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Application Note

Analysis of Doping Agents by UPC^2-MS/MS

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This is an Application Brief and does not contain a detailed Experimental section.

Abstract

UPC^2-MS/MS is a chromatographic technique orthogonal to GC and LC. A UPC^2-MS/MS method was developed for testing a variety of doping agents. Extremely polar compounds such as meldonium, amiloride, and ethyl glucuronide were well retained, and most other compounds displayed excellent chromatographic performance. Retention times were stable for all compounds within and between batches, with %RSDs <0.6%. The method had the analytical sensitivity and selectivity to accurately detect all compounds at, or below, WADA's Minimum Required Performance Levels (MRPL). This technique represents a valuable addition to GC and LC to more fully cover the chromatographic space required for anti-doping analysis.

Benefits

- Orthogonal selectivity and retention, allowing retention of compounds that perform poorly by GC or LC
- Rugged, reproducible chromatography for a variety of doping agents
- Analytical sensitivity required to meet Minimum Required Performance Levels (MRPL) of doping agents

Introduction

The Prohibited List of the World Anti-Doping Agency (WADA) [WADA, 2021] currently contains hundreds of specifically banned substances, as well as performance enhancing agents which are not explicitly named, but belong to banned classes of drugs. One of the greatest challenges for anti-doping labs is the physicochemical diversity of compounds that require analytical testing. Many of these are currently addressed by LC-MS (LC-HRMS and LC-MS/MS) and GC-MS (GC-HRMS and GC-MS/MS). However, there remain many substances for which the current technologies are challenged for reliable identification and confirmation. Many of these substances are polar, with minimal retention on traditional chromatographic platforms, or have poor peak shape due to their chemistry. UPC^2-MS/MS is a separation technique that is orthogonal to both GC and LC, often providing separation, resolution and selectivity that is not attainable by the other chromatographic techniques [Nováková, 2015; Losacco, 2020]. This application brief details the chromatographic method development and analysis of a wide variety of banned substances with a diversity of physicochemical properties, by UPC^2-MS/MS. These included substances such as stimulants, steroids, drugs of abuse, glucocorticoids, diuretics, beta-blockers, and other banned substances. Using the UPC^2-MS/MS method it was possible to retain and resolve compounds such as meldonium, amiloride, and ethyl

glucuronide, which are challenging to analyze by other chromatographic techniques, as well as dozens of other test compounds. Analysis of 1000 anonymized anti-doping samples showed no adverse analytical findings. Retention times were stable for all analytes within and between batches, and the method had the analytical sensitivity to accurately identify all compounds at WADA's Minimum Required Performance Levels (MRPL). [WADA, 2019]

Experimental

Materials

Reference material for all analytes and internal standards were generously provided by the Drug Control Centre (DCC), King's College London (London, UK). Eight compounds were used for initial column screening and method development. These are listed in Table 1 together with their specific MS conditions.

For the second phase of the work, a larger group of compounds (also provided by the DCC at King's College London) were investigated (see Appendix). Individual reference materials were combined to yield two mixed solutions – QC1 and QC2. These were prepared in methanol for method development and retention time verification, and in blank urine as a spiked reference sample, for inclusion when analyzing the batches of authentic samples. The Appendix lists the compounds, relevant concentrations, retention times, and specific MS conditions.

An internal standard (IS) solution contained mefruside, ephedrine-d_3, and salbutamol-d_3 at a concentration of 10 µg/mL.

Authentic Samples

One-thousand authentic, anonymized anti-doping urine samples were generously supplied by the DCC and analyzed using the final conditions listed.

Sample Preparation

Sample preparation was adapted from Nováková *et al.* [Nováková, 2015]. Two hundred microliters of urine was diluted with 790 µL ACN and 10 µL of IS mixture (10 µg/mL) and centrifuged at 5000 rcf for 10 min; 2 µL of the supernatant were injected onto the column.

LC Conditions

UPC^2-MS/MS

LC system:	ACQUITY UPC^2 System
Detection:	Xevo TQ-XS
Column(s):	Torus Diol (OH) Column, 130 Å, 1.7 µm, 3.0 x 100 mm.
Column temp.:	35 ^{o}C
Sample temp.:	10 ^{o}C
Injection volume:	2 µL
Flow rate:	1.2 mL/min
Mobile phase A:	CO_2
Mobile phase B:	Methanol with 0.1% strong ammonia
Make up flow:	Methanol at 0.2 mL/min

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
Init	1.2	90	10	6
1.0	1.2	90	10	6
4.0	1.2	50	50	6
4.5	1.0	43.3	56.7	6
5.0	1.0	43.3	56.7	6
5.1	1.2	90	10	6
7.0	1.2	90	10	6

MS Conditions

MS system:	Xevo TQ-XS
Ionization mode:	ESI+ and ESI-
Capillary voltage:	2.0 kV (-2.0 kV)
Collision energy (CE):	Compound dependent (see Appendix)
Cone voltage (CV):	Compound dependent (see Appendix)

Initial Column Testing Conditions

Two chromatographic dimensions were screened in order to find the optimal conditions; these were the organic modifier composition and the column chemistry. The following mobile phase B (MPB) modifiers were screened: no modifier, 0.1% formic acid, 0.1% strong ammonia, and 10 mM ammonium formate. Each was added to methanol and used as MPB.

