Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (9)

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(Editors)

Sport und Buch Strauß, Köln, 2001

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In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (9). Sport und Buch Strauß, Köln, (2001) 321-326

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Diuretics Screening and Confirmation using LC/MS/MS

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INTRODUCTION

Diuretics promote the excretion of water and electrolytes by the kidneys and have been banned in sport since 1988. Diuretics can therefore be used as masking agents for banned substances as a result of dilution effects in urine. They may also be used to allow rapid weight loss simply by passing water and so have been used by athletes to participate in a lower weight class.

Diuretics have been screened using a number of methods such as HPLC and GCMS of methylated derivatives (1-12). Studies in our laboratory have shown that some diuretics such as benzthiazide are difficult to detect by GCMS using traditional methylation methods so this method of screening is limited and also while they may be detected using HPLC their confirmation is difficult. It was thus useful to investigate the use of LCMS as a multiresidue procedure for diuretics. The data for four diuretics which are the most difficult to detect using extractive alkylation are presented here.

The procedure evaluated involved:

- Combining a high recovery extraction method[1)] with a relatively new, powerful analytical tool.
- pH controlled liquid-liquid extraction and wash.
- RP HPLC with MS/MS detection using internal standard quantification.
- Confirmation according to IOC criteria.

EXPERIMENTAL

Sample preparation was as follows:

- Urine (2mL) to which mefruside internal standard had been added was buffered at pH 5 with acetate buffer (2M),
- Extract diuretics by shaking for 30mins with ethyl acetate (4mL), centrifuging and removing the organic layer.
- Add 1 ml of phosphate buffer 0.2 M (pH 6) to the organic layer and shake for 15 minutes, centrifuge and remove organic layer.
- Evaporate to dryness under nitrogen
- Reconstitute in 50:50 methanol/water (200uL)
- Analyse by LC/CM/SMS

LC/MS/MS Instrumentation and Conditions

HPLC System	Waters 2690 Separations Module – Alliance
Mass Spectrometer	Micromass Quattro LC triple quadrupole
Sample inlet mode	Electrospray
Ionisation mode	Positive

HPLC Conditions

SCREEN RUN

HPLC Column	Supelco Discovery C18, 5 mm, 5 cm x 2.1 mm i.d
Guard column	Opti Guard C18, 1.0 x 1.5 mm (Alpha Resources)
Mobile Phase	A = 2% Formic Acid C = MilliQ water D = Methanol
Flow rate	0.2 mL/min
Injection volume	10 uL
Column Temperature	30 °C
HPLC run time	6.5 min
LC run program	10% A, 50% C, 40% D: Hold for 0.5min then increase to; 10% A, 10% C, 80% D: after 1.5min, hold constant for 1min; 10% A, 50% C, 40% D: after 3 mins until stop at 6.5min

