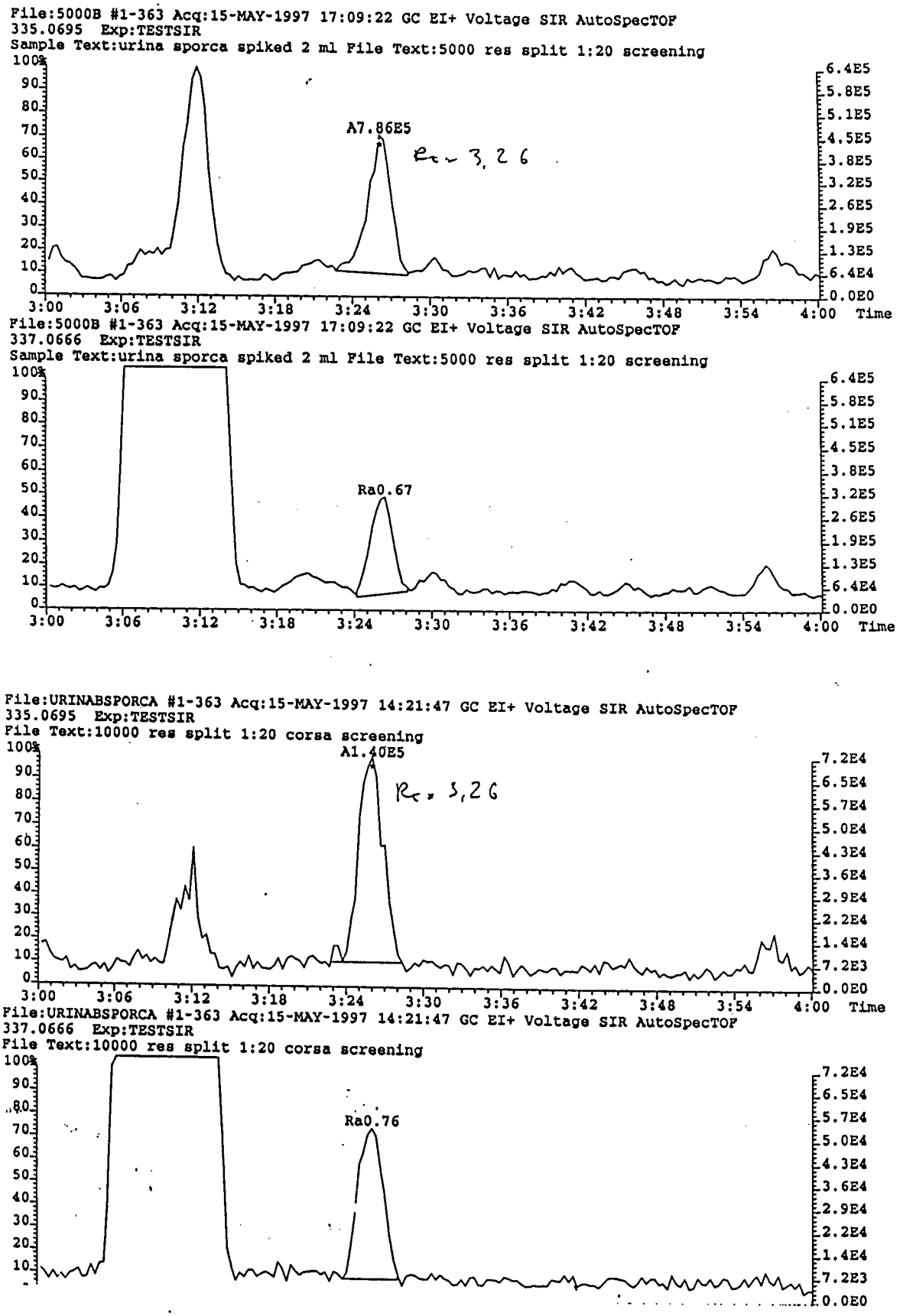

Fig. 2 spiked urine (2 ng/ml): clenbuterol: Rt 3.26 min. top: 5000 res, ions 335-337; bottom: 10000 res same ions.