Four columns were also screened, all with the same dimensions and particle size (130 Å, 1.7 µm, 3.0 x 100 mm). Stationary phases included: the Viridis BEH 2-Ethylpyridine (2-EP), Torus 2-PIC, the Torus 1-AA, and the Torus Diol (OH) Column. All columns used the solvent ramp detailed in final method, except that the flow rate for all columns other than the Diol Column was 1.5 mL/min.

Compound	RT (min)	Ionization mode	CV (V)	$[M+H]^+$/ $[M-H]^-$	Quantifier ion (*m/z*)	Qualifier ion (*m/z*)	CE1 (eV)	CE2 (eV)
Danazol	1.46	Pos	20	338.2	303.3	321.3	15	15
Fluticasone propionate	1.77	Pos	20	501.2	293.2	313.1	20	15
Probenecid	3.25	Neg	20	284.1	240.1	140.1	20	20
GW 1516	3.33	Pos	20	454.2	188.1	256.1	45	45
Bumetanide	4.10	Neg	20	363.1	207.1	80.0	20	20
Meldonium	4.26	Pos	25	147.1	59.1	132.1	20	15
Ethyl glucuronide (EtG)	4.62	Neg	25	221.1	85.0	75.0	15	15
3' OH stanozolol glucuronide	5.28	Neg	20	519.3	343.2	175.1	40	20

Table 1. Compounds used for initial UPC^2 testing.

Results and Discussion

Column and Modifier Testing

Four UPC^2 Columns and four modifiers were initially evaluated using a limited test mix of compounds. Initial testing using the Viridis 2-EP Column revealed that using 0.1% strong ammonia resulted in superior peak shape and retention compared with 0.1% formic acid, 10 mM ammonium formate, or no modifier at all. Further testing with the additional columns showed that the Torus Diol Column outperformed the other three with regards to retention, peak shape, reduced tailing, and analytical sensitivity, particularly for 3-OH stanozolol-glucuronide and meldonium. An example of the chromatography on the Diol column is shown in Figure 1. Excellent chromatographic performance was seen for the eight initial doping compounds tested. Ethyl glucuronide and meldonium, both of which are difficult to retain by reversed-phase LC or GC were well retained and exhibited very good peak shape with minimal tailing. The other compounds also demonstrated good retention and symmetrical peak shape, despite their chemical variety. The Diol Column was therefore used to analyze the larger panel of doping substances as well as 1000 authentic anonymized athlete samples.

