Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (9)

W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck-Engelke
(Editors)

Sport und Buch Strauß, Köln, 2001

J. MARCOS, J.A. PASCUAL, X. DE LA TORRE, J. SEGURA:
Fast and Comprehensive Screening of Anabolic Agents and other Compounds in Human Urine by GC/MS/MS
In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (9). Sport und Buch Strauß, Köln, (2001) 99-106

J. Marcos[1], J.A. Pascual*[1,2], X. de la Torre [1,3] and J. Segura[1,2]

Fast and Comprehensive Screening of Anabolic Agents and other Compounds in Human Urine by GC/MS/MS.

[1] Pharmacology Research Unit, Institut Municipal d'Investigació Mèdica. IMIM. Dr. Aiguader, 80, 08003-Barcelona, Spain.

[2] Dept. of Experimental and Health Sciences, Universitat Pompeu Fabra. Dr. Aiguader, 80, 08003-Barcelona, Spain

[3] Autonomous University of Barcelona, UAB, Barcelona (Spain)

Introduction

Since the introduction of *"high sensitivity analysis"* for some anabolic agents by the IOC in 1996, some changes were implemented in the analysis for anabolic agents. For screening purposes, the main change lied in the use of more sensitive instruments, like high resolution mass spectrometers (GC/HRMS) and, in a similar extent, cheaper instruments able to do tandem mass spectrometry (GC/MS/MS) through the ion trap technology.

As a starting point, a completely new screening for just a few compounds was implemented. Later, some attempts have been made to extend its use so that it could in the end replace the previous screening methods (1-4).

On the other hand, endogenous steroids are becoming screened by IRMS given its ability to draw definitive conclusions. These circumstances allow a change in the analytical strategy regarding the traditional screening for anabolic agents allowing it to be faster and more sensitive.

The aim of this work was the development of a fast, sensitive and comprehensive method for the screening of anabolic steroids (free and conjugated) and β_2-agonists, as well as some narcotics, stimulants and diuretics, using GC/MS/MS with an ion trap mass spectrometer. The routine sample preparation procedure already used for the classical screening of steroids was applied so that extracts could be analysed by re-injection with the present procedure.

Experimental

Sample preparation

Screening of anabolic androgenic steroids and their metabolites includes the analysis of conjugated (as glucuronides) and unconjugated excreted steroids, the so-called total fraction. Briefly, 25 µl I.S. solution was added to 2.5 ml of urine. The urine was passed through an XAD-2 column. The column was washed with 2 ml of de-ionised water and eluted with 2 ml of methanol. After evaporation to dryness (under nitrogen stream), the extract was reconstituted in 1 ml of 0.2 M phosphate buffer, pH 7.2 and hydrolysed using 30 µl of β-glucuronidase from *Escherichia coli* . The mixture was incubated at 55°C for 1 h in a water bath. The hydrolysate was left to cool at room temperature and 250 µl of potassium carbonate solution, pH 9 were added. The sample was extracted with 5 ml of tert-butylmethyl ether by rocking mixing for 20 min, and centrifuged for 5 min at approximately 3500 rpm. The organic phase was separated and evaporated to dryness. The extract was reconstituted and derivatised with 50 µl of MSTFA/NH_4I/mercaptoethanol (1000:2:6 v/w/v) for 20 min at 60°C.

GC-MS/MS instrument and conditions

A GC Top 8000 gas chromatograph coupled with a GCQ, and equipped with an A200S GC autosampler (Finnigan MAT, San Jose, USA) was used to carry out all experiments. The whole system was controlled by Excalibur software (revision 1.1). An HP Ultra 1 cross-linked methyl silicone capillary column was used; length 25 m, I.D. 0.2 mm, film thickness 0,11 µm, helium carrier gas at a constant pressure of 204 kPa. A 3 µl aliquot of the derivatised extract was injected into the system, which was operated in split (1:10) mode. The GC temperature was ramped as follows: initial 180°C to 310°C at 25°C/min, hold 2.3 min (total run time 7.4 min). The injector, transfer line, and ion source temperatures were 280°C, 280°C and 225°C, respectively.

Results and discussion

GC conditions were chosen to minimize time of analysis allowing distributing all analytes in

acquisition groups containing 5 or less parent ions. This limitation was established to ensure that an adequate number of data points were collected in order to fully define the chromatographic peaks. The final oven program enables the detection 38 exogenous anabolic steroids, 4 β_2-agonists, 4 stimulants, 2 diuretics, 1 narcotic and other forbidden agents as probenecid and cannabis in less than 7.4 minutes. Tables 1 and 2 lists all the compounds included in the screening together with their assigned abbreviation. Co-elutions of different analytes didn't significantly affect detection capability because of the selectivity obtained by working in selected reaction monitoring (SRM) mode.