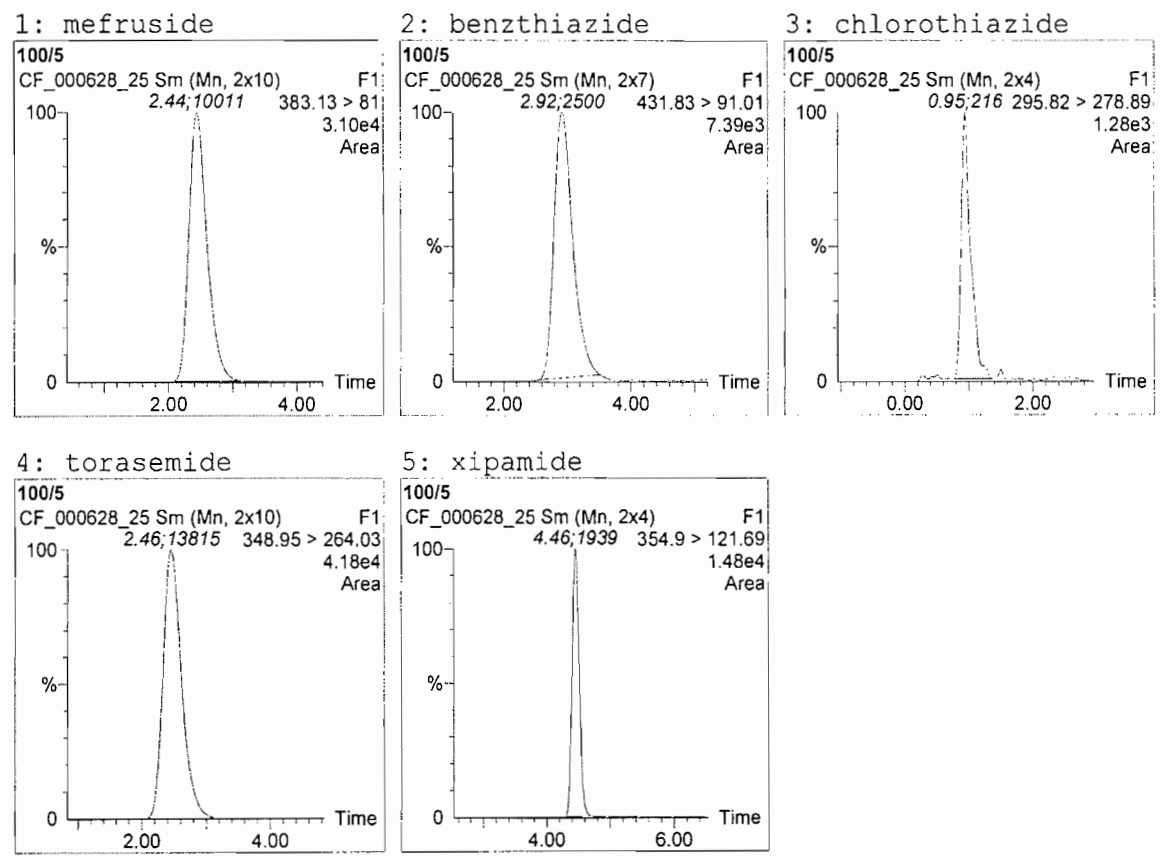
Multiple Reaction Monitoring

Mass Scanning Mode for screening	Multiple Reaction Monitoring (MRM) m/z: 295.82 > 278.89 (Chlorothiazide) m/z: 348.95 > 264.03 (Torasemide) m/z: 354.90 > 121.69 (Xipamide) m/z: 383.13 > 81.00 (Mefruside ISTD) m/z: 431.83 > 91.01 (Benzthiazide)
Mass Scanning Mode for confirmation	Multiple Reaction Monitoring (MRM) m/z: 295.82 > 279, 205, 215, 296 (Chlorothiazide) m/z: 348.95 > 349, 264, 125, 168, 183, 219, 230, 290 (Torasemide) m/z: 354.90 > 355, 122, 107, 234 (Xipamide) m/z: 383.13 > 81.00 (Mefruside ISTD) m/z: 431.83 > 91, 432 (Benzthiazide) monitoring includes parent ion
Retention Time	Chlorothiazide - 0.94 min Torasemide – 2.48 min Xipamide – 4.45 min Mefruside ISTD – 2.46 min Benzthiazide – 2.93 min

RESULTS

The mass spectral data for the screening process is shown in the example below (FIG 1) for a 0.1ug/mL spiked urine sample. This is shown at the minimum level at which diuretics detection is monitored in the IOC reaccreditation. It can be seen that the signal to noise at this concentration is excellent and the final detection limits are well below this. The mass spectral fragmentation for these transitions can be monitored by measuring several ions or by scanning the appropriate range.

FIG 1 Example LC/MS/MS Chromatograms (0.1ugml^{-1} spike in urine)



The results for detection limits, repeatability and recovery are shown in the Table1 below.

Table 1 Validation Data

Analyte	Recovery	Method Detection Limit (μgml^{-1})	Instrument Repeatability (%CV, n=20)	Method Repeatability (%CV, n=7)
Benzthiazide	99	0.005	2.3	6.2
Chlorothiazide	54	0.020	6.8	9.0
Torasemide	86	0.002	3.5	7.3
Xipamide	99	0.002	3.4	8.7
Mefruside (IS)			3.7	

Recovery based on 0.1 μgml^{-1} fortified urines.
Method repeatability determined using low level fortified urines (0.010 or 0.020 μgml^{-1}).

