Masato Okano, Mitsuhiko Sato, Ayako Ikekita, Shinji Kageyama

A rapid ultra-performance liquid chromatography (UPLC™)/mass spectrometry– Applications in doping control

Anti-doping Center, Mitsubishi Chemical Medience Corporation, Tokyo, Japan

Introduction

Most laboratories, including our laboratory, make efforts to simplify analytical methods without increasing the number of instruments or the number of personnel. Our laboratory will aim to perform doping control tests on 750 urine samples for the 11th World Championships in Athletics Osaka 2007, within 10 days. Thus, screening procedures have to be modified to obtain the analytical results within a required period of time. Several methods have been published for the detection of diuretics and corticosteroids by means of HPLC/MS/MS [1-4]. The UPLC™ has recently been used as a way to conduct a rapid throughput analysis as an alternative to traditional high-performance liquid chromatography (HPLC)[5-6]. In this study, the potential use of UPLC™ MS/MS for diuretics, masking agents, stimulants and corticosteroids in our laboratory were described.

Experimental

All reagents were analytical grade. The reference materials were obtained from Sigma (St. Louis, MO, USA), Steraloids (Newport, USA), USP (MD, USA) or NARL (Pymble, Australia).

Sample Preparation for Diuretics: According to our screening procedure for diuretics, 2 mL of 0.2 M acetate buffer (pH 5.2) and 0.5 μg of mefruside as internal standard were added to 2 mL of urine samples, and the analytes were extracted by passage of the sample through a solid-phase extraction cartridge (ABS NEXUS-ELUT 650 mg/3 mL, Varian), followed by washing with 2 mL of distilled water and elution with 2 mL of methanol. The extract was evaporated to dryness under N_2 stream at 60 °C and reconstituted in 100 μL of a aqueous solution of CH_3COOH (1 %) and CH_3CN (90/10, v/v). After centrifugation a volume of 5 μL of the supernatant was injected into the UPLC™/MS/MS system.

Sample Preparation for corticosteroids: Urine samples were prepared according to a previous published screening procedure for corticosteroids[7]. Injection volume was 5 µL for UPLC™/MS/MS and 20 µL for HPLC/TOF-MS respectively.

Instrumentation: The UPLC™/MS/MS system was a Quattro Micro API with a ESI-Source (Z-Spray) from Waters (Tokyo, Japan). The HPLC/TOFMS system was a QSTAR XL MS/MS system (Applied Biosystems, CA, USA). The detailed instrumental parameters are described in Tab. 1.

Validation: The methods were validated according to the International Standard of Laboratories (ISL) of WADA. The limit of detection is shown in Tab. 2.

Table 1 UPLC™/MS/MS and HPLC/TOFMS parameters

	UPLC		**HPLC**
	Screening for diuretics	**Screening for corticosteroids**	
Instruments	ACQUITY UPLC(Waters)	ACQUITY UPLC(Waters)	Agilent 1100 series
Column	ACUITY UPLC BEH C18 2.1 mm x 50 mm , 1.7 µm	ACUITY UPLC BEH C8 2.1 mm x 50 mm , 1.7 µm	ZORBAX Eclipse XDB-C8 4.6 mm x 150 mm , 5 µm
Mobile phase	A: 1 % CH_3COOH B: CH_3CN	A: 1 % CH_3COOH B: CH_3CN	A: 1 % CH_3COOH B: CH_3CN
Gradient	0-0.5 min A:90 % B:10 % 8 min A:20 % B:80 % 8.01 min A:90 % B:10 %	0-0.5 min A:65 % B:35 % 4 min A:40 % B:60 % 4.01-5.0 min A:10 % B:90 % 5.1 min A:65 % B:35 %	0-3 min A:65 % B:35 % 10 min A:40 % B:60 % 15 min A:35 % B:65 % 16-21 min A:10 % B:90 % 25 min A:65 % B:35 %
Run time	10 min	6 min	27 min
Flow rate	0.2 mL/min	0.35 mL/min	0.25 mL/min
Column Temp.	25 °C	25 °C	25 °C
Column Pressure	4000 psi	6000 psi	300 psi
	MS/MS		**TOF-MS**
Instruments	Quattro Micro API with a Z-spray(Waters)	Quattro Micro API with a Z-spray(Waters)	QSTAR XL MS/MS System
Ionization	ESI (Z-spray)	ESI (Z-spray)	Turbo Ion Spray
Acquisition mode	Multiple reaction monitoring(MRM)	Multiple reaction monitoring(MRM)	m/z 140 to 600
Dwell(s)	0.1	0.05	-
Desolvation Temp	350 °C	350 °C	-
Desolvation Gas Flow	600 L/hr(N_2)	600 L/hr(N_2)	Nebulizer Gas 2.85 L/min
Ionspray Temp	120 °C	120 °C	450 °C
Cone Gas Flow	50 L/hr(N_2)	50 L/hr(N_2)	Auxially Gas 4.80 L/min
Capillary Voltage	3.0 kV	3.0 kV	Ion spray Voltage 5500 V
Collision gas	3.6 x 10^{-3} mbar(Ar)	3.6 x 10^{-3} mbar(Ar)	-
Ion Polarity	Table 2	Table 2	Positive
Cone Voltage	Table 2	Table 2	Declustering Potential 50 V
Collision Energy	Table 2	Table 2	Focusing Potential 250 V