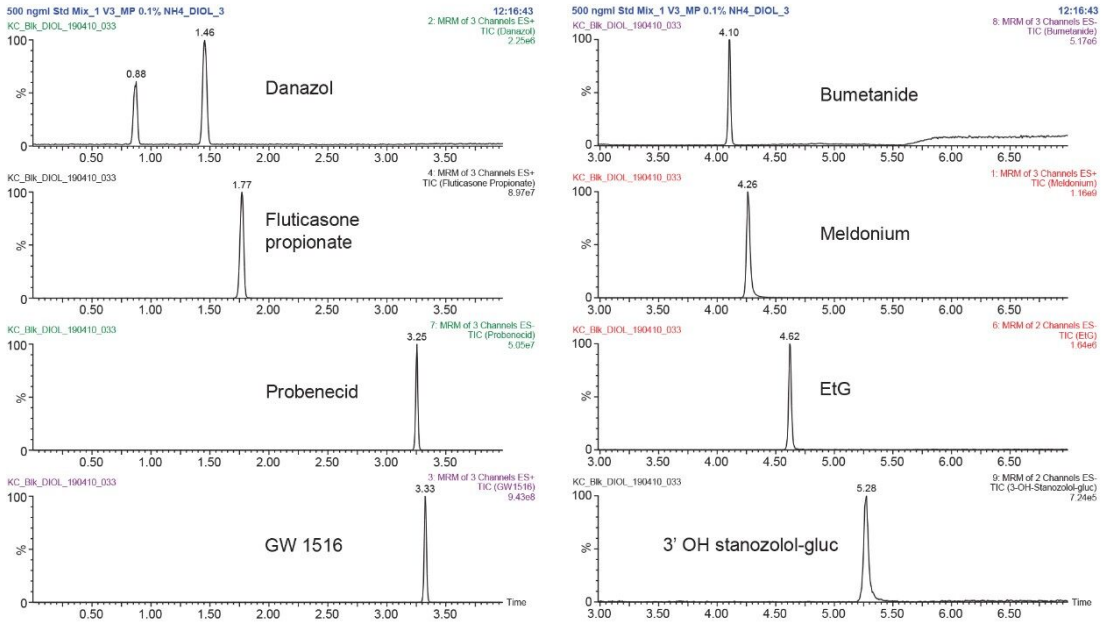

Figure 1. Final chromatography of doping compounds from the initial screening experiments. This separation was achieved on a Torus Diol (OH) Column (130 Å, 1.7 µm, 3.0 x 100 mm). The concentration of all analytes was 500 ng/mL.

Analysis of Authentic Samples

The expanded panel of compounds listed in the Appendix was used to screen 1000 authentic anti-doping samples. This list of substances was compiled by scientists from anti-doping laboratories; compounds were selected to ensure representation for several key drug classes from the WADA prohibited list. The chromatography of these can be seen in Figure 2. Most compounds demonstrated good chromatographic performance regarding retention, peak shape and selectivity. Nikethamide, for example, eluted early, but displayed good peak shape and retention time stability, unlike some of the retention time stability issues described by Losacco ***et al***. [2020] when using a BEH Column with ammonium formate as a mobile phase modifier. Most of the other peaks displayed excellent chromatographic characteristics. Some exceptions were compounds such as fentanyl, which consistently displayed a peak doublet. Octopamine had significant tailing and minor tailing was seen for oxymorphone and cathine. Many of the sulfated steroids either co-eluted or were not fully baseline resolved from their structural analogs. Nevertheless, most compounds representing a wide variety of chemotypes displayed excellent chromatographic performance.


Cortisol
Cortisone

Figure 2. Chromatography of the compounds in the expanded panel used for the second phase of experiments. Cortisol is specifically labelled at 2.74 min and cortisone is labelled in the prednisolone MRM channel at 2.83 min. All other compounds are named in the upper right corner of their traces. All retention times are listed in the Appendix.

One key advantage of UPC^2 is its orthogonality to other chromatographic separation techniques. This can be seen in the retention and selectivity for extremely polar compounds such as ethyl glucuronide, amiloride, and meldonium. Other polar and moderately polar compounds such as morphine, salmeterol, etilefrine, and amphetamine were also very well retained and resolved, demonstrating the overlap between UPC^2 and LC. This broad, alternative selectivity should allow UPC^2 to be an important complementary method, expanding the reach of traditional chromatographic methods such as LC and GC and offering confirmation by an alternative chromatographic technique.

Retention Time Stability

Reference standards (QC1 and QC2) injected in the beginning, middle, and end of each batch revealed stable retention times for all analytes. All compounds had between batch retention time %RSDs <0.6%. The majority had %RSDs under 0.5% and 63% were under 0.3%. This easily meets WADA's retention time criteria for positive identification [WADA, 2015]. In addition, the internal standards included in each sample were monitored and were found to all have retention time %RSDs <0.3% within a batch.

Sensitivity

WADA defines analytical thresholds as Minimum Required Performance Levels (MRPL). These values are listed in the Appendix and were the concentrations used in the urine QC standards (QC1 and QC2) with the exception of hydrochlorothiazide, propranolol, and bendroflumethiazide, which were spiked at 50% of MRPL. Ketoconazole and tramadol have no established MRPL and were spiked at the concentrations listed in the Appendix. With the exception of ketoconazole, all the compounds investigated could easily be identified by the system at the noted concentrations. Responses for ketoconazole were close to the detection limit at 50 ng/mL, but it was still detected in all spiked QC samples in all 23 batches. Buprenorphine was easily detected at 5 ng/mL as was fentanyl at 2 ng/mL.

Conclusion

Waters UPC^2-MS/MS System using the Xevo TQ-XS has been demonstrated to be a reliable, orthogonal alternative to GC and LC-MS assays, especially for polar compounds that do not retain well by other chromatographic methods. Retention times were stable across 23 batches (>1200 injections) for all analytes. Method development revealed that of those investigated, the Torus Diol Column combined with a mobile phase modifier of 0.1% strong ammonia resulted in the best chromatography for nearly all the compounds. Even using a simple dilute and inject method, the system has the sensitivity and selectivity to positively identify all spiked compounds at the MRPL and in many cases, even at 50% MRPL in both positive and negative ESI.