Regarding sensitivity, one of the main goals of the method was detecting all those compounds at the concentration required by the IOC (i.e 2 ng/ml for 5 anabolic agents, 10 ng/ml for the rest of anabolic agents, 15 ng/ml for THC-m1 and 100 ng/ml for stimulants, diuretics, salbutamol and probenecid. For morphine, a detection limit of 150 ng/ml (NIDA regulations) was chose rather than 1 μg/ml required by IOC.

Conditions were optimised for each individual compound in order to reach those low conditions required. Candidates for precursor and product ions were selected by studying pure reference materials in full scan mode under different collision energies. Then, final conditions were chosen by analysing extracts from urines spiked at 5 times the limit of detection required. When pure reference material was not available, urine obtained from controlled excretion studies was used instead. From the conditions studied, those with the maximum signal to noise ratio were chosen rather than absolute response. Three different blank urine samples were used to estimate the noise (biological and instrumental) in each condition and for each compound. Figure 1 shows the optimisation curves for norandrosterone, as an example of the process. The transition m/z 405 $\rightarrow$ m/z=225 was chosen in that case because of its favourable S/N ratio in the presence of urine matrix.

Apart from the optimised ion selected, when possible, 2 more product ions were acquired in order to improve the identification power. Moreover, in some cases, more than one metabolite was also monitored.

Table 1. Gas chromatographic and mass spectrometric data of exogenous anabolic steroids and/or their metabolites.

PRECURSOR	EXCRETED SUBSTANCE	ABBR.	RRT[a]	Precursor ION (m/z)	Monitored IONS[b] (m/z)	C.E.[c] (V)
Bolasterone	7α,17α-dimethyl-5β-androstane-3α,17β-diol	BOL-m1	0.954	374	**284**, 374, 269	0.9
Boldenone	Boldenone	BOD	0.928	206	**206**	0.0
	5βα-androst-1-en-17β-ol-3-one	BOD-m1	0.789	417	**327**, 237, 417	1.2
	5β-androstan-1-en-3α-ol-17-one	BOD-m2	0.837	417	**275**, 185	1.0
Calusterone	7β,17α-dimethyl-5α-androstane-3α,17β-diol	CAL-m1	0.933	374	**284**, 374, 269	1.0
Clostebol	4-chloroandrost-4-en-3α-ol-17-one	CLO-m1	0.950	451	**415**,451, 361	1.1
	4-chloro-5ζ-androstan-3α-ol-17-one	CLO-m2	0.990	470, 468	**453**, 455, 468	1.0
Clormetandienone	4-chloro-17α-methylandrosta-1,4-dien-6β,17β-diol-3-one	CMD-m1	1.162	317, 315	**241**, 227, 315	1.4
Danazol	Ethisterone (17α-ethynyl-4-androsten-17β-ol-3-one)	DAN-m1	1.008	456	**441**, 456, 351	1.2
	2ξ-hydroxymethylethisterone (17α-ethynyl-2ξ-hydroxymethyl-4-androsten-17β-ol-3-one)	DAN-m2	1.124	558	**468**, 543, 453	1.5
Drostanolone	2α-methyl-5α-androstan-3α-ol-17-one	DRO-m1	0.859	433	**253**, 343, 433	1.1
Fluoxymesterone	Fluoxymesterone	FLU	1.109	552	**462**, 552, 447	1.1
	9α-fluoro-17α-methylandrost-4-ene-3α,6β,11β,17β-tetraol	FLU-m1	1.038	552	**462**, 416, 286	1.0
	9α-fluoro-18-nor-17,17-dimethylandrosta-4,13-dien-11β-ol -3-one	FLU-m2	0.893	462	**357**, 372, 337	1.0
Furazabol	Furazabol	FUR	1.114	387	**297**, 387	1.0
	16β-hydroxyfurazabol	FUR-m1	1.282	490	**231**, 490, 143	1.0
Mesterolone	1α-methyl-5α-androstan-3α-ol-17-one	MES-m1	0.899	433	**253**, 343, 433	1.0
Metandienone	6β-hydroxymetandienone	MED-m1	1.071	517	**337**, 517, 317	1.5
	17α-epimetandienone	MED-m2	0.920	444	**339**, 444, 206	1.2
	17β-methyl-5β-androst-1-ene-3α,17α-diol	MED-m3	0.789	358	301, 343, 358	1.1
Methenolone	Methenolone	MTN	0.962	446	**446**	0.0
	1-methylen-5α-androstan-3α-ol-17-one	MTN-m1	0.880	431	**251**	1.1
	1-methyl-5α-androst-1-en-16z-ol-3,17-dione	MTN-m2	1.095	517	**427**, 337, 517	1.5
Methyltestosterone	17α-methyl-5α-androstane-3α,17β-diol	MET-m1	0.901	435	**255**, 345	1.3
	17α-methyl-5β-androstane-3α,17β-diol	MET-m2	0.901	435	**255**, 345	1.3
Mibolerone	Mibolerone	MIB	0.975	446	**431**, 446, 301	1.0
Nandrolone	Norandrosterone (5α-estran-3α-ol-17-one)	NAN-m1	0.779	405	**225**, 315, 405	1.1
	Noretiocholanolone (5β-estran-3α-ol-17-one)	NAN-m2	0.813	405	**225**, 315, 405	1.2
Norethandrolone	17α-ethyl-5β-estrane-3α,17β-diol	NOE-m1	0.952	421	**331**, 421, 241	0.9
	17α-ethyl-5β-estrane-3α,17β,20-triol	NOE-m2	1.044	421	**331**, 421	1.0
Oxandrolone	Oxandrolone	OXA	1.048	363	**213**, 273, 363	0.8
	17α-epioxandrolone	OXA-m1	0.960	363	**213**, 273, 363	0.8
Oxymesterone	Oxymesterone	OXM	1.118	534	**389**, 444, 534	1.4
Oxymetholone	2ξ-hydroxymethylene-17α-methyl-5α-androstane-3ξ,(4/6)ξ,17β-triol	OXY-m1	1.143	550	**370**, 460, 550	1.0
	2ξ-hydroxymethyl-17α-methyl-5α-androstan-3ξ,17β-diol	OXY-m2	1.074	462	**267**, 282, 462	1.3
Stanozolol	3'-hydroxystanozolol	STA-m1	1.295	545	**455**, 545	1.7
	4β-hydroxystanozolol	STA-m2	1.303	560	**560**	0.0
Trenbolone	17-dehydroepitrenbolone	ETB-a1	0.905	307	**275**, 293, 307	1.1

