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Screening of doping agents using liquid-chromatography/time-of-flight mass spectrometry

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Introduction

The appearance of new abused molecules has increased the number of substances on the prohibited list of WADA and has given to the doping control laboratories a big challenge to keep their analytical methods updated. Occasionally totally new analytical methods should be developed and applied, which is time consuming and may also require additional and sometimes expensive instrumentation or reagents. Furthermore, the increase in the number of separate analytical procedures renders the laboratory analysis more complex, delays reporting, increases the workload, and raises the cost of a single test. Addition of new drugs and their metabolites in screening procedures is sometimes slow or impossible due to a lack of reference substances.

For a long time, doping analytics has mainly been based on different gas chromatography – mass spectrometric techniques (GC/MS) [1]. However, recently, the excellent suitability of liquid chromatography – mass spectrometry (LC/MS) has been demonstrated for multi-analyte screening of many classes of prohibited substances. Actually, many new compounds added to the list of banned substances can be effectively screened only by LC/MS. In theory, LC/MS would have capabilities “almost for an all-in-one screening procedure”. Unfortunately, at the moment, this approach is greatly restricted by the use of non-universal sample preparation procedures and by the use of scanning-type of mass spectrometers.

Recently, a novel toxicological screening method for urine samples based on liquid chromatography/time-of-flight mass spectrometry (LC/TOFMS) has been established [2]. In the method, acidic, neutral, and basic drugs are extracted in urine and analyzed by

LC/TOFMS with positive-ion ionspray and continuous accurate mass measurement. The method has been used effectively for screening of several different drugs, drug metabolites, and pesticides.

The aim of this on-going World Anti-Doping Agency (WADA) funded project is to develop a general LC/TOFMS-based screening method for several chemically and pharmacologically different doping agents. Included in the study are compounds belonging to the following classes of prohibited substances: agents with anti-estrogenic activity, cannabinoids, beta-blockers, beta2-adrenergic agonists, diuretics, narcotics and stimulants.

Experimental

LC/TOFMS

The analysis set used was similar with the method described by Pelander et al [2]. An Agilent 1100 series LC system with a Bruker Daltonics micrOTOF mass spectrometer equipped with an electrospray ion source (ESI) was used in the study. All experiments were carried out in positive ion mode. The applied capillary voltage was 4500 V. The m/z range was set from 50 to 800 and the average mass resolution was 8000. Separation was carried out on a Phenomenex Luna C-18(2) 100 x 2 mm (3 µm) column and a 4 x 2 mm precolumn. Injection volume was 10 µl. The mobile phase contained 5 mM ammonium acetate in 0.1 % formic acid (solvent A) and acetonitrile (solvent B). A gradient with a flow rate of 0.3 ml/min was run at 40 °C from 10 to 40% B in 10 min, to 75% B in 13.5 min, to 80% B in16 min and held at 80% for 6 min.

Construction of database

The database contained theoretical monoisotopic exact masses of protonated compounds, their molecular formula and retention times. Theoretical exact masses were calculated with the Bruker IsotopePattern software. The database was constructed by running mixtures of reference substances dissolved in LC-eluent. The final concentration of each analyte in the mixture was 1 µg/ml. Different compounds (n=124) belonging to different classes of prohibited drugs (agents with anti-estrogenic activity, cannabinoids, beta-blockers, beta2-adrenergic agonists, diuretics, narcotics and stimulants) were analyzed.

Sample preparation

Urine sample (1 ml) containing 100 ng of dibenzepin as an internal standard was hydrolyzed enzymatically with β-glucuronidase at 56 °C for two hours and applied to a preconditioned IST Isolute HCX-5 (100mg) mixed mode solid phase extraction cartridge. The column was rinsed with 1 ml of water and 10 mM hydrochloric acid. The elution was performed with 1 ml of methanol and methanol/ammonia (98:2, v/v). The eluates were evaporated to dryness and the residues were reconstituted in 150 µl of LC-eluent.

Data analysis

Acquired data was processed with the DataAnalysis software and an application macro program created by Bruker Daltonics. The application searched for target masses included in the established database. The search criteria were ppm mass tolerance, sigma value, retention time and minimum area count. The sigma value is the relationship between theoretical and measured isotope pattern. The application created automatically a Microsoft Excel-based result report on all findings.

Method evaluation

The applicability of the method in doping analysis was investigated with blank urine samples and urine samples spiked with the investigated compounds at concentrations corresponding the minimum required performance limits (MRPL) established by WADA [3]. The suitability of the method was also demonstrated with four external quality control urine samples prepared from urine samples collected after administration of pemoline, methylphenidate, spironolactone and oxprenolol.

Results and discussion

Different doping agents (n=124) were studied and retention times and mass spectral data of 106 substances could be added to the database (Table 1). Preliminary results indicate that the majority of substances in the database can be detected in urine at concentrations corresponding the MRPLs set by WADA. It was observed that some drugs (e.g. cannabinoids

and diuretics that contain double bonded sulphur atoms) could not be analyzed by this method since they did not ionize in the aspplied conditions (positive ESI).

Analysis of drug-free urines from six volunteers verified the specificity of the method; no false identification of any drug of the database was observed.