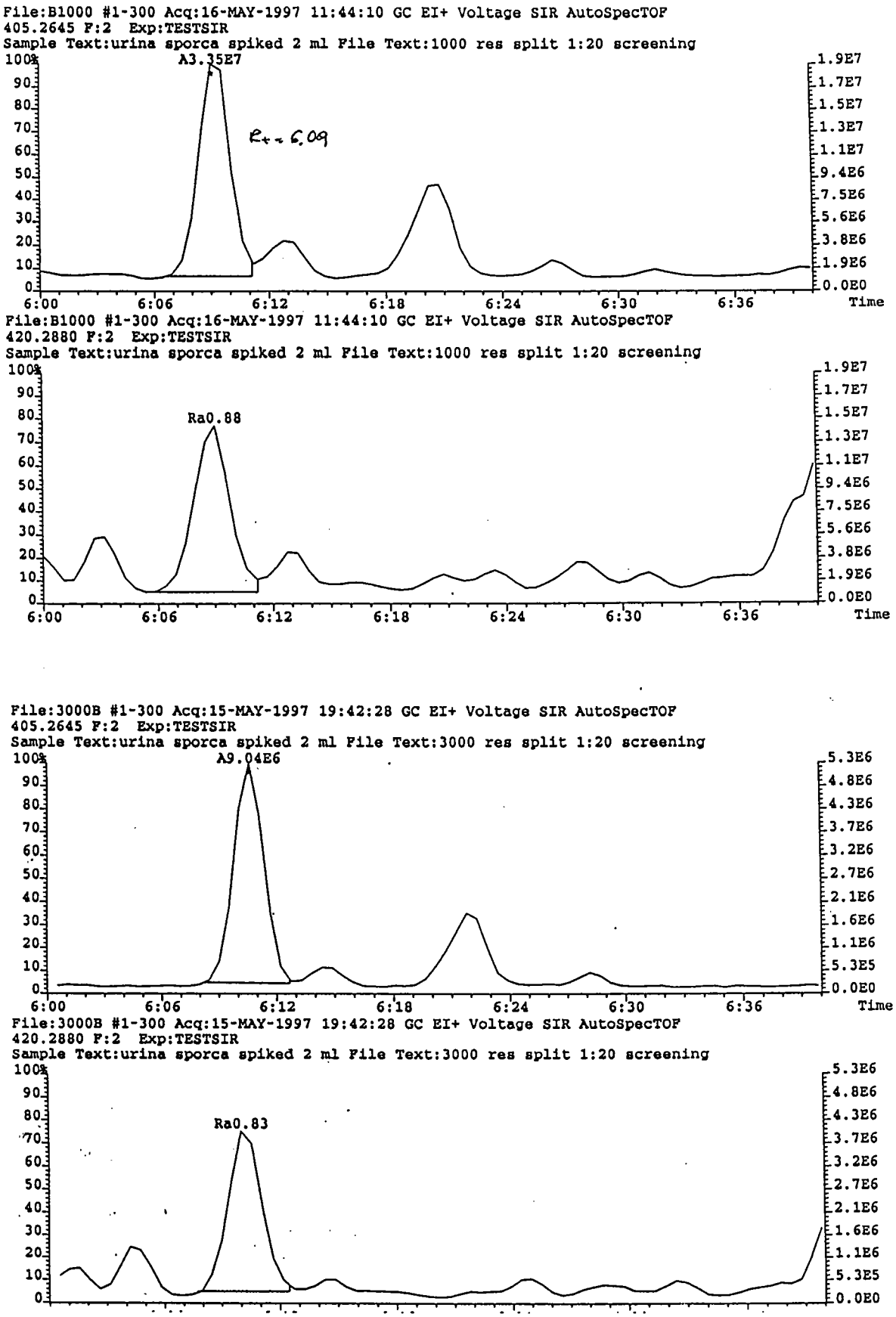

Fig 3 spiked urine (2ng/ml): norandrosterone: Rt 6.09 top 1000 res, ions 405-420, bottom: 3000 res same ions

