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Analysis of Synthetic Cannabinoids in Urine by UPLC-MS/MS and K2 (Synthetic Cannabinoids-1) Urine Enzyme Immunoassay

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Abstract

Cannabimimetics are forbidden since 1 January 2011 according to the WADA Prohibited List, group S8. The new UPLC-MS/MS method has been developed for routine analysis of metabolites of "Spice" in urine.
Five hundred µL of urine sample is extracted after enzymatic hydrolysis and injected to the UPLC-MS/MS system applying electrospray ionization in positive mode. "Spice" metabolites can be detected at urinary concentrations lower than 0.5 ng/mL. Confirmation of the "Spice" metabolites can be performed with the same extraction and chromatography system using a modified mass spectrometry method.
We tested the Immunalysis K2 EIA Kit for screening of synthetic cannabinoids. According to the manufacturer the kit is sensitive to detect the presence of JWH-018, JWH-073, AM-2201 and their metabolites in human urine samples.

Introduction

Cannabimimetics are forbidden in sports since 1st of January 2011 according to the WADA Prohibited List [1], group S8. The recently marketed cannabimimetics activate the same receptors as THC. It is a challenge for doping control laboratories to analyse the cannabinoids as vast number of compounds on the market and the number is still growing. Another problem is to have appropriate reference material as only metabolites are excreted in urine. There are several methods available to analyse this group of compounds using different techniques and instrumentation [2-4].

Experimental

Reference material

Reference standards of metabilites (M) and internal standards (IS) including JWH-018N-pentanoic acid M, JWH-018 N-(5-hydroxypentyl) M, JWH-018 5-hydroxyindole M, JWH-018 6-hydroxyindole M, JWH-073 N-butanoic acid M, JWH-073 5-hydroxyindole M, JWH-081 N-(5-hydroxypentyl) M, JWH-122 N-(5-hydroxypentyl) M, JWH-210 N-(5-hydroxypentyl) M, JWH-250 N-(hydroxypentyl) M, JWH-398 N-(5-hydroxypentyl) M, AM2201 N-(4-hydroxypentyl) M, JWH-018 N-(5-hydroxy-pentyl)-d5 (IS) and JWH-073 N-butanoic acid-d5 (IS) were provided by Cayman Chemicals (Ann Arbor, USA).

Sample pre-treatment

500 µL of urine sample is mixed with 200 µL of 1M ammonium acetate buffer pH 6.9, 10 µL (2 µg/mL), internal standard mixture (JWH-018 N-(5-hydroxypentyl) metabolite-d5 and JWH-073 N-butanoic acid metabolite-d5) and 10 µL β-glucuronidase from *E.coli*.
After hydrolysis (1 hour, 50°C) 300 µL of a solution (30% NaCl, 7,5% K_2CO_3) and 5 mL *tert*-buthyl methyl ether are added. The samples are mixed and centrifuged for 5 min at 3000 rpm.
The ether fraction is separated and after evaporation reconstituted in 100 µL of acetonitrile (ACN)/water, (50:50, v/v) solution. 5 µL is injected onto Waters UPLC-MS/MS system.

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Chromatographic conditions
The analytes are separated on an Acquity UPLC BEH Phenyl 1.7 µm (1.0x50 mm) column. The mobile phase constitutes of water with 0.1% formic acid (A) and acetonitrile (B). The flow rate used was 0.3 mL/min at 60°C. The gradient was as follows: initial mobile phase composition was 25% B, followed by a linear gradient to 55% B up to 3 min, followed by another increased gradient up 95% of B up to 3.5 min, hold for 0.5 min and then decreased to 25% of organic phase B and hold until the run is completed. Run time was 4.5 min.

Mass spectrometer
The Waters Quattro Premier tandem mass spectrometer with electrospray ionization ESI ion source was operated in the positive ionization mode with multiple reaction monitoring and 0.01 s dwell time. All compounds produce $[M+H]^+$ ions. Capillary voltage was 1 kV, source and desolvation temperature were 120°C and 350°C, respectively. Desolvation gas flow was 1000 L/h and Cone gas flow 50 L/h.

