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Simultaneous extraction and detection of diuretics, beta-blockers and other xenobiotics in human urine by HPLC-MS/MS and UPLC-MS/MS

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Introduction

Diuretic compounds, beta-blockers and stimulants are banned in competition by the World Anti Doping Agency (WADA) because of their performance enhancing effects and/or their ability to mask performance enhancing drugs. [1,2] Over the years, analysis of these classes of compounds has moved from GC/MS to tandem LC/MS systems which tend to be better suited to their polar nature.[3-7] Previously within our laboratory, diuretics and beta-blockers were screened using separate extraction and analysis procedures. The aims of this work were twofold. The first aim was to develop and validate a combined screening method for the simultaneous identification of 48 xenobiotic compounds (diuretics and masking agents, beta-blocker, and stimulants) in urine. A secondary goal was to compare conventional HPLC/MS/MS methodology to UPLC/MS/MS methodology, which makes use of higher pressure tolerances and smaller stationary phase particle sizes to minimize analysis times and improve chromatographic resolution.

Methods - Chemicals and reagents

Analytical standards were obtained from Sigma-Aldrich (St. Louis, MO, USA), Cerilliant (Austin, TX, USA), Toronto Research Chemicals, Inc (North York, ON, Canada), Abbott (Chicago, IL, USA), MP Biomedicals (Solon, OH, USA), TOCRIS (Elisville, MO, USA), and Cephalon (Frazer, PA, USA). Celiprolol and oxprenolol, as well as urine containing excreted metabolites of mesocarb were kind gifts from the Montreal Doping Control Laboratory, Canada. The following products were extracted from their therapeutic preparations: bisoprolol (Concor™, Merck-Sorono, Geneva, Switzerland); carteolol (Bausch & Lomb Pharmaceuticals, Inc, Tampa, FL, USA); esmolol (Brevibloc™, Baxter Healthcare Corp., Deerfield, IL, USA); labetalol (Normodyne™, Schering Corp., Berlin-Wedding, Germany); metipranolol (OptiPranolol™, Bausch & Lomb Pharmaceuticals, Inc, Tampa, FL,

USA); sotalol (Sotalex™, Bristol-Myers Squibb, New York, NY, USA). Reagents and solvents were all of analytical grade or better.

Sample Preparation

Urine (2 mL) was fortified with internal standard mixture (50 µL) and buffered with sodium acetate (1.5 mL, 0.05 M, pH 5.0). Varian Nexus™ SPE columns (60 mg, 3 mL) were conditioned with methanol (5 mL) followed by Milli-Q water (5 mL). After loading the sample, the sorbent bed was washed with Milli-Q water (1 mL) followed by 20% methanol/water (v/v) (1 mL). After vacuum drying, samples were eluted with methanol (2 mL), evaporated at 40 °C using a TurboVap (Caliper Life Sciences, MA, USA) and reconstituted in 95:5 (v/v) 0.1% formic acid/methanol (300 µL). 20 µL and 2 µL aliquots were injected on to the LC-MS/MS and UPLC-MS/MS systems respectively.

Instrumentation and chromatographic conditions

LC-MS/MS experiments were performed using an Agilent 1200 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with a Waters Atlantis T3™ column (100 mm x 2.1 mm ID, 3 µm particle size) and interfaced to an Applied Biosystems API 3200 triple-quadrupole mass spectrometer (Foster City, CA, USA) using an electrospray ionization (ESI) source. Chromatographic conditions are summarized in Table 1. The ion source was operated at 550 °C. GS1 and GS2 gas pressures were set to 50 arbitrary units each. Ion spray voltage was set at -4500 V for negative ions and 5000 V for positive ions.

