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RECENT ADVANCES IN DOPING ANALYSIS (9)

W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck-Engelke
(Editors)

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B. YAP, R. KAZLAUSKAS:
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Bin Yap and Ray Kazlauskas

Rapid HPLC Screening of Chlorothiazide, Torasemide, Xipamide and Benzthiazide in the Sydney 2000 Olympic Games

Australian Sports Drug Testing Laboratory
1 Suakin Street, Pymble. NSW. 2073. Australia.

Introduction

Methods for screening diuretics in urine have involved analytical techniques ranging from high performance liquid chromatography (HPLC) (1,2) and gas chromatography-mass spectrometry (GCMS) (3,4) to liquid chromatography-mass spectrometry (LCMS) (5,6). These techniques have been combined with various sample preparation protocols involving solid-phase extraction and liquid-liquid extraction steps. Most of these procedures are lengthy and appear to suffer from difficulties in resolving chlorothiazide and benzthiazide in the same urine matrix.

Recently, a rapid screening method using gradient HPLC was developed for targeting chlorothiazide, torasemide, xipamide and benzthiazide in the urine samples of athletes competing in powersports in the Sydney 2000 Olympic Games. The method was based on a qualitative procedure involving a solid-phase clean-up step using a mixed bed of acidic and basic matrix packed in a cartridge (Varian abselut cartridge with Nexus bonded phase) followed by a liquid-liquid extraction step. Analysis by HPLC was performed using a Rocket C18 column (Alltech Platinum Rocket) with photodiode array detection. This method was successfully employed to complement the extractive-alkylation and GCMS techniques presently used for screening diuretics in the Australian Sports Drug Testing Laboratory. This method has been accredited to the ISO/IEC Guide 25-1990 by the National Association of Testing Authorities, Australia.

Methodology

- Transfer the urine sample (2.5mL) containing Mefruside surrogate standard (1 ug/mL) to the abselut-Nexus bonded phase cartridge (60 mg) on a vacuum manifold. Gently draw the urine through the cartridge using low vacuum.

- Wash the cartridge by drawing demineralised water through it. Maintain the vacuum for 1 minute to dry the sample.
- Elute the analytes into a clean tube by drawing methanol (1mL) through the cartridge using low vacuum. Evaporate the eluate to dryness under gaseous nitrogen.
- Buffer the residue with aqueous phosphate buffer (pH 7) and extract with t-butyl methyl ether by vertical mixing for 15 min.
- Centrifuge the mixture and transfer the ether layer to a clean tube. Evaporate the ether to dryness under gaseous nitrogen.
- Transfer 0.05M Ammonium acetate solution (200 uL) at pH 6.8 and 17β-hydroxyethyltheophylline internal standard (equivalent to 1 ug/mL) to the analyte.
- Transfer the analyte to a glass vial for injection into the HPLC.

Instrument parameters

HPLC Conditions

Instrument: Waters 2690 Alliance HPLC connected to the Millennium 32 Management System.

Solvents: A: Demineralised, filtered (0.2 um) water

B: Acetonitrile (filtered)

C: 0.05M Ammonium acetate solution, pH 6.8

Flow rate: 1.0 mL/min.

Table 1: HPLC Gradient Programme

Time	Flow	%A	%B	%C
0.0	1.0	0	10	90
3.0	1.0	0	30	70
5.0	1.0	0	45	55
7.0	1.0	0	60	40
9.0	1.0	0	50	50
10.0	1.0	0	10	90

Photodiode Array (PDA) Conditions

Wavelength monitored	nm
Chlorothiazide	312
Xipamide	326
Torasemide	287
Benzthiazide	297
Hydroxytheophylline	273
Mefruside	240

Wavelength range	240-400
PDA threshold	0.001 absorbance

Injector Conditions

Injector volume	20 uL

Automatic syringe flush before each injection

Results

The method validation data are summarised in Table 2.

Table 2: Validation Data from samples spiked at 0.5 ug/mL in urine.

Analyte	Recovery N=9 (%)	LOD N=10 ug/mL	LOQ N=10 ug/mL	Intra-assay CV N=9 (%)	Inter-assay CV N=9 (%)
Chlorothiazide	36.4±0.9	0.1	0.1	8.1	2.4
Xipamide	91.4±4.3	0.1	0.3	4.8	1.3
Torasemide	74.4±3.8	0.1	0.1	5.2	2.8
Benzthiazide	89.9±7.6	0.1	0.3	8.5	2.5

