



# Longterm Detection of Anabolic Steroid Metabolites in Urine

## Application Note

Forensics/Doping Control

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### Abstract

A method for the longterm detection of anabolic steroid metabolites in urine was developed using both the Agilent 6890N network GC system with an Agilent 5975B Series GC/MSD and the Agilent 7000 Series Triple Quadrupole GC/MS. Monitoring the sulfate conjugates of these metabolites, instead of the routine glucuronide metabolites allowed detection of methenolone for a post-administration period that was almost twice as long. This was achieved using GC/MS in SIM mode. Performing the analysis on the Triple Quadrupole GC/MS in SRM mode extends the detection period even further, to almost three times as long as the conventional glucuronide approach.



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## Introduction

The nonmedical use of anabolic/androgenic steroids (AAS) is illegal in the US and banned by most sport organizations. However, AAS “doping” has long been a problem in some amateur and professional sports, and is becoming a growing problem in high school and collegiate athletics. The detection of stanozolol in a urine sample led to the disqualification of sprinter Ben Johnson from the 1988 Olympic Games, and the loss of his gold medal and two world records. The detection of an elevated testosterone/epitestosterone ratio in a screening test eventually led to loss of the 2006 Tour de France title by Floyd Landis, and a two-year ban.

Sophisticated violators of drug doping regulations know that AAS can be “safely” used in the “training” period, if their use is stopped long enough prior to a scheduled test to avoid detection. The timing between the last use of an AAS and urine sample collection for doping control is carefully adjusted by the violator for each drug to ensure negative test results. Retrospective detection of AAS is therefore essential to successful doping control, and doping laboratories around the world are looking for ways of detecting AAS for longer periods of time after cessation of use.

Typical doping control analysis for anabolic steroids and related substances in urine includes screening by gas chromatography/mass spectrometry (GC/MS). However, traditional analytical methods can typically detect AAS for only five days or so after administration. Detection of anabolic steroids for longer periods of time after drug use can be achieved by finding and monitoring longterm metabolites in urine [1-3], and using instrumentation capable of detecting very low concentrations of AAS.

This application note describes methods developed on both the Agilent 6890N network GC system with an Agilent 5975B Series GC/MSD and the Agilent 7000 Series Triple Quadrupole GC/MS that extend the time period over which some AAS compounds can be detected. This is made possible by analyzing for sulfate conjugates of the AAS metabolites in urine, which persist much longer than the metabolites that are routinely analyzed [4, 5]. Performing this analysis on the 6890N network GC system with Agilent 5795B GC/MS platform enables detection of some AAS metabolites almost twice as long as conventional GC/MS methods. However, the Agilent 7000 Series Triple Quadrupole GC/MS provides less interferences and high sensitivity to detect these metabolites for post-administration time periods that are almost three times longer than those obtained with the conventional GC/MS methods.

## Experimental

### Standards and Reagents

The standards and reagents used are listed in Table 1.

### Instruments

The GC/MS experiments were performed on an Agilent 6890N gas chromatograph equipped with a split/splitless capillary inlet and an Agilent 5795B GC/MSD. The GC/MS/MS experiments were performed on an Agilent 7890A gas chromatograph equipped with a split/splitless capillary inlet and an Agilent 7000 Series Triple Quadrupole GC/MS. The instrument conditions are listed in Tables 2 and 3.

Table 1. Standards and Reagents

Internal Standard	4-chloro-testosterone	Fluka	33755
Reagents	N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSFTA)	Campbell Science	Cat. number DR100
	Methanol	Burdick & Jackson	GC230-4
	Acetonitrile	Burdick & Jackson	015-4
	Ammonium Iodide	Fluka	09874
	Mercaptoethanol	Sigma	63689

Table 2. Agilent 6890N Network GC and Agilent 5975B Series GC/MSD Gas Chromatograph and Mass Spectrometer Conditions

GC Run Conditions	
Analytical column	Custom HP-1ms 10 m × 0.15 mm, 0.12 µm film
Injection	1 µL
Carrier gas	Hydrogen, Constant Flow, 1.0 mL/min
Oral-Turinabol oven program	150 °C (0.4 min), 60 °C/min to 192 °C; 5.4 °C/min to 217 °C; 47 °C to 310 °C (0.29 min)
Methenolone oven program	180 °C (0.4 min), 62 °C/min to 235 °C; 42 °C/min to 290 °C; 40 °C to 310 °C (1.9 min)
Transfer line temp	280 °C
MS Conditions	
Tune	Autotune
EMV Offset	+400
Acquisition parameters	El; selected ion monitoring
Solvent delay	1.5 min
MS temperatures	Source 230 °C; Quadrupole 150 °C

