A Sensitive and Reliable Method for Anabolic Agents in Human Urine on the Agilent 7000 Triple Quadrupole GC/MS

Application Note

Forensics/Drug Doping

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Abstract

A screening method for the detection of selected anabolic agents in human urine was developed on the Agilent 7890 Series GC coupled to the Agilent 7000 Series Triple Quadrupole GC/MS System [1]. After validating the compounds at the performance limits required by the World Anti-Doping Agency (WADA), the method was applied to the analysis of 1367 samples collected during the doping control of the XVI PanAmerican Games. High sensitivity was sustained during the analysis of more than 80 analytical batches, proving the outstanding ruggedness of the method.

Introduction

The misuse of drugs in sports events, in order to illicitly improve an athlete's performance, is controlled by the World Anti-Doping Agency (WADA). Once a year, WADA publishes a list of prohibited substances [2], and establishes a minimum required performance limit (MRPL) for each prohibited compound [3]. That list includes hundreds of different compounds divided into 11 different categories. In spite of all the efforts made by WADA since its foundation in 1998, anabolic agents persist as a category of drugs with an increasing prevalence of positive cases. Detection of this type of compound is a difficult analytical challenge owing to the large number of anabolic androgenic metabolites, both exogenous and endogenous, that might be found in urine samples.

Agilent Technologies

Since WADA-accredited laboratories are required to demonstrate that they can routinely screen all the forbidden substances at the MRPL, each laboratory has created a particular analytical strategy. Most of these anti-doping strategies are currently based on the use of two types of instruments: GC coupled to single quadrupole mass spectrometers (MS), and LC coupled to triple quadrupole MS.

However, this general scheme might be changing, as the 7000 Series Triple Quadrupole GC/MS System enables the sensitive detection of doping agents in SRM mode. Applications have already been developed on this instrument platform for the long term detection time of some specific steroids (methenole and dehydrochloromethyltestosterone metabolites) [4], and for the comprehensive detection of 150 doping analytes in less than 8 minutes [5].

From a practical point of view, a Triple Quadrupole GC/MS screening method has to fulfill two aspects:

1. It has to be able to detect compounds which are not detectable by other GC/MS or LC/MS/MS approaches at the MRPL, for instance, 17α-methyl-5β-androstane-3α,17β-diol (the main metabolite of methyltestosterone) at 2 ng/mL.
2. Its ruggedness has to be proven under the most demanding situations.

This application note describes a 7.3-minute screening method developed and validated on the 7000 Series Triple Quadrupole GC/MS System for the detection of seven anabolic agents [1]. The selection of analytes includes 17α-methyl-5β-androstane-3α,17β-diol, as well as other anabolic agents for which detection by triple quadrupole GC/MS/MS enhances the information obtained by classical GC/MS and LC/MS/MS approaches. The robustness of the method has been proven by the analysis of 1367 samples in the span of two weeks as part of the doping control at the XVI PanAmerican Games.

Experimental

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, epimethendiol, 19-norandrosterone and 19-noretiocholanolone were purchased from the National Measurement Institute (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, potassium hydroxide, ammonium iodide, ethanethiol and methanol were obtained from Merck (Darmstadt, Germany). N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) was obtained from *Macherey-Nagel* (Germany). Milli Q water was obtained using a Millipore Milli-Q Integral A 10 purification system.

Instruments

The study was performed on a 7890 Series GC equipped with a split/splitless capillary inlet and coupled to a 7000 Series Triple Quadrupole GC/MS System, using the Selected Reaction Monitoring acquisition mode. The instrument conditions are listed in Table 1.

Table 1. Agilent 7890/ 7000 Gas chromatograph and Mass Spectrometer Conditions

GC Run Conditions	
Analytical column	Agilent J&W HP- Ultra 1 Inert 25 m × 0.2 mm, 0.11 µm film (p/n 19091A-002)
Injection	2 µL; Split mode 1:10
Carrier gas	Helium, constant flow, 1 mL/min
Column temperature program	185 °C 30 °C/min to 230 °C 15 °C/min to 290 °C 70 °C/min to 310 °C (1.5 minutes hold)
Transfer line temperature	280 °C
Solvent delay	2 minutes
MS conditions	
Tune	Autotune
EMV Gain	20
Acquisition parameters	EI, selected reaction monitoring
Collision gas flow	N_2 collision gas: 1.5 mL/min
Quenching gas flow	Helium, 2.25 mL/min
MS temperatures	Source 220 °C; Quad 180 °C

