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Screening and confirmation results of three interesting cases in 2012 at the Doping Control Laboratory, Karolinska University Hospital.

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Abstract

► Case 1. In September 2012, Swedish National Health Institute banned all sales of Methylhexaneamine (DMAA) and all Pre-Work-Out, performance enhancers, (PWO) containing the substance e.g. Crack™ and Jack 3-D™. A new PWO, Craze™, was on sale at 2012. In the mid of 2012, some samples were suspicious for an amphetamine derivative which appeared as a possible mephentamine detection in the screening method. Craze was declared on some of those athletes' control forms.
► Case 2. In one athlete sample an abundant peak was found in the window for pipradrol in the screening 5 method. A small shift in retention time was observed, compared to the calibrator. Terfenadine, an antihistamine, was declared on the athlete's control form. It was shown that the metabolite (azacyclonol, which is an strucural isomer of pipradrol) was the reason to the positive screening result.
► Case 3. Ostarine, SARM, and metabolites were found in one sample 2012 in the screening 5 method. A LC-MS/MS method was developed to confirm the sample.

Introduction

A screening method was published in JMS 2008 [1] and is used routinely at the Stockholm doping control laboratory. The method is based on direct injection of diluted urine samples to UPLC-MS/MS (Waters, Acquity and Quattro Premier). The method includes diuretics, CNS-stimulants, opiates and SARM. A total of 150 different prohibited substances is analyzed within the chromatographic time of 7.5 min. Two ion transitions per analyte are used for the automatic evaluation program in the SDMS (Scientific Data Management System) software. Three interesting cases found during 2012 are presented here.

Experimental

Screening procedure in all three cases was according to the published method in JMS 2008 [1].
In case 2 the confirmation was preformed by high resolution mass spectrometry (Thermo, Q-Exactive) and the separation was performed on a Syncronis aQ column (Thermo) 2.1mm x 100 mm with 1.7 µm particle size. A linear gradient from 2% to 99% Methanol (10 mM ammonium formate) at 0.0 min respectively 7.5 min was used for the separation. Flow rate and column temperature was set at 0.45 ml/min respectively 55°C. Electrospray was operated in positive mode and the MS resolution and scan range was 70000 and 100-1000 m/z, respectively.
In case 3 the confirmation was performed by the same equipment and gradient as described in the screening method [1]. MS parameters and sample preparations (free and one total fraction analysis) was according to Table 1:

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A	Analyte	Precursor ion (m/z)	Cone (V)	Product ion (m/z)	Collision energy (V)
	OH-ostarine gluc.	579,8	35	134	35
				404,4	20
				309,7	20
	ostarine gluc.	564	45	444,9	20
				184,9	45
				117,6	40
				287,2	30
	OH-ostarine	404,4	25	134	50
				269	20
				185	50
	ostarine	387,8	30	117,5	20
				269,3	20
				185,3	30
	d3-testosterone	292,5	40	108,9	25
	d3-testosterone-gluc	468,5	40	108,9	33

B	Sample Preparation	
	"Free fraction"	"Total fraction"
	0.5 ml urine sample	0.5 ml urine sample
	0.1ml ISTD d3-Testosterone gluc. (4.56µg/ml)	0.1ml ISTD d3-Testosterone gluc.(4.56µg/ml)
	0.25 ml H2O	0.5 ml Phosphate buffer , pH7
	5µl inj UPLC-MS/MS	50µl β-glucuronidase
		50°C 60 min,
		2 ml tert-BME extraction,
		Evaporate to dryness,
		Reconstitute in 500µl 5% MeOH
		5µl inj UPLC-MS/MS

Table 1: A MS parameters; B Sample preparation

Results and Discussion

► Case1. At 2012 some samples were suspicious to contain some higher homologue of amphetamine, due to a peak in the mephentermine window in the screening method, with a retention shift of + 0.1 min compared to a calibrator. Craze™ was declared on some of those athletes' control forms and we started an investigation. In addition to the peak in the screening method we also found an intense peak at 2.8 min (M+H at 178 m/z). Similar fragmentations were observed for the two unknown substances. Chromatograms, product ion scan and proposed structures and names are shown in Figure 1.
► Case 2.In one sample a chromatographic peak was appeared close to the predicted retention time for Pipradrol. On the athlete's form the drug fexofenadine was declared. The reason for the suspicious result was a metabolite, azacyclonol [2]. Figure 2 shows data from the confirmation method of the parent drug and the metabolite, by a high resolution mass spectrometer (Thermo, Q-Exactive).
► Case 3.One sample was found suspicious to contain ostarine with the screening method. The sample was confirmed to be adverse analytical finding for ostarine. The confirmation was performed by analyzing the free and the total fractions of ostarine and the hydroxylated metabolite. At least three ion transitions [3] were used for each analytes in the confirmation method. An urine from WAADS, distributed as educational sample (WAADS QA 2012-1L) was used as a reference. A certified reference substance, a customs seizure, obtained from SKL (Swedish National Laboratory of Forensic Science) was used for quantification of the total fraction of ostarine. Testosterone glucuronide-d3 was used as internal standard for both the free and the total fraction method. Hence, in the latter method, the internal standard was hydrolyzed to free testosterone-d3 to check the hydrolyzed step. Chromatograms and reports of the positive sample are shown in Figure 3.

