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USE OF ION TRAP (GC-MS-MS) MASS SPECTROMETRY FOR DETECTION AND CONFIRMATION OF ANABOLIC SUBSTANCES AT LOW CONCENTRATION LEVELS IN DOPING ANALYSIS

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INTRODUCTION

In 1980 the International Olympic Committee (IOC) included anabolizant steroids in the list of forbidden substances. The use of these drugs affected performances, giving rise to fraudulent sportive results and above all causes harm to the health of the athlete (1). Quadrupole mass spectrometry has been the most frequently used technique in the analysis of these compounds (2,3,4). Nevertheless, it is nearly impossible to do a full scan analysis at low concentrations without considerable sample cleanup and unequivocal confirmation is impossible using selected ion monitoring. With regard to this situation, the IOC has obliged recently to its credited laboratories to use High Resolution Mass Spectrometry or MS-MS. The intention is to absolutely identify the presence of anabolic steroids at very low concentration levels.

Urine is the biological fluid used in doping control for sports. A large number of components are found in this matrix. Many of these are endogenous steroid substances in a wide range of concentrations. During the sample preparation process, these endogenous components are extracted together with the possible exogenous anabolic substances and introduced into the GC/MS system. As a result, coeluting interferences produce deformed chromatographic peaks as well as distortions in the mass spectrum and that lead us to erroneous interpretations. Recently, the GC-MS-MS ion trap system has proven to be an important technique in drug screening (5,6,7). In the case of analyzing samples with complex matrices, this technique allows a selective elimination of the interference arising from the

matrix and therefore, by eliminating the background, improves the detection limits. This makes it possible to operate in full scan mode at low ng/ml levels. The objective of this work has been to develop an analysis technique for 25 substances and /or metabolites with anabolizant effects using GC-MS-MS with an ion trap mass spectrometer.

EXPERIMENTAL

Sample preparation

The samples were prepared according to the procedure described by Schänzer *et al.* (4). The procedure can be described as follows: 250 ul I.S. solution (Methyltestosterone 10ng/ml in methanol) was added to 2 ml of urine. The urine was passed through a Pasteur pipette (230 x 7 mm) which contained 20 mm Serdolit AD-2 resin. The resin was washed with 2 ml de-ionized water, eluted with 2 ml of methanol and evaporated until dry. The residue was dissolved in 1 ml of a 0.2 M sodium phosphate buffer to pH 7 where 25 uL b-glucoronidase from Escheriquia coli was added. The mixture was extracted with 5 ml of diethyl ether on a mechanical shaker for 5 minutes. The organic phase was centrifuged and dried under vacuum. The dried residue was derivatized with 50 ul MSTFA/NH_4I/dithioerythritol (1000:2:4,v/w/w) for 30 minutes at 60°C.

Instrumentation

A Saturn 4D GC-MS-MS System (Varian Associates, Walnut Creet CA) coupled with a Varian 3400 gas chromatograph equipped with a 8200 CX autosampler and a model 1078 programmable temperature split/splitless injector port was used to carry out all the experiments. The whole system was controlled by standard Varian software (version 5.2), MS-MS conditions operating CID non-resonant and CID resonant excitation form were developed using Ion Trap MS-MS ToolKit software for Windows (Version 1.0). The instrumental conditions are shown in Table 1.

RESULTS AND DISCUSSION

Before the screening of anabolic substances with MS-MS using ion trap mass spectrometry, they were injected and subjected to electronic impact analysis. The majority of compounds revealed certain differences between their mass spectrum obtained using ion trap mass spectrometry and that obtained with quadrupole spectrometry: fragments of greater m/z were generally more abundant in the ion trap spectrometer than in the quadrupole. Furthermore, in some cases the proportions among these fragments were changed due to the time the ions remained in the trap, with the most unstable ions becoming the most stable as a result of the geometry of the molecules.

The parent ion for each anabolic substance was chosen with the aim of obtaining the greatest possible selectivity, so parent ions were chosen that were not present in the background and did not commonly present coeluate interferences coming from the urine sample. Moreover, in order to make confirmation possible with structurally informative spectra, ions with the highest possible m/z were selected. Finally, another important criterion for selecting the parent ion was that it should have a more intense ionic current to permit greater sensitivity.

Fragmentation of the precursor ion was performed by collision-induced dissociation (CID) with helium molecules, the carrier gas which fills the ion trap. CID fragmentation can be performed using non-resonant or resonant excitation mode. Once the excitation mode has been selected, the main parameters determining the fragmentation behavior of an ion are: excitation time, dissociation energy and the storage radio frequency, the last two parameters being the ones which had to be carefully optimized to ensure the best performance of the technique.

