PRELIMINARY INVESTIGATIONS OF A DIURETIC SCREENING METHOD CONVERTED FROM HPLC/MS/MS TO UPLC/MS/MS

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AIM

The conversion of a qualitative diuretic screening method used in sports doping analysis from HPLC/MS/ms (total run time of 10 min) to Ultra Performance Liquid Chromatography™ — UPLC™/MS/MS —where the analysis time is reduced without any loss of sensitivity and selectivity.

INTRODUCTION

- Diuretic compounds are listed as prohibited substances on the 2006 World Anti-Doping Code
- Diuretics can be used to mask the use of other banned substances such as steroids by speeding up the excretion of the compound via the urine
- Diuretics are used in sports with weight categories, such boxing and weight lifting, to rapidly lose weight via increased urination, allowing participation in a lower weight class
- World Anti-Doping Agency (WADA) states all laboratories must be able to confidently detect the minimal required performance limit (MRPL) of 250 ng/mL
- The increased number of athlete samples being tested each year has led to a need for a high-throughput rapid screen for prohibited substances
- Here we describe a rapid and sensitive UPLC/MS/MS method for the screening of diuretic compounds

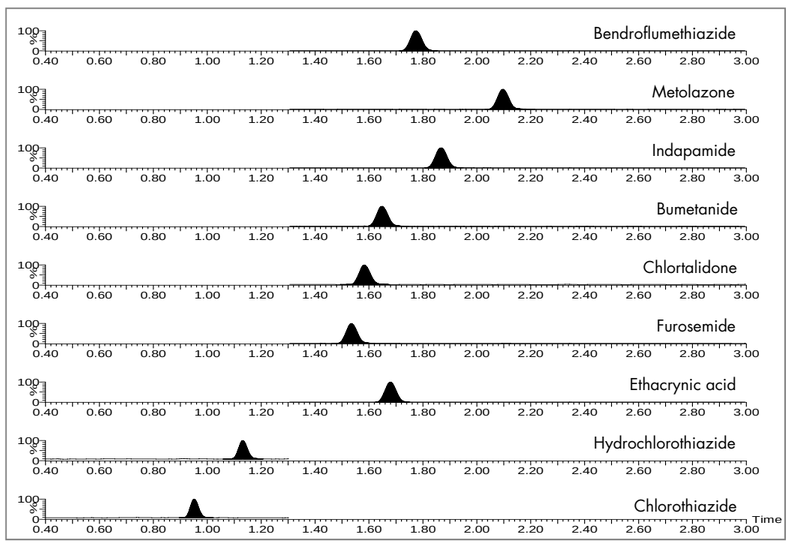

Figure 1. MRM chromatograms of the selected diuretic compounds. Responses obtained from a 10 µL injection of a 100 ng/mL QC sample.

METHODS

SAMPLE PREPARATION

Sample pre-treatment: 500 µL sample + 500 µL buffer (containing 500 ng/mL ISTD Clorsulon).

SPE sample clean-up: Oasis® HLB 1cc (30mg)

Condition	1 mL Methanol
	1 mL H_2O
Load	1 mL Pre-treated sample
Wash	200 µL Hexane
Elution	1 mL Ethyl Acetate

Evaporate 500 µL of eluent to dryness and reconstitute in 100 µL 50:50 Acetonitrile:H_2O.

CHROMATOGRAPHY

Waters® ACQUITY UPLC™ System

Column:	ACQUITY UPLC BEH C18 (2.1 x 100 mm, 1.7 µm)
Column temp:	50 °C
Autosampler temp:	10 °C
Injection vol:	10 µL
Solvent A:	0.1% Ammonium Hydroxide in Water
Solvent B:	0.1% Ammonium Hydroxide in Acetonitrile

Gradient program:

Time	% B	Flow	Curve
0	10	0.25	1
2.0	95	0.25	6
3.0	10	0.25	1

MASS SPECTROMETRY

Mass spectrometer:	Waters Quattro micro™ tandem mass spectrometer
Ionisation mode:	ES negative ion
Capillary voltage:	2.5 kV
MS-MS:	Collision gas: Argon at 2.0 x 10^{-3} mbar

Multiple reaction monitoring (MRM) transitions:

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
Chlorothiazide	294	214	45	32
Hydrochlorothiazide	296	269	40	20
Ethacrynic acid	301	243	18	10
Furosemide	329	285	28	15
Chlortalidone	337	190	32	20
Bumetanide	363	207	35	25
Indapamide	364	189	40	25
Metolazone	364	257	40	20
Clorsulon (ISTD)	380	344	25	15
Bendroflumethiazide	420	289	45	25

RESULTS

The chromatographic separation (figure 1) was performed using a 3 minute gradient with the diuretics identified using compound specific MRM transitions. A limited validation was undertaken. For all compounds, responses were linear (figure 2) over the investigated range (50–500 ng/mL). Intra-assay precision was satisfactory with CV's for spiked QC samples < 20%. The use of the SPE procedure was demonstrated to be very efficient and gave reproducible extraction recoveries *i.e.* > 78% for all analytes. Limits of quantitation were estimated to be 50 ng/mL with limits of detection ranging from 0.1 to 25 ng/mL, which meets the minimum required performance limits, as specified by WADA, of 250 ng/mL for the detection of diuretics compounds within athlete urine specimens. The method was applied to the analysis of blind spiked samples (*n* = 6) provided by the WADA accredited laboratory, HFL.

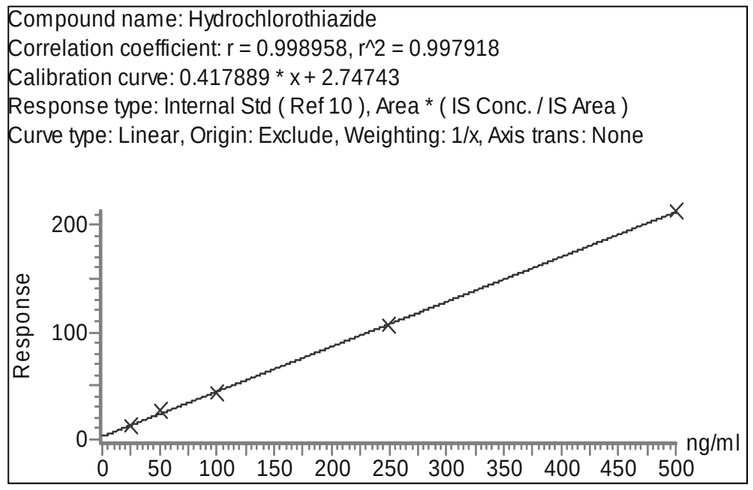

Figure 2. Typical response for urine containing Hydrochlorothiazide. All compounds were quantified by reference to the internal standard Clorsulon.

CONCLUSION

- This method shows the successful conversion of a qualitative diuretic screening method from HPLC/MS/MS to UPLC/MS/MS
- UPLC/MS/MS can provide a rapid, robust and sensitive solution for high-throughput sports doping analysis, particularly when screening for prohibited diuretics
- Preliminary investigations have shown a three-fold decrease in cycle time when using UPLC compared to the traditional HPLC method
- The method was successfully applied to the analysis of blind samples from a WADA accredited laboratory

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