Journal of Chromatography B, 897 (2012) 85–89

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Sensitive and robust method for anabolic agents in human urine by gas chromatography–triple quadrupole mass spectrometry

Miguel A. Delgadillo[a], Lorena Garrostas[b], Óscar J. Pozo[b], Rosa Ventura[b,c], Benjamín Velasco[a,d], Jordi Segura[b,c], Josep Marcos[b,c,*]

[a] National Laboratory of Prevention and Doping Control Mexico, Comisión Nacional de Cultura Física y Deporte, Camino a Santa Teresa # 482, Colonia Peña Pobre, Delegación Tlalpan C.P. 14060 México D.F., Mexico
[b] Bioanalysis Research Group, IMIM, Hospital del Mar, Doctor Aiguader 88, 08003 Barcelona, Spain
[c] Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Doctor Aiguader 88, 08003 Barcelona, Spain
[d] Department of Chemistry, Faculty of Superior Studies Cuautitlán, Field 1, Autonomous National University of Mexico, Av. May 1st s/n, Z.C. 54740, Cuautitlan Izcalli, State of Mexico, Mexico

ARTICLE INFO

Article history:
Received 10 February 2012
Accepted 25 March 2012
Available online 1 April 2012

Keywords:
Doping
Gas chromatography/tandem mass spectrometry
Human urine
Triple quadrupole
Anabolic agents

ABSTRACT

A rapid, sensitive and robust gas chromatography–triple quadrupole mass spectrometry method was developed for the determination of seven anabolic agents in human urine. The selection of analytes includes the main metabolites of all anabolics with higher sensitivity requirements. After optimizing the fragmentation conditions for each compound, a validation procedure for qualitative analysis was performed. The selectivity of the method showed that no interfering peaks were observed at the retention time of the compound. Adequate intermediate precision, below 14%, was observed for all of the compounds at the lower concentration tested. The concentrations assayed were in accordance with the performance limits required by the World Anti-Doping Agency (WADA). Unlike a previously published GC/QqQ method, detection of 17α-methyl-5β-androstane-3α,17β-diol (the main metabolites of methyltestosterone) at 2 ng/mL was accomplished under routine conditions. The qualitative method was applied to the analysis of 1367 samples in the span of 2 weeks, as part of the doping control of the XVI Pan American Games which took place in Mexico (14th–30th October, 2011). The high sensitivity was maintained during the analysis of all analytical batches, proving for the first time the excellent ruggedness of GC/QqQ methods.

1. Introduction

The use of performance-enhancing drugs in sports events is controlled by the World Anti-Doping Agency (WADA), which publishes a list of prohibited substances every year, and establishes a minimum required performance limit (MRPL) for each prohibited compound [1,2]. Within the different classes of prohibited drugs, the anabolic agents are the most frequent category of prohibited substances detected in the urine of athletes both globally and at the Olympic Games [3,4]. Detection of this family of compounds is a difficult analytical challenge owing to the large number of anabolic androgenic steroids and their metabolites, the similarity of exogenous and endogenous steroid structures and the low concentrations found in urine [5,6].

Given that WADA-accredited laboratories are required to demonstrate that they can routinely perform analyses at the MRPL, each laboratory has developed its own strategy to cope with the long list of prohibited substances. For low molecular mass substances, the majority of screening methods are currently based on hyphenated chromatographic mass spectrometric methods, which essentially mean the use of GC coupled to single quadrupoles, and LC coupled to triple quadrupoles [7].

Anabolic steroids are typically monitored by using GC/MS instruments in selected ion mode (SIM). In this way, it is possible to achieve both the qualitative determination of the majority of exogenous steroids with limits of detection in the range of 2–10 ng/mL, and the quantification of the endogenous steroid profile.

Re-analysis of the GC/MS extracts in a more sensitive method is still performed by some laboratories. The historical reasons for this strategy have to do with the imposition of a 2 ng/mL detection limit for five anabolic compounds in 1998 [8]. Back then, the use triple quadrupoles in doping control was still limited, thus researchers developed complementary new methods based on GC/HRMS

* Corresponding author at: Bioanalysis Research Group, IMIM, Hospital del Mar, Doctor Aiguader 88, 08003 Barcelona, Spain. Tel.: +34 933160472; fax: +34 933160499.
E-mail address: jmarcos@imim.es (J. Marcos).

