

Quality control of the completeness of solid-phase extraction and hydrolysis using the deuterated internal standards –Screening procedure for stimulants, narcotics, diuretics, β -blockers and other dope agents in human urine by means of UPLC/MS/MS–

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Introduction

It is important to control the daily assay processes, especially key-steps such as extraction, hydrolysis or chromatographic condition on the routine doping tests. The use of an internal standard is useful tool not only for quantification but also for monitoring any human errors. To monitor an enzymatic hydrolysis with β -glucuronidase on the deconjugation of steroids, androsterone- d_5 -glucuronide or androsterone- d_4 -glucuronide has been used as deuterated internal standard [1]. In 2007, a rapid screening procedure by means of ultra-performance liquid chromatography (UPLC)/tandem mass spectrometry has been reported [2]. In this presentation, a simultaneous screening procedure of 146 prohibited substances and their metabolites (55 stimulants, 11 narcotics, 39 diuretics, 37 β -blocking agents and 4 other agents) in urine using UPLC/triple quadrupole TQD mass spectrometer will be described and the quality control of the routine assay will be discussed.

Materials and Methods

β -Glucuronidase from *E. Coli* K12 (140 U/mL) was from Roche Diagnostics GmbH (Manheim, Germany). ABS NEXUS-ELUT 60 mg/3 mL for solid-phase extraction (SPE) was purchased from Varian Inc. (CA, USA). Caffeine- d_3 (99.8 atom%D, Figure-1a) was from C/D/N Isotopes Inc. (Quebec, Canada). Acetazolamide- d_3 (*d*ACZ, 99% atom%D, Figure-1b), (\pm)-nicotine-N-methyl- d_3 (*d*NM, 98%) and nicotine-N- β -glucuronide-methyl- d_3 (*d*NMG, 98%, Figure-1c) were purchased from Toronto Research Chemicals Inc. (ON, Canada). Urine sample were prepared and analyzed as shown in Figure-2 and Table-1.

