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A New Approach for Screening, Verifying and Confirmation of Prohibited Doping Substances

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Introduction

Diuretics and other masking agents are prohibited for samples taken out of and in competition according to World Anti-Doping Agency's 2006 prohibited list, group S5. The field of diuretic drugs development have been productive with a number of more than 30 different active substances commercial available on the market. Year 2006 WADA put up a number of 39 stimulants, group S6, to the monitoring program for out of competition samples. That time was a starting point to implement a new screening method for both group S5 and S6 simultaneously. One way to manage that challenge was usage of high throughput screening (HTS) methodologies.

One of the newest HTS techniques is Ultra performance liquid chromatography, UPLC. This technology utilizes sub 2 µm particles, performing peak capacity to separate two components within a few seconds. These small particles increases the back-pressure a factor of 6-27 times compared to traditional HPLC-columns (3 and 5 µm, respectively), so the system pump is constructed to handle pressures up to 15000 psi.

Tandem mass spectrometry combined with UPLC gives selectivity and sensitivity to perform direct injections of diluted urine samples, without any time consuming pre-treatments. Ion-suppression, due to matrix effects, in the electrospray ionization can be reduced by the efficient UPLC-columns due to separation of matrix substances from the analytes.

This study presents routine HTS , verifying and confirmation methods by UPLC-MS/MS of two different classes of doping agents, diuretics and stimulants.

Experimental

Chemicals and reagents

Some of the diuretic and stimulant reference substances were purchased from following manufactures:
European Pharmacopoeia, US Pharmacopoeia, Cerilliant, A/S Alfred Benlon, Merck, Novo Industri AS, Sigma Aldrich, Fluka, Radian, Apoteksbolaget
Others were gifts from different laboratories (Clinical pharmacology at Huddinge sjukhus, German Sport University at Cologne)
4-phenoxy-3-(1H-pyrrol-1-yl)-5-sulphamoylbenzoic acid, from European Pharmacopoeia, was used as internal standard.
All chemicals used for the method were analytical grade. Buffer solutions were prepared using deionized water.

Sample preparation

All samples were diluted with 20mM ammoniumacetate buffer including internal standard, IS, (100µl sample +100µl IS) by Xiril 100 workstation into 96 well microtiterplates. Calibrators, blank, and control samples were manually diluted with 20mM ammoniumacetate buffer including internal standard into 250 µl vials, separated from the samples to avoid contamination.

Chromatographic separation

UPLC-system from Waters and a 50mm x 2.1 mm C18-shield column with 1.7µm particles was used for the chromatographic separation. Gradient profile used to separate the substances in the method is shown in table 1 below.

Table 1. Gradient profile.

Time (min)	Methanol (%)	10mM ammonium acetate(%)	Flowrate (ml/min)
0	5	95	0.4
0.5	5	95	0.4
4	40	60	0.4
5	95	5	0.4
6	95	5	0.4
6.1	5	95	0.4
7.5	5	95	0.4

Mass spectrometric method

A Waters Quattro premier triple-quadropole instrument with an electrospray interface operating with fast polarity switching in multiple reaction mode (MRM) was used to detect the substances in the method. Twenty MRM-channels with different ion transitions and time intervals, at least one specific transition for each compound, were used for recording the data for the screening method. Three specific ion transitions per substance (one specific ion transition for the IS) were used for verification and confirmation methods.

Data evaluation

All data evaluation was performed by the Target lynx program embedded in the MassLynx software. Three different screening reports was printed to Adobe PDF-documents, one for diuretics, the second for stimulants (see figure 1) and the third for stimulants with reporting limits (ephedrine, cathine and methylephedrine) according to WADA Prohibited list 2007 and the substances included in the monitoring program 2007 (Caffeine, Synephrine and Pseudoephedrine). All three printed reports included the chromatograms and the data report (retention times, transitions, evaluation flags and concentrations).

Result and discussion

A screening batch analysis consisted of a calibrator, injected in triplicate before and after the sample batch. At every twelve sample a control sample were injected followed by a blank sample, to calculate the batch deviation and verify no injection carry-over, respectively. p-hydroxyamphetamine, cyclothiazide and norfenfluramine are found frequent as false

screening positive results (background interferences) and by using two ion-transitions, these samples can be rejected as false positive without confirmation methods.

The method sensitivity for the screening method was determined to less than 50 ng/ml for all substances. The limit of quantification was determined to less than 1 ng/ml for some of the substances (e.g. benzoylecgonine, see figure 2).

