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GC/HRMS-ION TRAP Screening and Confirmation of Anabolic Steroids

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Introduction

Gas chromatography coupled with high resolution mass spectrometry (GC/HRMS) is the method of choice for trace level analysis of anabolic steroids due to the high degree of specificity afforded by high mass-resolution and the high sensitivity of the sector field mass spectrometer. With GC/HRMS there is a 10- to 100-fold lowering of the detection limit for anabolic steroids extracted from urine, as compared to quadrupole, quadrupole MS/MS and ion trap mass spectrometers. Since employment in 1993 of HRMS for routine doping control in the Cologne laboratory, there has been a significant increase in the number of samples found positive for anabolic steroids [1]. Based on recommendation of the IOC, HRMS is now used in many of the accredited laboratories [2].

Improved analytical performance is realized by addition of a second stage of mass spectrometry, viz. tandem mass spectrometry. The Finnigan MAT 95 XL-Trap tandem mass spectrometer allows for high selectivity through high-resolution precursor ion selection, high specificity through MS/MS and MS^n, and ultimate MS/MS sensitivity due to the well-matched coupling of the sector field instrument to the ion trap mass spectrometer. The tandem mass spectrometer significantly reduces chemical noise, allowing for lower detection limits. It also offers a highly useful means to reanalyze suspicious samples, saving time and reducing the overall workload. Most importantly, the tandem mass spectrometer often provides scientific evidence that can be used as proof of identity of forbidden substances. Finally, the MAT 95 XL-Trap is ideally suited for routine steroid screening as it is under full data system control and the entire analysis, from sample injection to data printout, is automated.

The Finnigan MAT 95 XL-Trap mass spectrometer is depicted in Figure 1. The sector field mass spectrometer (MS1) is based on a reverse Nier-Johnson (BE) geometry. The GC (not shown) is connected to the EI ion source. For normal operation, 3,000 to 5,000 resolution is employed and the instrument is run in the electric scan mode [3]. An intermediate detector, located between the sector field and ion trap analyzer, is used for conventional GC/HRMS analyses. For MS/MS and MS^n analyses, precursor ions selected in MS1 are passed to the Finnigan LCQ ion trap mass spectrometer (MS2).

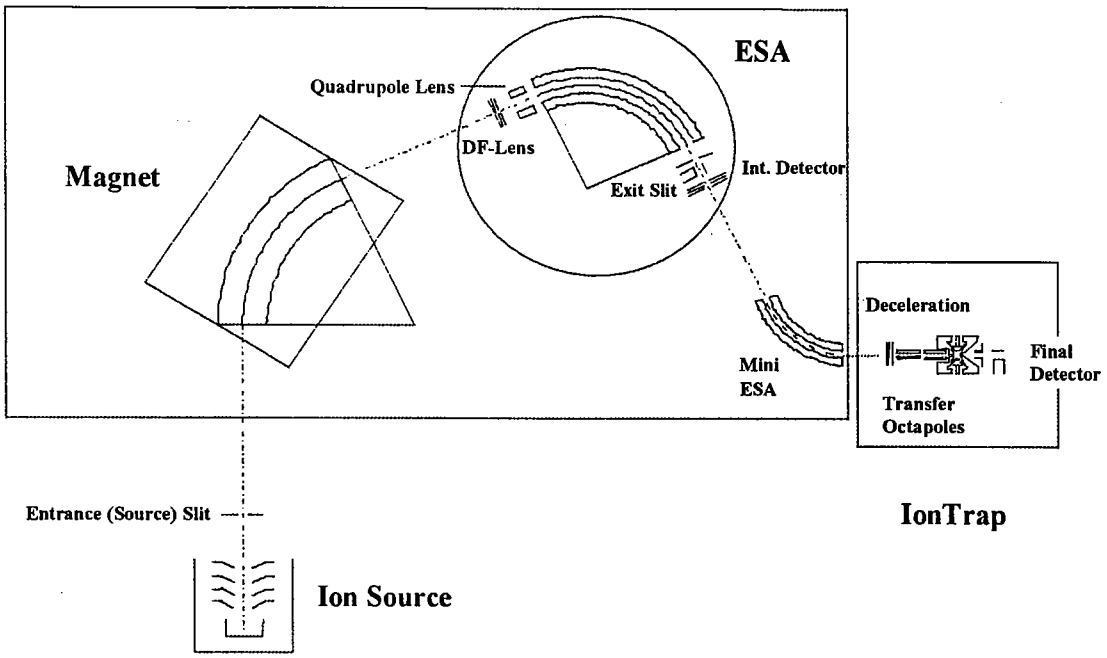

Figure 1. Finnigan MAT 95 XL-Trap mass spectrometer.

