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Comparison of sensitivity between gas chromatography–low-resolution mass spectrometry and gas chromatography–high-resolution mass spectrometry for determining metandienone metabolites in urine

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Abstract

In doping control laboratories the misuse of anabolic androgenic steroids is commonly investigated in urine by gas chromatography–low-resolution mass spectrometry with selected ion monitoring (GC–LRMS–SIM). By using high-resolution mass spectrometry (HRMS) detection sensitivity is improved due to reduction of biological background. In our study HRMS and LRMS methods were compared to each other. Two different sets were measured both with HRMS and LRMS. In the first set metandienone (I) metabolites 17α-methyl-5β-androstan-3α,17β-diol (II), 17-epimetandienone (III), 17β-methyl-5β-androst-1-ene-3α,17α-diol (IV) and 6β-hydroxymetandienone (V) were spiked in urine extract prepared by solid-phase extraction, hydrolysis with β-glucuronidase from *Escherichia coli* and liquid–liquid extraction. In the second set the metabolites were first spiked in blank urine samples of four male persons before pretreatment. Concentration range of the spiked metabolites was 0.1–10 ng/ml in both sets. With HRMS (resolution of 5000) detection limits were 2–10 times lower than with LRMS. However, also with the HRMS method the biological background hampered detection and compounds from matrix were coeluted with some metabolites. For this reason the S/N values of the metabolites spiked had to be first compared to S/N values of coeluted matrix compounds to get any idea of detection limits. At trace concentrations selective isolation procedures should be implemented in order to confirm a positive result. The results suggest that metandienone misuse can be detected by HRMS for a prolonged period after stopping the intake of metandienone.

Keywords: Metandienone; Anabolic steroids

1. Introduction

The long term misuse of anabolic androgenic steroids in human sports is still a challenge for analytical laboratories engaged in urine drug testing. In doping control, the anabolic steroids are commonly detected in urine by gas chromatography–low-resolution mass spectrometry (GC–LRMS) after a sample preparation procedure discussed in literature [1,2]. To urine are added a buffer and an internal

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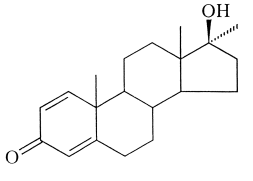
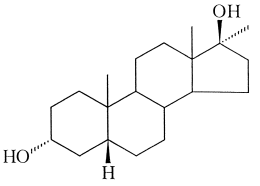
standard and unconjugated steroids are extracted with diethyl ether. Diethyl ether is evaporated to dryness and steroids are derivatized to yield trimethylsilyl (TMS) ether and enol ether derivatives [3] for GC–MS analysis with electron impact (EI) ionization. If both conjugated and unconjugated steroids need to be determined an adsorption resin and β-glucoronidase enzyme are needed for sample preparation.

Improvement of sensitivity and specificity is achieved by selected ion monitoring (SIM) technique, and consequently GC–LRMS–SIM method is commonly used in doping control laboratories for analysing anabolic steroids. However, sometimes biological background can cause detection problems, especially when monitoring trace amounts of steroids. The use of high-resolution mass spectrometry (HRMS) has proved to improve sensitivity and speficity compared to LRMS in the analysis of anabolic steroids. Therefore also the International Olympic Committee (IOC) has decided in 1997 that the accredited doping laboratories have to have the capability for confirmation of positive samples with either HRMS or tandem mass spectrometry. For example in Cologne laboratory in 1995 Schänzer et al. [4] identified 75 of the 116 positive cases via GC–HRMS screening. Upon screening identification, confirmation of a positive case was done by reanalysis of the urine, where a HPLC isolation procedure was used.

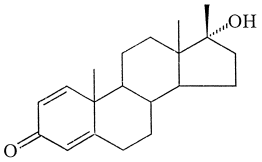
Metandienone (17α-methyl-androsta-1,4-dien-17β-ol-3-one) (I) is one of the most commonly abused anabolic steroids in sports. It is strongly metabolized in human body and most of the metabolites are excreted in urine either in free or conjugated forms [1]. In doping control, 17α-methyl-5β-androstan-3α,17β-diol (II), 17-epimetandienone (III), 17β-methyl-5β-androst-1-ene-3α,17α-diol (IV) and 6β-hydroxymetandienone (V) (Fig. 1) are most often used as target metabolites for metandienone. The aim of this study was to compare the sensitivity and specificity of the GC–LRMS technique to the GC–



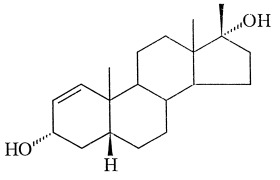
Metandienone (I)



17α-methyl-5β-androstan-3α,17β-diol (II)



17-Epimetandienone (III)



17β-methyl-5β-androst-1-en-3α,17α-diol (IV)

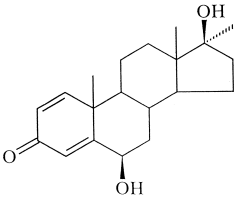


Fig. 1. Metandienone (I) and the target compounds analysed by GC–MS–SIM method.