Table 3. Agilent 7000 Series Triple Quadrupole GC/MS Gas Chromatograph and Mass Spectrometer Conditions

GC Run Conditions	
Analytical column	Custom HP-1ms 10 m × 0.15 mm, 0.12 µm film
Methenolone injection	1 µL
Oral-Turinabol injection	0.2 µL
Carrier gas	Hydrogen, Constant Flow, 1.0 mL/min
Oven program	180 °C (0.4 min), 62 °C/min to 235 °C; 42 °C/min to 290 °C; 40 °C to 310 °C (1.9 min)
Transfer line temp	280 °C
MS Conditions	
Tune	Autotune
EMV Gain	20
Acquisition parameters	El, selected reaction monitoring
Collision gas flows	N <sub>2</sub> Collision Gas: 1.5 mL/min
Solvent delay	1.5 min
MS temperatures	Source 230 °C; Quad 150 °C

## Sample Preparation

After initial cleanup (removal of urine matrix) on solid phase extraction cartridges, the extract is incubated with β-glucuronidase to effectively cleave glucuronide conjugates and produce free steroids. The mixture is then applied to a C-18 solid phase extraction cartridge and the uncleaved steroid sulfates as well as any remaining contaminants are washed off with acetonitrile. Free steroids that were formerly bound as glucuronides remain on the cartridge and are then eluted with methanol and derivatized for GC/MS analysis.

The acetonitrile wash fraction, which contains steroid sulfates, is dried down and then incubated with

β-Glucuronidase/Arylsulfatase to hydrolyze the sulfate conjugates. The hydrolyzed extract is then applied again to a C-18 solid phase extraction cartridge, which is rinsed with acetonitrile in order to wash off any remaining contaminants. The free steroids are then eluted with methanol, dried and derivatized for GC/MS analysis.

Derivatization was achieved by dissolving the dried sample in 100 µL of N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA)-NH<sub>4</sub>I-ethanethiol (100:2:3, v/w/v) and heating for 15 minutes at 65 °C [3].

## Analysis Parameters

The GC/MS parameters used in the analysis of the various AAS metabolites are shown in Table 4, and the Triple Quadrupole GC/MS parameters used are shown in Table 5.

## Results

### Detection of Longterm Steroid Metabolites

Anabolic steroids and their metabolites are excreted in urine as conjugates with glucuronic acid or sulfate, and the steroids must first be deconjugated before analysis.  $\beta$ -Glucuronidase from *E.coli* is currently used for routine steroid analysis in doping control. However, this enzyme does not cleave the sulfate conjugates, which are then “invisible” during routine testing. The sulfate conjugates persist in urine much longer than the glucuronide conjugates, allowing longer-term detection if a method is used to “visualize” these sulfate conjugates.

The anabolic steroids and their metabolites can be released from the sulfate conjugates using  $\beta$ -Glucuronidase/ Arylsulfatase and a selective extraction procedure. Resultant chromatograms of the extracted urine show excellent

separation of glucuronide and sulfate conjugated methenolone metabolites using conventional GC/MS in selected ion monitoring (SIM) mode (Figure 1). The lower chromatogram of the glucuronide fraction shows the presence of the 3 $\alpha$ -hydroxy metabolite and the parent (17 $\beta$ -hydroxy) conjugate. These two are commonly targeted in routine screening and confirmation procedures in doping control.

The upper chromatogram in Figure 1, showing the sulfate fraction from the extraction, reveals a metabolite highlighted in red that matches the 16-hydroxy 3,17-dione structure. In this case, 18% of this hydroxydione metabolite is glucuronide bound (red peak at 6.9 minutes in the lower chromatogram). The majority, 82%, is excreted as the sulfate conjugate (upper chromatogram). The commonly targeted methenolone metabolites in the glucuronide fraction were detectable in urine for only five days after oral drug administration. However, the hydroxydione sulfate conjugated metabolite can be detected much longer, for at least nine days, by conventional GC/MS. Sulfate-conjugated 16-hydroxy-3,17-dione metabolites are major metabolites and are common for a group of anabolic steroids with a methyl group in the A-ring, including stenbolone, drostanolone and mesterolone (data not shown).