Positive samples indicated from the screening results were verified by reinjecion of the sample with three diagnostic ions for each substance data acquired. Approximately less than 5 % of the samples required verification analysis due to false positive screening results. Positive verification results were reanalysed and confirmed by a new sample preparation and isotope labelled internal standard addition, if available. Figure 3 shows a confirmation report of the positive cocaine/benzoylecgonine sample (from figure 1).

This new screening method has capacity to perform 96 sample injections including blank, calibrator and control sample injections within 16 hours. Three reports give the opportunity to separate in and out of competition samples for evaluation. The sensitivity of the method is far below (5 times or more) the minimum required performance limit (MRPL) according to WADA technical document TD2004MRPL.

In the next future all substances in group S5, S6 and S7 in WADAs prohibited list will be included in the method.

References

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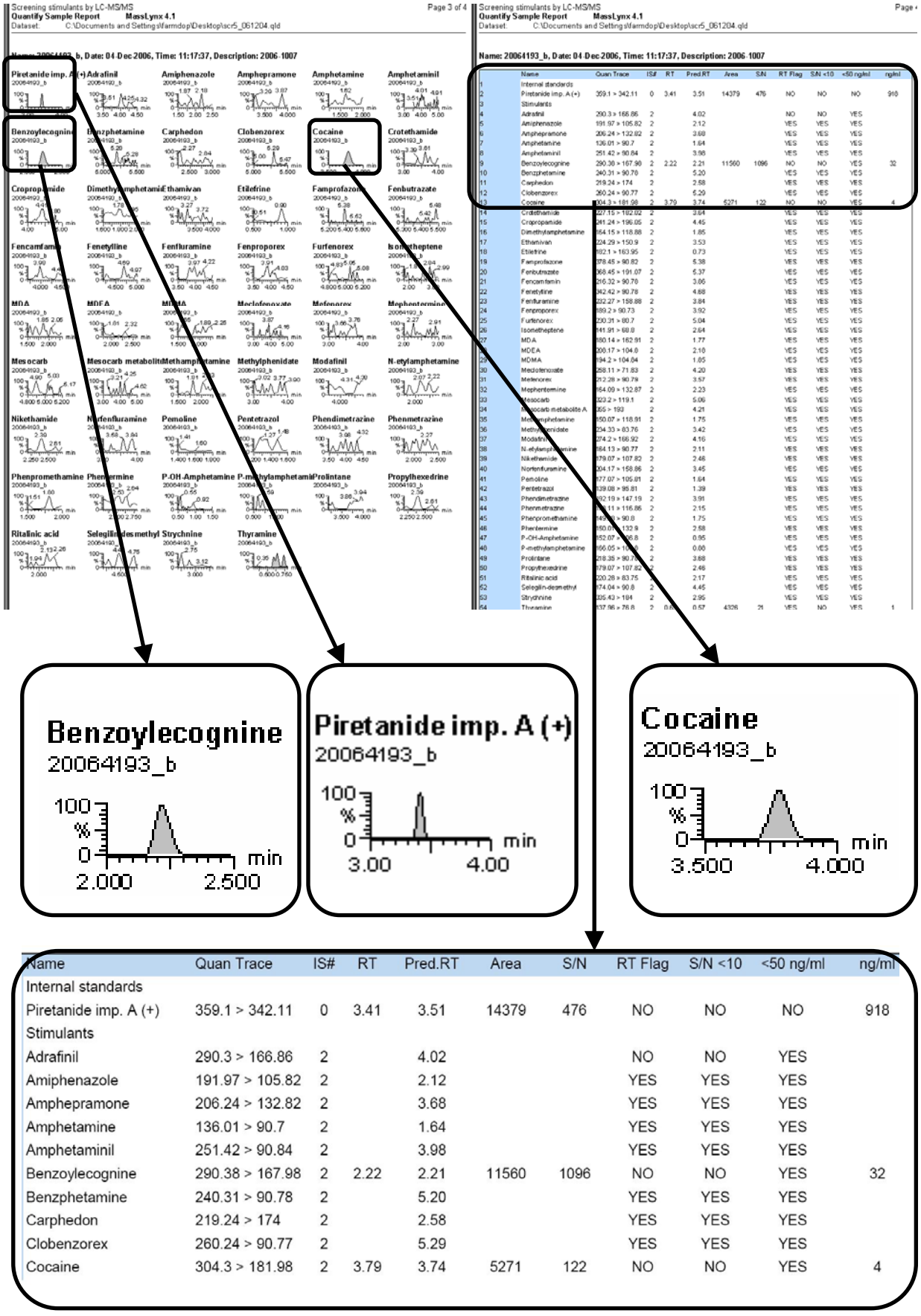