HRMS technique for determination of anabolic steroids in urine. Special attention was paid to the reduction of the background noise caused by urine matrix by increasing mass resolution and so increasing the signal-to-noise (S/N) ratio. First the GC–HRMS–SIM conditions (GC-program, ions measured in SIM, MS-parameters) were optimized and the same conditions were transferred to LRMS in order to compare the sensitivity and selectivity of HRMS and LRMS equipment. Then spiked urine samples were analysed with both GC–HRMS and GC–LRMS techniques and the results were also compared.

2. Experimental

2.1. Steroids and reagents

17α-Methyl-5β-androstan-3α,17β-diol (II), 17-epimetandienone (III), 17β-methyl-5β-androst-1-ene-3α,17α-diol (IV) and 6β-hydroxymetandienone (V) were synthesized by Schänzer group in Institute of Biochemistry, German Sports University, Cologne, Germany. Methyltestosterone (internal standard) was purchased from Steraloids Inc. (Newport, Rhode Island, USA). All reagents and solvents used were of analytical grade.

2.2. Preparing the spiked samples

We analysed two different spiked sets. In the first set spot urine samples were collected from seven healthy male volunteers (aged 35–44), who had been free of any medical treatment at least two weeks before urine collection. Each of the urine samples was first pooled and prepared by using a sample preparation procedure which is described as follows: A Sep-Pak C_{18} cartridge (Waters Co, Milford, Massachusetts, USA) was conditioned with 2.5 ml of methanol and then with 5 ml water. Then 2.5 ml of urine was introduced into the cartridge and after washing with water (3 ml) the sample was eluted with 3 ml methanol and evaporated to dryness. The residue was dissolved in 1 ml of 0.1 *M* phosphate buffer (pH 7) and hydrolyzed enzymatically with 20 μl of β-glucuronidase from *Escherichia coli* (K12, 200 U/ml, Boehringer, Mannheim, Germany) for 1 hour at 60°C. After adjusting pH to 11 with solid potassium carbonate, the sample was extracted with 5 ml of diethyl ether. The organic layer was finally separated and evaporated to dryness. The residue was trimethylsilylated (see Section 2.3) and analysed with GC–LRMS. After establishing that samples were drug-free, they were combined together.

Mixtures of the metandienone metabolites (1000 ng/ml or 100 ng/ml stock solutions in methanol) were spiked in urine matrix extract so that final concentrations in samples (V=100 μl) were 0, 5, 12.5, 25, 125 and 250 pg/μl. Because the urine volume extracted was 2.5 ml, the amounts added produced concentrations of 0, 0.1, 0.5, 1, 5 and 10 ng/ml in real urine samples. Methyltestosterone (internal standard) was spiked in every urine sample at a concentration of 50 ng/ml. Samples were evaporated to dryness and derivatized.

In the second set the urine samples were collected from four drug-free male persons. Metandienone metabolites were spiked in the original male urine samples in concentrations mentioned above. Then the samples were pretreated in the same way as mentioned above.

2.3. Derivatization for GC–MS analysis

For GC–MS analysis, TMS–ether or TMS–enol ether derivatives were formed depending on the steroid structure [3]. The dried sample was dissolved in 50 μl of MSTFA–NH_4I–dithioerythritol mixture (1000:2:4, v/v/v) and incubated at 60°C for 15 min. Then 50 μl of *n*-octane was added and the sample was transferred to an autosampler vial.

2.4. GC–HRMS screening analysis

HRMS experiments were performed with a JEOL SX 102 (JEOL, Tokyo, Japan) double focusing mass spectrometer equipped with HP 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA). A HP-1 column (length 16 m, 0.2 mm I.D., film thickness 0.11 μm) was employed with helium as carrier gas (flow 1 ml/min at 120°C). A 3-μl volume of sample was split injected (split ratio 1:7) into the GC. The GC temperature was programmed as follows: first from 120°C to 181°C at 40°C/min, then to 230°C at 3°C/min and finally to 310°C at

25°C/min. The injector and transfer line temperatures were 290°C and the ion source temperature was 250°C.

The samples were analysed by HRMS–SIM method with 70 eV EI-ionization. According to EI-spectra of the metabolites (Fig. 2), two ions/metabolite were selected for HRMS–SIM program (Table 1). The ions chosen were monitored in three different acquisition groups (scan cycle time, 50 ms each) because with electric field switching the mass range analyzed must be as narrow as possible to attain maximum sensitivity. The method was carried out by electric field switching using perfluorokerosin (PFK) for peak adjustment and during analysis for mass locking. Lock masses were m/z 354.9792 for group 1, m/z 430.9729 for group 2 and m/z 530.9664 for group 3. The mass resolution was 5000. The electron multiplier voltage was 1.7 kV.