Table 4a. Agilent 6890N Network GC with Agilent 5975B Series GC/MSD Analysis Methenolone Parameters

Compound	RT (min)	SIM	Dwell Time (ms)
Methenolone Metabolite (16 $\beta$ -hydroxy-1 $\alpha$ -methyl-5 $\alpha$ -androst-1-en-3,17-dione)	2.48	517	40
		518	40
		532	40
4-Chlorotestosterone (ISTD)	2.46	466	40
		468	40

Table 4b. Agilent 6890N Network GC with Agilent 5975B Series GC/MSD Analysis Oral-Turinabol Parameters

Compound	RT (min)	SIM	Dwell Time (ms)
Oral-Turinabol (Dehydrochloromethyltestosterone) Metabolite	7.06	656	40
		658	40
4-Chlorotestosterone (ISTD)	6.82	466	40
		468	40

Table 5. Agilent 7000 Series Triple Quadrupole GC/MS Analysis Parameters

Compound	RT (min)	SRM	Dwell Time (ms)	Collision Energy (EV)
Methenolone Metabolite (16 $\beta$ -hydroxy-1 $\alpha$ -methyl-5 $\alpha$ -androst-1-en-3,17-dione)	2.60	532 $\rightarrow$ 517	40	10
		517 $\rightarrow$ 207	40	17
Oral-Turinabol Metabolite (Dehydrochloromethyltestosterone Metabolite)	2.42	656 $\rightarrow$ 244	40	10
		658 $\rightarrow$ 244	40	10
4-Chlorotestosterone (ISTD)	2.34	466 $\rightarrow$ 431	40	10

## Methenolone Urinary Metabolites

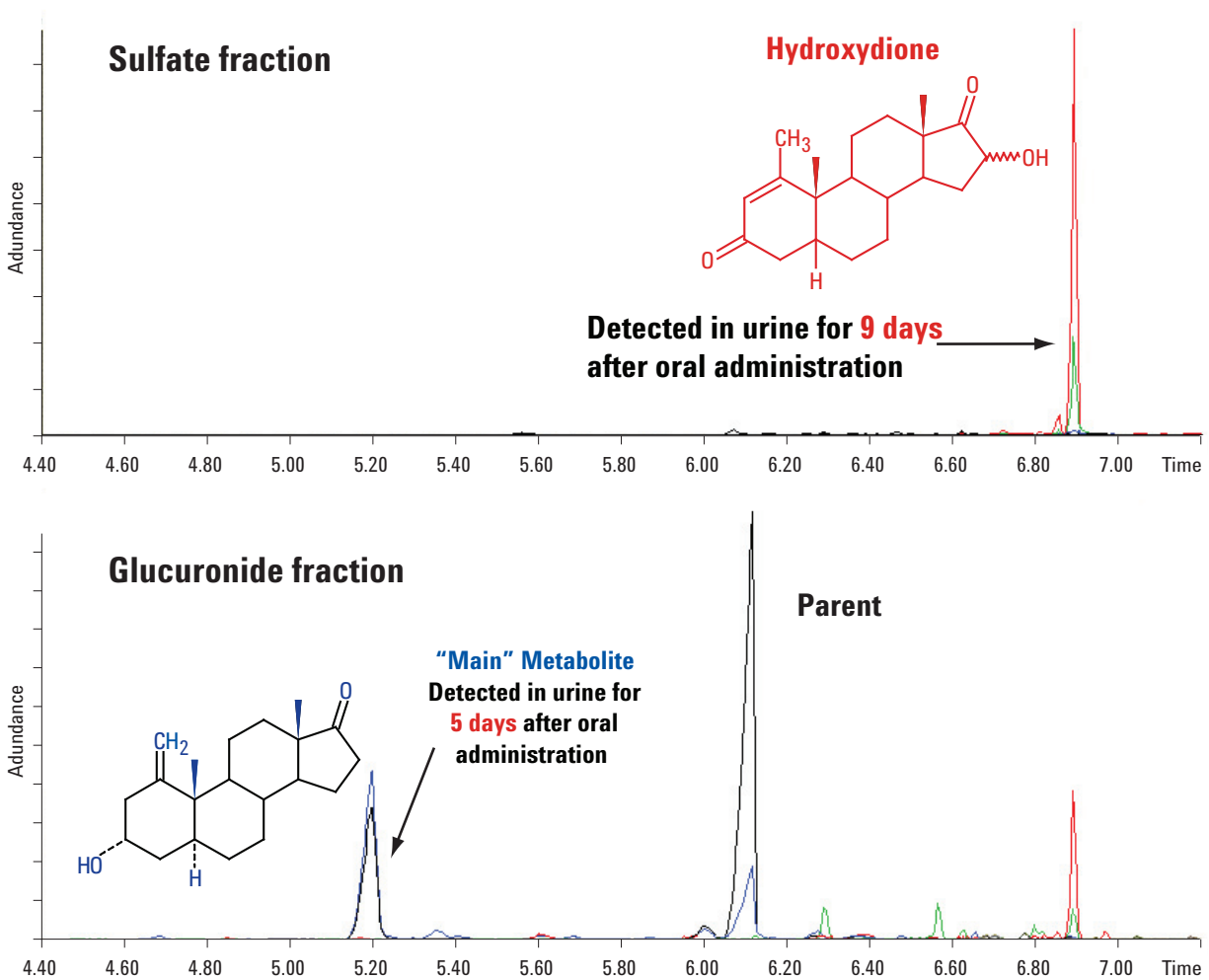
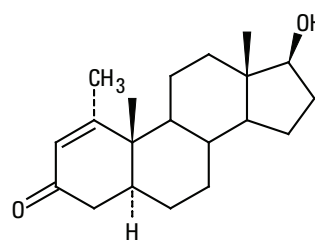