a :Relative retention times to methyltestosterone (RT: 4.77 min) obtained under the conditions of the method.
b : Bold m/z value corresponds to the optimised ion.
c : collision energy (volts)

Table 2. Gas chromatographic and mass spectrometric data of non-steroidal anabolic agents and other substances and/or metabolites detected.

PRECURSOR	EXCRETED SUBSTANCE	ABBR.	RRT[a]	Precursor ION (m/z)	Monitored IONS[b] (m/z)	C.E. (V)
Buprenorphine	Buprenorphine	BUP	1.522	554	**450**, 522, 554	1.4
	Nor-buprenorphine	BUP-m1	1.374	572	**468**, 482, 540	1.0
Ethamivan	Ethamivan	ETH	0.451	294	**264**, 294, 249	1.0
Morphine	Morphine	MOR	0.825	429	**324**, 401, 429	1.2
Pemoline	Pemoline	PEM	0.469	392	**205**, 301, 392	1.0
Bambuterol	Bambuterol	BAM	0.857	354	**309**, 354	1.1
Clenbuterol	Clenbuterol	CLE	0.535	335, 337	**300**, 335, 337	1.2
Salbutamol	Salbutamol	SAL	0.483	369	**207**, 191, 369	1.1
Terbutaline	Terbutaline	TER	0.434	356	**281**, 356, 267	1.1
Canrenone	Canrenone	CAN	1.265	412	**412**	0.2
Triamterene	Triamterene	TRI	1.013	454	**340**, 324, 454	2.3
THC	11-nor-9-carboxy-Δ^9-tetrahydrocannabinol	THC-m1	0.975	371	**289**, 305, 371	1.0
Probenecid	Probenecid	PRO	0.655	328	**257**, 193, 328	1.0
INTERNAL STANDARD: Methyltestosterone		I.S.	1.000	446	**356**, 431, 301	1.2

a :Relative retention times to methyltestosterone (RT: 4.77 min) obtained under the conditions of the method.
b : Bold m/z value corresponds to the optimised ion.
c : collision energy (volts)

Regarding the number of "microscans", this parameter was fixed depending on the number of precursor ions in the acquisition group. For groups containing only one precursor ion, 3 microscans were acquired, for those containing 2 precursors 2 microscans, and for those containing 3, 4 or 5 precursors only 1 microscan was acquired. Under this conditions, at least 5 data points were collected to define each peak.