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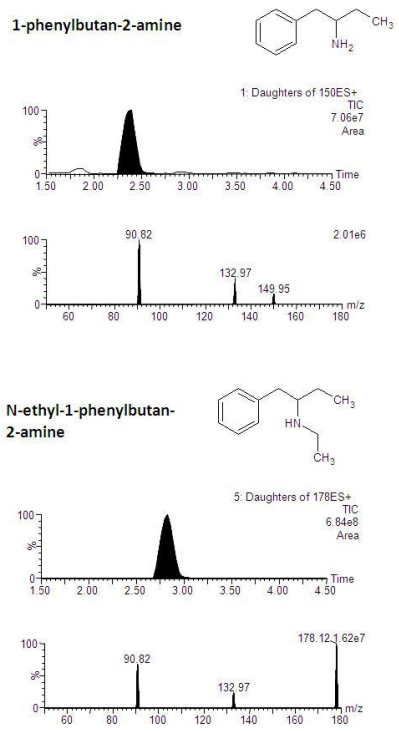

Figure 1. Chromatograms, mass spectra (MS/MS), proposed structures and names of the two analytes found in one of the investigated urine sample.

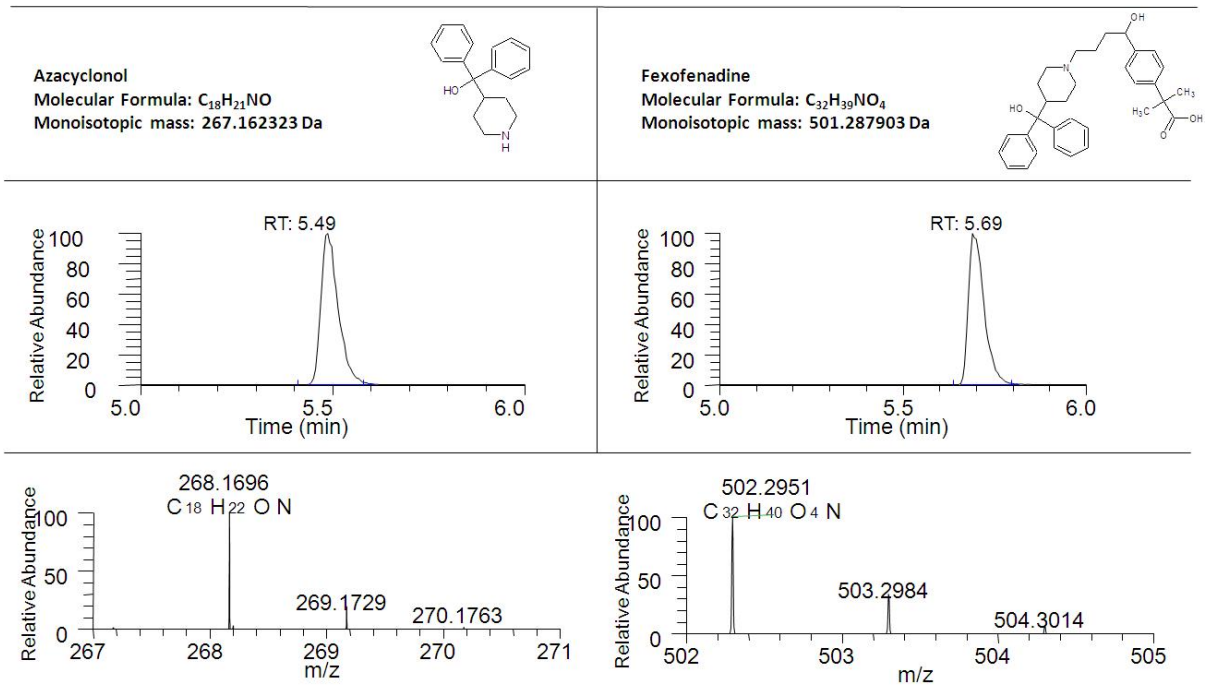

Figure 2. Chemical data, chromatograms and mass spectra of a confirmed sample containing the drug fexofenadine and the metabolite azacyclonol.