Figure 1. GC/MS SIM chromatograms of the glucuronide and sulfate fractions extracted from urine of a subject injected with methenolone. The lower chromatogram of the glucuronide fraction shows the presence of the 3 $\alpha$ -hydroxy metabolite and the parent (17 $\beta$ -hydroxy) conjugate in the glucuronide fraction. The upper chromatogram shows the sulfate fraction from the extraction, revealing a peak highlighted in red that matches the 3,17-dione 16-hydroxy metabolite (hydroxydione), 82% of which is excreted as sulfate. The methenolone metabolites in the glucuronide fraction were detectable in urine for only five days after oral drug administration, while sulfate conjugated metabolite can be detected for at least nine days. The blue trace is the 431 m/z ion indicative of the 3 $\alpha$ -hydroxy metabolite, and the red trace is the 517 m/z ion indicative of the hydroxydione metabolite.

## Triple Quadrupole GC/MS Extends the Detection Window

Selected reaction monitoring (SRM) analysis of urine performed on the Triple Quadrupole GC/MS instrument can extend the detection window for anabolic steroids even further. This is due to a lack of matrix interferences and increased sensitivity over conventional GC/MS SIM analysis. Methenolone detection is possible 14 days after steroid administration using SRM, versus nine days with GC/MS SIM (Figure 2). After 12 days, GC/MS SIM analysis cannot distin-

guish the methenolone metabolite peak from the background. Detection of oral turinabol in urine by GC/MS SIM analysis is problematic due to interferences that can be interpreted as the metabolite peak, even before administration. As a result, detection thresholds must be increased to a level at which chemical noise no longer interferes with the signal. Analysis of the same urine samples on the Triple Quadrupole GC/MS using SRM is very selective, revealing the absence of a metabolite peak before administration due to a flat baseline, and can detect the metabolite as much as nine days after administration of the steroid (Figure 3).