Using the software it is possible to change the dissociation conditions of the precursor ion, scan by scan, during the same analysis, thus permitting a rapid optimization of the fragmentation conditions. The fragmentation parameters were programmed into an ion preparation method (IPM). Each analysis was divided into segments, and within each segment a different IPM was used, thus in each analysis segment, optimal dissociation conditions existed for one or more ions. It is also possible to keep the specific fragmentation conditions of up to ten ions in each IPM, although to achieve the highest possible degree of selectivity it is advisable not to include more than three or four ions in each segment. Fragmentation studies were performed to ascertain the CID behavior of each precursor ion. Fig. 1 shows the dissociation behavior of m/z ion 358 of epimetendiol. The Y axis shows the variations of the reconstructed ionic current (RIC), and those of the m/z 358 precursor ion and the m/z 301 and

343 product ions, while the X axis shows the increase in voltage, while radio frequency was fixed at 130 m/z (upper/left) and 100 m/z (upper/right). Initially when zero volts were applied there was no fragmentation, thus permitting measurement of the initial ionic current of the precursor ion, which coincided with reconstructed ionic current. When voltage increased, m/z ion 358 began to dissociate into its product ions, which gradually gained ionic intensity. The voltage selected was the one which made it possible to obtain a spectrum with a very abundant base peak, that of m/z ion 301, a minimal initial ionic current loss, and the presence of representative fragments m/z ions 343 and 358. Also from the comparison of both graph, we can realize that it is possible to obtain two very similar MS-MS spectra of the chosen parent ion using different combinations of CID Radio Frequence and CID Amplitude values but with a good selection of this couple of parameters it is possible to increase the sensitivity level by a factor of two, fig. 1 (lower).

Having carried out a similar study of dissociation behavior for each of the precursor ions of each anabolic compound included in the screening, the dissociation conditions shown in Table 2 were selected. In all cases non-resonant excitation mode was used except for 3'-hydroxystanozolol, for which, given the high stability shown by its precursor ion, the much more aggressive resonant excitation mode was used. Table 3 shows the MS-MS screening under the conditions shown in Table 2, with the names of the compounds, their retention time, the parent ion and lastly the main daughter ions with their abundance. Fig. 2 shows the first page of the macro analysis after applying it to a sample spiked with a mixture of anabolic compounds at a concentration of 10 ng/ml in urine. To visualize each anabolic compound in the macro, the three most abundant fragments were chosen for each one, bearing in mind that since a full-scan screening was being used, as well as these fragments, all the fragments of the precursor ion were recorded during the analysis, with their corresponding abundancies, so that if a suspect signal appeared in the macro, it could always be sought in the analysis itself to confirm whether it was an interference or a positive result, fig. 3 shows the result of analyzing a positive sample containing epimetendiol obtained from an athlete with the macro procedure (left). The presence of aligned fragments made us suspect the presence of the anabolic substances in the sample, so that without needing to inject again, the signal was sought in the same analysis and the mass spectrum shown in the figure (right) was obtained, confirming the presence of the anabolic substance in the sample.

The use of these parameters to measure the spectral correlation between reference spectra (library or pure standards) and experimental spectra permits the establishment of cut off criteria for confirmation. In our case it was shown that a value of 800/1000 ensured a

spectral similarity between sample and reference which was sufficient to guarantee the presence of the anabolic compound in the urine. Fig. 4 shows an MS-MS spectrum for a sample spiked with 2 ng/ml of 5α-methyltestosterone (upper), the MS-MS spectrum for pure standard 5α-methyltestosterone (middle), and the difference between them (lower). The upper part of the figure shows the spectral comparison parameters.

Table 4 shows the sensitivity study for all the anabolic compounds included in the screening. Sensitivity was measured in terms of confirmation limits, this being understood as the concentration of the anabolic compound needed to permit a matching of more than 800/1000 when compared with the pure standard.

Fig. 5 shows two injections of the same sample with epimetendiol, (upper) by MS-MS, m/z ion 301 and (lower) by MS, m/z ion 358. Comparing these two signals shows how in this case thanks to the selective expulsion of the unwanted ions with the MS-MS technique the signal-to-noise ratio can be enhanced more than twenty-fold.