GC/HRMS-Ion Trap analyses were performed on anabolic steroids (and their metabolites) extracted from urine. The urine (2 mL) was spiked with clenbuterol (200 pg/mL), 3'- and 4β-hydroxy-stanozolol (400 pg/mL, each), 18-nor-17,17-dimethyl-5β-1,13-dien-3α-ol (400 pg/mL) and 17β-methyl-5β-androst-1-ene-3α,17α-diol (200 pg/mL). The urine was hydrolyzed (β-glucuronidase in pH 7 phosphate buffer), the steroids extracted (pH 9.6 in K_2CO_3-$KHCO_3$ buffer) in tert.-butylmethyl ether, and after drying, derivatized with 100 μL MSTFA-NH_4I-ethanethiol (100:0.2:0.6 v/w/v). GC analyses (2 μL aliquot) were performed in split (1:20) mode with a 20 m length fused-silica capillary column (J&W DB1 column, 0.25 mm i.d., 0.1 μm film thickness) with He carrier gas at 0.7 mL/min constant flow. The eluting

species were ionized (EI at 65 eV) and ions of interest were selected for MS/MS analysis via electric field mass locking (perfluorophenanthrene reference) at 3,000 to 5,000 resolution. The precursor ion mass and other ion trap parameters (ion injection time, scan range, collision energy, etc.) were controlled with the LCQ ion trap data system.

Discussion

Figure 1 depicts GC/HRMS and GC/HRMS-Ion Trap (MS/MS) results for analysis of the long-term excreted metandienone metabolite, 18-nor-17,17-dimethyl-5β-1,13-dien-3α-ol. The concentration of the metabolite in urine was 400 pg/mL (in the derivatized sample 8 pg/μL). Insert (a) shows the selected ion trace for the base peak ion (m/z 253.1956) which was recorded in MS1 via electric field jumping (MID). In the mass spectrum, recorded over the