Results and Conclusions

A total of 44 dope agents including both acidic compounds and basic compounds (eg. amiloride, triamterene) could be analysed within 10 min by employing UPLC™/MS/MS in simultaneous positive and negative cone voltage switching (Tab. 2). The total ion chromatograms of 23 corticosteroids in Fig. 1 clearly showed the reduced analysis time by using UPLC™ (bottom) and HPLC (top). By employing UPLC™ over traditional HPLC, the analysis time of corticosteroid screening reduced by a factor of 5.2 (26 min →5 min) (Fig. 1). This UPLC™ method for corticosteroids could allow a high throughput analysis with good

chromatographic separation of betamethasone and dexamethasone (Fig. 1). Thus, UPLC™/MS/MS is a very useful technique for rapid throughput screening of dope agents.

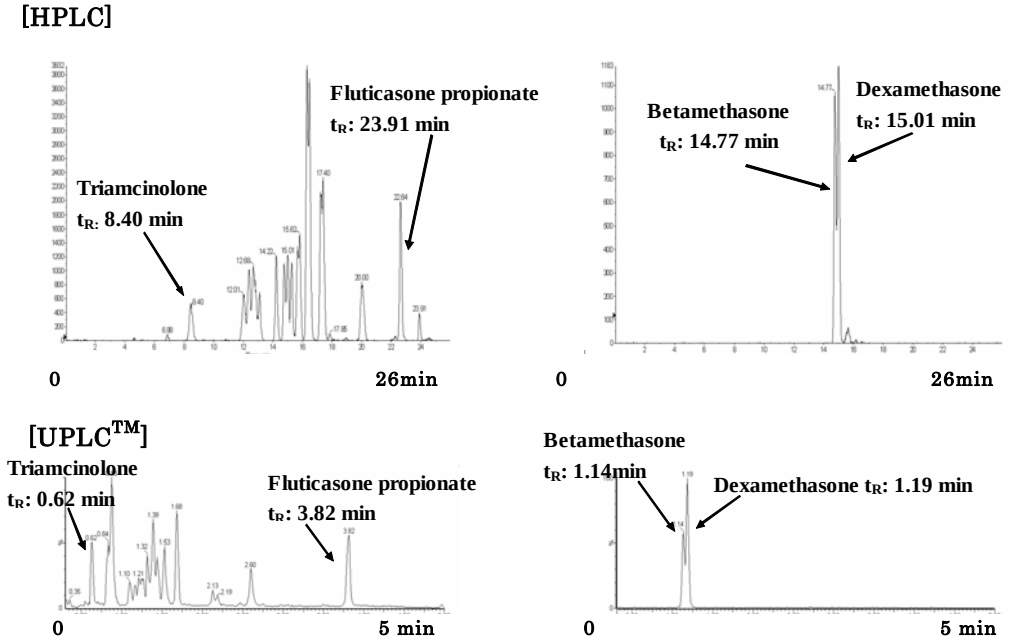

Figure 1 TIC of 23 corticosteroids (left) and dexamethasone/betamethasone (right)

Acknowledgments

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Table 2 Summary of diuretics and stimulants screening by UPLC™/MS/MS