CONCLUSION

These results prove that the use of the GC-MS-MS ion trap system is effective in the analysis of complex matrices such as urine. This technique has been shown to be a powerful tool for the detection and confirmation of anabolic substances at low concentrations in urine samples proceeding from sporting competitions. The analytical method used was highly sensitive, easy to optimize, time saving and allows to confirm anabolic substances at concentrations levels at which injecting by Single Ion Monitoring it is only possible detection. Therefore GC-MS-MS is a good complement to the SIM or similar GC-MS methods for analyzing anabolic compounds.

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Figure 1. Ion intensities as a function of CID voltage for precursor ion and product ions, (upper/left) CID Radio Frequence 130 m/z, (upper/right) CID Radio Frequence 100 m/z.(Lower) epimetendiol peak from two injections with different CID conditions.

Figure 2. First macro analysis page after applying it to a urine sample spiked with a mixture of anabolic agents at a concentration level of 10 ng/ml.

Figure 3. GC-MS-MS macro analysis of a positive urine sample containing epimetendiol with the three more abundant ion chromatogram monitorizated (left) and mass spectra of epimetendiol obtained from the same sample in the same injection.

Figure 4. Mass spectra from a urine sample of TMS derivative of 5a-methyltestosterone at a concentration level of 2 ng/ml (upper), mass spectra from a pure standard of TMS derivative of 5a-methyltestosterone (middle) and difference between both mass spectra (lower).

Figure 5. GC-MS-MS analysis of a positive urine sample containing epimetendiol (upper) and GC-MS analysis of the same sample (lower).

Instrumental conditions	
Gas Chromatograph	
Column:	HP Ultra-1 (Crosslinked Methyl Silicone Gun) 25m x 0.2mm x 0.11 um film
Flow Rate:	1 mL/min
Oven Program:	120°C hold 1.6 sec, 50°C/min until 200°C at 2°C/min until 245°C 245°C at 25°C/min until 300°C, hold 5 min
Injector Temperature.	280°C
Transfer Line Temperature:	300°C
Injection Mode:	Splitless, hold 1 min
Injection Volume:	2 uL
Mass Spectrometer (MS)	
Mass range:	130-600
Scan Rate:	2 scan/sec
Background Mass:	45 m/z
Ion Trap Temperature:	220°C
Axial Modulation:	4 volts
RF level:	100 m/z
Filament:	60 uAmps
Multiplier:	Autotune + 100 V
AGC Target:	Autotune
Mass Spectrometer (MS-MS)	
Filament:	90 uAmps
Multiplier:	Autotune + 200 V
AGC Target:	5000

Table 1.

GC-MS-MS parameters used for selective fragmentation of parent ions in substances with anabolic effect

Substances with anabolic effect	Main excreted substance: parent or metabolite	Parent Ion	CID parameters		
		m/z	Amplitude (volts)	RF m/z	Form
Bolasterone	Bolasterone PC	445	55	100	NR
	7α,17α-Dimethyl-5β-androstane-3α,17β-diol	374	65	180	NR
Boldenone	Boldenone PC	430	85	140	NR
	5β-Androst-1-en-17β-ol-3-one	432	78	160	NR
4-Chlorodehydro-methyltestosterone	6β-Hydroxy-4-chloro-dehydromethyl-testosterone	315	82	115	NR
Clenbuterol	Clenbuterol PC	300	85	110	NR
Clostebol	4-Chloro-androst-4-en-3α-ol-17-one	451	80	130	NR
Drostanolone	2α-Methyl-5α-androstan-3α-ol-17-one	433	65	100	NR
Fluoxymesterone	9α-Fluoro-17α-methyl-androst-4-ene-3α,6β,11β,17β-tetrol	552	80	150	NR
Furazabol	Furazabol PC	387	73	130	NR
Mesterolone	1α-Methyl-5α-androstan-3α-ol-17-one	433	62	100	NR
Metandienone	17β-Methyl-5β-androst-1-ene-3α,17α-diol	358	80	130	NR
Metenolone	Metenolone PC	446	62	105	NR
	1-Methylene-5α-androstan-3α-ol-17-one	431	62	103	NR
Methyltestosterone	17α-Methyl-5α-androstane-3α,17β-diol	435	75	150	NR
	17α-Methyl-5β-androstane-3α,17β-diol	435	75	150	NR
Nandrolone	5α-Estran-3α-ol-17-one	405	70	110	NR
	5β-Estran-3α-ol-17-one	405	70	110	NR
Norethandrolone	17α-Ethyl-5β-estrane-3α,17β-diol	421	75	150	NR
Oxandrolone	17-Epioxandrolone	363	75	130	NR
Salbutamol	Salbutamol PC	369	63	100	NR
Stanozolol	3'-Hydroxystanozolol	545	0.35	135	R
	16β-Hydroxystanozolol	560	72	140	NR

The CID window and the CID time were fixed and their values were 3 m/z and 20 msec respectively
NR: CID Non-Resonant excitation form; R: CID resonant excitation form

Table 2.

