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Application Note

Assessing the Chromatographic Performance of Small Polar Compounds when Using HILIC-based LC-MS Chromatography for Metabolomic Studies

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This is an Application Brief and does not contain a detailed Experimental section.

Abstract

This application brief assess the performance and robustness of HILIC and BEH Amide Column chemistries for the separation of hydrophilic polar metabolites and their suitability for metabolic profiling studies.

Benefits

The BEH Amide Column demonstrated excellent polar compound retention, chromatographic peak shape, and reproducibility for the analysis of standard mixtures and human urine thus proving to be suitable for large scale metabolomic studies.

Introduction

Biological matrices typically employed in metabolic profiling studies consist of a wide variety of small molecules pivotal in many biochemical processes. Pathways such as the citric acid cycle and those associated with amino acid metabolism contain very polar compounds that are difficult to retain and resolve by reversed-phase (RP) chromatographic conditions. Hydrophilic interaction liquid chromatography (HILIC) is a technique better suited to the analysis of these polar metabolites which relies on the hydrophilic nature of these compounds to retain them on column. HILIC columns tend to comprise of unbonded silica particles with some stationary phases containing polar functional groups to improve retention and separation of polar analytes that span a wide range of polarity, pKa, and structural moiety. Due to the variability of molecules present in biological matrices and fundamentally their differences in physiochemical properties, the true benefit of bonded versus unbonded stationary phases for profiling experiments is assessed.

Results and Discussion

To assess the differences in polar retention/separation for unbonded and bonded stationary phases, two HILIC columns were selected for investigation, the Waters BEH HILIC unbonded Silica Column (p/n: 186003461) and BEH Amide Column (p/n: 186004801). In order to assess differences in column characteristics, the Waters LCMS QC Reference Standard Mixture (p/n: 186006963) and a custom mixture of polar endogenous compounds were analyzed under four different mobile phase conditions (Table 1). The custom polar compound mixture contained phenylalanine, taurine, betaine, creatinine, hippuric acid, and benzoic acid. Each standard solution supplied in 100% water was diluted 1:4 with acetonitrile and injected in replicate (n=6). The column temperature and gradient profile was maintained across all column and mobile phase conditions at 40 ºC and 0–100% A for 7 mins respectively. The mobile phase flow rate was also maintained at 0.5 mL/min for all conditions.

	Mobile Phase	
	A	B
MP 1	95:5 Water:acetonitrile with 0.1 % formic acid	5:95 Water:acetonitrile with 0.1 % formic acid
MP 2	95:5 Water:acetonitrile with 0.1 % formic acid and 5 mM ammonium acetate	5:95 Water:acetonitrile with 0.1 % formic acid and 5 mM ammonium acetate
MP 3	95:5 Water:acetonitrile with 0.1 % formic acid and 10 mM ammonium acetate	5:95 Water:acetonitrile with 0.1 % formic acid and 10 mM ammonium acetate
MP 4	95:5 Water:acetonitrile with 0.1 % formic acid and 20 mM ammonium acetate	5:95 Water:acetonitrile with 0.1 % formic acid and 20 mM ammonium acetate

Table 1. Mobile phase (MP) conditions used to investigate the column characteristics for the BEH HILIC and BEH Amide columns.

All data were acquired using a Waters SYNAPT XS System, operated in resolution mode and data collected in positive ion mode. Extracted ion chromatograms for each of the standards were processed using MassLynx and Skyline (MacCoss Lab, University of Washington, Seattle, USA), where characteristics of chromatographic performance were calculated, including peak widths (Wb), retention factor (K'), selectivity (α), and theoretical plate count (N) in order to determine the optimal column across all the conditions evaluated.

Examining the range of peak widths generated, indicated that the amide column provided much greater consistency, with average widths ranging between 4 and 5 sec across all four mobile phase conditions (Figure 1). The BEH HILIC Column exhibited greater variation in peak widths which was shown to improve with the addition of ammonium acetate (Figure 2), but ultimately ranged from 2 to 9 sec peak widths.