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Acknowledgements

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Appendix. Retention times (RT), concentrations and MS Conditions for all analytes.

Compound	RT (min)	Conc. (ng/mL)	Ionization mode	$M+H]^+$/ $[M-H]^-$	CV (V)	Quantifier ion (*m/z*)	Qualifier ion (*m/z*)	CE1 (eV)	CE2 (eV)
Amiloride	4.40	100	Pos	230.0	4	171.0	116.1	18	30
Amphetamine	2.54	100	Pos	136.1	10	91.1	119.0	10	10
19 nor-Androsterone sulfate	3.81	50	Neg	355.1	30	355.1	231.1	25	40
19 nor-Etiocholanolone sulfate	3.83	50	Neg	355.2	30	355.2	231.1	25	40
5a-DHT-sulfate	3.81	50	Neg	369.2	30	369.1	285.2	25	40
Androsterone-sulfate	3.81	200	Neg	369.2	30	369.2	259.1	25	40
Atenolol	3.51	100	Pos	267.2	10	116.0	190.2	18	20
Bendroflumethiazide	3.51	100	Neg	420.1	56	289.0	328.2	22	30
Benzoylecgonine	3.07	100	Pos	290.2	2	168.1	105.1	30	18
Betamethasone	2.82	30	Pos	393.2	20	355.0	279.0	20	20
Buprenorphine	1.55	5	Pos	468.3	58	414.2	55.0	32	4
Cathine	3.16	100	Pos	134.1	44	117.1		25	
Codeine	2.05	50	Pos	300.1	28	215.1	165.0	24	38
Cortisol	2.74	30	Pos	363.2	25	121.1	91.1	22	50
Cortisone	2.83	30	Pos	361.2	25	343.2	325.2		
Dexamethasone	2.79	30	Pos	393.2	20	355.0	279.0	20	20
DHEA-sulfate	3.81	200	Neg	369.2	30	369.1	285.2	25	40
Ephedrine-d3 (IS)	***2.65***	***100***	***Pos***	***169.1***	***2***	***151.1***	***115.1***	***10***	***25***
Ephedrine	2.64	100	Pos	166.1	2	148.1		10	
Etilefrine	3.32	100	Pos	182.1	20	135.0	164.1	12	30
Fenoterol	4.17	20	Pos	304.2	38	135.1		16	
Fentanyl	0.92	2	Pos	337.2	30	188.2	105.1	20	35
Formoterol	3.61	20	Pos	345.1	10	149.1		18	
Hydrochlorothiazide	4.19	100	Neg	295.9	62	269.0		20	
Ketoconazole	2.33	50	Pos	531.3	20	489.0		20	
Mefruside (IS)	***2.21***	***100***	***Pos***	***383.3***	***20***	***285.0***	***190.0***	***10***	***25***
Meldonium	4.26	200	Pos	147.1	25	59.1	132.2	20	15
Methamphetamine	2.05	100	Pos	150.1	18	91.1	119.1	16	9
Morphine	3.04	50	Pos	286.1	25	201.1	165.1	25	35
Nandrolone sulfate	3.94	50	Neg	353.2	30	353.1	271.1	25	40
Nikethamide	0.66	100	Pos	179.1	44	108.1		18	
Octopamine	4.09	1000	Pos	136.0	40	91.1		16	
Oxilifrine	3.51	100	Pos	182.0	20	105.0		20	
Oxymorphone	2.08	50	Pos	302.1	34	227.1	242.1	25	25
Prednisolone	2.25	30	Pos	361.2	25	343.2	325.2	20	20
Probenecid	2.95	100	Neg	284.1	50	240.1	139.9	16	24
Propranolol	2.62	50	Pos	260.2	10	116.1	183.2	16	16
Pseudoephedrine	2.64	100	Pos	166.1	2	148.1		10	
Ritalinic acid	3.54	100	Pos	220.1	25	84.0	56.0	40	40
Salbutamol-d3 (IS)	***3.43***	***500***	***Pos***	***243.2***	***20***	***225.1***	***151.1***	***7***	***25***
Salbutamol	3.43	500	Pos	240.1	20	222.0	166.0	7	10
Salmeterol	3.56	20	Pos	416.2	50	380.2		18	
Testosterone-epi-sulfate	3.87	50	Neg	367.2	30	367.2	351.1	25	30
Testosterone-sulfate	3.90	50	Neg	367.2	30	367.2	351.1	25	30
THC-COOH	2.51	150	Pos	345.2	25	193.0	299.2	25	25
Tramadol	1.67	50	Pos	264.2	25	58.0		15	
Tuaminoheptane	2.18	100	Pos	116.0	18	57.1		10	

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