2.5. GC–LRMS screening analysis

LRMS experiments were performed with a HP 5972A quadrupole mass spectrometer (Hewlett-Packard, Palo Alto, CA USA) equipped with HP 5890 E gas chromatograph. The column and the GC-program were identical to those used GC–HRMS screening analysis. The ions monitored were the same as those with the HRMS but they were monitored as integer mass values instead of exact masses in HRMS. In routine analysis, a widely accepted criteria in LRMS is that more than two ions are used for identification of compounds. In this study, however, we wanted to use exactly identical conditions as in HRMS and that is why only two ions were used in both techniques. Identification criterion in our study was that the area ratios of the SIM ions of the metabolites can vary within 30%.

3. Results and discussion

3.1. Influence of urine matrix on detection

The signal to noise relationships of metabolites with GC–HRMS–SIM and GC–LRMS–SIM methods measured from the first set (metabolites spiked in extracted pooled matrix), are presented in Tables 2–5 and LRMS–HRMS comparison curves (S/N/concentration) are illustrated in Fig. 3. The relative standard deviation (RSD) for S/N values were in both techniques $<20\%$ ($n=4$, Tables 2–5). Urine matrix caused problems with both MS techniques at trace concentrations (<1 ng/ml), as interfering compounds from the matrix were coeluted with metabolites (II) and (III). That is why the S/N values had to be first compared to the S/N values of interfering peaks in the blank urine matrix sample to get any idea of sensitivity. In our experiments, the criteria of detection limit was three times of background signal. Typically with HRMS–SIM method, the S/N values of spiked samples raised more drastically compared to LRMS–SIM method (Fig. 3), which made the peak detection easier at lower concentrations (<1 ng/ml).

In the second set, where the metandienone metabolites were spiked in authentic urine samples before pretreatment, the detection limits of the metabolites varied man by man. With some men, the interfering peaks, which coeluted with metandienone metabolites, were so intense, that detection limits were higher. That is why the results are discussed man by man and the detection limit range between the men was given for every metabolite.

Schänzer et al. [4] have studied only the metabolite (IV). However, it is also important to use other metabolites of metandienone as target compounds to get any idea of their behavior in urine and the influence of the matrix for their analysis. In general, in routine screening, with LRMS the metabolites (II), (III) and (V) in addition with metabolite (IV) are often used as target metabolites of metandienone and therefore we also included these compounds in HRMS analysis.

3.2. 17α-Methyl-5β-androstan-3α,17β-diol (II)

In the set, where the metabolites were spiked in extracted matrix (first set), with both the HRMS and the LRMS methods a compound from the matrix coeluted with the TMS-metabolite (II), when monitoring the ions m/z 435.3115/360.2848 with HRMS and m/z 435/360 with LRMS. At the concentration of approximately 0.3 ng/ml in urine the S/N value was $>3\times$ the S/N value of blank matrix when monitoring m/z 435.3115 with HRMS, but with m/z 360.2848 the value was at the same level as in the