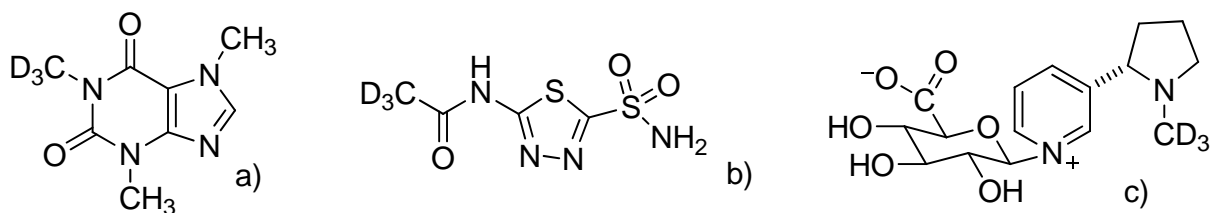


Figure-1. Chemical structure of deuterated internal standards

Table-1. UPLC/MS/MS condition

	UPLC Condition	MS/MS Condition
Urine sample 3 mL		
50 μ L internal standard (IS) ethanol solution	UPLC : ACQUITY UPLC (Waters)	MS/MS : Triple Quadrupole TQD
0.75 μ g caffeine- d_3 , 0.15 μ g acetazolamide- d_3	Column: ACQUITY UPLC BEH C18,	with a Z-spray (Waters)
0.5 μ g nicotine-N- β -glucuronide-methyl- d_3	2.1 mm x 50 mm , 1.7 μ m (Waters)	Ionization: Electro spray (120°C)
pH 6.0 \pm 0.1 0.5 mL 1M-Phosphate	Mobile phase A: 1% CH ₃ COOH	Desolvation temp.: 350°C
25 μ L β -glucuronidase (<i>E.Coli.</i>), 50°C 60 min	B: CH ₃ CN	Desolvation gas flow: 600 L/hr (N ₂)
↓ Centrifuge 2,500 rpm 5 min	A% : B%	
supernatant 3 mL	Gradient: 0-2 min. 90 : 10	Cone gas flow: 50 L/hr (N ₂)
ABS ELUT-NEXUS 60 mg/3 mL	8 min. 20 : 80	Cappillary voltage: 3.5 kV(Positive)
↓ 2 mL 0.5% Glycerol in CH ₃ OH	8.01min 90 : 10	3.0 kV(Negative)
dried residue (Dry up under N ₂ at 60°C)	Run Time: 10 min.	Collision gas: 3.6 x 10 ⁻³ mbar (Ar)
↓ 130 μ L A/B=90/10 (v/v)	Flow rate: 0.20 ml/min.	Acquisition mode: MRM
↓ Centrifuge 13,000 rpm 5 min	Column Temp. 30°C	Mass Transition: grouping: 25 (Table-2)
supernatant UPLC/MS/MS 10 μ L Injection		

Figure-2. Sample preparation

Results and Discussion

As shown in Table-2, a total of 146 dope agents could be analyzed within 10 min by employing UPLC/MS/MS in simultaneous positive and negative cone voltage switching. The limit-of-detection ranged from 0.1 ng/mL to 100 ng/mL. The method allows the detection of prohibited substances in urine sample fortified with 50 % of WADA required MRPL. Control of the hydrolysis step using *d*NMG with 167 ng/mL-urine and caffeine- d_3 with 250 ng/mL-urine was performed. *d*NM as aglycone after enzymatic hydrolysis was 81 ng/mL urine. We selected to monitor the intensity of the aglycon (*d*NM) because intact nicotine-glucuronide (*d*NMG) could not be recovered by this SPE method. If the hydrolysis is complete, the signal to noise (S/N) ratio for *d*NM (m/z 166>132) is an intensive as shown in Figure-3A. Example chromatogram occurred with human error (*e.g.* skip of addition of enzyme) was shown in Figure-3B. In other hands, it is well known that the recovery of acetazolamide and methazolamide from alkali urine on solid phase extraction is very poor [3]. Control of the SPE step with *d*ACZ was employed 50 ng/mL urine (20 % of WADA MRPL). If the SPE is complete, the S/N ratio for *d*ACZ (m/z 224>86) is an intensive as shown in Figure-3A. The S/N ratio of *d*ACZ generated from alkali urine (Figure-3C) is less intensive than that of normal urine (Figure-3A) by one-tenth.

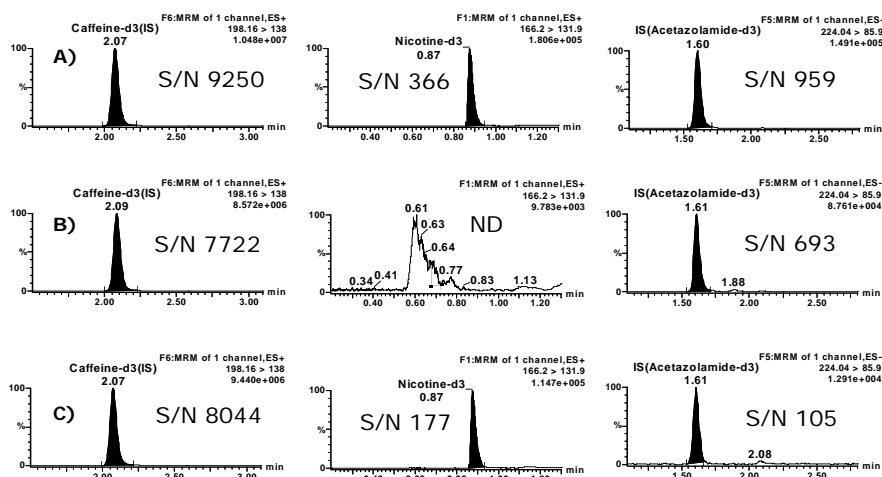


Figure-3. Typical MRM chromatograms; A), B), C)