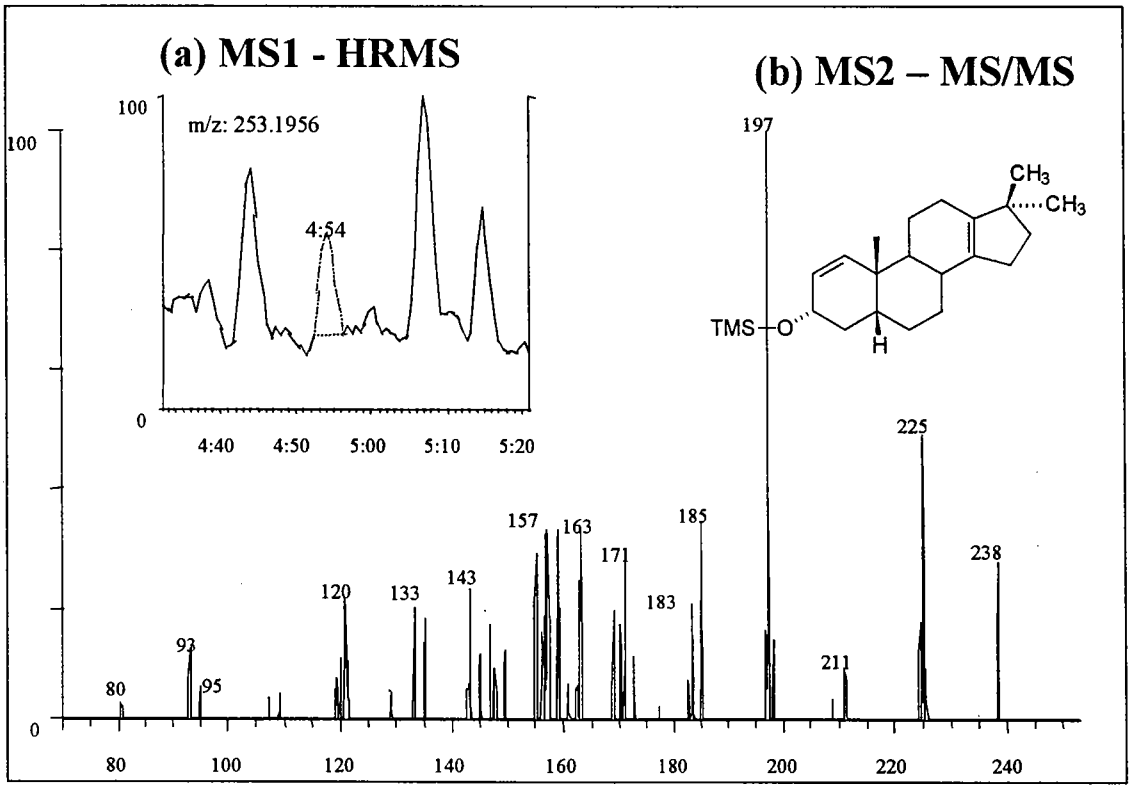

Figure 2. Insert (a), GC/HRMS analysis of metandienone metabolite, 18-nor-17,17-dimethyl-5β-1,13-dien-3α-ol, extracted from urine (2 mL). 2 μL aliquot analyzed in splitless mode (1:20 split). Peak shown corresponds to 0.8 pg. The base peak EI fragment ion, m/z 253.1956, was recorded using electric field jumping (MID) at 3,500 resolution. Insert (b), GC/HRMS-Ion Trap (MS/MS) analysis of the same sample (reinjected). MS/MS was performed on the ion 253.1956 with AGC off (500 ms injection time) using 1 μscan, and 30% collision energy.

range m/z 60 – 400 (not shown), the only ions observed are those from stearic acid-TMS which coelutes with the metandienone metabolite. Insert (b) shows the MS/MS fragment ion mass spectrum for the same sample (reinjected) recorded in the ion trap. MS/MS was performed on the base peak EI fragment ion m/z 253.1956. The precursor ion was selected with MS1 at 3,500 resolution using electric field mass locking. In the ion trap (MS2) the ion was selected with AGC off (fixed 500 ms injection time) at 30% collision energy. The MS/MS spectrum compares well with that recorded on the Finnigan GCQ mass spectrometer (see contribution by Schänzer and coworkers in this Proceeding).

The stanozolol metabolites, depicted in Figure 3, are another example of long-term excreted metabolites that are usually found in very low concentration. Normally these species