Substances with anabolic effect	Main excreted substance: parent or metabolite	Parent Ion	R.T	Main daughter Ions m/z (abundance %)
		m/z	min.	
Bolasterone	Bolasterone PC	445	22,41	225(100%)/355(95%)/265(30%)/445(10%)
	7α,17α-Dimethyl-5β-androstane-3α,17β-diol	374	24,49	284(100%)/374(92%)/269(60%)/214(32%)/247(30%)
Boldenone	Boldenone PC	430	16,05	430(100%)/325(58%)/415(20%)
	5β-Androst-1-en-17β-ol-3-one	432	21,20	417(100%)/432(85%)/19430%)/327(12%)/342(10%)
4-Chlorodehydro-methyltestosterone	6β-Hydroxy-4-chloro-dehydromethyl-testosterone	315	29,52	227(100%)/241(80%)/315(65%)/279(15%)
Clenbuterol	Clenbuterol PC	300	9,24	284(100%)/300(40%)/210(35%)/285(15%)/226(5%)
Clostebol	4-Chloro-androst-4-en-3α-ol-17-one	451	22,39	325(100%)/451(90%)/361(70%)/235(50%)/271(40%)
Drostanolone	2α-Methyl-5α-androstan-3α-ol-17-one	433	18,69	253(100%)/343(75%)/181(42%)/171(32%)/271(40%)
Fluoxymesterone	9α-Fluoro-17α-methyl-androst-4-ene-3α,6β,11β,17β-tetrol	552	26,86	462(100%)/319(55%)/407(32%)/447(20%)/552(18%)
Furazabol	Furazabol PC	387	28,06	297(100%)/387(57%)/161(8%)
Mesterolone	1α-Methyl-5α-androstan-3α-ol-17-one	433	20,11	253(100%)/261(63%)/343(55%)/171(42%)/433(42%)
Metandienone	17β-Methyl-5β-androst-1-ene-3α,17α-diol	358	16,06	301(100%)/358(22%)/343(12%)/357(8%)/268(8%)
Metenolone	Metenolone PC	446	22,61	208(100%)/193(70%)/446(40&)/177(12%)/341(10%)
	1-Methylene-5α-androstan-3α-ol-17-one	446	19,44	431(100%)/446(30%)/432(20%)/341(17%)/251(12%)
Methyltestosterone	17α-Methyl-5α-androstane-3α,17β-diol	435	20,39	255(100%)/435(50%)/345(12%)/213(10%)/199(5%)
	17α-Methyl-5β-androstane-3α,17β-diol	435	20,71	345(100%)/255(70%)/435(60%)/269(15%)/
Nandrolone	5α-Estran-3α-ol-17-one	405	15,72	225(100%)/315(45%)/155(25%)/143(18%)/169(15%)
	5β-Estran-3α-ol-17-one	405	16,97	315(100%)/225(85%)/169(22%)/143(22%)155(20%)
Norethandrolone	17α-Ethyl-5β-estrane-3α,17β-diol	421	22,48	331(100%)/241(60%)/421(40%)/199(5%)
Oxandrolone	17-Epioxandrolone	363	21,81	273(100%)/363(98%)/213(37%)/161(30%)/227(18%)
Stanozolol	3'-Hydroxystanozolol	545	31,3	455(100%)/277(12%)/387(10%)/439(10%)/347(8%)
	16β-Hydroxystanozolol	560	33,5	560(100%)/231(8%)/328(6%)