Compound	MRM Group Polarity	RT (min)	Precursor ion	Product ion	Cone V	CE V	LOD(ng/mL)
acetazolamide		1.56	220.90	82.80	30	16	5.0
clofenamide	**1** 0-2.4 min ES-	2.10	269.00	77.80	40	24	20.0
hydrochlorothiazide		2.14	295.93	268.90	45	20	10.0
amilolide		1.10	230.02	171.00	30	18	1.0
dorzolamide		1.61	325.06	199.00	30	20	1.0
chlorothiazide	**2** 0-2.8 min ES+	1.93	295.98	278.90	45	16	20.0
methazolamide		2.37	237.00	195.00	25	16	1.0
triamterene		2.40	254.09	237.00	50	26	1.0
ritalinic acid		2.55	220.11	83.90	30	20	1.0
brinzolamide		2.73	382.06	77.80	50	28	40.0
meticrane		2.87	274.03	117.90	50	32	100.0
hydroflumethiazide	**3** 2.4-3.7 min ES-	2.94	330.02	238.90	45	26	5.0
ethiazide		3.38	324.01	204.80	50	22	45.0
dichlorphenamide		3.43	302.91	77.80	40	30	15.0
carphedon		3.13	219.14	174.10	20	14	1.0
5-oxo-mefruside		3.38	397.00	83.80	50	30	-
chlorthalidone		3.44	339.01	321.90	20	14	5.0
norbuprenorphine		3.50	414.23	83.00	50	48	1.0
clopamide		3.62	346.10	250.00	45	22	1.0
modafinil	**4** 2.8-4.5 min ES+	3.86	167.06	152.00	50	22	15.0
torasemide		3.98	349.12	264.00	25	18	1.0
carboxy-finasteride		4.12	403.00	335.00	50	40	2.0
buprenorphine		4.28	468.21	54.90	50	46	1.0
trichlormethiazide		4.05	379.91	305.80	35	12	5.0
methylchlothiazide		4.28	357.96	321.80	30	14	5.0
metolazone	**5** 3.7-5 min ES-	4.51	364.07	257.00	45	22	1.0
furosemide		4.62	329.02	284.90	30	14	10.0
althiazide		4.67	381.95	340.80	35	16	5.0
ethoxzolamide		4.78	259.09	178.00	30	18	1.0
indapamide		4.88	366.03	132.00	25	16	1.0
tripamide	**6** 4.5-7 min ES+	5.42	370.07	136.10	50	30	1.0
piretanide		5.51	363.11	282.00	40	22	1.0
bumetanide		5.87	365.10	240.10	35	18	1.0
canrenone		6.15	341.21	107.00	40	34	1.0
benzthiazide		5.07	429.96	307.90	45	24	1.0
mefruside (I.S.)		5.11	381.07	189.00	50	30	-
polythiazide	**7** 4.7-6.0 min ES-	5.44	437.97	397.80	35	16	1.0
bendroflumethiazide		5.45	420.04	289.00	45	24	1.0
cyclopenthiazide		5.56	378.07	204.80	50	28	5.0
xipamide		5.64	353.03	273.90	50	26	1.0
probenecid		6.01	284.11	240.10	30	16	1.0
penflutiazide		6.04	400.07	269.00	50	24	1.0
ethacrynic acid	**8** 5.7-10.0 min ES-	6.18	301.02	242.90	20	12	1.0
benzbromarone		8.37	422.93	250.80	50	32	10.0

Table 3 Summary of glucocorticosteroid screening by UPLC™/MS/MS

Compound	MRM Group Polarity	RT (min)	Precursor ion	Product ion	Cone V	CE V	LOD(ng/mL)
triamcinolone		0.60	395.23	375.10	24	8	4.8
20β-Dihydroprednisolone		0.63	363.29	345.10	24	8	0.9
prednisolone		0.82	361.15	343.10	22	10	0.7
hydrocortisone (cortisol)		0.85	363.29	121.10	34	28	-
predonisone		0.85	359.21	341.10	26	12	2.1
cortisone		0.88	361.29	163.20	42	24	-
fludrocortisone	**1** 0-2.0 min ES+	0.87	381.22	239.20	48	26	3.2
modafinil		0.90	167.10	152.10	46	26	1.0
6a-methylprednisolone		1.10	375.22	357.10	24	14	1.0
d4-dexamethasone(IS)		1.24	397.25	377.10	22	8	-
betamethasone		1.18	393.23	373.10	24	8	0.7
dexamethasone		1.24	393.23	373.10	24	8	0.7
corticosterone		1.29	347.27	121.00	36	30	2.4
flumethasone		1.29	411.24	253.20	26	18	0.6
beclomethasone		1.35	409.18	391.00	22	10	0.5
desonide		1.40	417.24	399.00	26	12	0.4
triamicinolone acetonide		1.43	435.25	415.10	24	12	0.7
flunisolide	**2** 1.0-2.1 min ES+	1.48	435.25	339.10	24	12	0.8
19-nortestosterone(IS)		1.59	275.23	109.00	38	30	-
fluocortolone		1.74	377.00	303.06	20	12	-
fluorometholone		1.74	377.22	279.20	24	20	0.8
budesonide		2.20	431.25	413.10	24	10	0.3
medrysone	**3** 1.9-6.0 min ES+	2.66	345.26	327.20	34	16	0.4
fluticasone propionate		3.85	501.23	313.20	26	14	0.1

-: not estimated