Ion trap mass analysers working in MS/MS mode were shown to be a very useful and simple tool for the fast screening of many simultaneous compounds. In this work, a comprehensive screening was developed for exogenous anabolic agents, complying with the specific "high sensitivity" requirements for some of the compounds (e.g. 2 ng/ml for norandrosterone, epimethendiol, etc.) but also speeding up the analysis thanks to the selectivity offered by the MS^n system. As in previous analytical strategies, other convenient compounds, apart from anabolic agents, were also included in the screening (see table 2).

The method takes profit of the new capabilities incorporated like the unlimited number of precursor ions simultaneously monitored, for example. Using al these features and after optimisation for each individual compound with respect to the chemical background of blank urines, the method developed is able to detect more than 50 compounds (see tables 1 and 2) in

less than 7.4 minutes. Figure 2 shows an example of print-out of the analysis of different compounds.

The continuous use of the method showed good robustness and suitability for routine use in doping control.

Bibliography

1. Schänzer W, Delahaut P, Geyer H, Machnik M, Horning J. *J. Chromatogr. B.* 1996; 687: 93.
2. Trout G, Soo S, Kazlauskas R. in *Recent Advances in Doping Analysis (7).* p. 249 .
3. De Brabander HF, Batjoens P, Courtheyn D, Vercammen J, De Wasch. *J. Chromatogr. A.* 1996; 750: 105.
4. Muñoz-Guerra J, Carreras D, Soriano C, Rodriguez C, Rodriguez AF. *J. Chromatogr. B.* 1997; 704: 129.

Acknowledgement

This work was partially funded by grant SAF97-0068 from the Comisión Interministerial de Ciencia Tecnologia (CICYT), Spain.

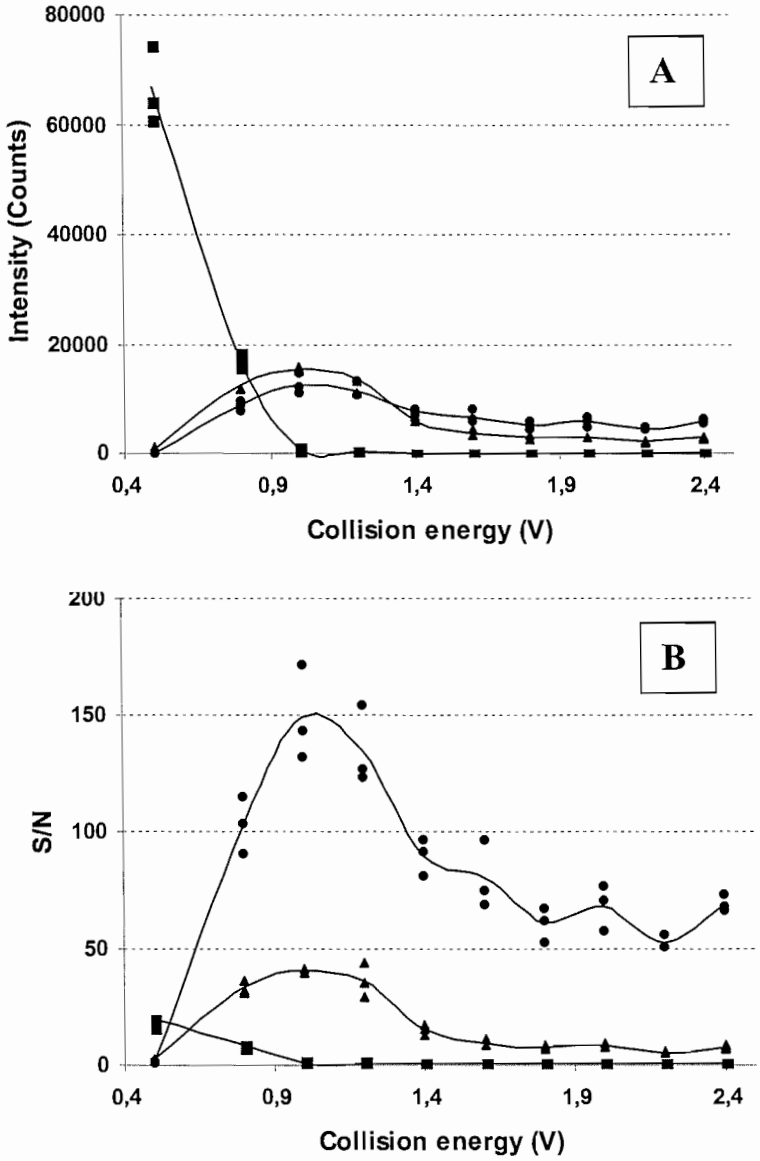

Figure 1. Effect of collision energy (V) on precursor and product ion abundances (A) or signal to noise (S/N) ratio (B) for norandrosterone (as bis-O-TMS derivative). Precursor ion: ■, m/z 405. Product ions: ●, m/z 225; ▲, m/z 315.