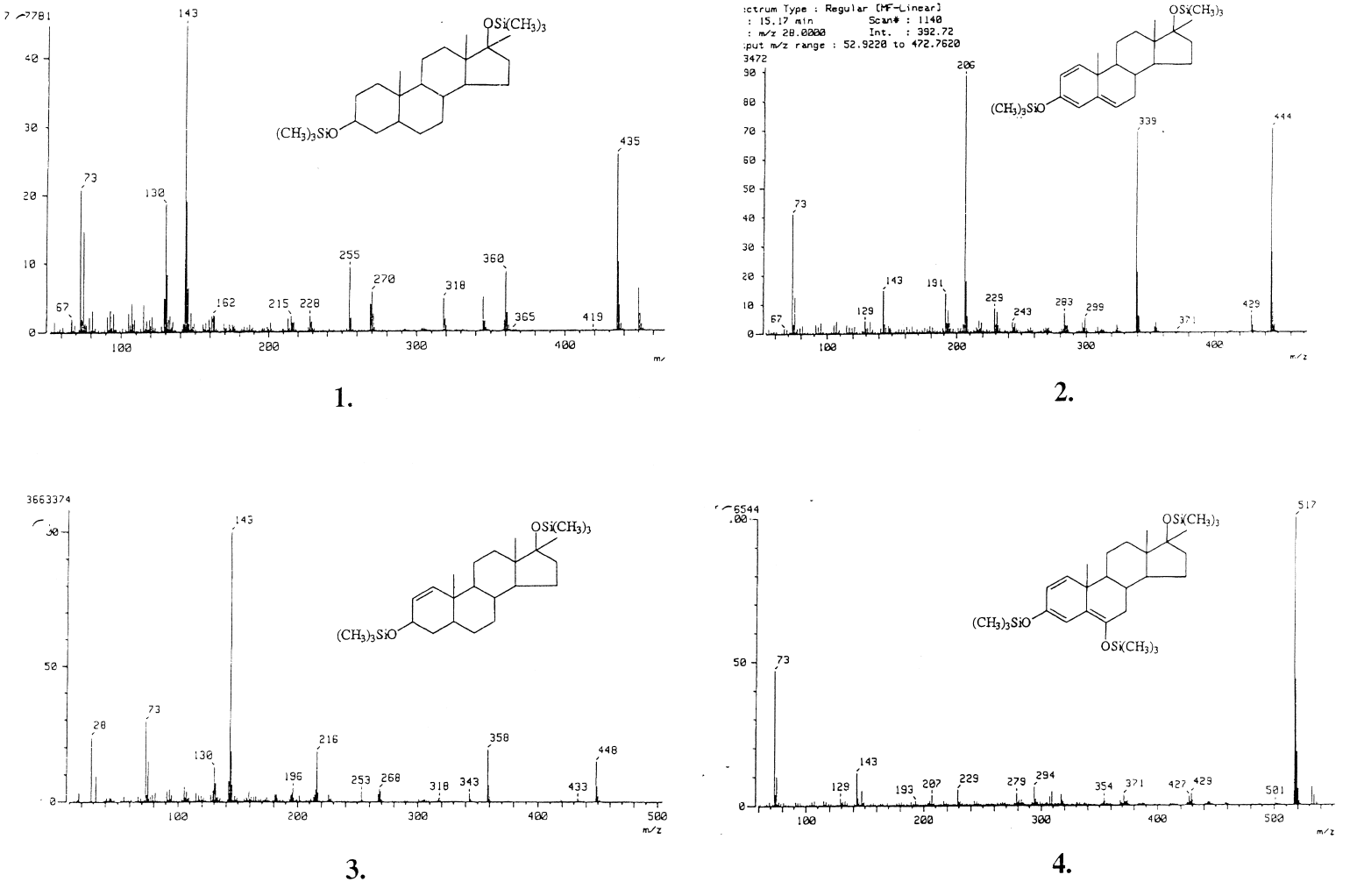


Fig. 2. EI-spectra of trimethylsilylated metandienone metabolites. (1) metabolite (II), (2) metabolite (III), (3) metabolite (IV), (4) metabolite (V).

Table 1
The silylated metabolite ions monitored with HRMS, the group where the ions are monitored, composition of ions and fragmentation. Lock masses were m/z 354.9792 for group 1, m/z 430.9729 for group 2 and m/z 530.9664 for group 3. With LRMS ions were monitored as integer masses

Compound	Ions monitored (HRMS) m/z	RT (min)	Group	Composition	Fragmentation
17α-methyl-5β-androstan-3α,17β-diol (II)	360.2848	11.8	2	$C_{23}H_{40}OSi$	$M^+-C_3H_{10}OSi$
	435.3115			$C_{25}H_{47}O_2Si_2$	M^+-CH_3
17-epimetandienone (III)	339.2144	12.2	2	$C_{22}H_{31}OSi$	M^+-CH_3
	444.2880			$C_{26}H_{44}O_2Si_2$	M^+
17β-methyl-5β-androst-1-en-3α,17α-diol (IV)	216.1909	8.9	1	$C_{12}H_{28}OSi$	$M^+-C_{14}H_{20}OSi$
	358.2692			$C_{23}H_{38}OSi$	$M^+-C_3H_{10}OSi$
6β-hydroxymetandienone (V)	517.2990	17.1	3	$C_{28}H_{49}O_3Si_3$	M^+-CH_3
	532.3224			$C_{29}H_{52}O_3Si_3$	M^+
methyltestosterone (ISTD)	301.1988	14.8	2	$C_{19}H_{29}OSi$	$M^+-C_7H_{17}OSi$
	446.3036			$C_{26}H_{46}O_2Si_2$	M^+

Table 2
Average S/N values of 17α-methyl-5β-androstan-3α,17β-diol (II) measured by HRMS and LRMS ($n=4$)

Conc. ng/ml	HRMS				LRMS			
	S/N for m/z 435.3115	RSD %	S/N for m/z 360.2848	RSD %	S/N for m/z 435	RSD %	S/N for m/z 360	RSD %
0	19	23	32	17	9	11	<1	–
0.2	51	6.5	33	3	12	5	<1	–
0.5	57	9.3	41	7.3	14	6.7	<1	–
1	70	4	51	2	16	5.9	<1	–
5	208	0.6	116	5.2	35	3.7	3	6.3
10	371	2.5	194	2.1	51	3.7	5	3.7

blank matrix. With LRMS, the estimated detection limit was 3 ng/ml when monitoring the ion m/z 435. There were also many interfering peaks from matrix with strong intensity near the metabolite (II) which made detection more difficult with both MS methods. However, with HRMS the detection limit was about 10 times lower than with LRMS.