Table 1: LC-MS/MS chromatographic conditions

Time (min)	Solvent A (10 mM NH_4COOH; pH 3.5)	Solvent B (ACN)	Flow Rate (ml/min)
0.0	90	10	0.3
0.1	90	10	0.3
10.0	24	76	0.3
10.1	90	10	0.3
12.5	90	10	0.3

Table 2: UPLC-MS/MS chromatographic conditions

Time (min)	Solvent A (0.1% HCOOH)	Solvent B (MeOH)	Flow Rate (ml/min)
0.0	95	5	0.5
0.1	95	5	0.5
3.0	2	98	0.5
4.0	2	98	0.5
4.01	95	5	0.5
6.0	95	5	0.5

UPLC-MS/MS experiments were performed using a Waters Acquity™ liquid chromatograph (Waters Corporation, Milford, MA, USA) equipped with a Waters Acquity™ BEH Shield RP18 column (100 mm x 2.1 mm ID; 1.7 µm particle size), interfaced to a Waters Micromass Quattro Premier XE triple-quadrupole mass spectrometer (Manchester, England, UK). Chromatographic conditions are summarized in Table 2. The ion source was operated at 350 °C and ion spray voltages were set at 3250 V for positive ions and -2800 V for negative ions.

In each case, ion source parameters were optimized to achieve the best performance for all of the compounds in the assay. Specific MRM transitions for each method are listed in Table 3.

Results and discussion

We were able to successfully extract and analyze all of the target compounds via a single extraction and analysis. The total run time required to identify 49 exogenous compounds extracted from human urine by HPLC-MS/MS was 12.5 minutes. However, utilizing the greater pressure capacity and scanning speed of the UPLC-MS/MS instrument allowed the total run time for the analysis to be reduced to 6 minutes. Mass spectrometer properties such as overlapping multiple reaction monitoring (MRM) experiments, faster scanning parameters, and the ability to rapidly switch between positive and negative ionization modes allowed us to fully take advantage of the speed of the chromatographic system.

Fig. 1 shows a positive control sample (decision standard) of a selection of analytes of all classes analyzed using the Waters QPXE UPLC/MS/MS system. Retention times, recovery percentages, ionization modes, limits of detection (LODs) and transition ions for all compounds in the two methods are shown in Table 3. The higher LODs for some compounds analyzed by UPLC/MS/MS are most likely a function of the lower injection volume used (2 vs 20 μl), although many of the LODs are comparable or even lower than those found using HPLC/MS/MS. The extraction recovery of each analyte was determined from n=5 replicates spiked at the minimum required performance limits (MRPL) (250 ng/mL for diuretics and 500 ng/mL for stimulants and beta-blockers) set by WADA[8] and ranged from 30-110% with the majority being >75%. Given the variety of compounds analyzed this wide range of SPE recoveries is to be expected.