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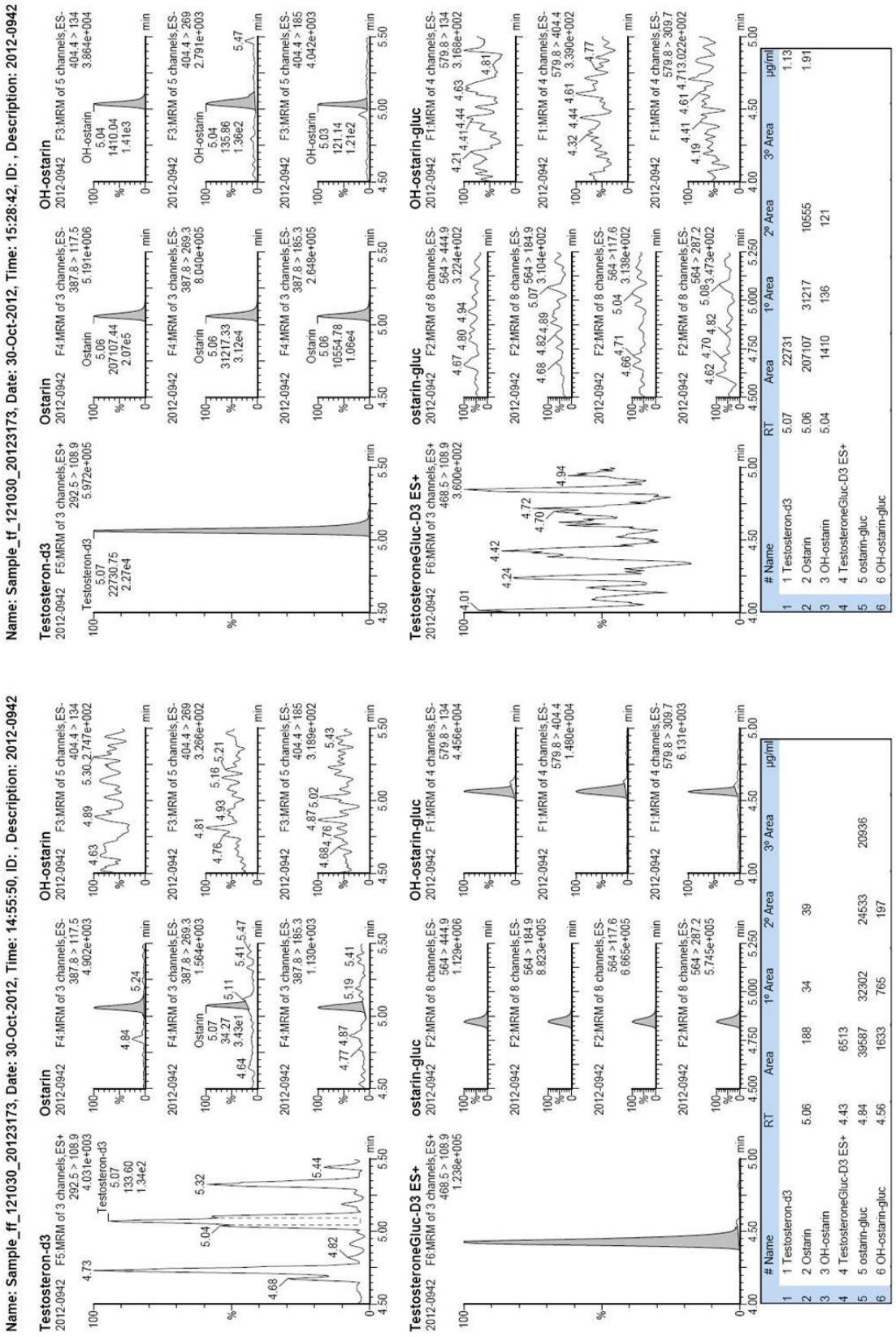

Figure 3. Chromatograms and reports of the sample from the ostarine confirmation method. The left report corresponding to the analyze of the total fraction in the sample.

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Conclusions

► Case 1. Further investigations will be performed to identify the substances in the PWO product Craze.
► Case 2. Azacyclonol is a metabolite of fexofenadine and a strucural isomer of pipradrol and can lead to false positive screening cases.
► Case 3. A confirmatory method for ostarine was successfully developed.

References

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