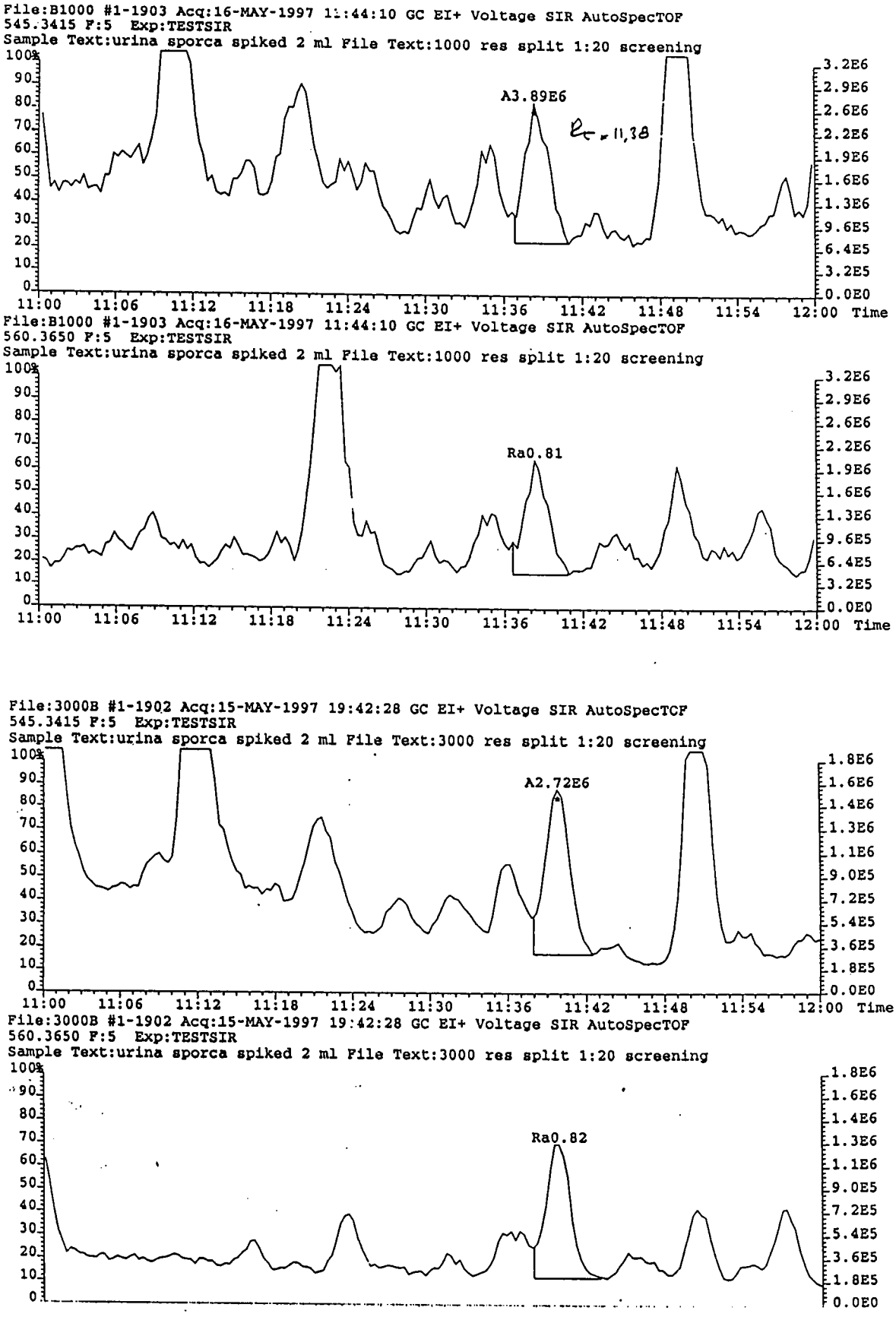

Fig 4 spiked urine (3 ng/ml): stanozolol: Rt 11.38 top 1000 res, ions 545-560; bottom 3000 res same ions

