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Comparison between reverse phase and hydrophilic interaction liquid chromatography techniques in doping analysis: a case study on phenolalkylamines

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Introduction

The analysis of highly hydrophilic, ionic and polar compounds often requires a highly aqueous mobile phase to achieve retention, which can cause (i) a decrease in sensitivity in electrospray ionization mass spectrometry (ESI/MS), because high-aqueous content mobile phase is not conducive enough to allow sufficiently adequate ionization, and (ii) a decrease in stationary phase performance and lifetime of operation. In 1990, Alpert proposed the hydrophilic interaction liquid chromatography (HILIC) technique [1]. HILIC is characterized by a hydrophilic stationary phase and an aqueous-organic solvent mobile phase with a high organic solvent content that has an increased solubility for the hydrophilic compounds and shows excellent compatibility with mass spectrometry [2-3]. Four mechanisms control analyte retention in HILIC: (a) partitioning between a water-rich layer on the surface of the stationary phase and the more hydrophobic mobile phase, (b) hydrogen bonding with the thin layer of water on the stationary phase, (c) electrostatic interaction with the negatively charged residual silanol groups in the stationary phase, and (d) dipole-dipole interactions [2-3]. In this study the suitability of unmodified silica stationary phase as an alternative to RPLC stationary phases for the analysis in human urine of phenoalkylamines was investigated. The effects of chromatographic parameters including the mobile phase acetonitrile percentage, column temperature and buffer pH, concentration and composition on the selectivity, sensitivity, peak shape and compounds chromatographic retention was examined.

Experimental

Etilefrine, buphenine (nylidrin), norfenefrine, octapamine, oxilofrine and pholedrine were supplied by Sigma-Aldrich (Milan, Italy). Deuterated salbutamol (salbutamol d3, used as

internal standard) was purchased from NMI (National Measurement Institute, Pymble, Australia). All chemicals (sodium bicarbonate, potassium carbonate, ammonium formate, ammonium acetate, formic acid, acetic acid, acetonitrile, ethanol, methanol, acetone, *tert*-butylmethyl ether) were from Carlo Erba (Milano, Italy).

All LC-MS/MS experiments were performed using an Agilent 1200 Rapid Resolution Series HPLC pump with binary gradient system and automatic injector (Agilent Technologies Milan, Italy). Reversed-phase liquid chromatography was performed using a Zorbax® RX-Sil column (150 x 2.1 mm, 5 µm) from Agilent (Agilent Technologies Milan, Italy). Different column temperatures were evaluated: 20, 40, 50, 60 and 80 °C. Different mobile phases composition were tested: water containing different percentages of formic or acetic acid (0.1, 0.2 or 0.4%) or different amounts of ammonium acetate or formate (5, 10, 20 and 50 mM) (A) and acetonitrile containing different percentages of formic or acetic acid (0.1, 0.2, 0.3 or 0.4%) (B). The HILIC gradient program started at 90% B and decreased to 70% B in 10 min. The column was finally re-equilibrated at 90% B for 4 min. The flow rate was set at 250 µL/min. The RPLC gradient program was as currently adopted by the accredited sports anti-doping laboratory of Rome [4].

Mass spectrometry was performed using an API4000 triple-quadrupole instrument (Applera Italia, Monza, Italy) with positive electrospray ionization. The ion source was operated at 450 °C, the applied capillary voltage was 5000 V, declustering potential was 60 V and selected monitoring reaction (SRM, Table 1) experiments were performed employing collision-induced dissociation (CID) using nitrogen as collision gas at 5.8 mPa, obtained from a dedicated nitrogen generator system Parker-Balston model 75-A74, gas purity 99.5% (CPS analitica Milano, Italy).

Sample pre-treatment includes liquid/liquid extraction at pH 9 (0.8 M carbonate buffer) with 10 mL of *tert*-butylmethyl ether. The organic layer was then separated and evaporated under nitrogen at 50 °C. The residue was reconstituted in 50 µL of mobile phase and 10 µL were injected into the LC-MS/MS system.

Results and Discussion

The best chromatographic conditions in terms of peak shape, selectivity and chromatographic retention were obtained using a high percentage of organic solvent: 90% at t=0, a column temperature of 40 °C, 5 mM of ammonium acetate and 0.1% of acetic acid (pH 4.5) as mobile phase. Good reproducibility of the relative retention times (CV% < 0.5) and of relative abundances of selected ion transitions (CV% < 10) were noticed for all compounds

considered in this study. The analyses on 100 negative samples confirmed that the method did not show significant interferences and therefore has an adequate selectivity. Carryover signals were not detected in blank urine samples that were injected after the analysis of the fortified urine samples at a concentration five times the Minimum Performance Required Limit (MRPL, 500 ng/mL) [5]. The test for ion suppression/enhancement effects by post column split-infusion of analytes yielded no significant matrix effects (< 15%) at the retention times of the target analytes and internal standard when 20 different urine samples were injected. The lower limits of detection (LLOD) were in the range of 80-200 ng/mL (see Table 1), thus significantly lower than the MRPL [5]. The recovery was higher than 70%, with a satisfactory repeatability (CV % < 10).