Name	Quan Trace	IS#	RT	Pred.RT	Area	S/N	RT Flag	S/N <10	<50 ng/ml	ng/ml
Internal standards										
Piretanide imp. A (+)	359.1 > 342.11	0	3.41	3.51	14379	476	NO	NO	NO	918
Stimulants										
Adrafinil	290.3 > 166.86	2		4.02			NO	NO	YES	
Amiphenazole	191.97 > 105.82	2		2.12			YES	YES	YES	
Ampheprамone	206.24 > 132.82	2		3.68			YES	YES	YES	
Amphetamine	136.01 > 90.7	2		1.64			YES	YES	YES	
Amphetaminil	251.42 > 90.84	2		3.98			YES	YES	YES	
Benzoylecognine	290.38 > 167.98	2	2.22	2.21	11560	1096	NO	NO	YES	32
Benzphetamine	240.31 > 90.78	2		5.20			YES	YES	YES	
Carphedon	219.24 > 174	2		2.58			YES	YES	YES	
Clobenzorex	260.24 > 90.77	2		5.29			YES	YES	YES	
Cocaine	304.3 > 181.98	2	3.79	3.74	5271	122	NO	NO	YES	4

Figure 1. A screening report for stimulants, showing a positive cocaine/benzoylecgonine case.

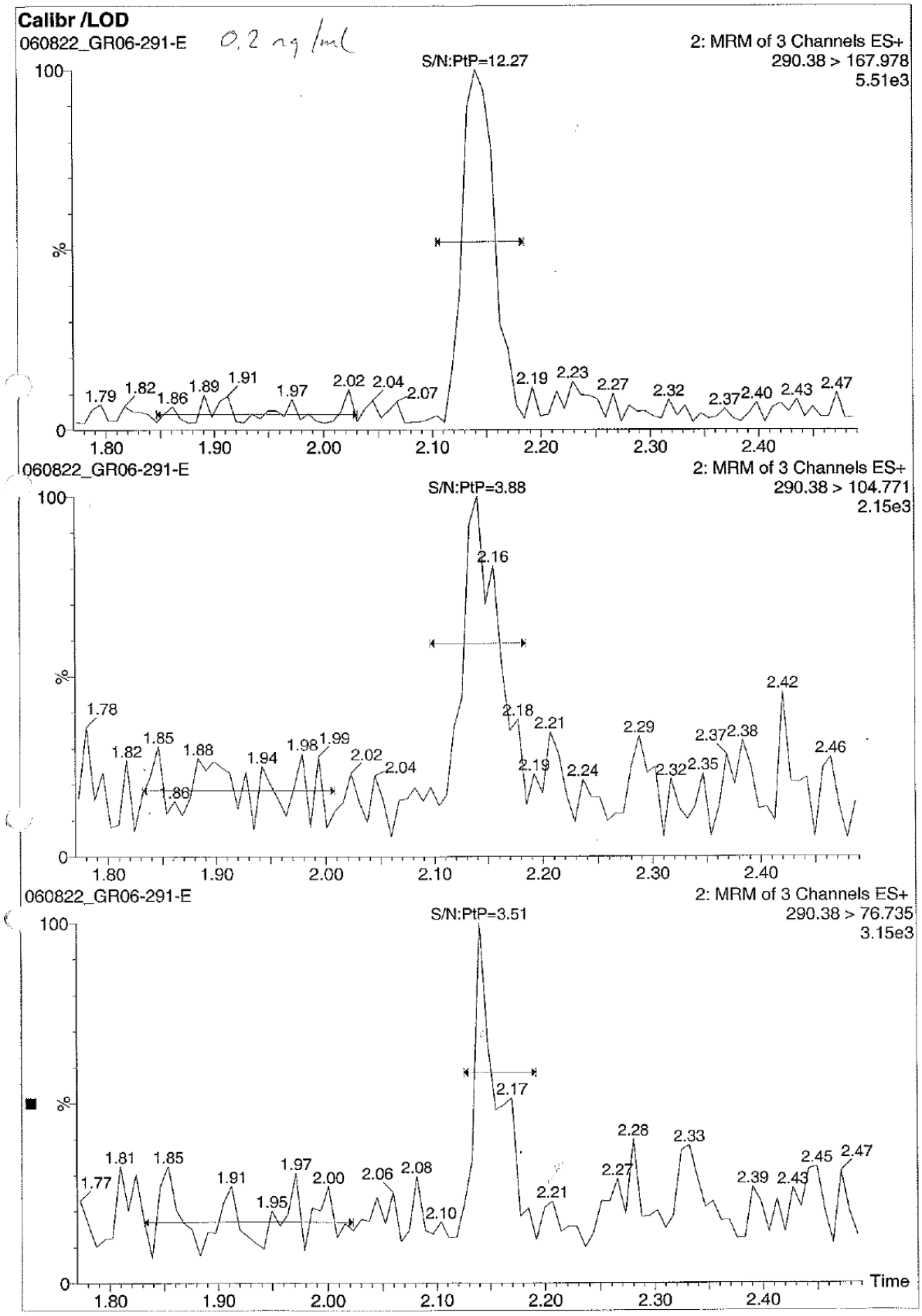
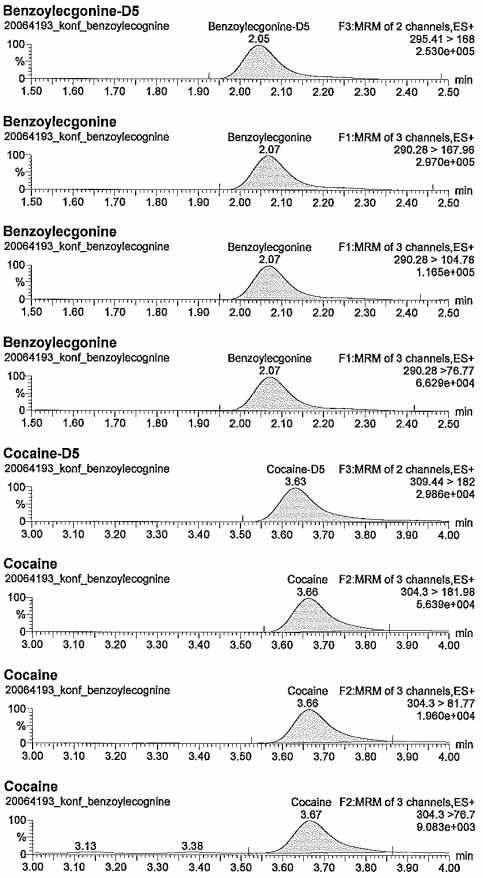