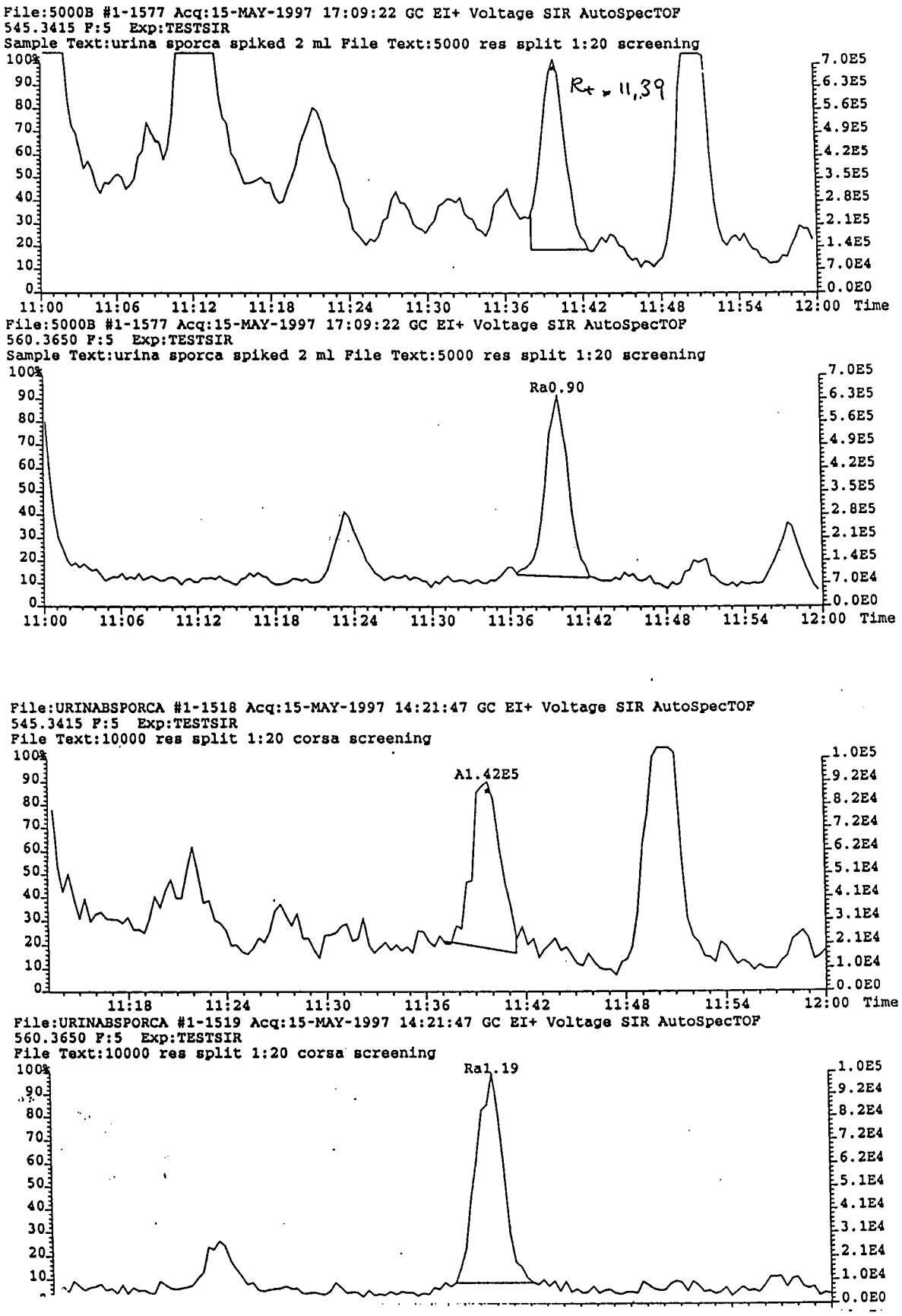

Fig 5 spiked urine (3 ng/ml): stanozolol: Rt 11.38 top 5000 res, ions 545-560; bottom 10000 res same ions