The method was also tested with four authentic urine samples collected after administration of pemoline, methylphenidate, spironolactone and oxprenolol. All samples were analyzed positive using the LC/TOFMS method. Pemoline was identified as parent drug and spironolactone as its metabolite, canrenone. In case of oxprenolol, parent drug and two hydroxylated metabolites were found. In case of methylphenidate, parent drug and its metabolite ritanilic acid were identified (Figure 1).

Conclusion

The developed screening method based on solid phase extraction and LC/MSTOF allowed identification of chemically and pharmacologically different banned drugs in urine in the same run. The approach has great potential in doping analytics and might dramatically simplify analytical screening strategies in anti-doping laboratories in the future.

The next stage in the project is to carefully validate the method for qualitative screening, and compare it with the standard screening procedures currently in use. After completing the project, the substance database containing accurate masses and retention behavior of the studied substances will be available for all WADA-doping control laboratories.

Table 1. List of substances measured with the LC/MSTOF method.

STIMULANTS

Amfepramone	Etilefrine	Pemoline
Amphetamine	Fencamfamine	Pentetrazole
Benzphetamine	Fenetylline	Phendimetrazine
Carphedon	Fenfluramine	Phenmetrazine
Cathine	Fenproporex	Phentermine
Chlorophentermine	Heptaminol	Phenylefrine
Clobenzorex	MDA	Phenylpropanolamine
Cocaine	MDMA	p-hydroxyamphetamine
Crotethamide	Mefenorex	Picrotoxin
Dimethylamphetamine	Mephentermine	Pipradol
Doxapram	Mesocarb	Prolintane
Ephedrine	Methamphetamine	Propylhexedrine
Etafedrine	Methoxyphenamine	Pseudoephedrine
Ethamivan	Methylephedrine	Ritalinic acid
Ethylamphetamine	Methylphenidate	Selegiline
	Nikethamide	Strychnine

NARCOTICS

Buprenorphine	Methadone	Oxymorphone
Dextromoramide	Morphine	Pethidine
Fentanyl	Norbuprenorphine	Pentazocine
Heroin	Noroxycodone	
Hydromorphone	Oxycodone	

DIURETICS ABD MASKING AGENTS

Amiloride	Clopamide	Metolazone
Bumetanide	Indapamide	Probenecid
Canrenone	Mefruside	Spironolactone

BETA2-ADRENERGIC AGONISTS

Clenbuterol	Rimiterol	Salmeterol
Fenoterol	Ritodrine	Terbutaline
Formoterol	Salbutamol	

BETA-BLOCKERS

Acebutolol	Carvedilol	Penbutolol
Alprenolol	Celiprolol	Pindolol
Atenolol	Esmolol	Practolol
Betaxolol	Labetalol	Propranolol
Bisoprolol	Metoprolol	Sotalol
Bunolol	Nadolol	Timolol
Carteolol	Oxprenolol	

ANTIESTROGENIC DRUGS

Clomiphene	Tamoxifen	Toremiphene
Exemestane		

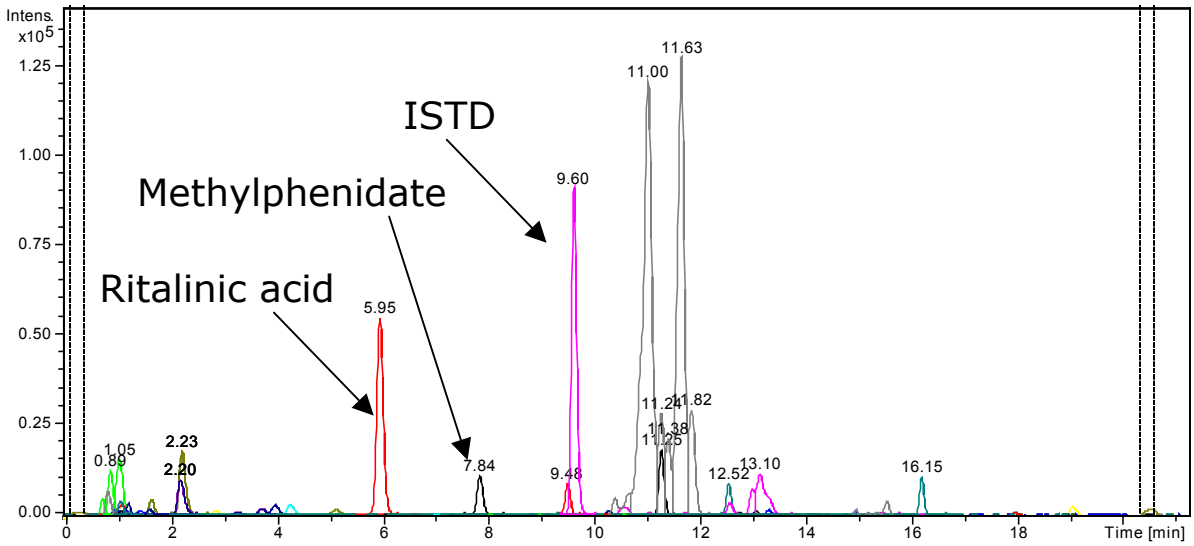

Figure 1. Merged extracted ion chromatograms produced automatically by the identification software after evaluation of the data obtained from LC/MSTOF analysis of the methylphenidate-urine. Extracted ions for methylphenidate, ritalinic acid and dibenzepin (internal standard) were m/z 234.1489, 220.1332 and 296.1757, respectively.

References

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