Sample Preparation

After addition of 1 mL of phosphate buffer (1 M, pH 7), urine samples (2.5 mL) were incubated with 25 µL of β-glucuronidase preparation for 1 hour at 55 °C. After cooling to room temperature, pH was adjusted to 9.5 by addition of 135 µL of KOH (5M), and a liquid-liquid extraction was performed by addition of 5 mL tert-butylmethylether. After centrifugation, the organic layer was evaporated, derivatized with 50 µL of MSTFA-NH_4I-ethanethiol (100:2:6, v/w/v) by heating at 60 °C for 30 minutes, and transferred to the injection vial.

Acquisition Parameters

The Agilent Triple Quadrupole GC/MS system parameters used in the analysis are listed in Table 2.

Results

Method Development

Optimization of the mass spectrometric conditions was effected by a two-step process. In the first step, a full-scan spectrum was obtained for each of the derivatized standards. Product-ion spectra at five different collision energies were then obtained for the main precursor ions of the full-scan spectra. Through these studies, candidates were chosen for both precursor and product ions in the Selected Reaction Monitoring (SRM) mode. The final optimization process was then carried out by analyzing extracts from blank urine samples and spiked urine samples prepared at a concentration five times higher than the minimum required performance limit (MRPL). Each possible precursor ion was fragmented again using five different collision energies bracketing the best value obtained from the previous experiment. The final conditions selected were those showing the maximum signal-to-noise (S/N) ratio 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 2 summarizes the results for the optimum conditions found for each compound.

Table 2. Agilent 7000 Series Triple Quadrupole GC/MS/MS Acquisition Parameters

Compound	Derivative	RT (min)	Precursor ion	Product ion	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
Epimethendiol (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
3′-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

RT: Retention Time
CE: Collision Energy

Method Validation

Validation protocols for qualitative screening methods are less demanding than those for quantitative measurements [6]. 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 three. For this purpose, six urine samples from different volunteers not taking any forbidden substance were analyzed.

Repeatability was evaluated at the MRPL established for each agent by WADA. A set of quality control samples containing the analytes of interest (six replicates each) was analyzed using the method described. Repeatability is expressed as the relative standard deviation (RSD) of the ratio of the areas of the main transition for the analyte and the internal standard. Table 3 lists the results obtained.

Table 3. Method Validation Results

Compound	MRPL (ng/mL)	Concentration (ng/mL)	Repeatability (RSD, %)
Clenbuterol	2	0.1	4.9
19-norandrosterone (metabolite of nandrolone)	2	1	10.2
19-noretiocholanolone (metabolite of nandrolone)	10	5	7.8
17β-methyl-5β-androst-1-ene-3α, 17α-diol (metabolite of methandienone)	2	1	13.4
17α-methyl-5β-androstane-3α, 17β-diol (metabolite of methyltestosterone)	2	1	9.4
3'-hydroxystanozolol (metabolite of stanozolol)	2	1	9.2
16α-hydroxyfurazabol (metabolite of furazabol)	10	5	7.0

MRPL: Minimum Required Performance Limit
RSD: Relative Standard Deviation

Application to Real Samples

During the XVI PanAmerican Games, 1367 urine samples collected from athletes were analyzed at the CONADE (Mexico's National Commission for Physical Culture and Sport) premises. All samples were analyzed with the described method as part of the strategy for screening all banned drugs. Each analytical batch included a positive control spiked with the analytes at the MRPL and a negative control.

The ratio of the areas of the two transitions monitored for the each compound, used as identification criteria, remained constant (RSD below 7%) throughout the series of 82 analyzed batches. Figure 1 illustrates the sensitivity achieved for both transitions for all compounds.