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http://dx.doi.org/10.1016/j.jchromb.2012.03.037

(magnetic sector high-resolution mass spectrometers) [9,10], and GC/ion trap detectors for a selected number of doping agents [11,12].

Since then, the presence of triple quadrupoles coupled to LC instruments has become common in the sport drug testing laboratories, and the number of substances that are routinely monitored with this technique has increased drastically [13–15], including some of the compounds which have to be detected at 2 ng/mL like clenbuterol [16], and 3′-hydroxystanozol [17]. However, some steroid metabolites are poorly detectable using LC–MS/MS with electrospray ionization (ESI) due to the lack of any easy ionizable moiety.

Although triple quadrupole GC/MS/MS systems have been available for a while, its use in the doping testing field is just starting out. A few applications are available for the detection of esters of steroids and metandienone metabolites in hair [18,19], and for the elucidation of the Δ6-methyltestosterone metabolism [20]. More recently, Van Eenoo et al. presented a comprehensive method for the detection of 150 doping agents in less than 8 min proving that, in addition to an improvement in the qualitative detection of exogenous compounds, GC/QqQ methods allow the adequate quantitative determination of the steroid profile [21]. Nevertheless, the limit of detection achieved for the main metabolite of methyltestosterone, i.e. 17α-methyl-5β-androstane-3α,17β-diol, was above the mandatory MRPL (2 ng/mL).

In the present work, the use of a complementary GC/QqQ qualitative method for the detection of a selected number of anabolic agents with special sensitivity requirements (2 ng/mL) including 17α-methyl-5β-androstane-3α,17β-diol was investigated.

The robustness of the method has been tested by analyzing all the samples collected for the doping control at the XVI Pan American Games.

2. Experimental

2.1. Standards and reagents

Methyltestosterone used as internal standard, and clenbuterol were from Sigma (St. Louis, MO, US). 17α-methyl-5β-androstane-3α,17β-diol, 3α-hydroxystanozolol, 16α-hydroxyfurazabol, 17β-methyl-5β-androst-1-ene-3α,17α-diol, 19-norandrosterone and 19-noretiocholanolone were purchased form NMI (Pymble, Australia). The β-glucuronidase preparation (type *E. Coli K12*) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Analytical grade di-sodium hydrogen phosphate, sodium hydrogen phosphate, tert-butylmethylether, sodium hydroxide, ammonium iodide, ethanethiol and methanol were obtained from Merck (Darmstadt, Germany). N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSFTA) was from Macherey-Nagel (Germany). Milli Q water was obtained using a Millipore Milli-Q Integral A 10 purification system.

2.2. Instruments

The study was performed on an Agilent 7890A gas chromatograph equipped with a 7693 autosampler, a split/splitless capillary inlet and an Agilent 7000A Series Triple Quadrupole GC/MS, using the selected reaction monitoring acquisition mode. The whole system was controlled by MassHunter workstation software (revision B.04.00). The GC was equipped with a capillary column (HP-Ultra 1, 25 m × 0.2 mm id with a 0.11 μm film thickness) from J&W (Agilent Technologies, USA). The oven temperature was programmed as follows: the initial temperature was 185 °C, increased at 30 °C/min to 230 °C, then at 15 °C/min to 290 °C, then at 70 °C to 310 °C, and maintained at the final temperature for 1.5 min. The transfer line was kept at 280 °C. Helium was used as carrier gas at a constant flow rate of 1 mL/min. Two microliters of the final extract were injected in split mode (split ratio 1/10).

Nitrogen was used as collision gas at a flow rate of 1.5 mL/min, and helium as a quenching gas at a flow rate of 2.25 mL/min. The electron impact source was kept at 220 °C and the quadrupoles at 180 °C.

2.3. Sample preparation

Urine samples (2.5 mL) were added with 25 μL of an internal standard solution containing methyltestosterone at 50 μg/mL, 1 mL of phosphate buffer solution (1 M, pH 7) and 25 μL of β-glucuronidase, and incubated for 1 h at 55 C. After cooling to room temperature, pH was increased to approximately 9.5 by the addition of 135 μL of NaOH (5 M). Subsequently samples were extracted with 5 mL of tert-butylmethylether and centrifuged. Finally the organic layer was evaporated, and derivatized with 50 μl of MSTFA-NH_4I-ethanethiol (100:1:2, v/w/v) by heating at 60 °C for 30 min. Those extracts were first analyzed by a GC/MS method which allows for the quantitation of endogenous steroids, and for the qualitative detection of many anabolic agents. Afterwards, the vials were moved to the GC/QqQ autosampler.