In the second set, the estimated detection limits varied between 0.3–0.6 ng/ml with HRMS and 3–5 ng/ml with LRMS.

3.3. 17-Epimetandienone (III)

In the first set, the trimethylsilylated 17-epimetandienone (III) also coeluted with a urine matrix compound in both the HRMS and LRMS analyses.

Table 3
Average S/N values of 17-epimetandienone (III) measured by HRMS and LRMS ($n=4$)

Conc. ng/ml	HRMS				LRMS			
	S/N for m/z 444.2880	RSD %	S/N for m/z 339.2144	RSD %	S/N for m/z 444	RSD %	S/N for m/z 339	RSD %
0	3	6.4	5	28	2	9.5	2	10
0.2	9	15	17	5.9	3	11	3	10
0.5	13	13	22	9.1	3	3	4	6.3
1	15	10	36	5.6	5	5.6	7	4
5	56	1	181	0.6	28	5.4	35	2.9
10	98	5.7	284	5.1	45	2.2	62	1.6

Table 4
Average S/N values of 17β-methyl-5β-androst-1-ene-3α,17α-diol (IV) measured by HRMS and LRMS ($n=4$)

Conc. ng/ml	HRMS				LRMS			
	S/N for m/z 358.2328	RSD %	S/N for m/z 216.1909	RSD %	S/N for m/z 358	RSD %	S/N for m/z 216	RSD %
0	<1	–	97	3.7	3	15	10	10
0.2	3	9.1	96	16	4	9.5	12	8.3
0.5	5	13	143	15	5	6.3	13	7.6
1	8	15	154	10	7	4.6	16	6.3
5	22	14	320	9.3	21	2.4	26	3.8
10	44	9.1	590	1.9	32	3.1	38	2.6

With HRMS, when monitoring m/z 444.2880 the S/N value of coeluted peak from urine matrix was 3 and with LRMS it was 2 (m/z 444). However, with spiked samples at the concentration of 0.2 ng/ml the S/N value was 11 with HRMS, but with LRMS it was only 3. The signal of the spiked metabolite can be distinguished from the matrix signal at an estimated concentration of 0.3 ng/ml with HRMS and at 2 ng/ml with LRMS (see Table 3 and Fig. 3).

In the second set the detection limits varied man by man especially with HRMS. With HRMS detection limits were 0.1–0.3 ng/ml. With LRMS the estimated detection limit was 3 ng/ml with every authentic sample.

3.4. 17β-Methyl-5β-androst-1-en-3α,17α-diol (IV)

The advantage of the HRMS method compared to the LRMS method was best seen in analysing the metabolite (IV). In metabolic studies, (IV) has been shown to be a metabolite being excreted over a long period of time [4].By monitoring m/z 358.2692 with HRMS, no signal from the biological matrix was coeluted with trimethylsilylated metabolite (IV), but with LRMS (m/z 358) there was a peak with a signal of S/N value of 3. With spiked samples at the concentration of 0.2 ng/ml a clear signal (S/N of 3) was detected with HRMS, but with LRMS the signal of the metabolite (IV) was distinguished from the matrix related noise at a concentration of 1 ng/ml (S/N value of 7). HRMS–SIM and LRMS–SIM chromatograms of ion m/z 358.2692 (m/z 358 with LRMS) at different concentrations are shown in Figs. 4 and 5. With both techniques ion m/z 216.1909 (m/z 216 with LRMS) was problematic: a huge peak from urine matrix coeluted with metabolite (IV). The reason for using this ion was that according to EI-spectra (Fig. 2) the ion m/z 216.1909 was more intensive than ion m/z 448.3192 and it suited better for grouping.

In the second set, when comparing between the men, the estimated detection limits varied between 0.3–0.8 ng/ml with HRMS and 2–4 ng/ml with LRMS.

3.5. 6β-Hydroxymetandienone (V)

In the first set, with HRMS, a compound from

Table 5
Average S/N values of 6β-hydroxymetandienone (V) measured by HRMS and LRMS ($n=4$)

Conc. ng/ml	HRMS				LRMS			
	S/N for m/z 5172990	RSD %	S/N for m/z 532.3224	RSD %	S/N for m/z 517	RSD %	S/N for m/z 532	RSD %
0	<1	–	<1	–	2	12	<1	–
0.2	16	8.8	<1	–	5	5.6	<1	–
0.5	27	9.3	<1	–	14	4.3	<1	–
1	48	2.7	<1	–	29	4.8	3	6.9
5	251	2	18	3.2	167	4.4	6	9.2
10	473	6.8	31	9.5	268	3.4	10	4.9

urine matrix was eluted as a shoulder peak next to metabolite (V) when monitoring ion m/z 517.2990. With spiked samples, at the concentration of 0.2 ng/ml both the signal of the metabolite (V) and the signal from urine matrix had a S/N value of 16, and they appeared as a double peak in SIM chromatogram. With LRMS, a broad peak coeluted with metabolite (V). However, the signal of the metabolite (V) could be distinguished from matrix at the concentration of 0.4 ng/ml with HRMS when monitoring ion m/z 517.2992. With LRMS (m/z 517) the estimated detection limit was 0.5 ng/ml. When monitoring ion m/z 532.3224 the detection limit was about 3 ng/ml with HRMS and approximately 5 ng/ml with LRMS.