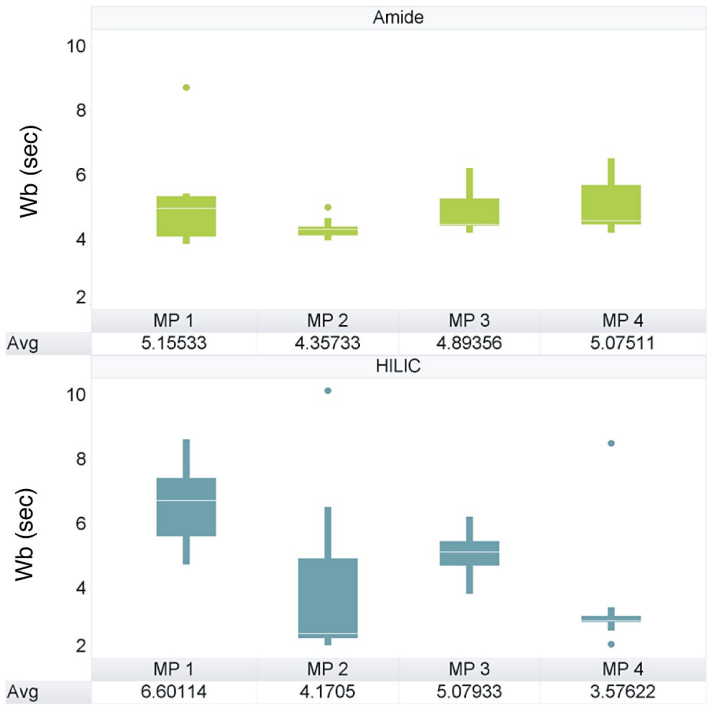

Figure 1. Comparison of peak widths for the LCMS QC Reference Standards at base (Wb). The BEH Amide (upper) and BEH HILIC (lower) columns are represented for the four mobile phase conditions assessed (MP1-MP4).

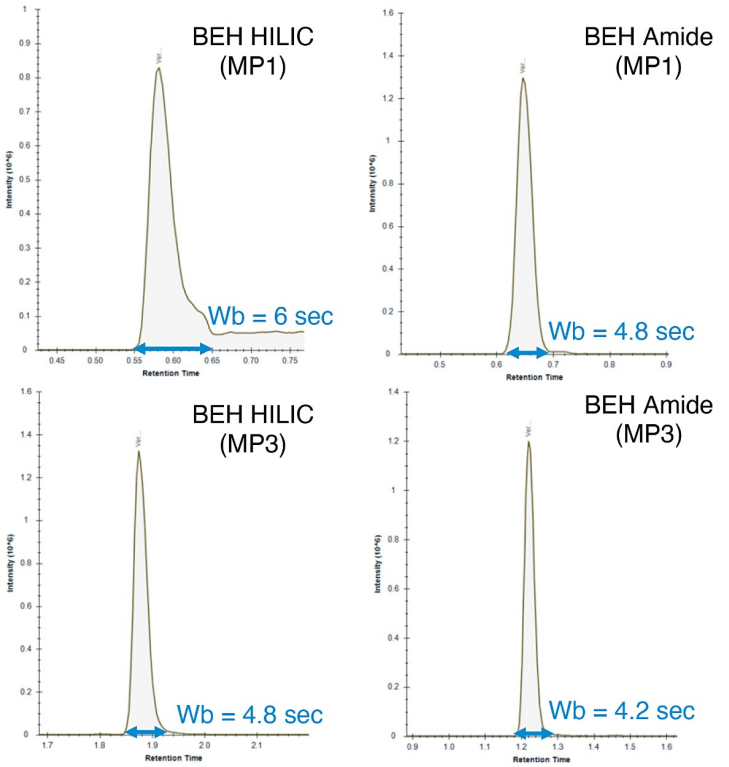

Figure 2. Chromatogram comparison showing variation in peak width at base (Wb) for the compound Verapamil using mobile phases 1 and 3 with the BEH HILIC and Amide columns.

The retention factor (K′) is a measurement of the retention performance and capacity of the column. K′ values between 1 and 10 are generally deemed acceptable during method development and validation.[1] Figure 3 shows the average K′ for the LCMS QC reference compounds, separated using both columns under all four mobile phase conditions. Both columns provided an average K′ of >1.5 for the majority of mobile phase conditions assessed, however, the BEH Amide Column is shown to be more consistent over all 4 mobile phase conditions.

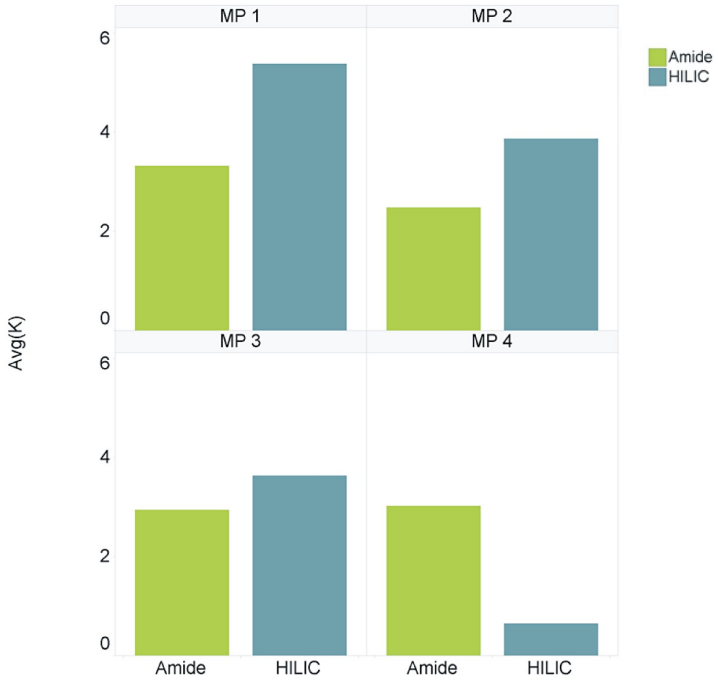

Figure 3. Average retention factor (K') based on the LCMS QC reference standard for the BEH Amide and HILIC columns under the four mobile phase conditions assessed.