2.4. Validation

Validation protocols for qualitative methods are less demanding than those for quantitative measurements. In general, only the assessment of selectivity and limit of detection are required. Other parameters are nevertheless essential for the full characterization of the method, such as repeatability and reproducibility particularly at the minimum levels required [22,23].

Selectivity was evaluated by analyzing a set of different blank urine samples and monitoring the absence of interferences with signal-to-noise (S/N) ratios higher than 3. For this purpose, ten urine samples from different volunteers not taking any forbidden substance (5 males: age 28.2 ±9.2 years, 5 females: age 25.9 ± 3.4 years) were collected early in the morning and analyzed. Limits of detection were estimated based on the (S/N) ratios obtained at the lower concentration tested.

Repeatability was evaluated at two different concentration levels. A set of quality control samples containing the analytes of interest (six replicates each) were analyzed using the method described. Reproducibility was evaluated at the MRPL for each analyte using the 82 control samples analyzed in two different instruments during the two weeks of competition of the XVI Pan American Games. Reproducibility and repeatability were expressed as the relative standard deviation of the peak areas ratios.

The extraction recovery of each target compound was evaluated by analyzing six samples representing 100% extraction recovery. To prepare these samples, six different urine extracts were spiked at the high concentration level assayed in the repeatability experiments. The addition was made after the liquid–liquid extraction, just before the evaporation and derivatization steps. The extraction recovery was calculated by comparing the peak areas ratios (analyte versus internal standard) with and without extraction.

3. Results and discussion

3.1. Method development

Optimization of the mass spectrometric conditions was performed following a two-step process. In the first step, a full-scan spectrum was obtained for each of the derivatized standard. Product-ion spectra at five different collision energies were then obtained for the main precursor ions of the full-scan spectra.

Table 1
Triple quadrupole GC/QqQ acquisition parameters.

Compound	Derivative	RT (min)	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	CE (V)
Methyltestosterone (ISTD)	Bis-O-TMS	5.00	446	301	20
		5.00	446	169	30
Clenbuterol	N-TMS,O-TMS	2.45	335	300	10
		2.45	335	227	15
19-Norandrosterone (metabolite of nandrolone)	Bis-O-TMS	3.68	405	315	15
		3.68	405	169	20
19-Noretiocholanolone (metabolite of nandrolone)	Bis-O-TMS	3.85	405	315	15
		3.85	405	169	20
17β-Methyl-5β-androst-1-ene-3α,17α-diol (metabolite of methandienone)	Bis-O-TMS	3.73	358	301	20
		3.73	358	196	5
17α-Methyl-5β-androstane-3α,17β-diol (metabolite of methyltestosterone)	Bis-O-TMS	4.38	435	345	10
		4.38	435	255	20
3α-Hydroxystanozolol (metabolite of stanozolol)	Bis-O-TMS,N-TMS	6.65	545	455	40
		6.65	545	147	30
16α-Hydroxyfurazabol (metabolite of furazabol)	Mono-O-TMS	6.57	490	231	15
		6.57	490	143	35

Through these studies, candidates were chosen for both precursor and product ions in the selected reaction monitoring (SRM) method. Once all transitions had been chosen for the SRM mode, the final optimization process was carried out by analyzing extracts from blank urine samples and also from spiked urine samples prepared at a concentration five times higher than the MRPL. Each potential precursor ion was fragmented again using 5 different collision energies bracketing the best value obtained from the previous experiment. The final conditions selected were those showing the maximum signal/noise ratio (S/N) rather than absolute response since matrix and endogenous steroidal background may have a significant influence. In order to improve the overall identification power of the method; two transitions per compound were optimized. Table 1 summarizes the results on the optimum conditions found for each compound.

3.2. Method performance

Results obtained for repeatability were below 19% at the two different concentration levels tested, proving the adequate intra-assay precision of the method. Table 2 lists the two levels of assayed concentrations, together with the RSD obtained for the seven analytes.

The RSD values obtained for reproducibility were between 11 and 25%, proving the robustness of the method even when using two different instruments. The recovery values obtained for each compound were between 60 and 120% for all compounds (Table 2).