In the major event, the laboratories are often necessary to help a new comer or temporary staff. To control the key steps in the method is especially important for a robust assay, and we could achieve the high quality and more robust test using the deuterated internal standard. One ion transition is set to monitor the substances in our described method, however, unknown peaks in blank samples for the early eluting analytes (e.g. Synephrine) can be identified by multiple ion transitions and it could also allow for the robust screening method. For the confirmation, the collision energy (CE) and cone voltage should be optimized to obtain minimum of three specific product ions generated from precursor ions of target substances.

Acknowledgements

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References

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Table-2. Retention time and mass parameters used of screening method

Compound	RT(min)	Polarity	Mass transition m/z	Cone V	CE eV	Compound	RT(min)	Polarity	Mass transition m/z	Cone V	CE eV
Synephrine	0.77	+	168.2 > 150.0	18	8	Propylhexedrine	4.56	+	156.3 > 69.0	28	16
Egocinemetylester	0.80	+	200.4 > 182.2	32	16	GHRP-2 metab.	4.57	+	358.0 > 169.8	26	28
Octopamine	0.80	+	154.1 > 135.9	14	6	5-oxo-mefruside	4.60	+	397.0 > 83.8	50	30
Nicotine-methyl-d3 (IS)	0.81	+	166.2 > 131.9	24	14	Ethiazide	4.54	-	324.0 > 204.8	50	22
Phenylephrine	0.85	+	Same as Synephrine			Dichlorphenamide	4.60	-	302.9 > 77.8	40	30
Morphine	0.93	+	286.4 > 152.2	60	62	Pethidine	4.66	+	248.4 > 174.2	34	20
p-OH-Amphetamine	0.97	+	152.1 > 135.0	20	10	Oxprenolol	4.67	+	266.1 > 116.0	25	25
Heptaminol	0.99	+	146.3 > 128.2	20	10	Norbuprenorphine-1	4.69	+	414.2 > 83.0	50	48
Atenolol	1.05	+	267.1 > 145.0	30	35	Norbuprenorphine-2	4.69	+	414.2 > 101.0	50	44
Amiloride	1.10	+	230.0 > 171.0	30	18	Norbuprenorphine-3	4.69	+	414.2 > 57.0	50	48
p-OH-EP/Etilefrine	0.79/0.93	+	182.2 > 164.1	22	12	chlorthalidone	4.71	+	339.0 > 321.9	20	14
Pholedrine	1.01	+	166.1 > 135.0	24	12	Isopropranolol	4.77	+	326.2 > 115.9	30	30
Hydromorphone	1.10	+	286.4 > 185.2	60	30	Indenolol	4.77	+	248.1 > 171.0	30	25
Sotalol	1.15	+	273.1 > 255.0	20	15	Bufetolol	4.78	+	324.2 > 268.0	30	25
Amiphenazole	1.38	+	192.1 > 116.8	36	24	Pipradolol	4.80	+	268.4 > 250.3	24	14
Dorzolamide	1.45	+	325.1 > 199.0	30	20	Labetalol	4.81	+	329.2 > 311.0	25	20