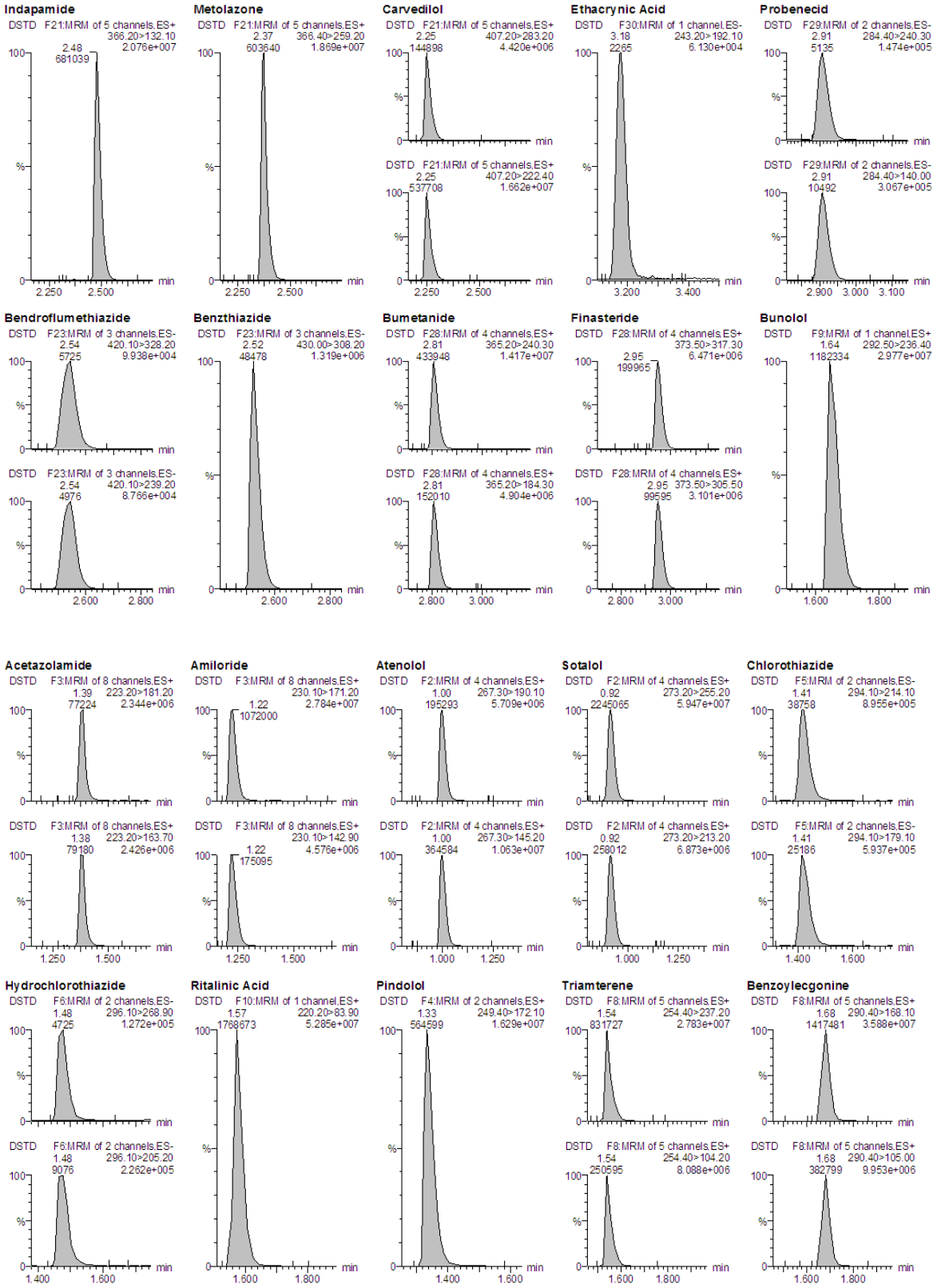
Both systems readily detected all compounds at the WADA MRPL[8] and analysis of six different blank urine samples showed no interfering peaks with either the target analytes or internal standards. We also analyzed 30 samples that had screened and confirmed positive for a variety of compounds. These samples also screened as positive using the methods detailed herein. Furthermore, thirty urine samples which had screened as negative using previous methods similarly screened as negative using the presented methods.

We have successfully combined extraction and analysis methods for 49 exogenous compounds (diuretics, stimulants and beta-blockers). Extraction recoveries exceeded 50% for all but one compound and LODs easily exceeded WADA requirements. We have also shown that utilizing the chromatographic properties of UPLC allows us to reduce analysis time by 50% while maintaining or improving the sensitivity and selectivity needed.

Table 3. Ionization modes, extraction recoveries, retention times, LODs and MRM transitions for HPLC/MS/MS and UPLC/MS/MS analyses.