Immunological method
The Immunalysis K2 EIA Kit (from IMMUNALYSIS Corp., Pomona, CA, USA) is used at the drug of abuse laboratory for screening purposes. The assay is based on the competition of a naphthoyl indole labeled enzyme, (glucose-6-phosphate dehydrogenase, G6PDH) the free drug and the drug metabolites. The enzyme G6PDH activity is determined at 340 nm spectrophotometrically by the conversion of NAD^+ to NADH. According to the manufacturer the kit is sensitive to detect the presence of JWH-018, JWH-073, AM-2201 and their metabolites in human urine samples. According to the table of Cross-Reactivity with related drugs the method could also be useful for analysis of JWH-007, JWH-015, JWH-019, JWH-022, JWH-200.

Results and Discussion

The presented UPLC-MS/MS method (Figure 1) is developed and used for screening of 13 metabolites of cannabinoids for antidoping purposes and for confirmation in drug of abuse laboratory after screening with the Immunalysis K2 EIA Kit.
The extraction procedure is similar to one already used in our laboratory for extraction of anabolic steroids with some modifications. Smaller amounts of the urine sample and the reagents are used. Ammonium acetate is used instead of phosphate buffer.
With this method set up, cannabinoids can be detected at urinary concentrations lower than 36 pg/mL and LOQ below 120 pg/mL for all but one, the metabolite of CP47,497 (LOD 450 pg/mL and LOQ 1.5 ng/mL). This is significantly lower than 1 ng/mL required by WADA in the Technical Document-TD2013MRPL.
The calibration curves for all measured compounds were obtained by analysing spiked urine samples in range 0-100 ng/mL. The correlation factor for all standard curves was better than 0.99. Recovery exceeded 80% for all hydroxy-metabolites and no less than 44% for carboxylic acid metabolites.
Immunological analysis of urine samples separately spiked with 1 ng/mL of 13 metabolites of cannabinoids tested negative for all but one, JWH-018 N-pentanoic acid. At 10 ng/mL the results were positive for metabolites of JWH-018, JWH-073, JWH-122 and AM2201. We conclude that this method in not sensitive enough for antidoping purposes. Results are presented in Figure 2.

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JWH 018 M -D5	363 > 155	25	38
JWH 073 M -D5	358 > 127	25	40
JWH 018 5-pentyl-OH M	358 > 155	25	40
	358 > 155	55	40
JWH 018 N-pentoic acid M	372 > 127	51	36
	372 > 155	24	36
JWH 018 5-indole-OH M	358 > 127	55	40
	358 > 155	25	40
JWH 018 6-indole-OH M	358 > 127	55	40
	358 > 155	25	40
JWH 073 N-butanoic acid M	358 > 127	55	40
	358 > 155	25	40
JWH 073 5-indole-OH M	344 > 127	51	39
	344 > 155	27	39
JWH 081 5-pentyl-OH M	388 > 157	45	42
	388 > 185	25	42
JWH 122 5-pentyl-OH M	372 > 141	40	42
	372 > 169	25	42
JWH 210 5-pentyl-OH M	386 > 155	40	42
	386 > 183	25	42
JWH 398 5-pentyl-OH M	392 > 161	50	42
	392 > 189	25	42
JWH 250 N-(5-hydroxypentyl) M	353 > 90.8	55	34
	353 > 121	20	34
AM2201 N-(4-hydroxypentyl) M	376 > 127	55	40
	376 > 155	25	40
(±)-CP 47,497-C7-hydroxy M	335 > 175	15	16
	335 > 317	10	16