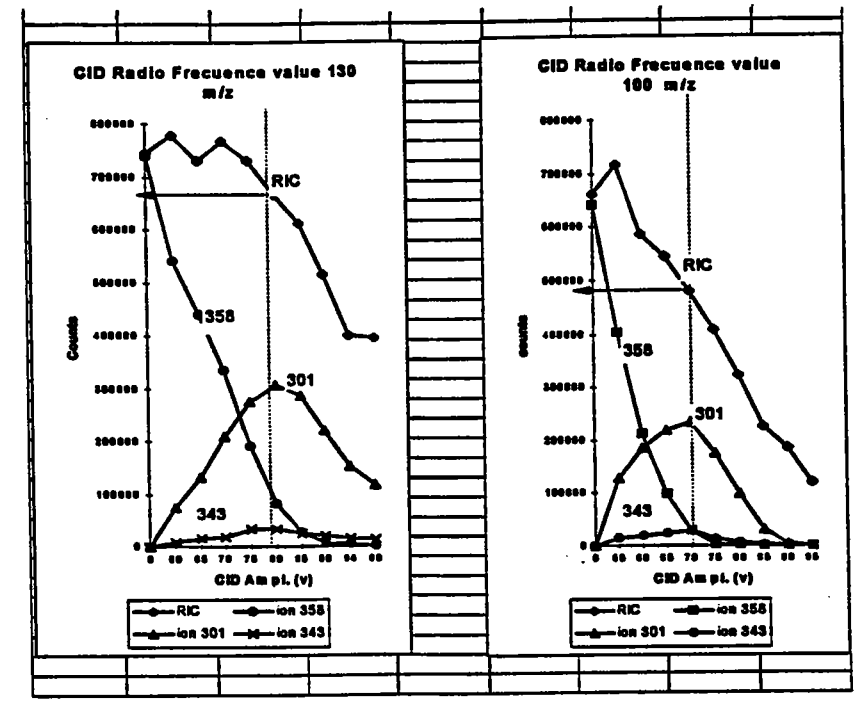
Table 3.

Retention times and limits of detection for the anabolic compounds listed below. The assay was carried out with spiked urine samples (n=10)

Substances with anabolic effect	Main excreted substance: parent or metabolite	R.T. (Min.)	Approximate limit of confirmation (ng/ml)
Bolasterone	Bolasterone PC	22.41	3
	7α,17α-Dimethyl-5β-androstane-3α,17β-diol	24.49	1.5
Boldenone	Boldenone PC	16.05	1
	5b-Androst-1-en-17b-ol-3-one	21.20	4
4-Chlorodehydro-methyltestosterone	6β-Hydroxy-4-chloro-dehydromethyl-testosterone	29.52	2
Clenbuterol	Clenbuterol PC	9.24	1
Clostebol	4-Chloro-androst-4-en-3α-ol-17-one	22.39	2
Drostanolone	2α-Methyl-5α-androstan-3α-ol-17-one	18.69	3
Fluoxymesterone	9α-Fluoro-17α-methyl-androst-4-ene-3α,6β,11β,17β-tetrol	26.86	2
Furazabol	Furazabol PC	28.06	1
Mesterolone	1α-Methyl-5α-androstan-3α-ol-17-one	20.11	3
Metandienone	17β-Methyl-5β-androst-1-ene-3α,17α-diol	16.06	1
Metenolone	Metenolone PC	22.61	*
	1-Methylene-5α-androstan-3α-ol-17-one	19.44	1
Methyltestosterone	17α-Methyl-5α-androstane-3α,17β-diol	20.39	1
	17α-Methyl-5β-androstane-3α,17β-diol	20.71	1
Nandrolone	5α-Estran-3α-ol-17-one	15.72	0.5
	5β-Estran-3α-ol-17-one	16.97	0.8
Norethandrolone	17α-Ethyl-5β-estrane-3α,17β-diol	22.48	1
Oxandrolone	17-Epioxandrolone	21.81	1
Salbutamol	Salbutamol PC	8.45	0.7
Stanozolol	3´-Hydroxystanozolol	31.74	3
	16β–Hydroxystanozolol	33.15	*

* It was not possible to calculate the LOC, no standard solution is available.