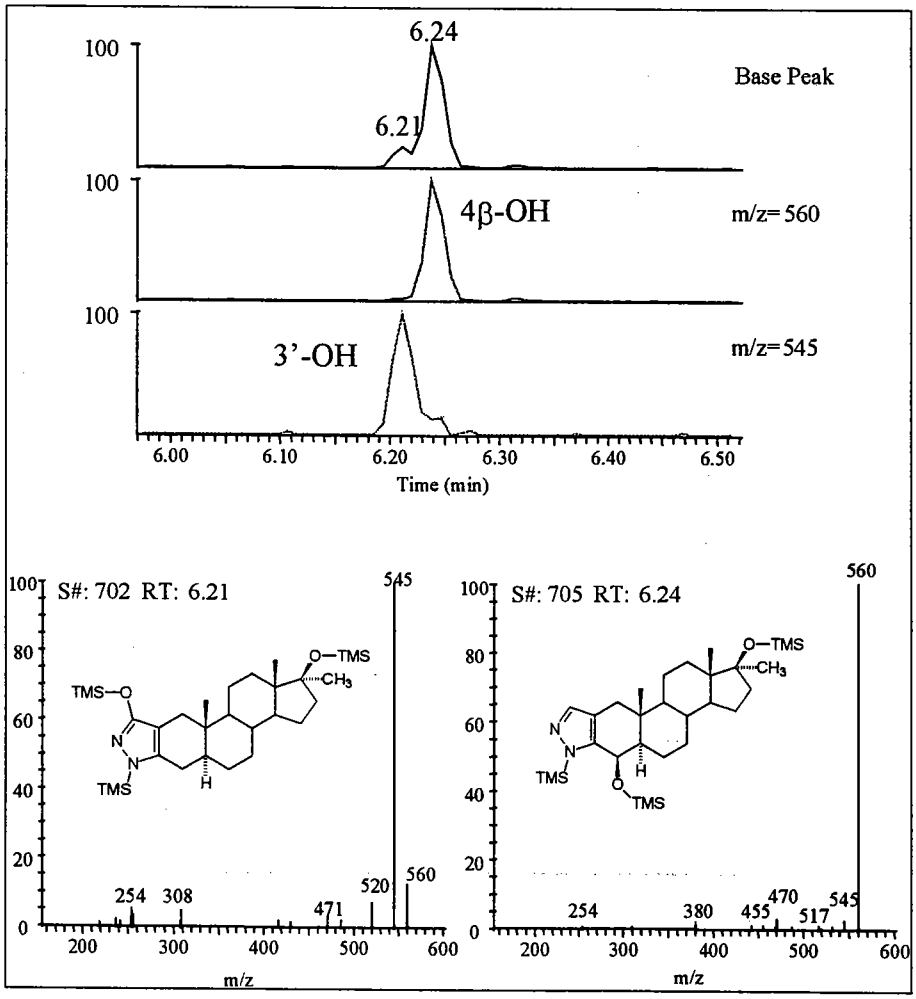

Figure 3. GC/HRMS-Ion Trap analysis of 3'-OH- and 4β-OH-stanozolol. MS/MS spectra recorded for 0.8 pg sample in the absence of urine matrix. The molecular ion, m/z 560.3650, was selected at 4,000 resolution. MS/MS performed as in Fig. 2 using 20% collision energy.

are effectively monitored by GC/HRMS. If a suspicious sample is found it is subjected to an extensive cleanup (using, for example, immunoaffinity chromatography) and reanalyzed. This takes considerable time and requires specialized treatment by skilled workers. Alternatively, the suspicious sample could simply be reanalyzed via GC/HRMS/MS. As in the case of the metandienone metabolite, a precursor ion is selected in MS1 at high resolution and passed to MS2 for MS/MS analysis. For the stanozolol metabolites, the molecular ion, m/z 560.3650, is selected at 4,000 resolution and subjected to collisional activation in the ion trap. At low collision energy 4β-OH-stanozolol is stable and very little dissociation of the molecular ion