Figure 1:Chromatogram of analytes spiked at 1 ug/mL in urine

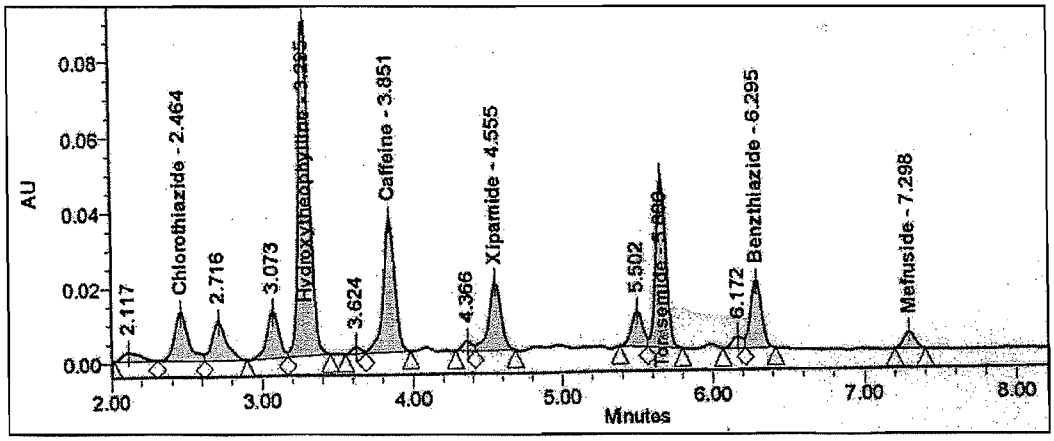
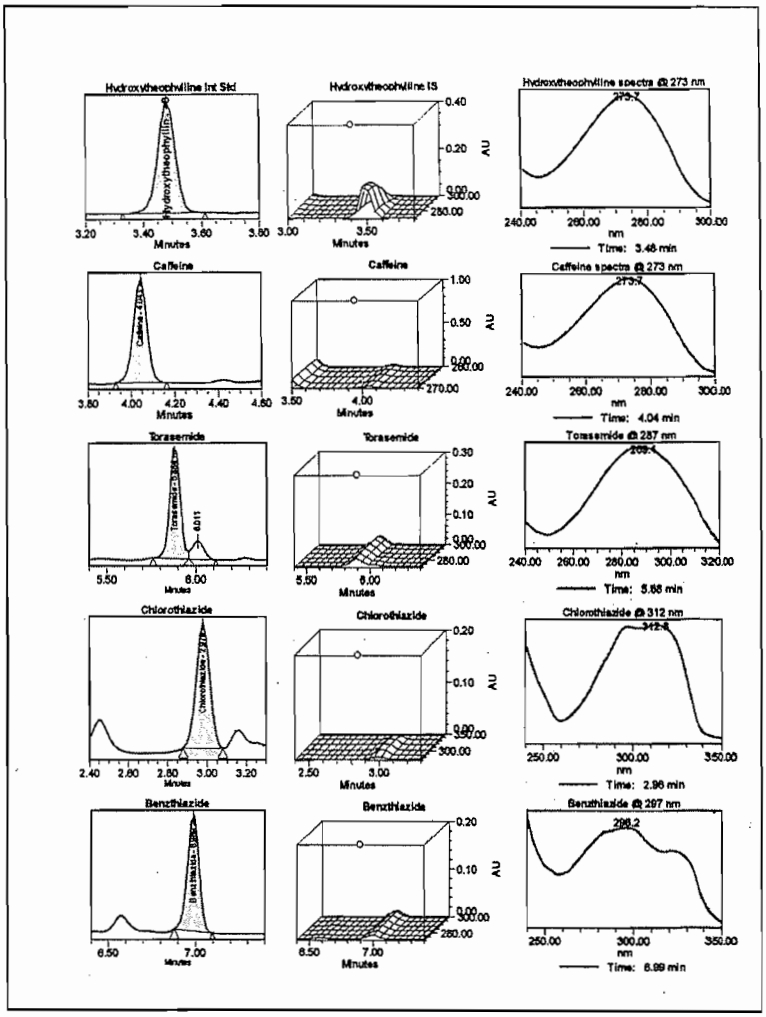
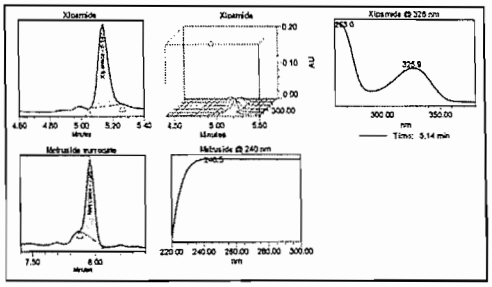


Figure 2: Photodiode array chromatographic profile and ultra violet spectra of analytes





Discussions

There was minimum interference by the matrix in the chromatography of the analytes tested. The low recovery of chlorothiazide could have been due to possible chemisorption of the analyte at a limited number of sites on the matrix. Use of the Abselut combined acid-base solid phase cartridge combined with the liquid-liquid extraction step in the clean-up process produced a clean sample extract. Chromatographic peak separation and run times were improved significantly using the Platinum Rocket reversed-phase C18 Column.

General Conclusions

- The method facilitates fast sample analysis in less than 8 minutes per run. This procedure enables a high sample throughput in a short analysis time.
- There are minimum interference and contamination from the sample matrix and analytical reagents on the analytes tested.

- The method provides good accuracy, precision, repeatability and reproducibility. The method can be applied in routine operation by suitably-trained technicians.

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