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Multi-residue extraction and analysis method for prohibited substances in urine

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Abstract

Currently there are several methods used to detect prohibited substances in urine. This study aimed to develop a single extraction and analysis method to detect the majority of substances on WADA's prohibited list. [1]
Samples, spiked with approximately 150 prohibited substances, were extracted by solid phase extraction (SPE) and analysed by LC-MS/MS. Of the 150 substances only 4 were not detected in urine matrix using this method, with the remaining detectable at or below the minimum required performance levels (MRPL). A number of compounds were found to have low recoveries, despite being detectable at the MRPL. Experiments indicated that they were being retained on SPE cartridges during extraction. To overcome this, the SPE elution solution was modified, resulting in significant improvement of recoveries for the majority of substances.
While it was determined that a single extraction and analysis method was unable to be developed to detect the majority of substances on WADA's prohibited list [1], a LC-MS/MS method was developed to detect those desired analytes in classes S1, S3, S4, S5, S6, S7, S8 and S9.

Introduction

The existing procedures used for detecting the range of WADA prohibited substances in urine rely on a combination of extraction methods and analysis techniques – between 2-3 analysis methods for out-of-competition samples and up to 5 different methods for in-competition.
A study was conducted with the aim of developing a single extraction and analysis method which has the ability to detect the 5 low level anabolic agents along with other compounds listed in the WADA Prohibited List [1], using SPE extraction techniques and analysis by LC-MS/MS. MS conditions for approximately 150 compounds were optimized (at least 2 transitions per analyte where possible) for the study and included S1. Anabolic Agents; S3. Beta-2-agonists; S4. Hormone and metabolic modulators; S5. Diuretics and other masking agents; S6. Stimulants; S7. Narcotics and S9. Glucocorticosteroids.

Experimental

Certified reference materials were purchased from Chemical Reference Materials (NMI, Australia) and reference materials were purchased from Steraloids (Newport, RI, USA) and Sigma Aldrich (St Louis, MO, USA). The β-glucuronidase enzyme (from *E. coli*) was purchased from Roche Diagnostics (Mannheim, Germany). Various laboratory chemicals and reagents were purchased from Merck (Darmstadt, Germany) and Ajax Finechem (Australia). All solvents were of HPLC-grade or higher and were purchased from Sigma-Aldrich (St Louis, MO, USA).
The LC-MS/MS experiments were performed using a Waters Aquity UPLC interfaced to an AB Sciex QTrap 5500 mass spectrometer. Chromatography was performed using an Acquity UPLC BEH C18 1.7 µm (1.0 x 100 mm) column. Mobile phases consisted of 0.2% formic acid in water (A) and 90% acetonitrile with 0.2% formic acid in water (B). The gradient consisted of a constant flow rate of 100 µL/min with solvent B increasing from 10% to 90% in 15 minutes and returning to starting conditions for 5 minutes.

 ISBN 978-3-86884-038-4

Appropriate concentrations of the internal standard mefruside, β-glucuronidase enzyme and phosphate buffer (pH 7.0) were added to 3 mL of urine prior incubation. Samples were then loaded onto an OASIS WCX SPE cartridge pre-conditioned with methanol (3 mL) then water (3 mL). The SPE cartridges were washed with water (2 mL) then eluted with 2 mL of 60% acetonitrile/40% methanol (containing 0.5% glycerol) with 2.0% formic acid. The eluate was evaporated to dryness and reconstituted with 200 µL of 10% methanol. 10 µL was injected into the LC-MS/MS.