Based on these observations, mobile phase 3 (10 mM ammonium acetate) produced the optimum chromatographic performance for both columns, resulting in similar peak widths and K' (Figures 1 and 3 respectively). As can be seen in the chromatograms from Figure 4, the BEH Amide Column showed greater separation for the LCMS QC Reference Standard and greater retention for many of the polar standards.

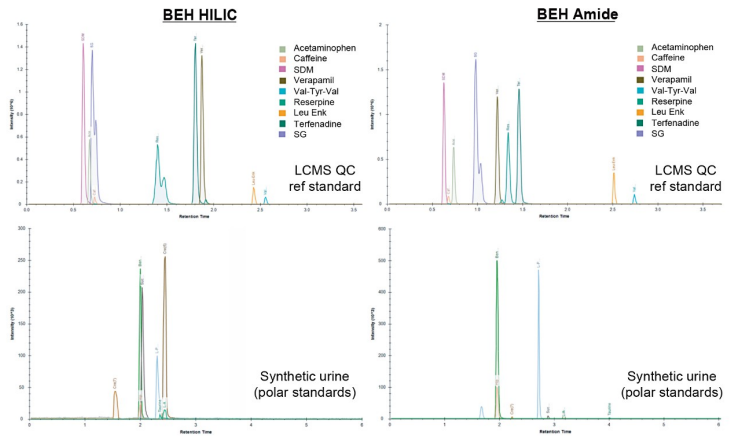

Figure 4. Example chromatograms representing the LCMS QC reference and synthetic urine standards injected on the BEH Amide (right) and BEH HILIC (left) columns using 10 mM ammonium acetate buffered mobile phase.

Based on the evaluation outlined, the BEH Amide Column was assessed for reproducibility by acquiring 200 injections (34 hrs of analysis time) of human urine using the optimised chromatographic conditions. The results demonstrated excellent reproducibility over the duration of the analysis (Figure 5).

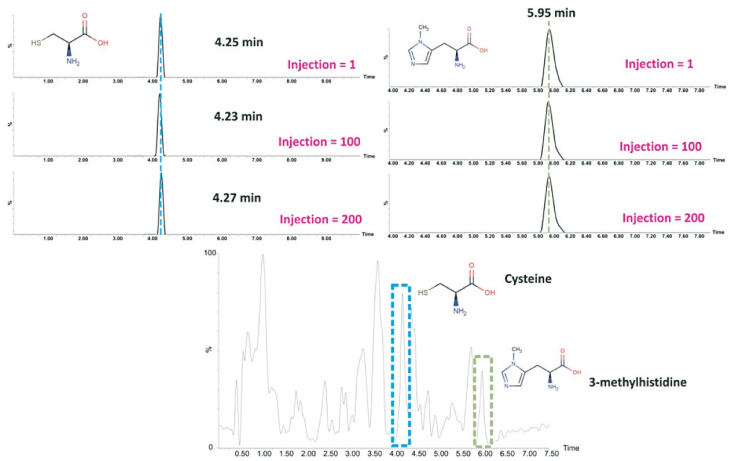

Figure 5. Retention time reproducibility for known endogenous metabolites from human urine based on the BEH Amide Column with a buffered mobile phase consisting of 10 mM ammonium formate. The coefficient of variance (CV) for cysteine and 3-methylhistidine was 2.8% and 0.6% respectively over the course of 200 consecutive injections.

Conclusion

This investigation highlighted the benefit of bound stationary phase columns for generic metabolic profiling studies when compared with unbound traditional HILIC columns. Improved compound retention, peak shape, and reproducibility for the analysis of polar compounds in complex matrices has been demonstrated. The BEH Amide Column demonstrated improved selectivity and higher peak resolution based on the LCMS QC Reference Standard.

Furthermore, the addition of a buffered mobile phase enhanced the compound-stationary phase interaction leading to sharper peaks and thereby improving chromatographic separation. Assessing the reference standards spiked into human urine showed excellent reproducibility across 200 injections based on the optimized method using the HILIC Amide Column.

References

1. Reviewer Guidance – Validation of Chromatographic Methods, Center for Drug Evaluation and Research (CDER), November 1994.

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