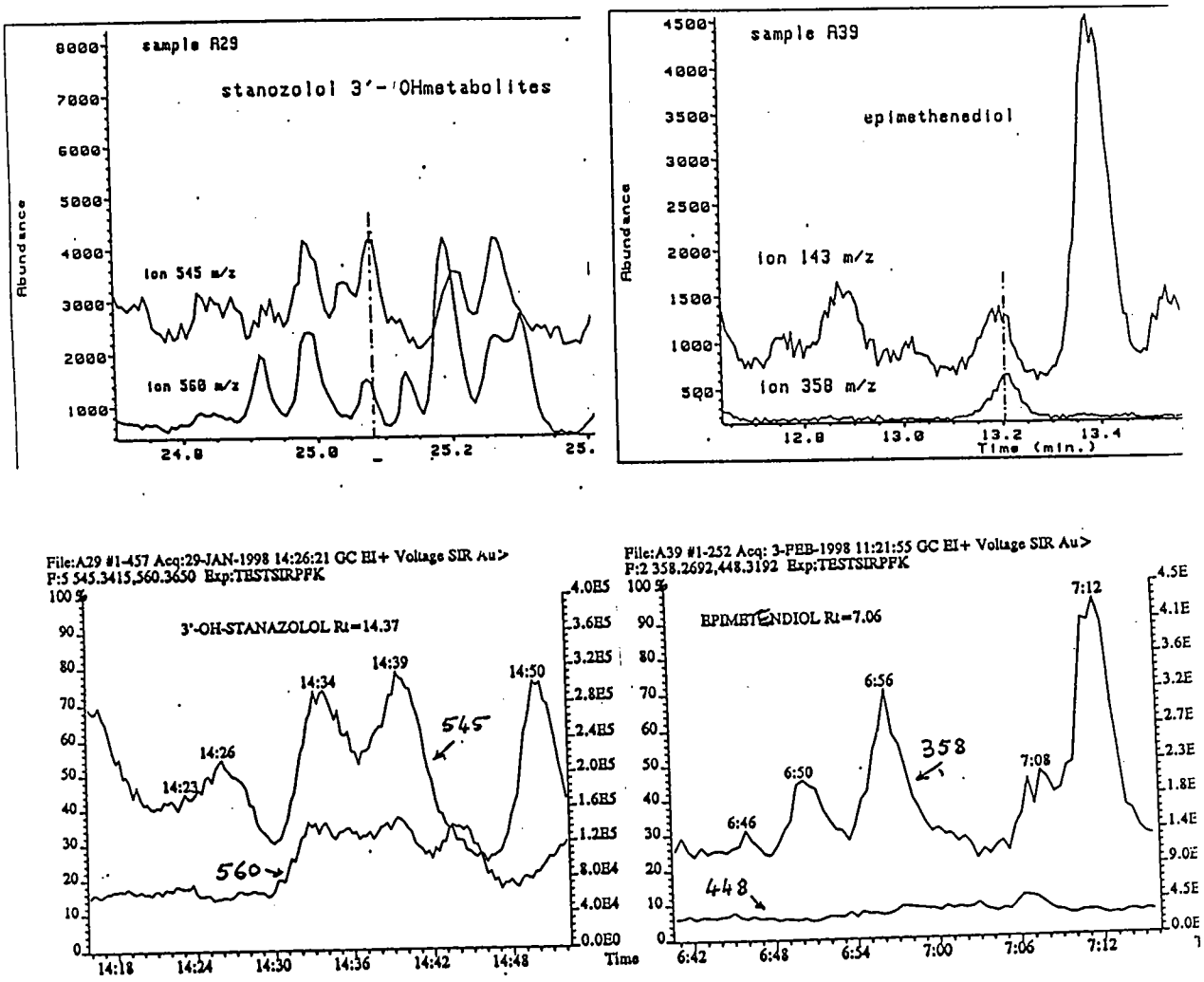

Fig.6 comparison between traces obtained with quadrupoles and traces obtained with AUTOSPEC at 5000 res; left: an example regarding possible stanozolol; right: an example regarding possible epimethene diol