In the second set, with HRMS detection limit was about 0.1 ng/ml with every male person. With LRMS the detection limit varied 0.3–1 ng/ml depending on the person.

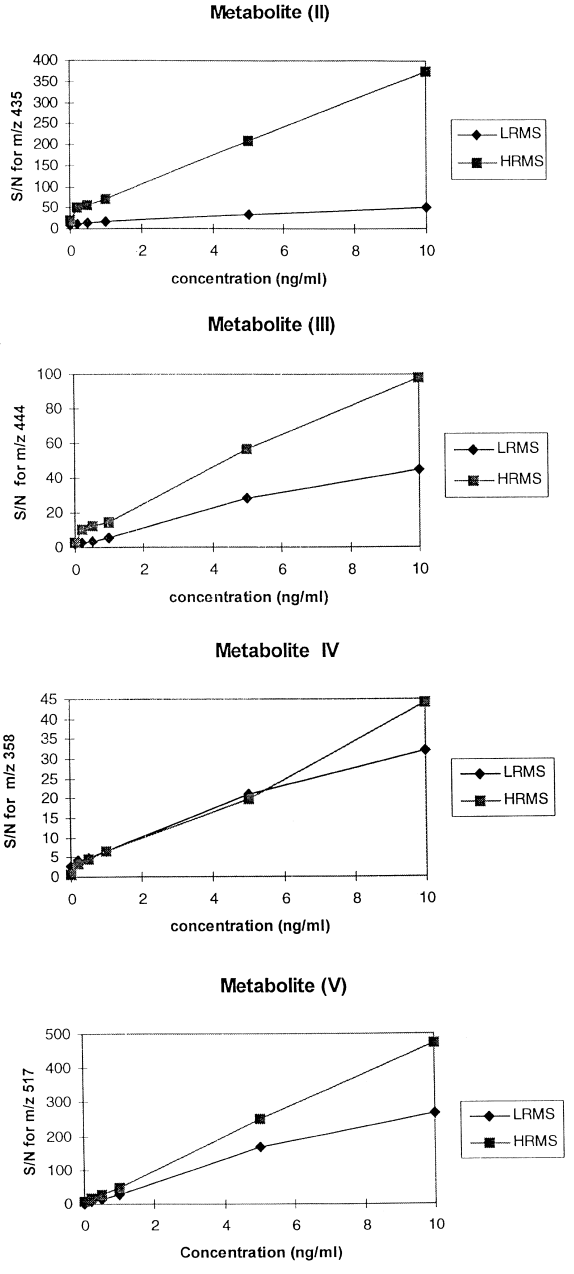


Fig. 3. LRMS–HRMS comparison curves (S/N/concentration) of metandienone target compounds.

4. Conclusions

In our experiments the sensitivities of GC–HRMS–SIM and GC–LRMS–SIM were compared. The metandienone metabolites 17α-methyl-5β-androstan-3α,17β-diol (II), 17-epimetandienone (III), 17β-methyl-5β-androst-1-ene-3α,17α-diol (IV) and 6β-hydroxymetandienone (V) were analysed with GC–HRMS–SIM technique at the resolution of 5000 with electric field switching and the results were compared with GC–LRMS–SIM results analysed by quadrupole instrument in same conditions as in HRMS. In the case of metandienone metabolites the detection limits were 0.2–0.4 ng/ml with HRMS and 0.5–3 ng/ml with LRMS, when the metabolites were spiked in the pooled extracted matrix. The best advantage of HRMS was seen when analysing the metabolite (IV): the detection limit was 0.2 ng/ml with HRMS compared to 1 ng/ml with LRMS.