Table 4.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RIC
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301
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ion 301
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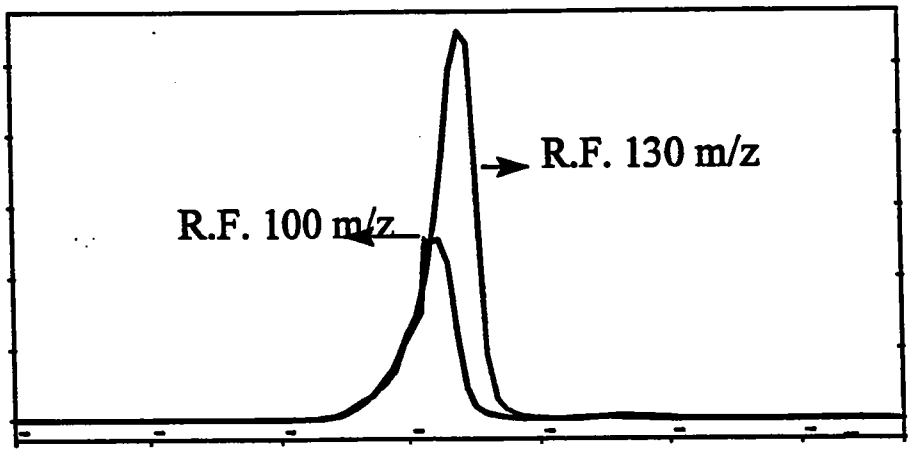
ion 358
ion 301
ion 343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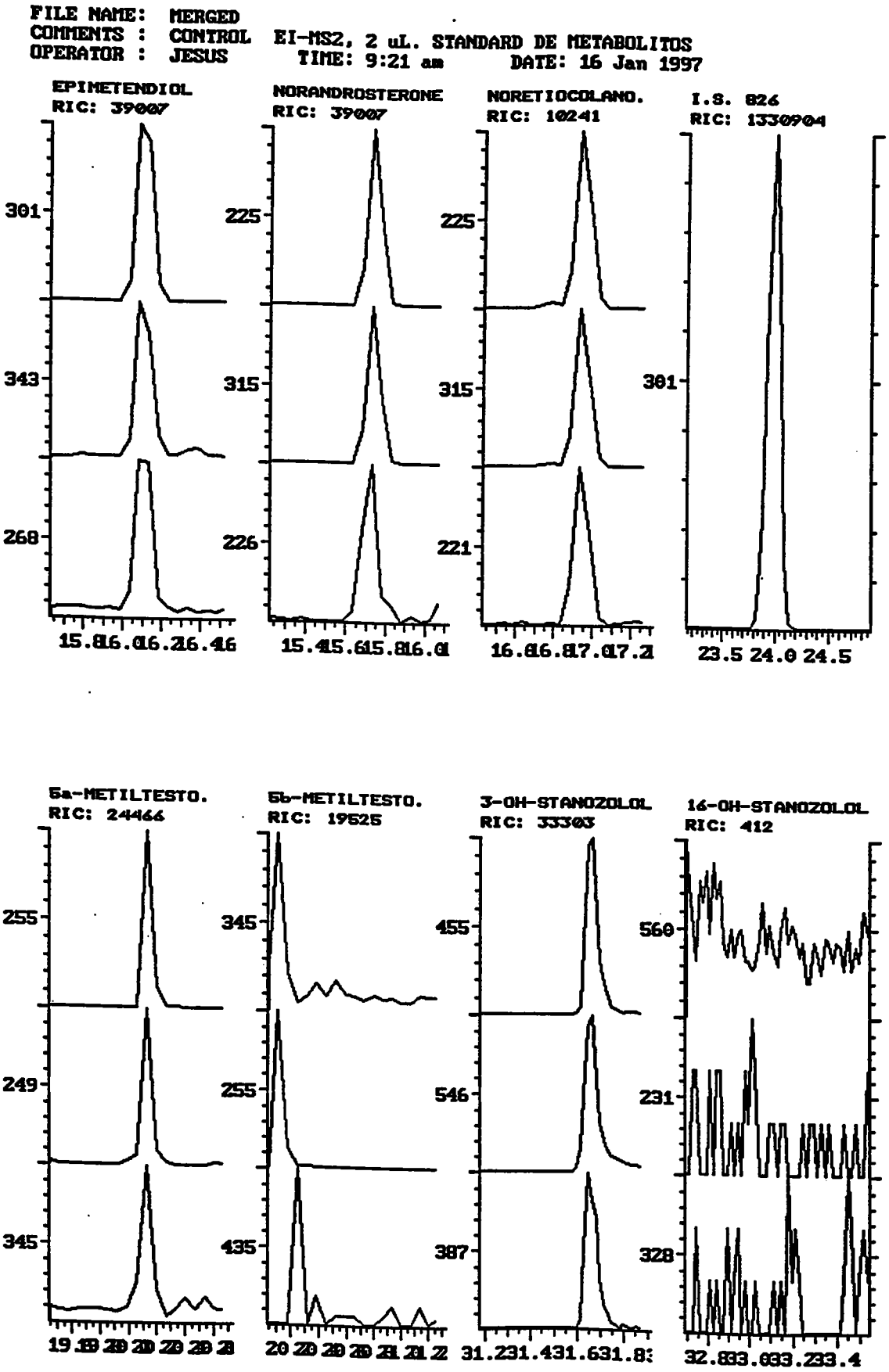
R.F. 100 m/z