Regarding selectivity, in general no significant interferences were observed in any of the two transitions monitored for each compound. Fig. 1 represents two chromatograms in each window, blank urine and a spiked control sample at a concentration below the MRPL.

Regarding limits of detection, according to the S/N ratios obtained, they were below those explicitly required (Table 2).

3.3. Application of routine real samples

During the XVI Pan American Games, 1367 urine samples collected from athletes were analyzed at the Comisión Nacional de Cultura Física y Deporte (CONADE) premises in Mexico City in a temporary WADA-accredited facility.

All these samples were analyzed employing the described method as part of the strategy for screening all banned drugs. Each analytical batch included a negative control, and a positive control containing all analytes at their respective MRPL.

As described in Section 2, two transitions were acquired for each substance. For all compounds the ratio of the areas of the two transitions, used as identification criteria, remained constant (RSD below 9% for all compounds) throughout the series of 82 analytical batches. More importantly, the S/N for the characteristic transitions corresponding to the compounds present in the positive control remained stable during the whole period.

The superior performance in terms of sensitivity, together with the adequate quantitation accomplished by GC/QqQ instruments might lead to the substitution of GC/MS single quadrupole instruments by triple quadrupoles in doping control laboratories.

Table 2
Performance results: limits of detection, repeatability and recovery ($n = 6$), and reproducibility ($n = 82$) obtained at the MRPL required by WADA.

Compound	Recovery (%)	LOD (ng/mL)	Repeatability				Reproducibility	
			ng/mL	RSD (%)	ng/mL	RSD (%)	ng/mL	RSD (%)
Clenbuterol	92.8	0.01	0.1	4.9	1	6.5	2	11.7
19-norandrosterone (metabolite of nandrolone)	101.3	0.3	1	10.2	10	13.9	2	23.4
19-Noretiocholanolone (metabolite of nandrolone)	98.1	0.2	1	7.8	10	14.9	10	15.9
17β-Methyl-5β-androst-1-ene-3α,17α-diol (metabolite of methandienone)	119.5	0.8	1	13.4	10	13.2	2	19.2
17α-Methyl-5β-androstane-3α,17β-diol (metabolite of methyltestosterone)	100.4	0.2	1	9.4	10	13.9	2	13.5
3α-Hydroxystanozolol (metabolite of stanozolol)	66.7	0.3	1	9.2	10	14.3	2	22.3
16α-Hydroxyfurazabol (metabolite of furazabol)	115.9	1	5	7.0	10	18.2	10	24.6