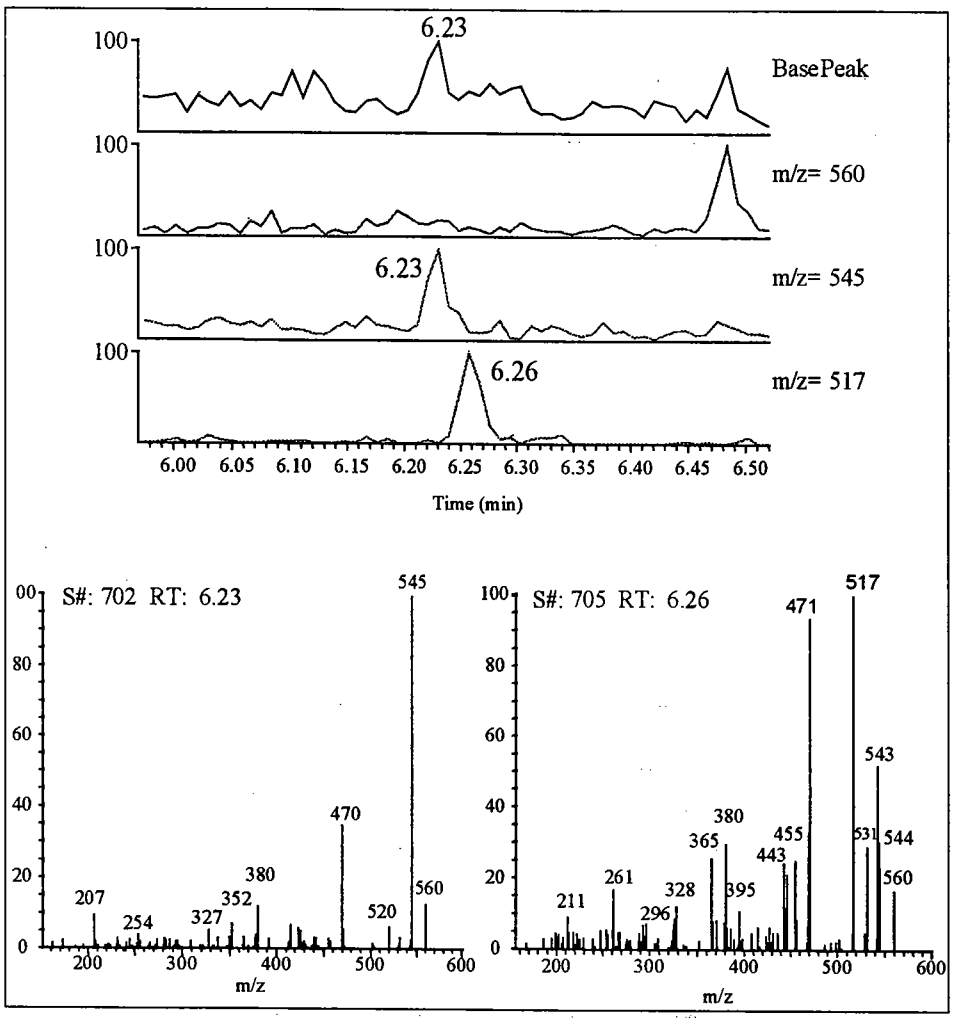

Figure 4. GC/HRMS-Ion Trap analysis of 3'-OH- (left side) and 4β-OH-stanozolol (right side). MS/MS spectra recorded for 0.8 pg sample in urine matrix (400 pg/mL concentration). The molecular ion, m/z 560.3650, was selected at 4,000 resolution. MS/MS performed as in Fig. 3 using 30% collision energy.

occurs. The 3'-OH species, however, readily dissociates to yield the $M^{+\cdot}$–15 ion (m/z 545), and to a much lesser extent ions at m/z 520, 471 and 254. As the collision energy is raised, the more stable species, 4β-OH-stanozolol, dissociates, yielding many interesting fragment and rearrangement ions, as depicted in Figure 4. Abundant and characteristic fragment ions include those at m/z 543 ($M^{+\cdot}$– 17), 531 ($M^{+\cdot}$– 29), 517 ($M^{+\cdot}$– 43) and 471 ($M^{+\cdot}$– 89). The MS/MS fragment ion chromatograms (shown in Figure 4) are very useful markers for the presence of stanozol in urine, particularly the m/z 517 ion trace, which is highly characteristic for 4β-OH-stanozolol. Using MS/MS ion chromatograms it is possible to determine the presence of stanozol metabolites in urine at levels below 100 pg/mL.

With the LCQ ion trap it is possible to perform higher order MS experiments, e.g., MS^3. Sequential MS/MS/MS analyses can yield highly specific ion fragments that allow for improved identity and confirmation of discrete substances. Figure 5 shows the sequential fragmentation spectra (560→545→•) of 3'-OH- and 4β-OH-stanozolol. The MS^3 experiment is a straight-forward extension of that described in Figure 4. In MS1 the precursor ion (the molecular ion, m/z 560.3650) is selected at 4,000 resolution and passed to the ion trap where it is subjected to collisional activation. As illustrated in Figure 4, there is substantial yield of the $M^{+\cdot}$–15 fragment ion (m/z 545) at 30% collision energy. The m/z 545 ion is mass-isolated then subjected to further collisional activation to yield the sequential fragment ions depicted in Figure 5. For 3'-OH-stanozolol the predominant ion formed in the sequential MS experiment is m/z 455, corresponding to a loss of 90 ($C_3H_{10}OSi$) from the m/z 545 ion. The much more stable 4β-OH-stanozolol species undergoes loss of 16 from the m/z 545 ion to give the ion at m/z 529. The nature of this ion at m/z 529 is not known.