Figure 1.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NORANDROSTERONE
RIC: 39007
NORETIOCOLANO.
RIC: 10241
I.S. 826
RIC: 1330904
301
343
268
225
315
226
225
315
221
301
15.8 16.0 16.2 16.4 16
15.4 15.6 15.8 16.0
16.0 16.8 17.0 17.2
23.5 24.0 24.5
6a-METILTESTO.
RIC: 24464
6b-METILTESTO.
RIC: 19525
3-OH-STANOZOLOL
RIC: 33303
16-OH-STANOZOLOL
RIC: 412
255
249
345
345
255
435
455
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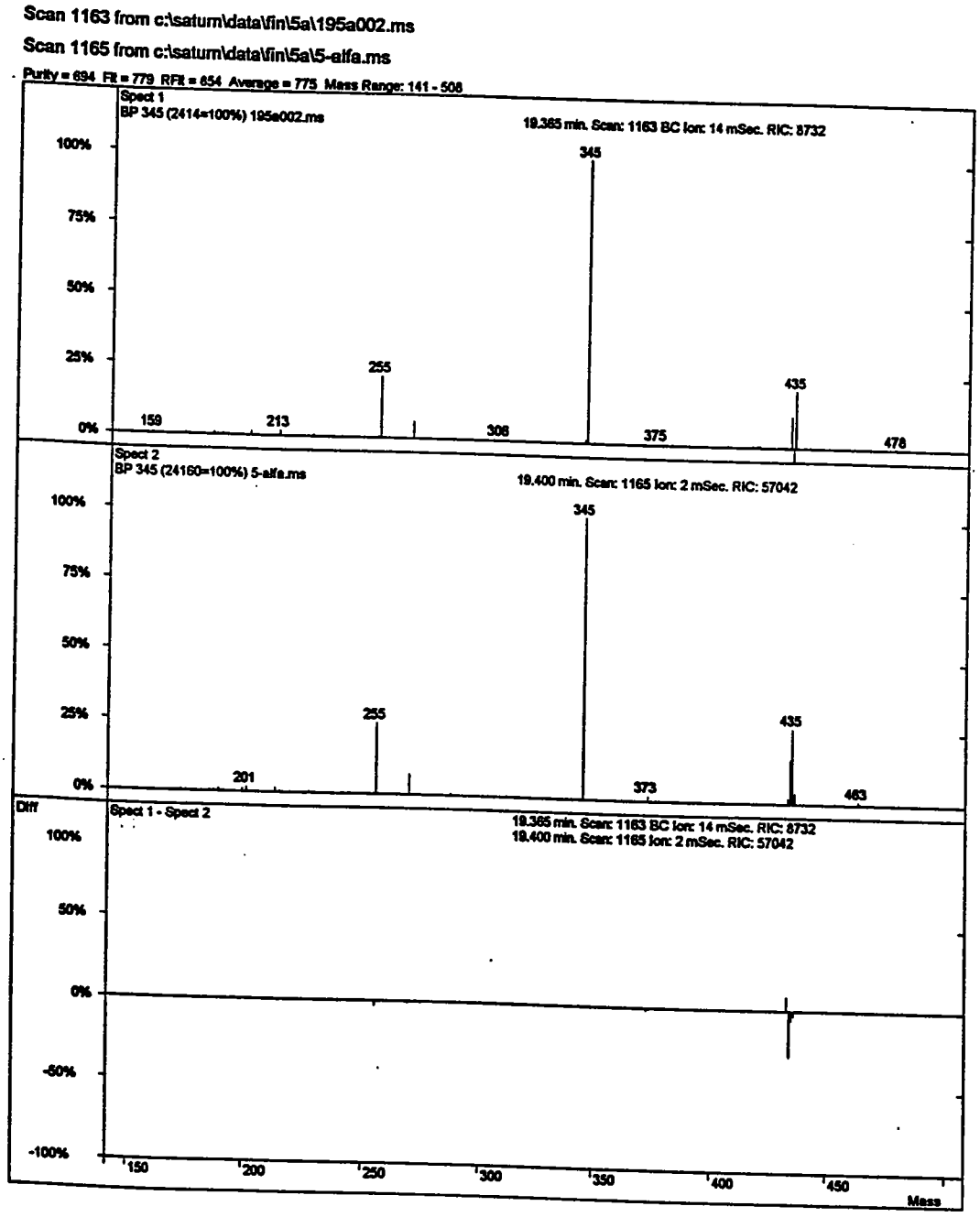
31.2 31.4 31.6 31.8
32.8 33.0 33.2 33.4