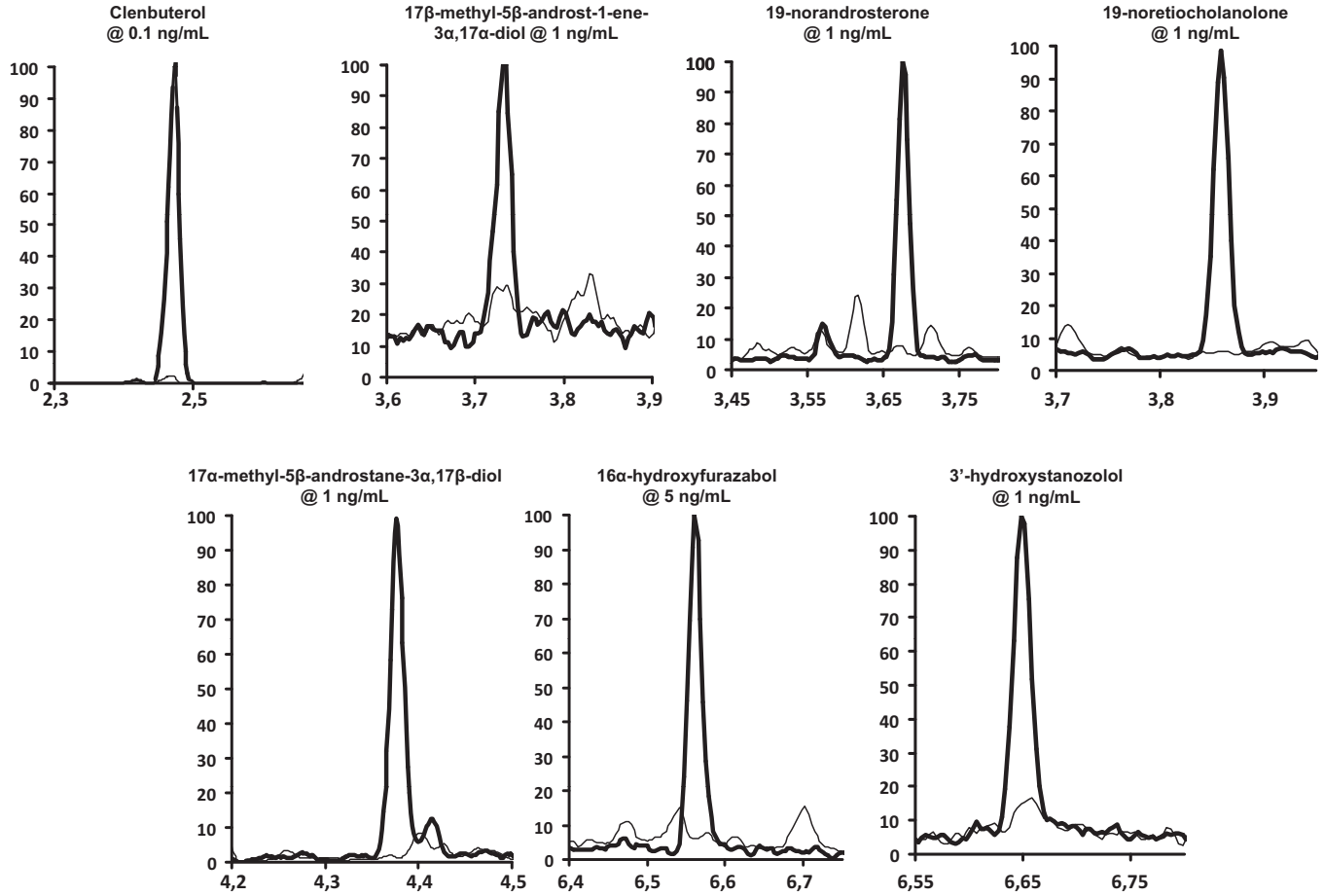

Fig. 1. Validation results: analysis of a sample spiked at low concentrations (bold line). Analysis of a negative control sample (thin line) is also given for comparison.

An already published method is a clear evidence of that fact. The only limitation that has to be noticed in that method is that the limit of detection achieved for the main metabolite of methyltestosterone was above the mandatory MRPL [21]. If not for that, that comprehensive method would make unnecessary the re-injection of the GC/MS extracts in those "higher-sensitivity" instruments that enter the laboratories in the late 1990s, i.e. GC-ion trap, or GC/HRMS.

In the present work, we have evaluated the strategy of using the GC/QqQ equipment as the complementary "higher sensitivity" tool for the qualitative determination of a selected number of anabolics only.

By using the developed method, the 2 ng/mL level for 17α-methyl-5β-androstane-3α,17β-diol was easily achieved in routine conditions. The results obtained for the rest of studied anabolic agents were also satisfactory, thus complementing the information obtained by other GC/MS and LC–MS/MS screenings.

With a final run-time of 7.3 min, and an injection volume of 2 μL, this GC/QqQ screening method fulfills two practical aspects: firstly, it is able to detect compounds which are not easily detectable by GC/MS or LC–MS/MS approaches at their MRPL. Secondly, the ruggedness of GC/QqQ methods has been proved for the first time, under the most demanding situations, i.e. when analyses have to be performed in a large number of samples, dealing with short reporting times (24 h).

4. Conclusions

In conclusion, this study presents a rapid, reproducible and robust GC/QqQ qualitative method for detection of anabolic agents in human urine, which has been developed, validated, and successfully applied to the analysis of 1367 urine samples collected during the XVI Pan American Games.

The novel procedure represents a valuable tool for screening anabolic agents with special sensitivity requirements in routine antidoping laboratories. A clear improvement compared to a previous GC/QqQ method was observed regarding the detection of low amounts of the main metabolite of methyltestosterone, 17α-methyl-5β-androstane-3α,17β-diol.

The screening method presented here may be easily extended to other compounds showing difficulties in their determination by standard GC/MS and LC/MS/MS approaches, so that a very cost-effective strategy could be implemented for doping control.

Acknowledgments

The authors thank the Comisión Nacional de Cultura Física y Deporte (CONADE), for financial support of the analysis of urine samples of XVI Pan American Games. Grant from Generalitat de Catalunya (Consell Català de l'Esport, DIUE 2009SGR492) is gratefully acknowledged.

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