Application Note 20710

HPLC for the Retention and Resolution of Very Polar Compounds

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Key Words
Hypercarb, polar compounds, hydrophilic, ionized

Abstract
Separation methods for extremely polar compounds have been developed using a Thermo Scientific™ Hypercarb™ HPLC column. Good retention and resolution was obtained. These methods demonstrate good reproducibility and robustness.

Introduction

The ability to retain and separate very polar, hydrophilic molecules in reversed-phase chromatography can be challenging and problematic. Separation generally requires the use of ion pair reagents, mobile phase pH modification, concentrated buffers, or highly aqueous mobile phases. Such options have potential detrimental effects upon low-wavelength UV detection and reduced sensitivity in electrospray (ESI) mass spectrometry and often still offer poor retention.

The application described here was developed for AstraZeneca at their request because their existing method exhibited poor retention of the compounds, eluting near the solvent front, and poor resolution of some components (Figure 1). UV detection at very low wavelengths was required, which limited the choice of the mobile phase and buffer. A range of columns, including polar end-capped phases, did not offer acceptable chromatography for the compounds of interest. This application demonstrates that extremely polar compounds can be retained using Hypercarb columns. In addition, this method is suitable for compounds that require detection at a low wavelength of 195 nm.

The drug referred to as 4 in this application is at an early stage of the development process within AstraZeneca and the name of this compound cannot be disclosed due to confidentiality issues. Compounds referred as 1, 2, 3, 5, and 6 are either by-products of the synthetic process, intermediate synthetic compounds, or degradation products. Table 1 includes some of the physicochemical properties of these compounds. High pK_a values show that the compounds are permanently charged, and the negative LogD values show that the compounds are extremely hydrophilic. These separation methods were developed for the analysis of these drugs, their degradation products, and impurities arising from the synthetic process.

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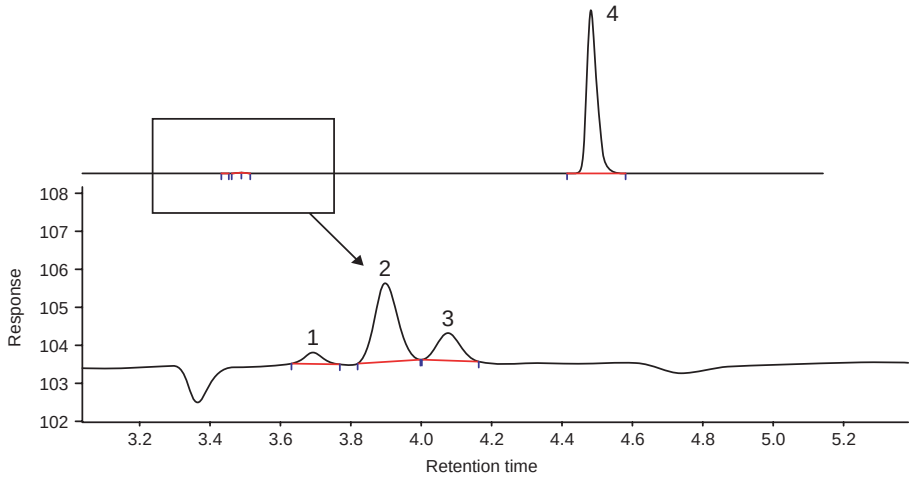

Figure 1: Separation of four extremely polar compounds using AstraZeneca's existing method [1]

Compound	Calculated pk_a	Calculated LogD (at pH 4.5)
1	pk_a (1) >14 pk_a (2) 7.4	-3.7
2	pK_a (1) >14 pK_a (2) 1.7	-7.0
3	pK_a (1) >14 pK_a (2) 1.8	-7.1
4	>14	-5.4
5	Neutral	n/a
6	>14	0.6

Table 1: Physicochemical properties of the test compounds (calculates values obtained using ACD 12.01 software). Compounds 5 and 6 were added later at the customer's request and are not shown in the chromatogram in Figure 1.

Hypercarb

Hypercarb media is 100% porous graphitic carbon (PGC). The particles are spherical and fully porous. The surface of the PGC is composed of flat sheets of hexagonally arranged carbon atoms as in a very large poly-nuclear aromatic molecule. The surface is crystalline and highly reproducible, with no micro-pores or chemically bonded phase.