Thiazide-ACB	1.57	+	286.0 > 268.7	24	10	Crothetamide	4.82	+	227.4 > 182.2	16	8
Hydroxymetprolol	1.70	+	284.1 > 115.9	35	25	Doxapram	4.82	+	379.5 > 292.2	34	20
Acebutolol	1.77	+	309.2 > 116.0	30	25	Clopidamide	4.89	+	346.1 > 250.0	45	22
Chlorothiazide	1.86	+	296.0 > 278.9	45	16	Diphenylpropylamine	4.90	+	212.3 > 90.9	25	18
Acetazolamide	1.56	-	220.9 > 82.8	30	16	Ethamivan	4.91	+	224.2 > 151.0	32	18
Acetazolamide-d3 (IS)	1.56	-	224.0 > 85.9	40	20	Fencamfamine	4.93	+	216.4 > 91.0	30	32
Clofenamide	2.07	-	269.0 > 77.8	40	24	Pentazocine	4.93	+	286.5 > 218.3	32	20
Hydrochlorothiazide	2.14	-	295.9 > 268.9	45	20	Hydroxyphenbutolol	4.95	+	308.2 > 252.0	30	25
Amphetamine	1.97	+	136.3 > 91.0	20	14	Metipranolol	4.95	+	310.2 > 191.0	35	35
Oxycodone	2.00	+	316.4 > 298.2	30	20	Prolintane	5.01	+	218.4 > 91.0	30	20
Caffeine-d3 (IS)	2.01	+	198.2 > 138.0	38	20	Propranolol	5.04	+	260.1 > 115.9	30	25
Carteolol	2.17	+	293.2 > 237.0	25	20	Fenfluramine	5.05	+	232.3 > 159.1	32	20
MDA	2.20	+	180.3 > 163.2	18	10	GHRP-2	5.02	+	409.7 > 169.9	26	26
Nikethamide	2.34	+	179.3 > 108.1	26	20	Mesocarb-m	5.09	+	355.0 > 193.0	26	12
Phendimetrazine	2.35	+	192.3 > 147.3	32	20	Alprenolol	5.10	+	250.1 > 116.0	30	20
Phenmetrazine	2.35	+	178.3 > 114.9	30	24	Amineptine-m	5.11	+	310.2 > 193.2	16	12
Pindolol	2.65	+	249.1 > 115.9	30	25	Betaxolol	5.12	+	308.2 > 71.8	35	30
MDMA	2.75	+	194.3 > 163.1	22	12	Bupranolol	5.13	+	272.1 > 216.0	25	20
Nadolol	2.77	+	310.2 > 254.0	25	25	Toraseamide	5.13	+	349.1 > 264.0	25	18
Methazolamide	2.99	+	237.0 > 195.0	25	16	Bevantolol	5.16	+	346.2 > 165.0	30	35
Hydroxybunitrolol	3.10	+	265.1 > 209.0	25	20	Trichlormethiazide	5.17	-	379.9 > 305.8	35	12
Methamphetamine	3.11	+	150.3 > 91.1	26	16	Methylchlorothiazide	5.39	-	358.0 > 321.8	30	14
Diethylpropion	3.22	+	206.2 > 105.0	34	22	Benzphetamine	5.20	+	240.4 > 91.1	30	22
Ethylamphetamine	3.30	+	164.3 > 91.1	24	18	Fentanyl	5.25	+	337.5 > 188.0	52	26
Dimethylamphetamine	3.39	+	164.2 > 90.9	28	18	Modafinil	5.32	+	167.1 > 152.0	50	22
Fenproporex	3.40	+	189.3 > 119.1	24	10	Amineptine	5.33	+	338.4 > 193.2	16	12
Triamterene	3.45	+	254.1 > 237.0	50	26	Carboxy-finasteride	5.33	+	403.0 > 335.0	50	40
Phentermine	3.52	+	150.3 > 91.1	18	18	Clobenzorex	5.38	+	260.2 > 90.8	32	22
Strychnine	3.62	+	335.4 > 184.2	56	38	Cropropamide	5.44	+	241.3 > 196.2	18	8
Mephentermine	3.69	+	164.3 > 91.0	20	24	Carvedilol	5.50	+	407.2 > 99.9	40	40