DISCUSSION

The method has been found to have a number of advantages:

- Rapid, low-cost sample preparation and handling
- High sample throughput
- Combines detection and confirmation methods (when required)
- High sensitivity, low method detection levels (sensitivity varies for individual diuretics and each needs to be determined separately).
- ISO G25 accredited
- Analytes are fully quantifiable
- While the data for four diuretics is presented here the method can be extended to include all currently monitored diuretics.

Bibliography

1) M. B. Barroso and H. D. Meiring. Gas chromatographic-mass spectrometric analysis of the loop diuretic torasemide in human urine. *J.Chromatogr.B.* 690:105-113, 1997.

2) P. Campins-Falcco, R. Herraez-Hernandez, and M. D. Pastor-Navarro. Analysis of diuretics in urine by column-switching chromatography and fluorescence detection. *Journal of Liquid Chromatography and Related Technologies* 20 (12):1867-1885, 1997.

3) S. Carda-Broch, J. R. Torres-Lapasio, J. S. Esteve-Romero, and M. C. Garcia-Alvarez-Coque. Use of a three-factor interpretive optimization strategy in the development of an isocratic chromatographic procedure for the screening of diuretics in urine samples using micellar mobile phases. *Journal of Chromatography A* 893 (2):321-337, 2000.

4) D. Carreras and C. Imaz. Comparison of derivatization procedures for the determination of diuretics in urine by gas chromatography-mass spectrometry. *Journal of Chromatography A.*, 1997.

5) S. D. Garbis, L. Hanley, and S. Kalita. Detection of thiazide-based diuretics in equine urine by liquid chrmatography-mass spectrometry. *Journal of AOAC International* 81 (5):948-957, 1998.

6) H. J. Guchelaar, L. Chandi, O. Schouten, and W. A. van-den-Brand. A high-performance liquid-chromatographic method for the screening of 17 diuretics in human urine. *Fresenius Journal of Analytical Chemistry* 363 (7):700-705, 1999.

7) Y. W. Li, J. Li, and T. H. Zhou. GC-MS Study on Diuretics in Urine II Detection Method Using Trimethylsilylation. *Chinese Chemical Letters* 2 (1):19-22, 1990.

8) A.M. Lisi, R. Kazlauskas, and G. Trout. Diuretic screening in human urine by gas chromatography-mass spectrometry: use of a macroreticular acrylic copolymer for the efficient removal of the coextracted phase-transfer reagent after derivatization by direct extractive alkylation. *Journal of Chromatography* 581:57-63, 1992.

9) S-J. Park, H-S. Pyo, Y-J. Kim, M-S. Kim, and J. Park. Systematic Analysis of Diuretic Doping Agents by HPLC Screening and GC/MS Confirmation. *Journal of Analytical Toxicology* 14:84-90, 1990.

10) A.Rosado-Maria, A. I. Gasco-Lopez, A. Santos-Montes, and R. Izquierdo-Hornillos. High-performance liquid chromatographic separation of a complex mixture of diuretics using a micellar mobile phase of sodium dodecyl sulfate. Application to human urine samples. *Journal of Chromatography A* 748 (2):415-424, 2000.

11) D. Thieme, J. Grosse, R. Lang, R. K. Mueller, and A. Wahl. Screening, confirmation and quantitation of diuretics in urine for doping control analysis by high-performance liquid chromatography-atmospheric pressure ionisation tendem mass spectrometry. *Journal of Chromatography B* 757:49-57, 2001.

12) R. Ventura, D. Fraisse, M. Becchi, O. Paisse, and J. Segura. Approach to the analysis of diuretics and masking agents by high-performance liquid-chromatography-mass spectrometry in doping control. *Journal of Chromatography* 562:723-736, 1991.

13) Guchelaar, H.-J.; Chandi, L.; Schouten, O.; van den Brand, W.A.; Fresenius J Anal Chem., 1999 (363), 700-705.