Compound	Before SPE solvent modification			After SPE solvent modification		
	Concentration (ng/mL)	Mean Recovery (%)	SD	Concentration (ng/mL)	Mean Recovery (%)	SD
19-norandrosterone	2	59	10.8	2	87	24.2
3OH stanozolol	2	57	5.3	2	84	12.5
Clenbuterol	2	4	1.7	2	95	9.4
EMD	2	48	3.7	2	76	7.7
Methyltestosterone M2 (17a-methyl-5b-androstane-3a,17b-diol)	2	65	8.3	2	101	12.2
Aminoglutethamide	10	107	13.4	4	52	7.9
Boldione	10	107	4.5	4	98	8.4
Codein	10	68	7.0	4	87	10.1
Fenoterol	10	4	3.7	4	90	9.1
Fluoxymesterone M2 (9a-fluoro-17,17-dimethyl-18-nor-androst-4,13-diene-11b-ol-3-one)	10	97	7.9	4	87	10.5
Stenbolone	10	119	18.6	4	86	10.4
Terbutaline	10	25	3.3	4	93	11.4
Amiloride	30	*	*	40	95	11.9
Bamethan	30	9	1.3	40	93	9.7
Carvedilol	30	2	0.6	40	81	9.8
Chlorexolone	30	100	6.4	40	100	7.3
Desonide	30	97	3.8	40	97	7.5
Etilefrine	30	33	5.7	20	91	7.4
Fluocortolone	30	100	5.1	40	98	8.8
Formoterol	30	6	3.7	40	96	7.9
Norbuprenorphine	30	1	0.3	40	94	8.7
Norfentanyl	30	1	0.1	40	96	7.4
Pholedrine	30	2	1.0	40	96	9.6
Para-hydroxyamphetamine	30	17	5.8	40	94	9.9
Probenecid	30	94	19.4	40	95	12.4
Salmeterol	30	6	4.3	40	98	5.2
Synephrine	30	26	5.1	40	77	7.4
Acetazolamide	30	46	20.6	40	22	7.1
Althiazide	30	30	1.4	40	28	1.8
Cyclopenthiazide	30	31	0.4	40	23	1.7
Cyclothiazide	30	31	0.8	40	24	2.0
Ethacrynic Acid	30	57	5.0	40	71	5.1
Hydrochlorothiazide	30	91	10.8	40	80	9.4

* *Didn't extract well initially*

Table 1. Mean recoveries of 33 compounds before and after modification of SPE elution solution

ISBN 978-3-86884-038-4

Results and Discussion

The method was developed to analyse approximately 150 compounds from WADA's prohibited list. All, except four compounds analysed (19-norandrosterone, methyltestosteroneM2(17a-methyl-5b-androstane-3a,17b-diol), 1-testosterone metabolite(1-androsterone) and oxabolone metabolite(4-hydroxyestrenedione)), were detectable at or below their MRPL's (80% with LOD≤1 ng/mL). Despite being difficult to ionise, 19-norandrosterone and methyltestosterone M2 were detectable at 1 ng/mL at the instrument in solvent but in urine matrix were detectable at 3.0 and 2.0 ng/mL, respectively. 1-Testosterone metabolite and oxabolone metabolite were undetectable in a urine matrix.
Despite some compounds being detectable at their MRPL, they had unacceptably high CV's with low recoveries. Norbuprenorphine at a concentration of 30 ng/mL, had a recovery of less than 10% with a CV of approximately 30% (Table 1). Those compounds with low recoveries were found to have similar chemical structures, secondary amines with a ring structure and attached hydroxyl groups. It was also noted that these compounds had even lower recoveries in the higher concentration spikes. These compounds with low recoveries were found to be retained on the SPE cartridge during extraction. To improve recoveries the SPE elution solution was modified to an acetonitrile/methanol/formic acid mix instead of methanol/formic acid.
While modification of the elution solution improved the recoveries of most analytes, no change was observed for 3 thiazide compounds, indicating that loss or degradation is occurring before extraction by the SPE cartridges. One compound, aminoglutethiamide, showed a decrease in recovery using the modified elution solvent. Despite this its LOD was still below 0.1 ng/mL, well below its MRPL of 50 ng/mL.
Given the sensitivity of the Waters Acquity UPLC interfaced to an AB Sciex5500, it is reasonable to consider the direct analysis of diluted urine [2] method for S3, S4, S5, S6, S7 compounds and probably P2 compounds given results obtained for carvedilol.

Conclusions

Although it was not possible to set up a single SPE based multiresidue method which can be used to screen all out of competition samples for the compound classes S1, S3, S4 and S5 which include anabolic steroids and diuretics, a method has been developed which can extract all the desired analytes and which using LC-MS/MS can detect most prohibited substances in the classes S1, S3, S4, S5, S6, S7, S8,S9 and P2. The compounds which are not able to be detected at the required MRPL, such as some anabolic androgenic steroid metabolites, will still require a second GC-MS or GC-MS/MS analysis of the derivatised SPE extract.

References

[1] World Anti-Doping Agency. The 2011 Prohibited List. International Standard, Montreal (2011) http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/WADA_Prohibited_List_2011_EN.pdf

[2] Guddat S., Solymos E., Orlovius A., Thomas A., Sigmund G., Geyer H., Thevis M., and Schanzer W. (2011), High-throughput screening for various classes of doping agents using a new 'dilute-and-shoot' liquid chromatography-tandem mass spectrometry multi-target approach. *Drug Test. Analysis* **3**,836-850.

Acknowledgements

This project was supported with funding from the WADA.