We also measured a set, where the metandienone metabolites were spiked first in blank urine of four male persons before pretreatment. In this experiment, both with HRMS and LRMS, detection limits of metabolites varied little between the men in same concentrations. For 17-epimetandienone (III) the detection limits were 0.1–0.3 ng/ml with HRMS and 3 ng/ml with LRMS. For 17β-Methyl-5β-androst-1-en-3α,17α-diol (IV), variation was 0.3–0.8 ng/ml with HRMS and 2–4 ng/ml with LRMS. For 6β-hydroxymeandienone (V) the detection limit was 0.1

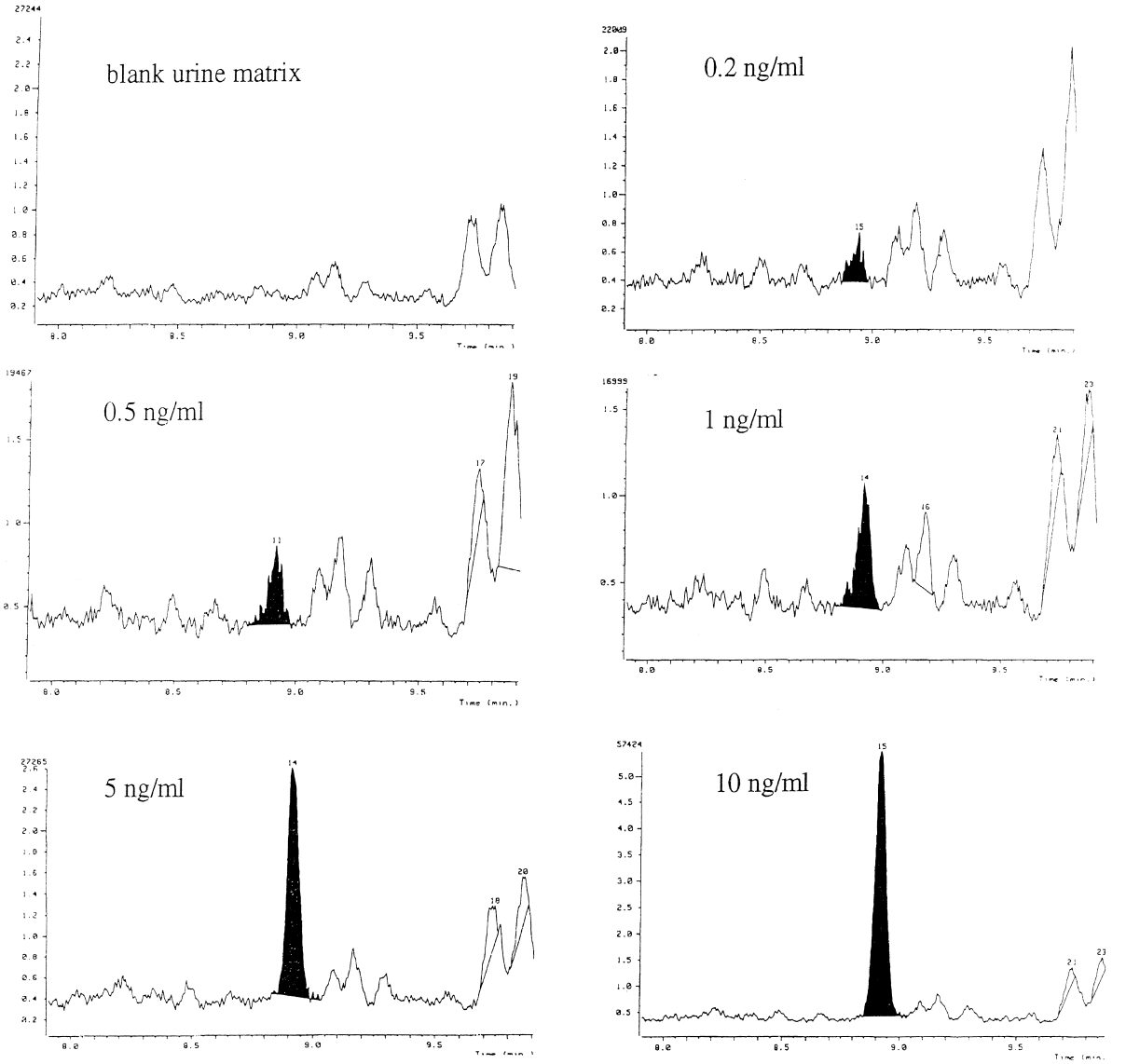


Fig. 4. HRMS–SIM chromatograms of trimethylsilylated metandienone metabolite (IV) (m/z 358.2692) at different concentrations spiked in urine.

ng/ml in every person with HRMS. With LRMS it varied between 0.3–1 ng/ml.

Both experiments show that HRMS method is 2–10 times more sensitive for metandienone metabolites than LRMS method. Although there is a detection limit variation between the persons at same spiked concentrations, the sensitivity difference between HRMS and LRMS methods is clearly observed. This is in accordance with previous studies [4].