Hydroxyalprenolol	3.70	+	266.1 > 115.9	30	25	Famprofazone	5.50	+	378.3 > 162.1	36	24
Hydroxyoxprenolol	3.70	+	282.1 > 241.0	25	20	Modafinil acid	5.62	+	167.3 > 152.1	60	24
Ritalinic acid	3.72	+	220.1 > 83.9	30	20	p-OH-Mesocarb	5.64	+	339.2 > 193.0	24	12
Hydroxypropranolol	4.28	+	276.1 > 199.0	35	20	Metolazone	5.63	-	364.1 > 257.0	45	22
Thiazide-AFB	3.37	-	318.1 > 238.8	46	22	Furosemide-1	5.70	-	329.0 > 284.9	30	14
Brinzolamide	3.88	+	382.1 > 77.8	50	28	Furosemide-2	5.70	-	329.0 > 204.9	30	26
Hydroflumethiazide	3.90	-	330.0 > 238.9	45	26	Althiazide	5.74	-	382.0 > 340.8	35	16
Meticrane	4.00	-	274.0 > 117.9	50	32	Benzthiazide	6.07	-	430.0 > 307.9	45	24
Dobutamine -1	3.75	+	302.3 > 137.1	34	24	Penbutolol	5.82	+	292.2 > 236.1	30	20
Dobutamine -2	3.75	+	302.3 > 106.9	34	24	Ethoxzolamide	5.86	+	259.1 > 178.0	30	18
Desmethylselegiline	3.85	+	174.1 > 119.0	26	10	Dextromoramide	5.90	+	393.4 > 306.2	30	22
Mepindolol	3.90	+	263.1 > 115.9	30	25	Propoxyphene	5.92	+	340.4 > 58.0	18	16
Benzoylcegonine	3.91	+	290.4 > 168.2	30	18	Indapamide	5.93	+	366.0 > 132.0	25	16
Methoxyphenamine	3.97	+	180.3 > 149.1	24	12	Methadone	6.00	+	310.5 > 265.2	28	14
l-Selegiline	4.04	+	188.2 > 90.8	28	18	Prenylamine	6.00	+	330.3 > 91.0	35	28
Timolol	4.09	+	317.1 > 261.0	25	20	Polythiazide	6.36	-	438.0 > 397.8	35	16
Acebutolol	4.12	+	337.2 > 116.0	35	30	Bendroflumethiazide	6.37	-	420.0 > 289.0	45	24
Biunitrolol	4.14	+	249.1 > 193.0	25	20	Cyclopentthiazide	6.47	-	378.1 > 204.8	50	28
Bemegride	4.22	+	156.2 > 128.1	28	12	Xipamide	6.56	-	353.0 > 273.9	50	26
Isometheptene	4.22	+	142.3 > 69.0	24	16	Penflutiazide	6.86	-	400.1 > 269.0	50	24
Metoprolol	4.22	+	268.1 > 115.9	30	30	Probenecid	6.88	-	284.1 > 240.1	30	16
Befunolol	4.23	+	292.1 > 214.9	35	30	Ethacrynic acid	7.06	-	301.0 > 242.9	20	12
Levobunolol	4.28	+	292.2 > 236.0	25	20	Tripamide	6.42	+	370.1 > 136.1	50	30
Carboxybupranolol	4.28	+	302.1 > 245.9	30	20	Piretanide	6.46	+	363.1 > 282.0	40	22
Carphedon	4.39	+	219.1 > 174.1	20	14	Fluticasone propionate metabolite	6.50	+	453.3 > 293.1	30	16
Bucumolol	4.40	+	306.1 > 250.0	25	20	Bumetanide	6.74	+	365.1 > 240.1	35	18
Esmolol	4.47	+	296.2 > 145.0	35	40	Mesocarb	6.85	+	323.4 > 177.2	26	12
Celiprolol	4.52	+	380.2 > 250.9	35	50	Benfluorex	6.85	+	352.3 > 230.0	25	18
Cocaine	4.55	+	304.4 > 182.2	26	20	Canrenone	7.05	+	341.2 > 107.0	40	34
Mefenorex	4.55	+	212.3 > 91.0	28	20	Benzbromarone	8.83	-	422.9 > 250.8	50	32