The effectiveness of the method was tested by analyzing routine samples positive for pholedrine and etilefrine (Figures 1A-B). As can be seen, all the analytes are clearly detected and distinguishable from matrix interferences, and can be identified by their characteristic fragment ions and RTs, satisfying the criteria for compounds identification established by the WADA in the technical document TD2010IDCR [6]. Furthermore the chromatographic retention, sensitivity and peak shape were very satisfactory.

Table 1: LLOD, retention time (Rt) and ion transitions.

	LLOD (ng/mL)		**Rt (min)**		**Ion transitions (m/z)**
Compound	*RPLC*	*HILIC*	*RPLC*	*HILIC*	
Etilefrine	150	80	0.9	7.9	182/56;182/65;182/77;182/91; 182/107;182/134
Nylidrin	120	100	0.8	5.9	300/91;300/150; 300/282
Norfenefrine	200	150	1.0	8.0	154/91;154/107; 154/119
Octapamine	250	200	1.0	10.3	154/91;154/107; 154/119
Oxilofrine	220	100	1,0	7.7	182/91;182/107; 182/135
Pholedrine	150	80	0.9	7.7	166/79;166/107; 166/151

In summary in this study it was demonstrated that HILIC is a powerful technique for the confirmation of phenoalkylamines offering a difference in selectivity compared to traditional RPLC. The use of type-B silica material for the analysis of basic compounds could significantly (i) improve desolvation with ESI mass spectrometry offering better sensitivity and consequently lower LLOD, (ii) decrease the matrix effect, (iii) decrease the column

backpressure permitting the use of faster flow rates or the use of longer columns and small particle size (less than 2µm) stationary phases without the need of expensive high-pressure pumps, and (iv) increase the RT repeatability and the peak resolution and shape. Furthermore, the increase in sensitivity permits the use of smaller sample volume and reduced sample preparation procedures, such as the direct dilution and the injection into the LC-MS/MS system.

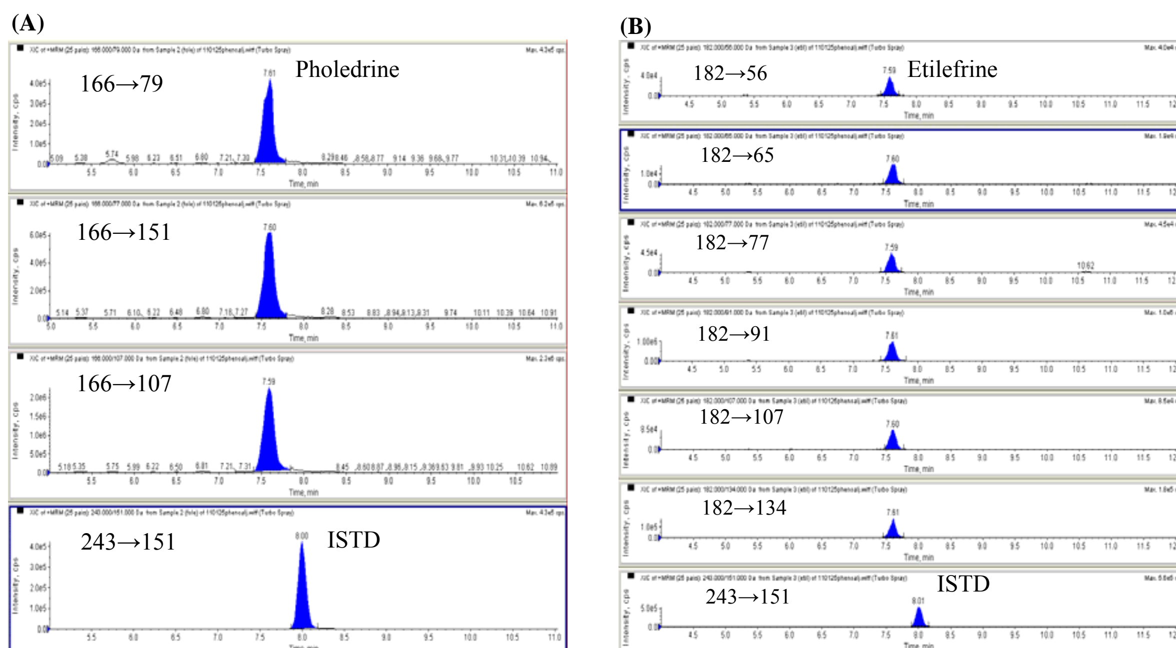

Figure 1: Extracted ion chromatograms of routine samples found to be positive for pholedrine **(A)** and etilefrine **(B)**.

References

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