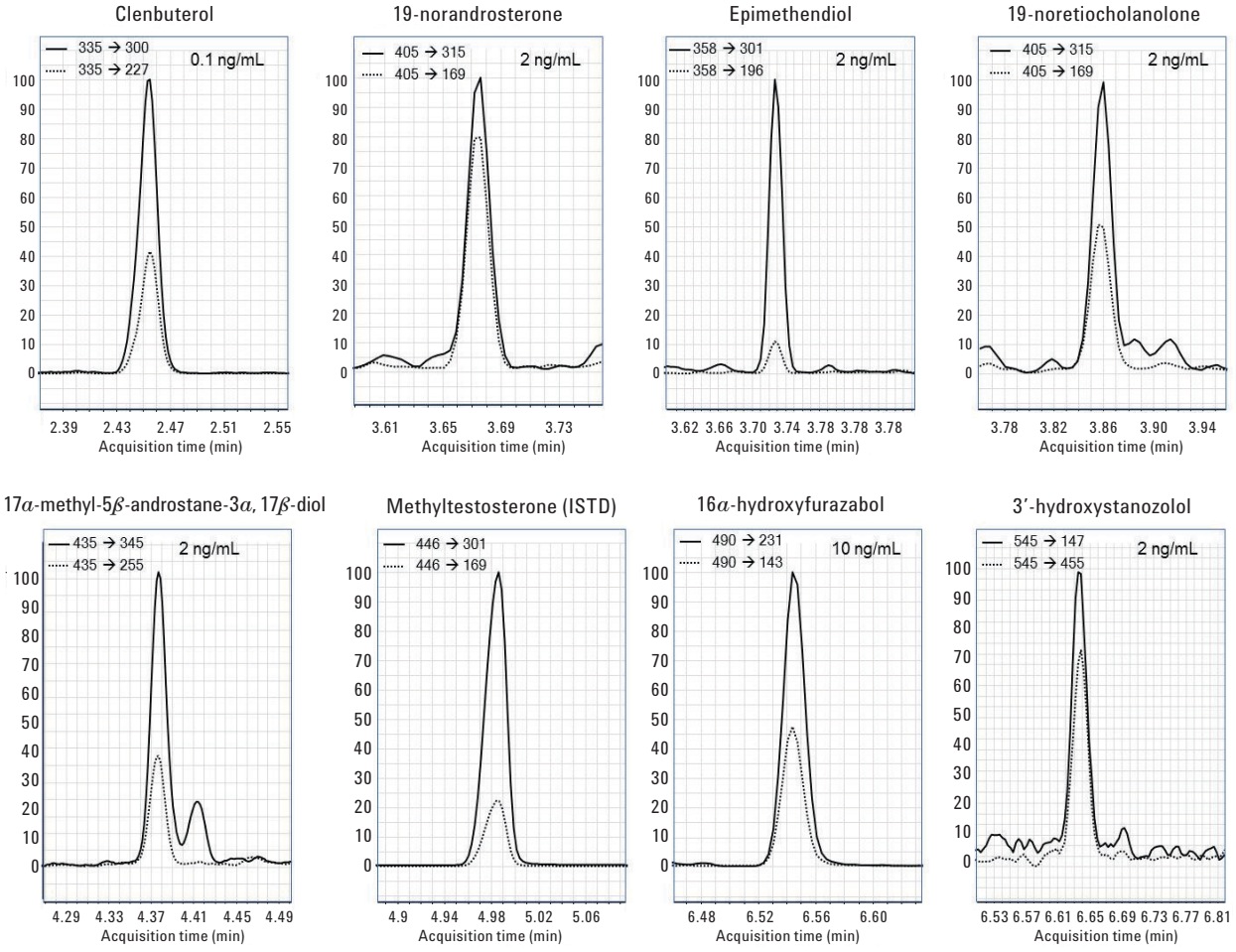

Figure 1. Representative chromatograms for the monitored transitions at the minimum required performance limits.

In addition, as shown in Figure 2, the S/N for the characteristic transition corresponding to each compound in the positive control remained stable during the entire analysis period.