Also with HRMS screening method detection is hampered by biological matrix and with metabolites (II) and (III) compounds from urine matrix were coeluted. With the metabolite (IV), when monitoring ion m/z 216.1909, a compound from urine matrix was coeluted too. In this experiment the ions of

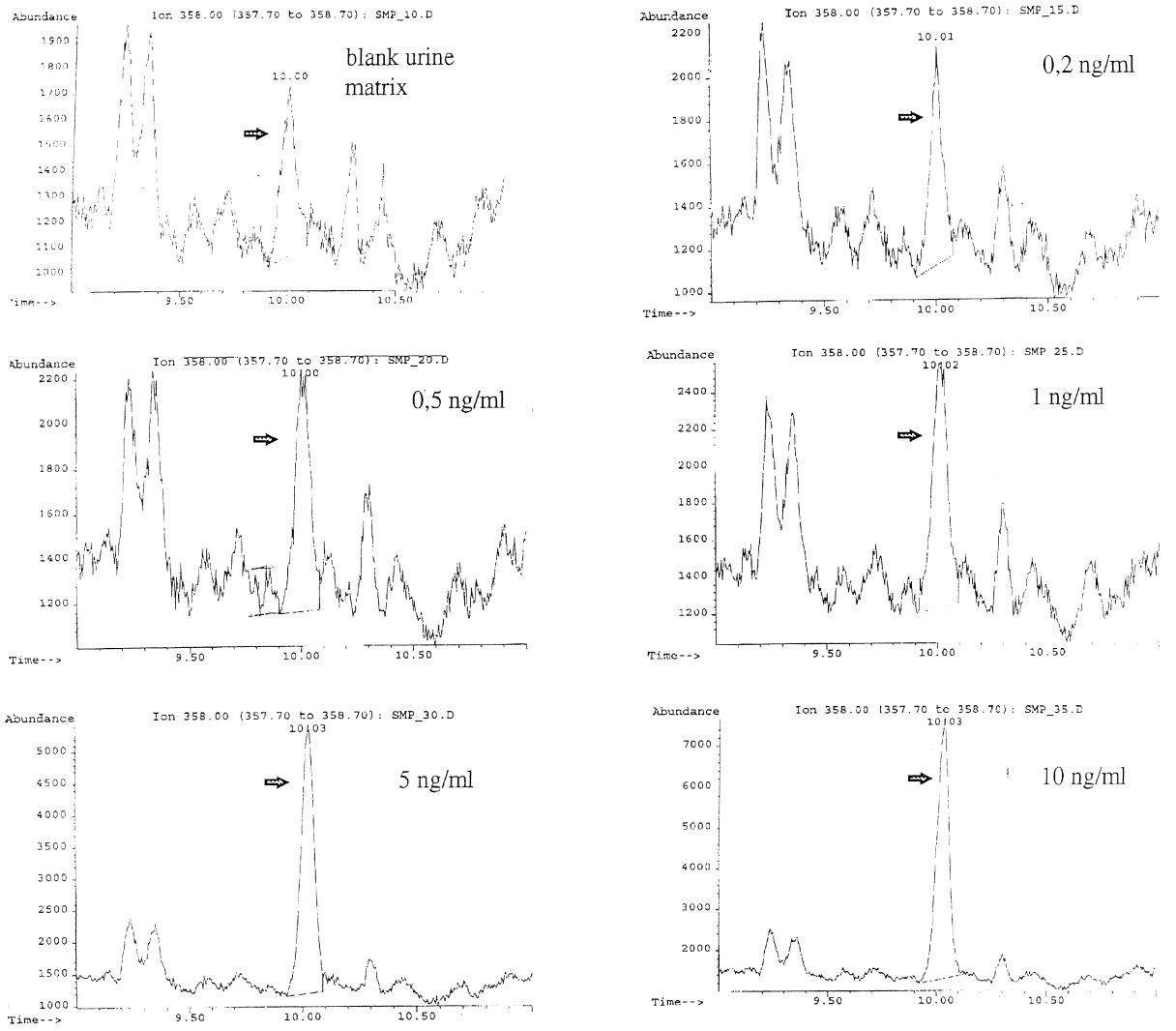


Fig. 5. LRMS–SIM chromatograms of trimethylsilylated metandienone metabolite (IV) (m/z 358) at different concentrations spiked in urine.

metandienone metabolites for the GC–HRMS–SIM method were chosen so that the mass range analysed is so narrow as possible to attain the maximum sensitivity with electric field switching. By selecting other ions the interference of urine matrix may be reduced and sensitivity improved. Also a selective isolation procedure, for example HPLC fractionation, should be implemented at trace concentrations in order to confirm a positive result.

Nowadays there is not so much HRMS equipment in doping laboratories, because it is quite expensive. However, according to the decision of the IOC, the accredited doping laboratories have to have capability to confirm positive sample with HRMS or tandem mass spectrometer. In our study we measured samples in two laboratories: one laboratory has LRMS- and the other HRMS-equipment. This study illustrates that the same screening conditions can be used with both HRMS and LRMS methods. That is why the costs per analysis do not differ from each other so much, only the investment of the HRMS-instrument is more expensive.

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