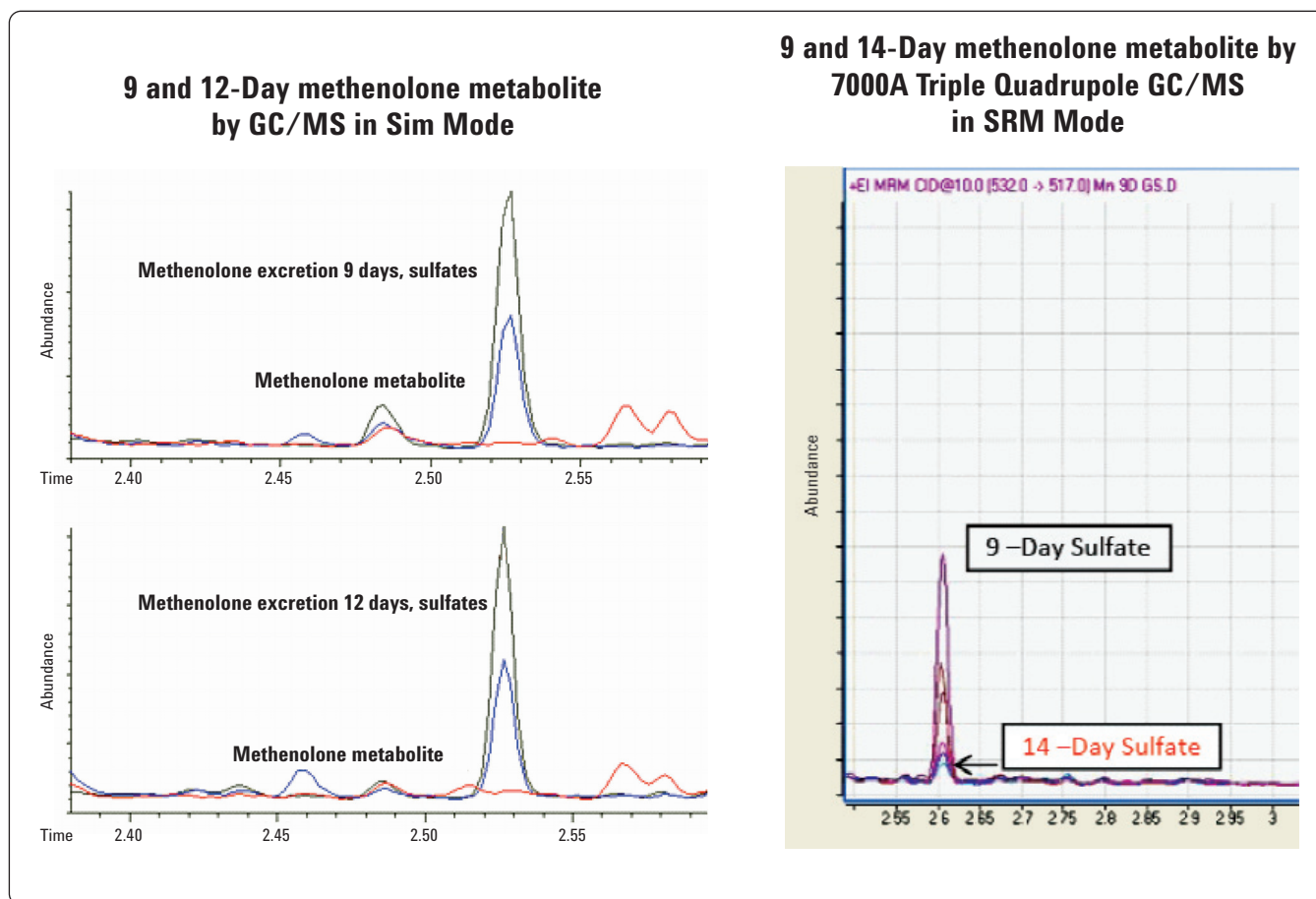


Figure 2. Comparison of analysis of the sulfate fraction of extracted urine for methenolone metabolite by GC/MS SIM and Triple Quadrupole GC/MS SRM. The GC/MS SIM analysis used the 532, 518, and 517 ions for detection, and the methenolone metabolite peak cannot be distinguished from the background after 12 days. In contrast, the Triple Quadrupole SRM analysis shows detectable metabolite peaks at 9, 10, 11, 12, 13 and 14 days, using the 532→517 transition.