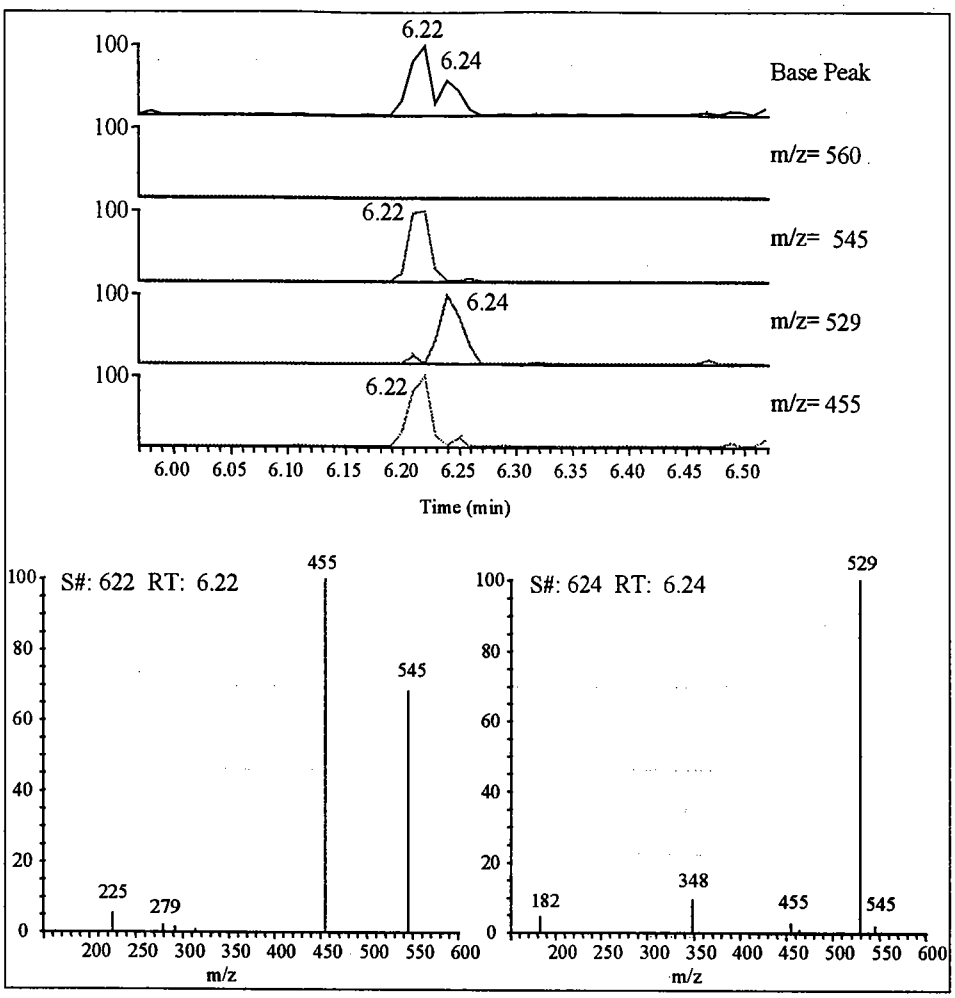

Figure 5. GC/HRMS-Ion Trap MS^3 analysis of 3'-OH- (left side) and 4β-OH-stanozolol (right side). Sequential ion (560→545→•) spectra recorded for 0.8 pg sample in urine matrix (400 pg/mL concentration). The molecular ion, m/z 560.3650, was selected at 4,000 resolution and subjected to collision at 30% collision energy (see Fig. 4). The fragment ion at m/z 545 was then isolated and subjected to a second collision at 20% collision energy to generate sequential fragment ions.

Figure 6 depicts MS^3 spectra of 3'-OH- and 4β-OH-stanozolol extracted from urine (upper panel) and from standards (lower panel). There is good agreement, however, the standard spectra show more fragment ion products. Note that in the standard spectra there appears to be some crossover due to slight GC coelution of the stanozolol metabolites (they were coinjected). Special care must be taken when evaluating MS/MS and MS^3 spectra, as the abundance of fragment ions can be influenced by several factors, including the number of stored ions (space charging), the background matrix (ion-molecule reactions), reaction times, coeluting isobaric and isomeric species, etc. For 4β-OH-stanozolol other sequential fragment sequences could be recorded, e.g., (560→517→•). It would even be possible to switch

between two different sequential MS^3 experiments (a) 560→545→• and (b) 560→517→• over the time course of the GC analysis. Likewise, even higher order (MS^4) sequential fragmentation processes, (560→545→529→•) could be monitored.