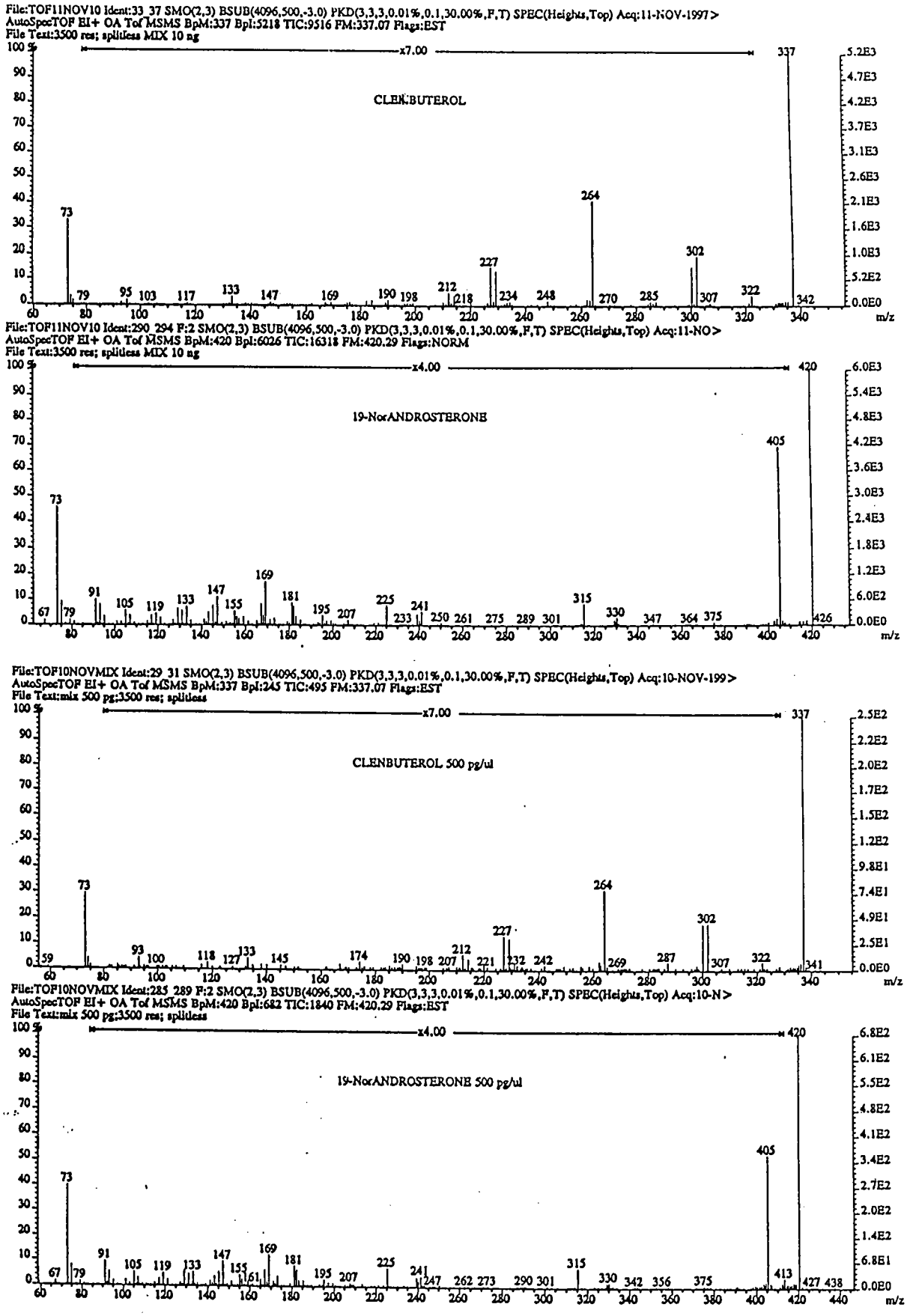

Fig.7 CID spectra of Clenbuterol and Norandrosterone: reproducibility with high and low amount.