The flat, highly crystalline Hypercarb surface leads to retention mechanisms that are different from those observed on silica-based bonded phases. The overall retention on Hypercarb media is a combination of two mechanisms:

1) Dispersive interactions between analyte-mobile phase and analyte-graphite surface, by which retention increases as the hydrophobicity of the molecule increases.
2) Charge-induced interactions of a polar analyte with polarizable surface of graphite (Figure 2).

(a) Analyte with electron-withdrawing properties approaching the graphite surface

b) Analyte with electron-donating properties approaching the graphite surface

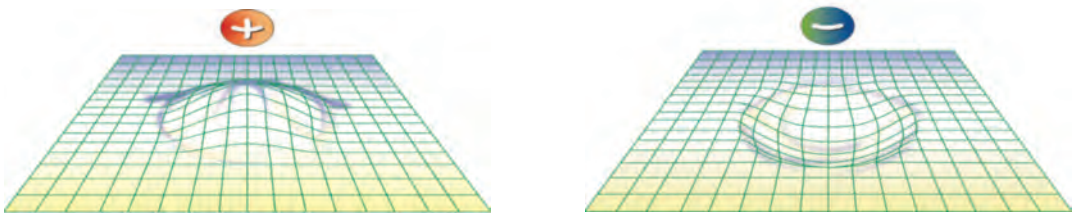

Figure 2: Schematic representation of charge-induced interaction on the PGC surface

This application describes two versions of the method. The first is suitable for systems with low dwell volume (less than 100 µL) and optimized (low) dead volume. This combination allows the use of narrow bore columns and fast gradients, resulting in faster methods using approximately 11 times less solvent. The second method was developed for systems with higher dwell volume (approximately 1100 µL) and higher dead volume, which meant that a 4.6 mm diameter column was essential. Due to changing customer requirements, the second method had two additional compounds (by-product 5 and compound 6) added.

Experimental Details

Consumables	Part Number
Fisher Scientific™ LC/MS grade water	W/0112/17
Fisher Scientific LC/MS grade acetonitrile	A/0638/17
Fisher Scientific Potassium dihydrogen orthophosphate (KH_2PO_4), HPLC grade	P/4810/50

Sample Handling Equipment	Part Number
Thermo Scientific™ Finnpipette™	9402151, 02-707-408, 02-707-423
Thermo Scientific™ National™ Mass Spec Certified Target DP 2 mL amber vial, ID patch, grey bonded cap PTFE/silicone pre-slit	MSCERT4000-41W

Sample and Calibration Preparation				Part Number
Method 1:				
Test mix preparation:	Approximately 3.00 mg of compounds 1, 2, and 3 were dissolved in 100 mL of sample diluents [water / acetonitrile (90:10 v/v)] to give a concentration of 0.03 mg/mL impurity solution. Then 15.0 mg of compound 4 was dissolved with 1 mL impurity solution to give the final ratio of 0.2% impurity to parent compound (4).			
Chromatographic method 1:				
Instrument:	Thermo Scientific™ Accela™ 600 Pump and Open Autosampler			
Column:	Hypercarb 5 µm, 100 × 2.1 mm			35005-102130
Mobile phase A:	100 mM KH_2PO_4 in LC/MS grade water			
Mobile phase B:	200 mM KH_2PO_4 / acetonitrile (LC/MS grade) (50:50 v/v)			
Gradient conditions	T/min	% A	% B	
	0.00	95.0	5.0	
	0.60	95.0	5.0	
	5.00	0.0	100.0	
	5.50	5.0	95.0	
	9.00	95.0	5.0	
Flow rate:	400 µL/min			
UV:	195 nm			
Wash solvent:	Water / acetonitrile (90:10 v/v)			
Injection volume:	1 µL			
Temperature:	Ambient			
Pressure (T=0):	252 bar			
Method 2:				
Test mix preparation:	Approximately 3.0 mg of compounds 1, 2, 3, 5 and 6 were dissolved in 100 mL of sample diluents diluents [water / acetonitrile (95:5 v/v)] to give a concentration of 0.03 mg/mL impurity solution. Then, 1.5 mg of compound 4 was dissolved in 1 mL impurity solution to give the final ratio of 2.0% impurity to parent compound (4).			
Chromatographic method 2:				
Instrument:	Competitor LC System			
Column:	Hypercarb 5 µm, 100 × 4.6			35005-104630
Mobile phase A:	100 mM KH_2PO_4 in LC-MS grade water			
Mobile phase B:	200 mM KH_2PO_4 / acetonitrile (LC-MS grade) (50:50 v/v)			