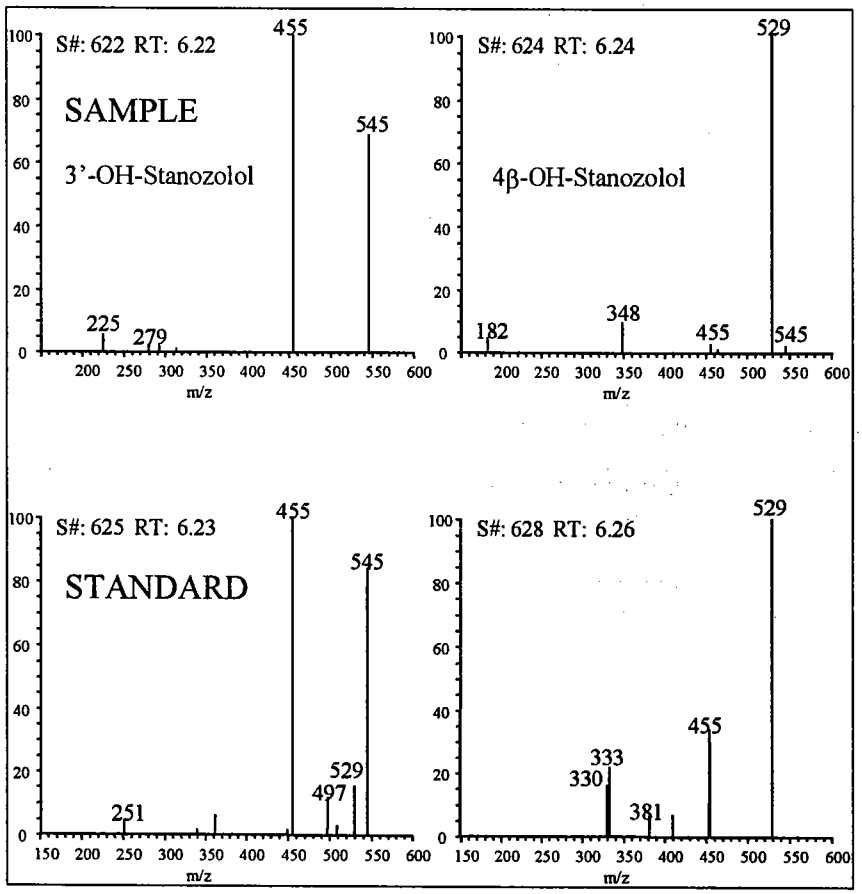

Figure 6. Comparison of MS^3 (560→545→•) spectra of 3'-OH- and 4β-OH-stanozolol in urine matrix at 400 pg/mL (sample) and as standards in the absence of matrix (0.8 pg injected). The sample spectra are the same as those in Fig. 5. For the sequential MS^3 experiment the molecular ion, m/z 560.3650, was selected at 4,000 resolution. Collision energies were 30% and 20% for MS^2 and MS^3, respectively.

Comprehensive GC/HRMS/MS anabolic steroid screening analyses can be performed using the MAT 95 XL-Trap mass spectrometer. In the screening analysis selected precursor ions are sent to the ion trap at predetermined times. The MS/MS data acquired can be plotted as fragment ion chromatograms or as fragment ion spectra, as illustrated in Figure 7. Here screening data are shown for clenbuterol and two metandienone metabolites. The MS/MS spectra were obtained for the most dominant EI fragment ion of the steroid TMS derivative.