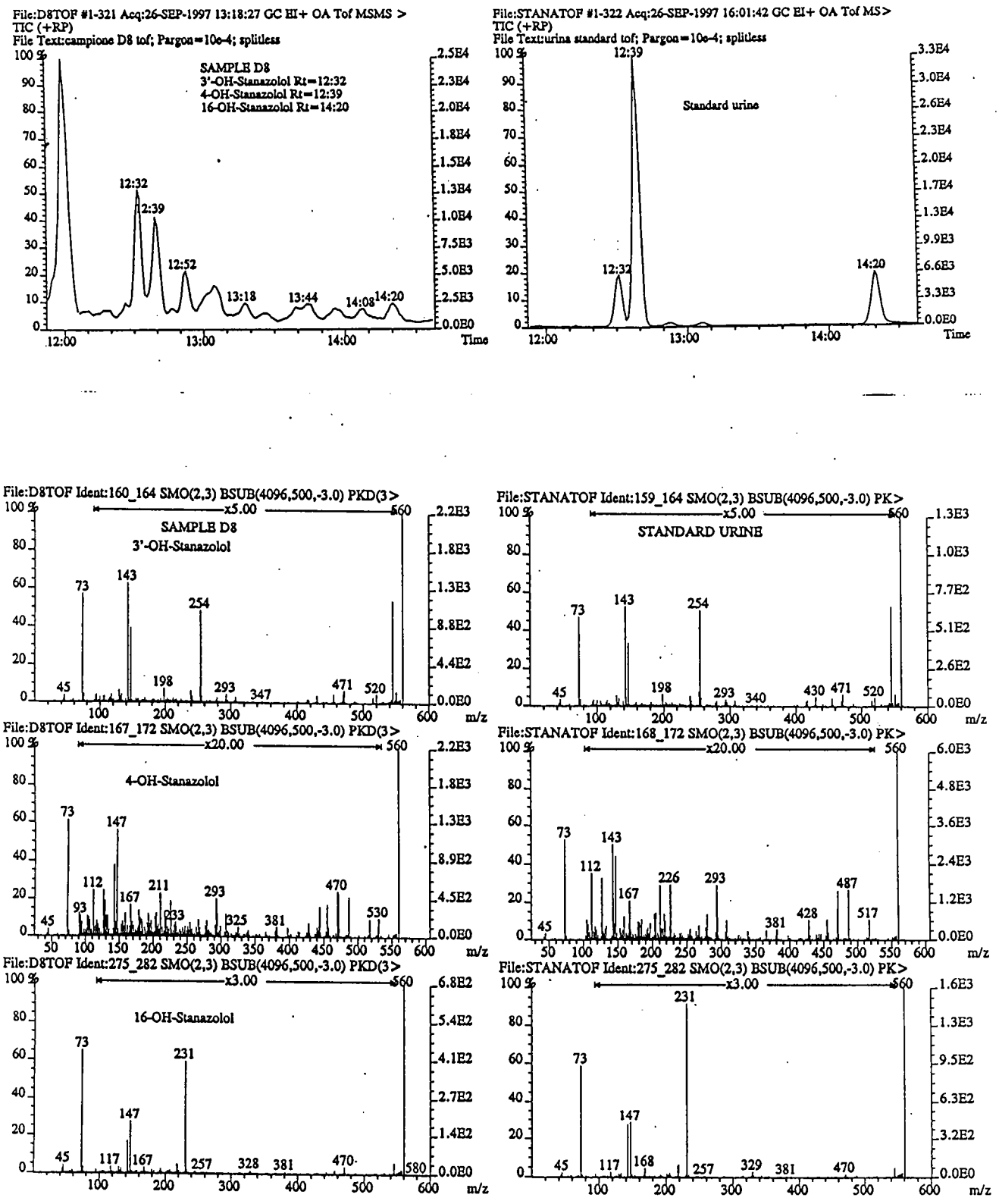

Fig 8 CID spectra of three metabolites of stanozolol. Top: traces of TOF detector. Left: sample D8 (lab's code). Right: standard urine.