Compound Class			**Agilent 1200 HPLC – API 3200**			**Waters Acquity UPLC – Quattro Premier XE**		
Diuretics and masking agents	Ioniz. Mode	% Rec	RT (min)	LOD ng/ml	Transition Ions	RT (min)	LOD ng/ml	Transition Ions
Acetazolamide	+	67	3.18	16	223>181	1.44	14	223>181, 164
Amiloride	+	87	1.86	16	230>171	1.26	27	230>171, 143
Bendroflumethiazide	-	81	9.25	4	420>289	2.62	54	420>328, 239
Benzthiazide	-	79	8.70	4	430>308	2.60	53	430>308
Bumetanide	+	74	9.59	9	365>240	2.89	52	365>240, 184
Canrenoic Acid	+	110	8.38	41	359>205, 145, 107	2.91	158	359>205, 173, 145
Chlorothiazide	-	96	3.85	9	294>214	1.48	17	294>214, 179
Chlorthalidone	+	83	6.27	22	322>243	2.12	51	322>241, 213
Clopamide	+	76	7.03	7	346>250	2.31	20	348>252, 171
COOH-Finasteride	+	73	7.38	7	403>335	2.72	7	403>335
Cyclothiazide	-	70	8.88	10	324>307	2.61	55	388>205, 78
Dichlorphenamide	+	73	5.61	20	302>188	2.06	32	303>188, 78
Ethacrynic Acid	-	85	9.20	6	243>192	3.27	13	243>192
Furosemide	-	81	7.80	8	329>205	2.55	85	329>285, 205
Furosemide d5 (IS)	-	*N/A*	*7.61*		*334>209*	*2.49*		*334>290*
Hydrochlorothiazide	-	83	4.24	29	296>269, 205	1.52	133	296>269, 205
Hydroflumethiazide	-	99	5.62	200	330>239	1.76	37	330> 239, 160
Idapamide	+	74	8.53	7	366>259	2.56	37	366>259
Methyclothiazide	-	77	7.63	15	358>322	2.29	43	358>322, 258
Metolazone	+	75	7.98	12	366>259	2.44	40	366>259
Probenecid	-	79	9.60	10	284>240	3.00	30	284>240, 140
Triamterine	+	88	4.63	22	245>237, 104	1.58	43	254>237, 104
Trichlormethiazide	-	79	7.29	28	306>242	2.25	24	380>308, 242
Beta Blockers								
Acebutolol	+	84	5.21	17	337>116	1.66	23	337>260, 116
Alprenolol	+	76	6.87	12	250>91	2.00	8	250>173, 116
Atenolol	+	53	1.56	23	267>145	1.04	62	267>190, 145
Betaxolol	+	77	6.96	5	308>74	2.02	10	308>98, 72
Bisprolol	+	81	6.34	3	326>116	1.86	11	326>116
Carvedilol	+	79	7.59	19	407>224	2.30	28	407>283, 222
Carteolol	+	89	4.03	18	293>237	1.38	21	293>237, 202
Celiprolol	+	82	5.88	6	380>251	1.77	14	380>307, 251
Esmolol	+	85	5.83	6	296>162	1.72	8	296>145
Labetolol	+	82	6.34	11	329>162	1.90	9	329>311, 207
Levobunolol	+	79	5.57	13	292>236	1.70	13	292> 236
Metipranolol	+	75	6.66	9	310>201	1.93	10	310>191, 165
Metoprolol	+	87	5.36	10	268>74	1.60	8	268>191, 74
Nadolol	+	83	4.16	7	310>201	1.40	17	310>254, 236
Oxyprenolol	+	78	6.14	4	266>72	1.79	18	266>72
Pindolol	+	78	4.35	21	249>116	1.38	11	249>172
Propranolol	+	72	6.78	18	260>155	2.00	10	260>183, 98
Sotalol	+	75	1.78	7	273>213	1.96	42	273>255, 213
Timolol	+	86	5.15	36	317>188	1.62	30	317>261, 244
Stimulants								
Benzoylecgonine	+	69	4.80	7	290>168	1.73	8	290>168, 105
Benzolecgonine d3 (IS)	+	*N/A*	*4.80*		*293>171*	*1.70*	-	*293>171*
Buproprion	+	80	6.24	18	240>184	1.81	21	240>184, 166
Carphedon	+	84	5.66	74	219>174	2.02	85	219>174
Modafinil	+	71	7.23	18	274>167	2.48	92	296>129
Modafinilic Acid	+	68	6.86	18	167>152	2.83	44	167>152
Ritalinic Acid	+	30	4.45	13	220>84	1.62	65	220>193
OH-Mesocarb	+	N/A	7.75	-	339>193	2.48	-	339>193

Fig. 1. Ion chromatograms of selected diuretics, beta blockers and stimulants analyzed by UPLC/MS/MS. Diuretics, beta-blockers and stimulants were fortified at 250, 500 and 500 ng/ml, respectively.



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