Gradient conditions	T(min)	%A	%B
	0.0	95.0	5.0
	15.0	0.0	100.0
	17.0	0.0	100.0
	17.1	95.0	5.0
	27.0	95.0	5.0
Flow rate:	1.50 mL/min		
UV:	195 nm		
Wash solvent:	Water: acetonitrile (95:5 v/v)		
Injection volume:	5 µL		
Temperature:	60 °C		
Pressure (T=0):	74 bar		

Results

Method 1

With the smaller ID column and four compounds, the analysis was carried out in less than 9 min. The peaks were eluted in less than 4 min and very high resolution could be achieved (Figure 3). To check the reproducibility of the method, over 40 runs were carried out. Four chromatograms are overlaid in Figure 4. The method is very robust and consistent. The RSD values from the 39 runs (excluding the first run) were calculated to be less than 0.2% for all four compounds. The results are recorded in Table 2.

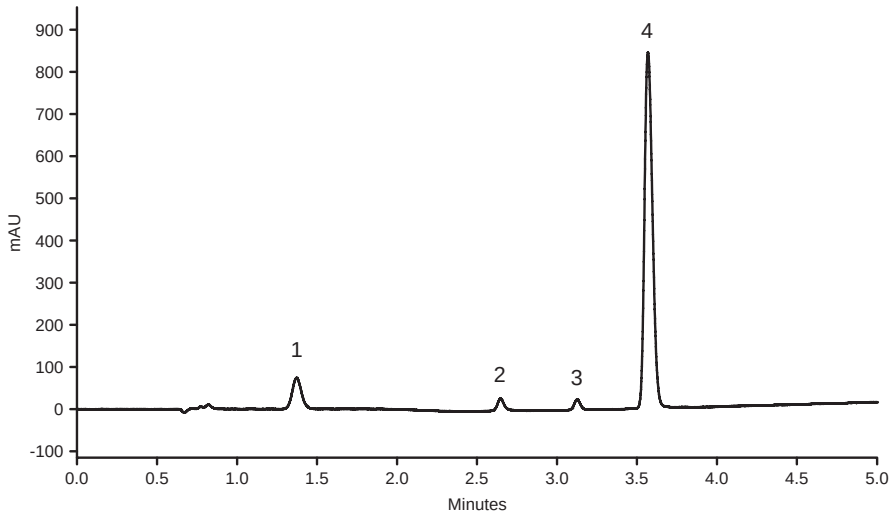

Figure 3: Separation of four extremely polar compounds on a Hypercarb 5 µm, 100 x 2.1 mm column

Compound	Retention time (min)	t_R RSD	Resolution
1	1.37	0.19%	N/A
2	2.65	0.11%	14.05
3	3.13	0.13%	6.58
4	3.57	0.10%	5.45

Table 2: Retention time, precision data, and resolution obtained using a Hypercarb 5 µm, 100 x 2.1 mm column

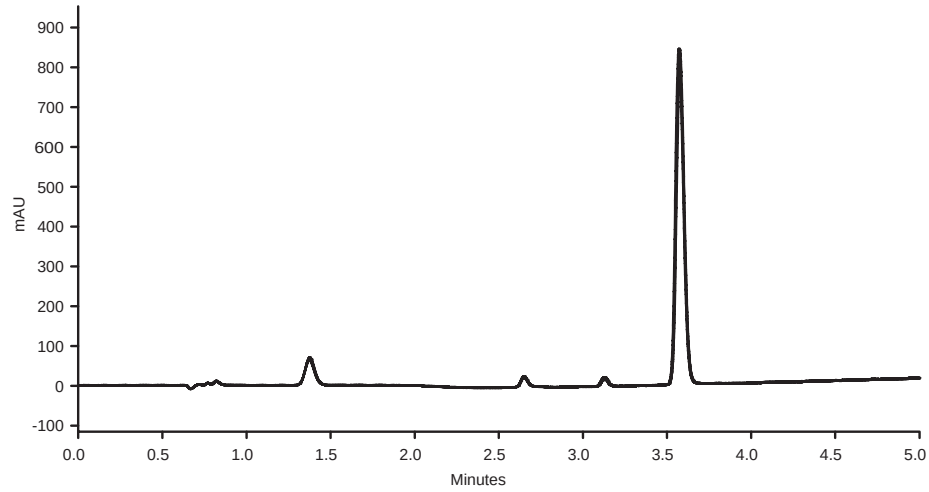

Figure 4: Overlaid chromatograms (injections 5, 20, 25, and 40) showing consistency and method robustness on a Hypercarb 5 µm, 100 x 2.1 mm column

Method 2

The customer requested that we develop a method suitable for use on a competitor HPLC system with high dwell volume and high dead volume. This required the use of a 4.6 mm ID column. Because the method looked so promising, the customer added two additional compounds. To get a stable and reproducible method using PGC, a long re-equilibration was needed and the analysis was extended to 27 minutes. With this method, all of the peaks were separated with good resolution (Figure 5).