Figure 2.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BP 345 (2414=100%) 195a002.ms
19.365 min. Scan: 1163 BC Ion: 14 mSec. RIC: 8732
100%
75%
50%
25%
0%
345
255
435
159
213
308
375
478
Spect 2
BP 345 (24160=100%) 5-alfa.ms
19.400 min. Scan: 1165 Ion: 2 mSec. RIC: 57042
345
255
435
201
373
463
Diff
Spect 1 - Spect 2
19.365 min. Scan: 1163 BC Ion: 14 mSec. RIC: 8732
19.400 min. Scan: 1165 Ion: 2 mSec. RIC: 57042
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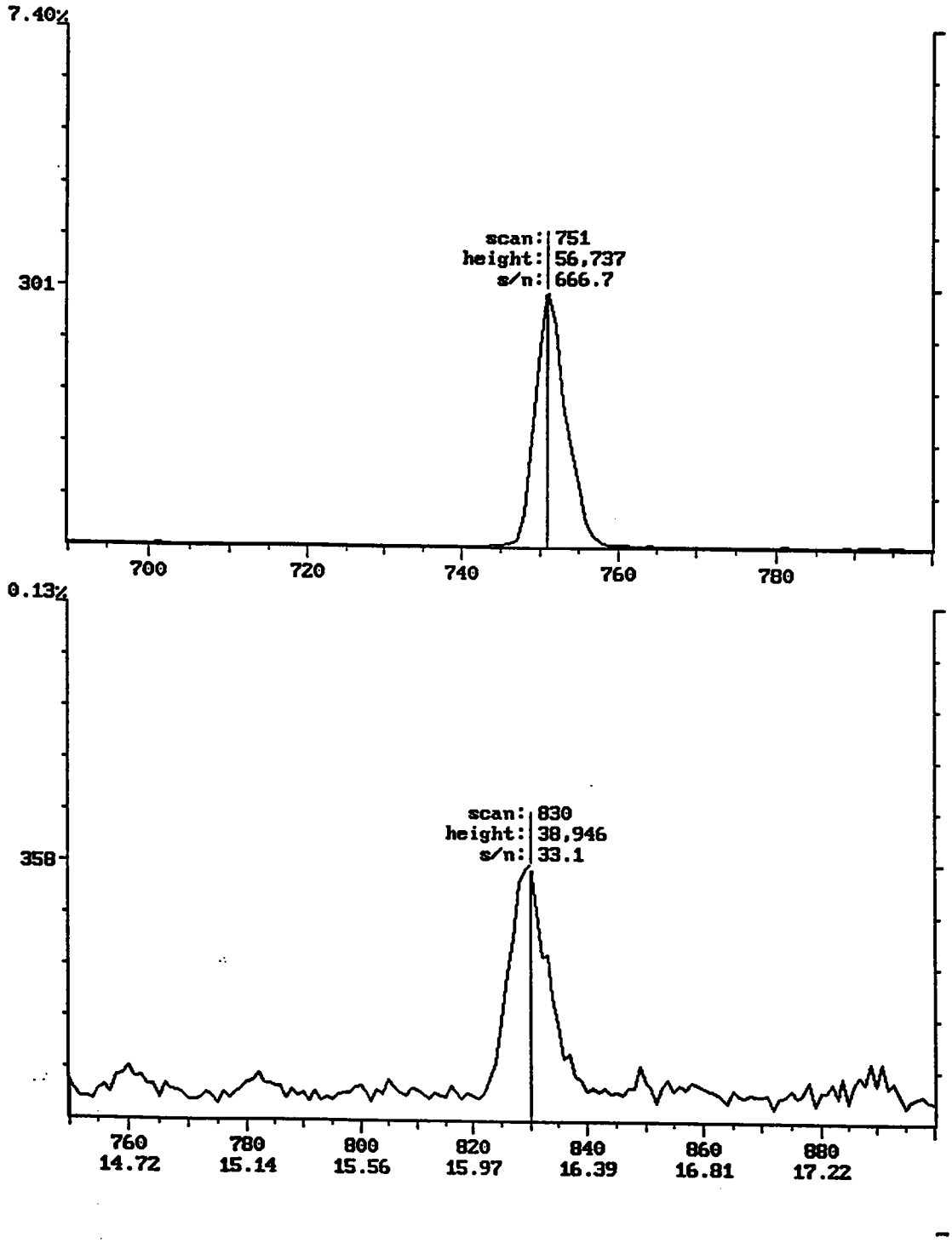
-100%
150
200
250
300
350
400
450
Mass

Figure 4.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740
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780
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height: 38,946
s/n: 33.1
760
14.72
780
15.14
800
15.56
820
15.97
840
16.39
860
16.81
880
17.22

Figure 5.