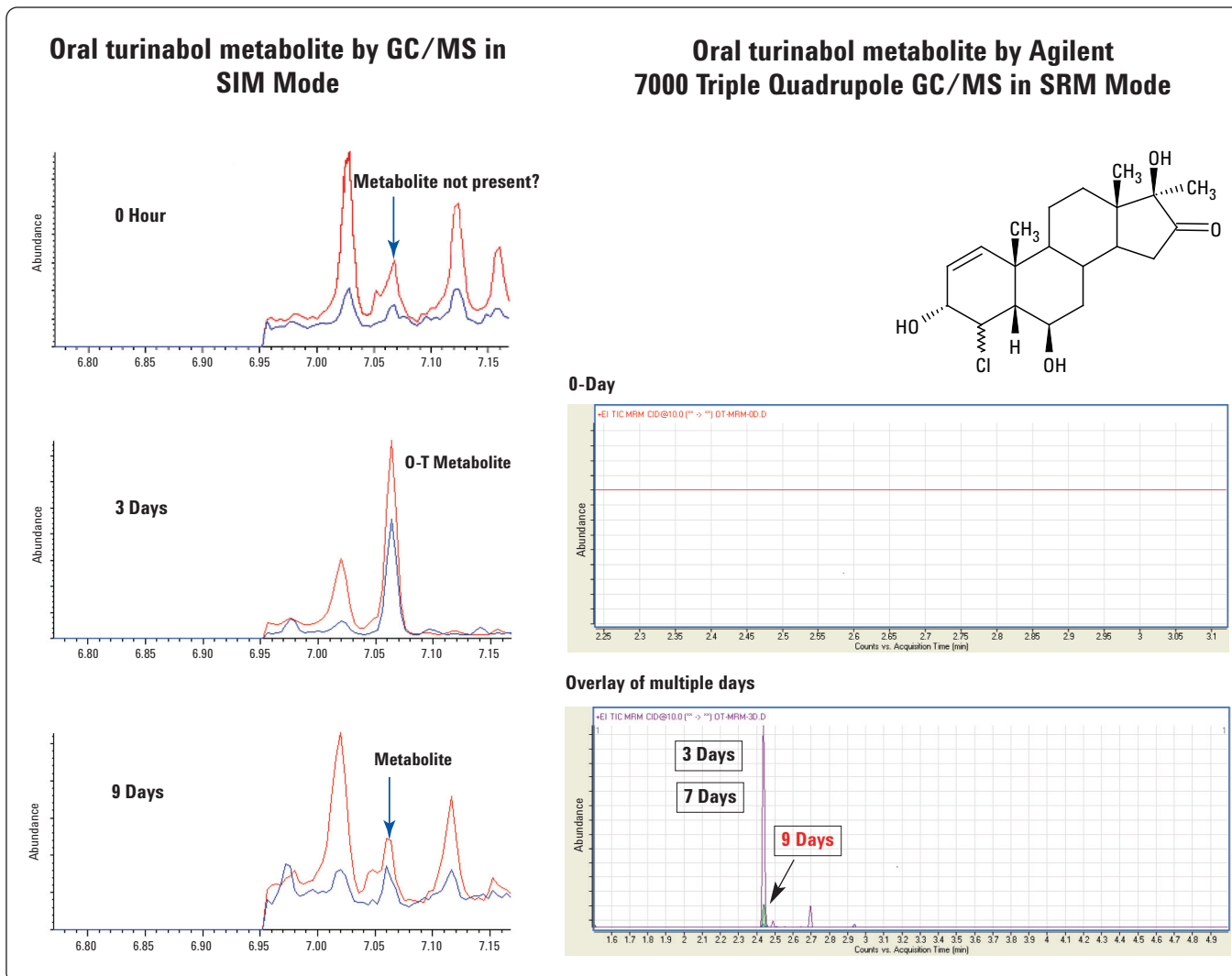


Figure 3. Comparison of analysis of the sulfate fraction ("Free Fraction") of extracted urine for oral-turinabol metabolites by GC/MS SIM and Triple Quadrupole GC/MS SRM. GC/MS SIM analysis is problematic due to interferences that can be interpreted as the metabolite peak, even before administration of the drug (0 hr). Analyzing the same urine samples on the Triple Quadrupole GC/MS using SRM reveals the absence of a metabolite peak before administration, and can detect the metabolite as much as nine days after administration of the steroid. The GC/MS SIM analysis utilized the 658 and 656 m/z ions for detection, while the Triple Quadrupole SRM analysis utilized the 656 → 244 m/z transition. The three day SRM trace is red, green is seven days, and blue is nine days.

## Conclusion

The detection window for anabolic steroids can be expanded significantly by monitoring sulfate-conjugated metabolites rather than those conjugated as glucuronides. A unique selective extraction procedure using  $\beta$ -Glucuronidase/Arylsulfatase provides metabolites cleaved from their sulfate conjugates and ready for derivatization and GC/MS analysis. This procedure can almost double the detection window for some anabolic steroids from five to nine days for

methenolone, using GC/MS SIM analysis. Performing analysis on the Triple Quadrupole GC/MS using SRM can extend the detection time after administration from nine to 14 days for methenolone due to the elimination of matrix interferences. This instrument system also allows the monitoring of some anabolic steroids that are not easily analyzed by traditional GC/MS in the SIM mode. The Triple Quadrupole GC/MS is therefore a preferable alternative to GC/MS SIM for longterm monitoring of anabolic steroids.

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