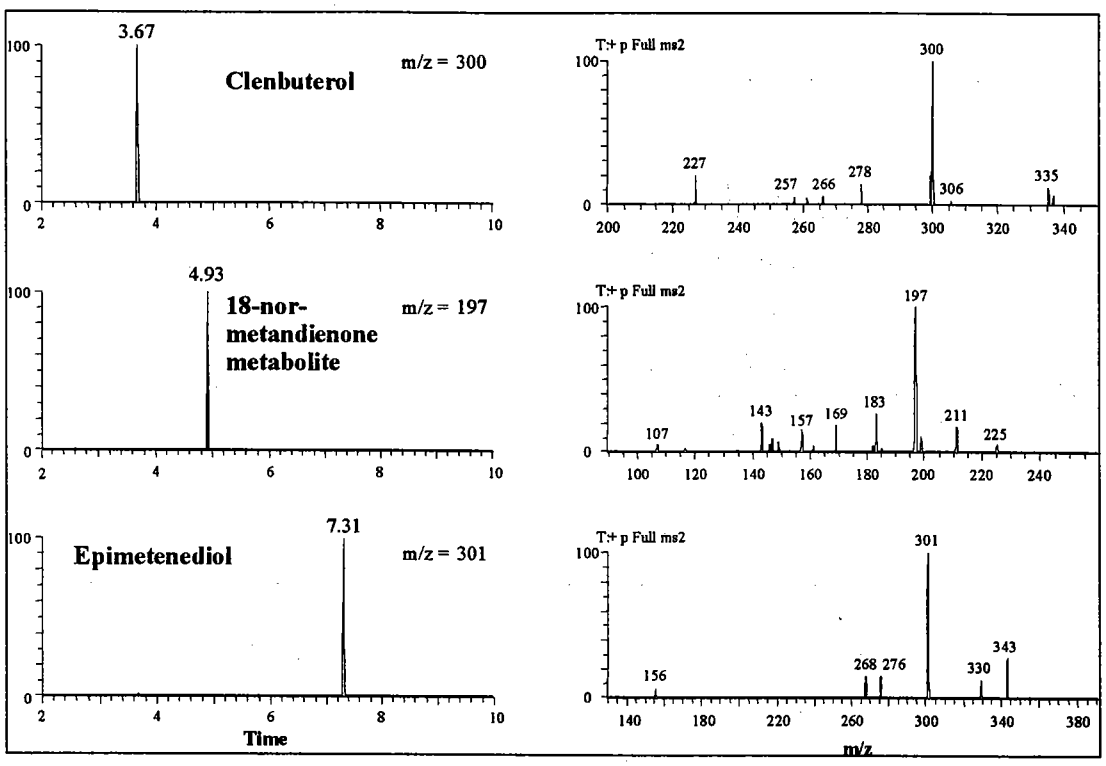

Figure 7: GC/HRMS-Ion Trap MS/MS screening analysis of anabolic steroids extracted from urine. MS/MS spectra obtained from most dominant EI fragment ion of the steroid TMS derivative. MS-1 resolution 3,000. MS-2 ACG off, with a fixed ionization (injection) time of 400 ms. 25% collision energy and 1 μscan.

Conclusion

The GC/HRMS-Ion Trap mass spectrometer is an ideal complement to the GC/HRMS instrument. It offers improved detection limits and additional proof of identity (MS/MS and MS^n) for trace levels of anabolic steroids extracted from urine. Lower detection and confirmation limits can be established for nearly all anabolic steroids (and their metabolites). For clenbuterol and metabolites of metandienone and stanozolol urinary concentrations below 500 pg/mL can be determined. High resolution MS combined with the high performance ion trap increases the specificity of the analysis. Suspicious findings from HRMS analyses can be reanalyzed with the HRMS-Trap saving time and reducing the overall workload. The HRMS/MS analysis allows for increased retrospectivity and helps assure that anabolic steroids are not misused. Most effectively, such analyses could be used in conjunction with out of competition testing. Finally, it is possible to devise comprehensive screening procedures for

routine analysis of anabolic steroids using automated control of the sector field mass spectrometer and the ion trap.

References

1. Schänzer W., Delahaut P., Geyer H., Machnik M. and Horning S., Long-term detection and identification of metandienone and stanozolol abuse in athletes by gas chromatography-high resolution mass spectrometry *J. Chromatogr. B* **687**, 93 (1996).

2. Horning S., Schänzer W. and B. Sample, HRMS Analyses Performed at the 1996 Summer Olympic Games, in W. Schänzer (Editor), *Recent Advances in Doping Analysis (5) - Proceedings of the 15th Cologne Workshop on Dope Analysis 1997*, Sport und Buch Strauß, Köln 1998, p. 329.

3. Horning S. and Schänzer W., Steroid screening using GC/HRMS, in W. Schänzer (Editor), *Recent Advances in Doping Analysis (4) - Proceedings of the 14th Cologne Workshop on Dope Analysis 1996*, Sport und Buch Strauß, Köln 1997, p. 261.