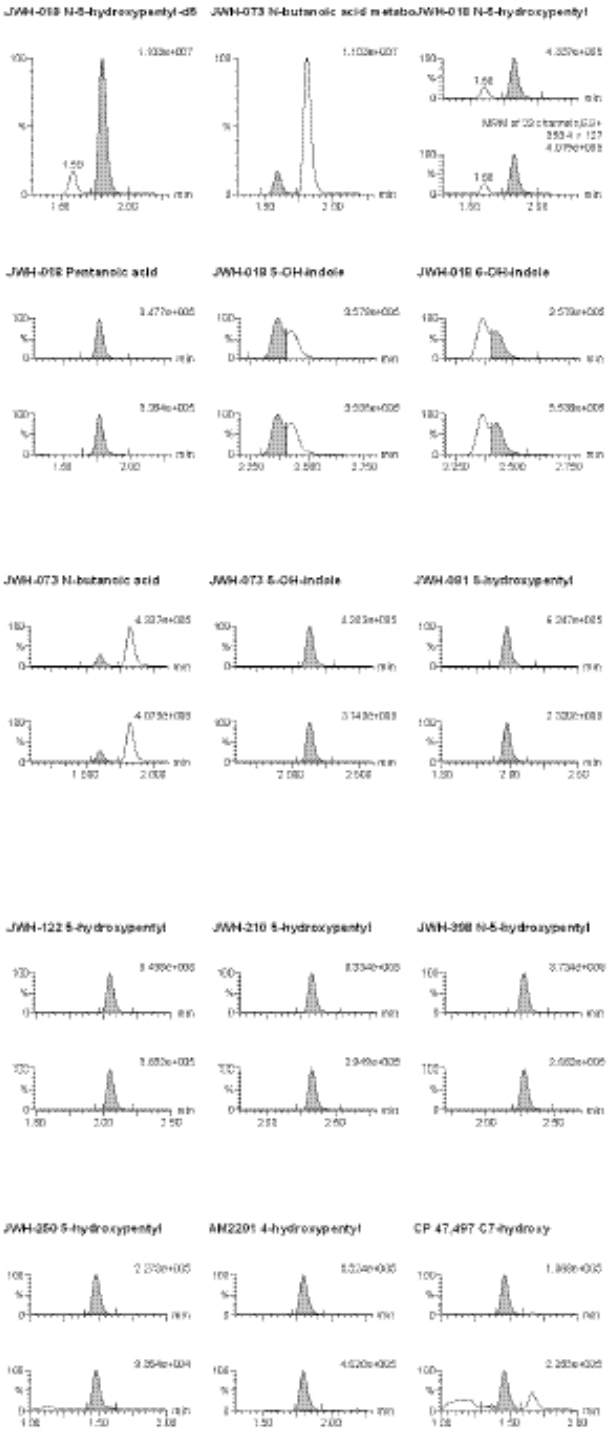

Figure 1. MRM method showing parent ion, daughter ion, collision energy and cone energy. The related chromatograms are injections of 5 ng/mL spiked urine except the metabolite of CP47,497 which is 50 ng/mL.

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Compound	Metabolite	1 ng/mL	10 ng/mL	100 ng/mL
JWH-073	N-butanoic acid	0.4	6.5	25.9
	5-OH-indole	0	1.6	12.3
JWH-018	N-pentanoic acid	1.1	6.2	27.3
	N-5-OH-pentyl	0	4.6	28.6
	5-OH-indole	0	1.1	8.7
	6-OH-indole	0.3	1.7	15.5
JWH-081	N-5-OH-pentyl	0	1.2	6.2
JWH-122	N-5-OH-pentyl	0.1	2	12.1
JWH-210	N-5-OH-pentyl	0	0.4	4.1
JWH-250	N-5-OH-pentyl	0.5	0	2.2
JWH-398	N-5-OH-pentyl	0	1.9	13.7
AM2201	N-4-OH-pentyl	0	5.9	28.8
CP47, 497	C-7-OH	0	0.2	0

Figure 2. Overview of the results from the immunological analysis of spiked urine. NOTE: The method saturates between 25-30 ng/mL, a value above 2 ng/mL is considered positive.

Conclusions

The immunological method was not sensitive enough to be used for doping analysis.
A sensitive UPLC-MS/MS screening method to determine metabolites of synthetic cannabinoids in urine was developed, fulfilling MRPL of 1 ng/mL required by WADA in the technical document – TD2013MRPL. Confirmation of cannabinoid metabolites is performed with the same extraction method and ESI-MS/MS method modified for specific compounds.

References

[1] World Anti-doping Agency. The 2013 Prohibited List International Standard, Montreal (2013) http://www.wada-ama.org

[2] Sobolevsky T, Prasolov I, Rodchenkov G. (2010) Detection of JWH-018 metabolites in smoking mixture post-administration urine. *Forensic Sci Int* **200**, 141-147.

[3] Hutter M, Broecker S, Kneisel S, Auwärter V. (2012) Identification of the major urinary metabolites in man of seven synthetic cannabinoids of the aminoalkylindole type present as adulterants in 'herbal mixtures' using LC-MS/MS techniques. *J Mass Spectrom* **47**, 54-65.

[4] Yanes DP, Lovett DP. (2012) High-throughput bioanalytical method for analysis of synthetic cannabinoid metabolites in urine using salting-out sample preparation and LC-MS/MS. *J Chromatogr B* **909**, 42-50.

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