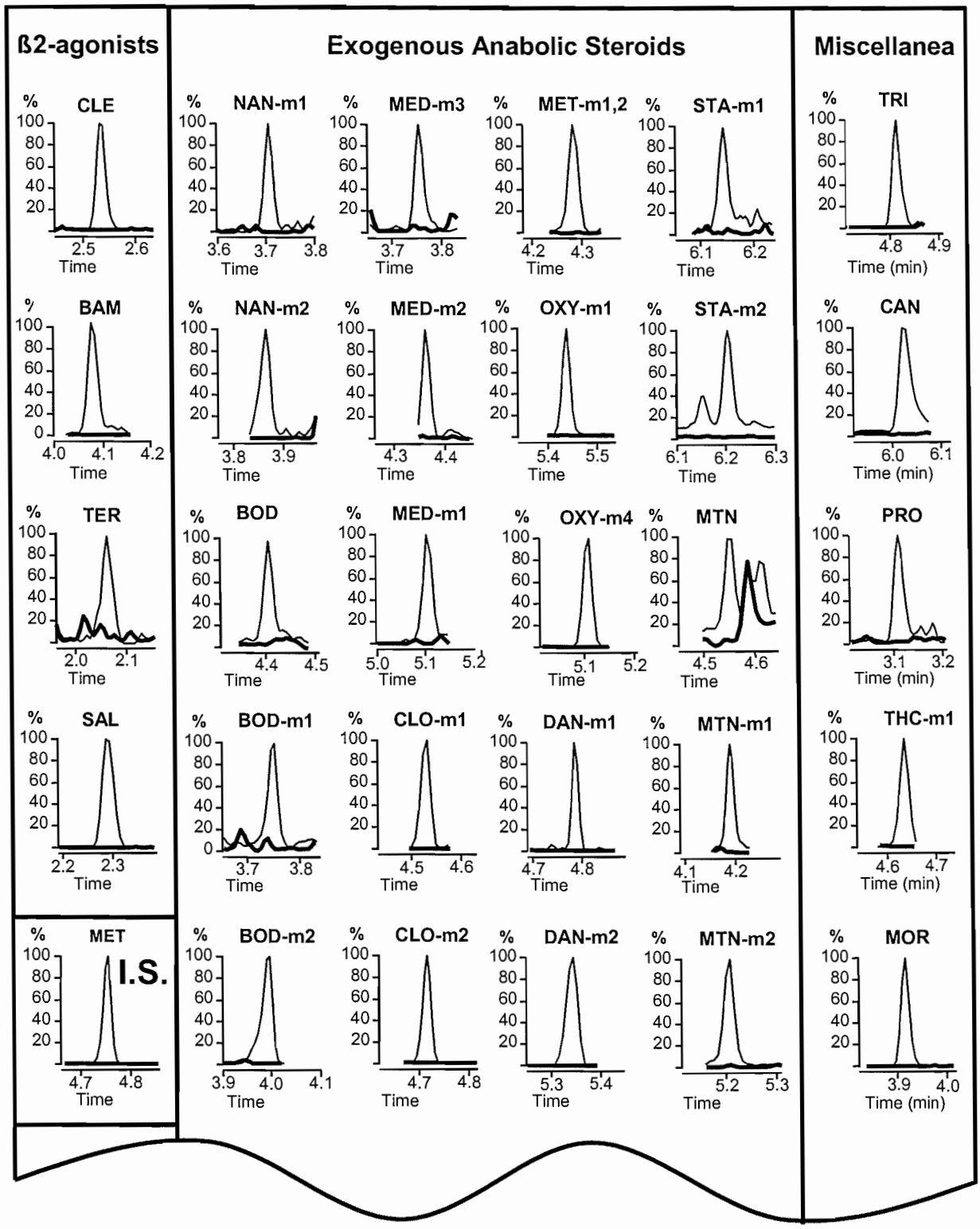

Figure 2. Example of part of the print-out of results obtained after the analysis of urines containing the compounds of interest at the limits of detection required by IOC. Excretion studies were used for those compounds no commercially available. Bold line corresponds to the analysis of a blank urine.