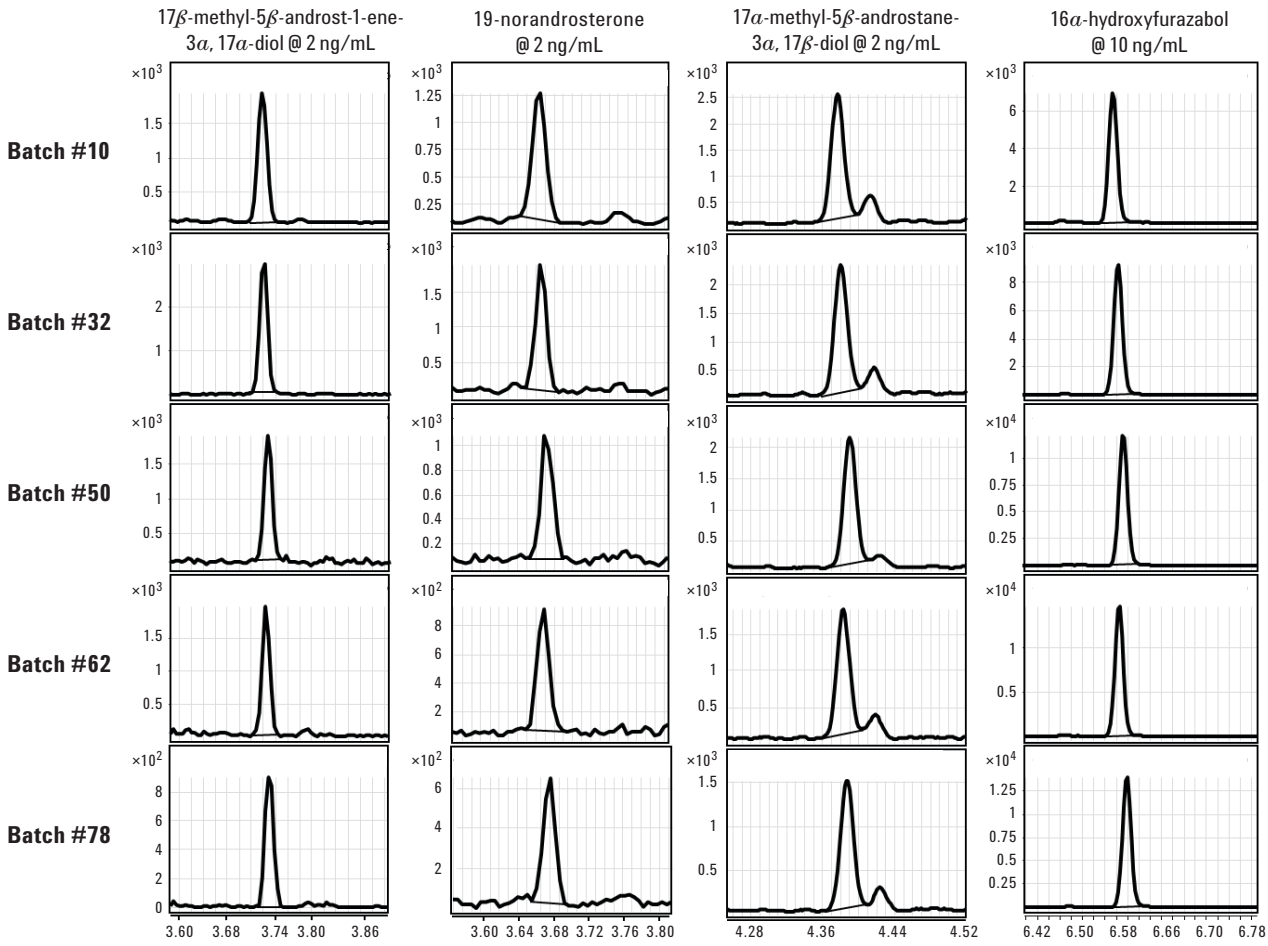

Figure 2. Stability of the S/N for the characteristic monitored transition of representative steroids present in the positive quality control in the course of the PanAmerican Games across 78 batches.

Conclusion

A fast and robust GC/MS/MS method that meets the MRPL requirements for the detection of WADA anabolic agents was developed on the 7000 Series Triple Quadrupole GC/MS System, validated, and applied to the analysis of more than 1300 samples collected during the XVI PanAmerican Games. Given that methods have also been developed on this instrument platform for the long term detection of anabolic steroids, as well as a comprehensive screening for doping agents [4,5], the 7000 Series Triple Quadrupole GC/MS System is an ideal solution for drug doping laboratories.

References

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