Figure 2. Limit of quantification determined to 0.2 ng/ml for benzoylecgonine with three ion-transitions.

Confirmation method for prohibited doping substances by LC-MS/MS
Quantify Sample Report **MassLynx 4.1**
Dataset: Untitled

Name: 20064193_konf_benzoylecognine, Date: 08-Dec-2006, Time: 14:26:15, Description: 2006-1007



	Name	RT	T Area	Q1 Area	Q2 Area	Q1/T	Pred	Q2/T	Pred	Q1/T Flag	Q2/T Flag	RT Fl...	ng/ml
1	Benzoylecgonine-D5	2.05	27502									NO	0.86
2	Benzoylecgonine	2.07	32278	12464	7173	0.386	0.396	0.222	0.213	NO	NO	NO	37.08
3	Cocaine-D5	3.63	3498									NO	1.17
4	Cocaine	3.66	5758	1985	936	0.345	0.355	0.163	0.144	NO	NO	NO	2.70

Cocaine
Standard CR06-036-KAL **5ng/ml** **Sample 20064193**

RULES for Acceptance Range
WADA Technical Document, TD2003IDCR.

Ions	m/z	Abundance	Relative Abundance	RT	Acceptance Range	Relative Abundance	Relative Abundance	RT (min)	Relative Abundance	Acceptance Range
$[M+1]^+$	**304.3**	**AREA**	**(%)**	**(min)**		**AREA**	**(%)**		**(% of Tgt)**	**LC/MSn**
Tgt	181.98	5464	100.0	3.66	-	5758	100.0	3.66	> 50%	± 15% (absolute)
Q1	81.77	1923	**35.2**		**26.4-44**	1985	**34.5**		25% to 50%	± 25% (relative)
Q2	76.7	888	**16.3**		**6.3-26.3**	936	**16.3**		< 25%	± 10% (absolute)

BenzoylEcgonine
Standard CR06-036-KAL **3ng/ml** **Sample 20064193**

RULES for Acceptance Range
WADA Technical Document, TD2003IDCR.

Ions	m/z	Abundance	Relative Abundance	RT	Acceptance Range	Relative Abundance	Relative Abundance	RT (min)	Relative Abundance	Acceptance Range
$[M+1]^+$	**209.28**	**AREA**	**(%)**	**(min)**		**AREA**	**(%)**		**(% of Tgt)**	**LC/MSn**
Tgt	167.96	5058	100.0	2.07	-	32278	100.0	2.07	> 50%	± 15% (absolute)
Q1	104.78	2003	**39.6**		**29.7-49.5**	12464	**38.6**		25% to 50%	± 25% (relative)
Q2	76.77	1075	**21.3**		**11.3-31.3**	7173	**22.2**		< 25%	± 10% (absolute)

Figure 3. A confirmation report with ion ratio comparison, showing the positive found sample from the screening method in figure 1.