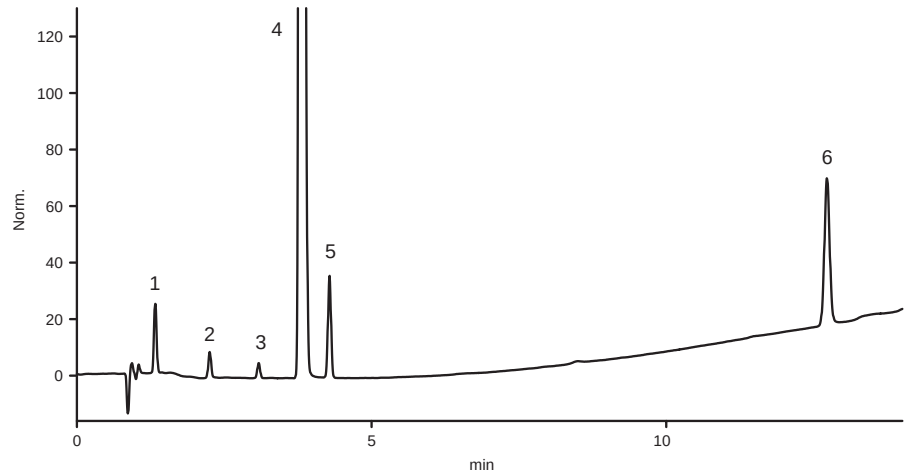

Figure 5: Separation of six extremely polar compounds on a Hypercarb 5 µm, 100 x 4.6 mm column

The method robustness was also established with 40 replicate injections and overlaid chromatograms of seven injections from through the run are shown in Figure 6. After the column equilibrated sufficiently, consistent results could be obtained.

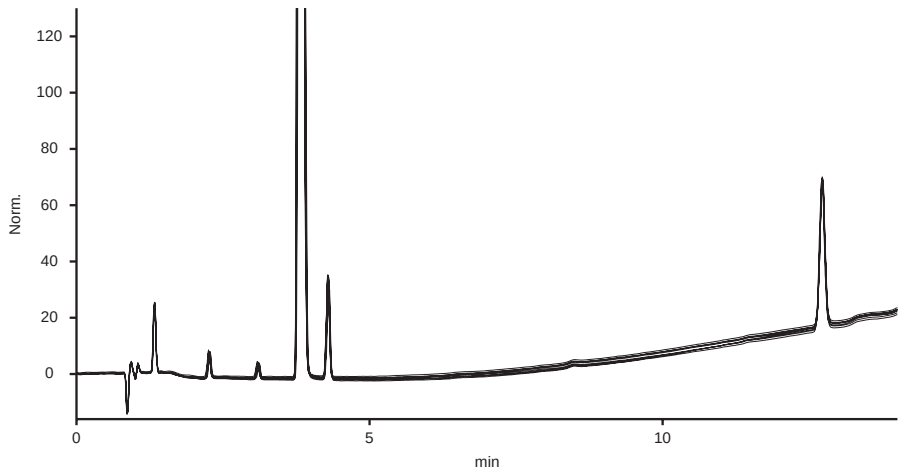

Figure 6: Overlaid chromatograms showing method robustness

Conclusion

Hypercarb columns can be used successfully for the retention and separation of extremely polar compounds, which are problematic to separate in reversed-phase conditions. This application has demonstrated that:

- Hypercarb columns offer good retention of polar compounds.
- Methods developed on Hypercarb columns are robust.
- Hypercarb columns give good resolution and efficiency.
- Methods using optimized HPLC equipment are faster and use less solvent, but methods can be tailored to compensate for limitations in the HPLC hardware available.

References

1. AstraZeneca Analytical Method Protocol
2. Method Development Guide for Hypercarb Columns, 2007 Thermo Fisher Scientific

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