How to Benefit from Different Column Selectivities in the Clinical Laboratory

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Introduction

Liquid chromatography, combined with mass spectrometric detection, has emerged as an important tool in many clinical laboratories and hospitals. Although when a modern LC-MS or LC-MS/MS is installed, dedicated methods and protocols are part of the package, many times these "complete methods" failed at meeting the expectation, due to lack of efficient chromatographic separations. How to select the appropriate column chemistry to suite for the separation of target molecules under study, becomes the most critical part in many projects.

In clinical sample testing, reversed phase liquid chromatography (RPLC) is the most used separation technique, and can be employed for a variety of applications in junction with all common detection principles. However for those polar and hydrophilic analytes, hydrophilic interaction liquid chromatography (HILIC) has emerged as an excellent alternative, as it is "orthogonal" to RPLC separation.

In this study, the use of RPLC and HILIC in routine clinical analysis will be discussed. The UHPLC-MS/MS analysis on Buprenorphine and Tramadol will be presented as RPLC examples. The HILIC-MS/MS and HILIC-fluorescence (FL) analysis on Catecholamines will be presented as well.

Analysis of Catecholamines with HILIC-FL and HILIC-MS/MS

Many endogenous molecules such as Catecholamines have been analyzed in clinical laboratories with lengthy methods using older techniques and where the data validity could be questioned. For this reason, there is a vast of interest in developing new assays at hospitals and labs. In many situations, LC-MS and LC-MS/MS methods are sought for reasons of sensitivity and specificity. However, in terms of sensitivity, for some compounds fluorescence detection can be an alternative.

Catecholamines such as dopamine, epinephrine and norepinephrine are hydrophilic compounds, and therefore very suitable for HILIC. They also have molecular backbones, which allows to the use of fluorescence (FL) detection. HILIC combine with FL provides higher sensitivity because of the high organic in mobile phase. Also the potential impurity contribution from water and reagents can be minimized, compare to RP-FL method.

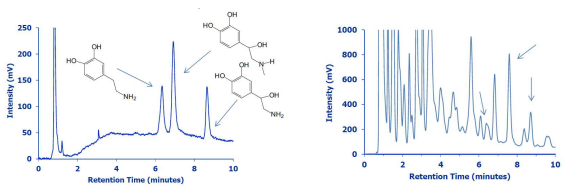
Experiment

Column: SeQuant® ZIC®-HILIC (5 µm, 200 A) 150x4.6 mm (PN 1.51455.0001)
Mobile phase (v/v): A: Acetonitrile/NH4formate 25 mM, pH 6.3 (90:10 v/v); B: Acetonitrile/NH4formate 100 mM, pH 3 (80:20 v/v)

Gradient:	Time (min)	% A	% B	
	0-10	100 -> 0	0 -> 100	Linear gradient
	10-15	100	0	Equilibration

Flow rate: 2.0 mL/min.
Chromatographic system: Hitachi VWR Chromaster with Fluorescence Detection (Ex=260 nm and Em=320 nm)
Injection Volume: 30 µL
Temperature: 40 C
Sample: 50 ppb of Dopamine and Adrenaline, 75 ppb of Noradrenaline diluted in mobile phase A
Sample preparation: 1. Pipette 100 µL urine and diluted to 10 mL with mobile phase A.
2. Fill autosampler vials with sample and perform direct injection.

Separation of Dopamine, Epinephrine, and Norepinephrine with HILIC-FL



(a) (b)

Figure 1. a) Separation of 50 ppb dopamine and epinephrine, and 75 ppb norepinephrine with a ZIC®-HILIC column (150x4.6 mm, 3.5 um, 100A) using gradient elution with 2.0 mL/min flowrate; b) Analysis of human urine sample diluted 100x in mobile phase A

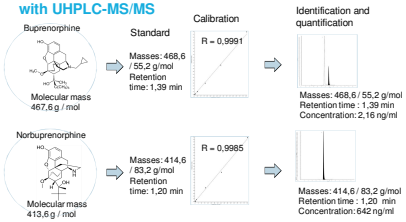
UHPLC-MS/MS Analysis of Buprenorphine and Tramadol

Buprenophine is a synthetic derivative of alkolid thebaine and is used for pain treatment and aversion therapy for heroin dependence. Tramadol (Ultram, Tramal) is a centrally acting opioid analgesic, used in pain treatment and other applications such as treatment for restless leg syndrome and fibromyalgia. In both studies, a newly developed 2 um particle packed high purity silica (Purospher® STAR RP-18e), which specifically designed for fast analysis, were used. The operation cost also dramatically reduced due to the less solvent consumption.

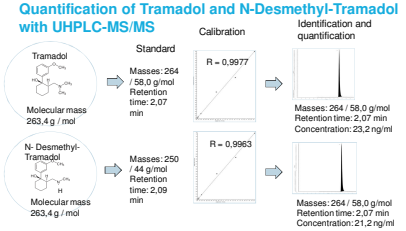
Experiment

Buprenorphin				Tramadol			
MS intrument: Sciex API4000				MS intrument: Sciex API4000			
HPLC: Agilent 1200 Autosampler: CTC HTC PAL				HPLC: Agilent 1200 Autosampler: CTC HTC PAL			
UHPLC Column: Purospher STAR RP-18e, 2 µm				UHPLC Column: Purospher STAR RP-18e, 2 µm			
Mobile phase A: 0,1% formic acid in Milli-Q water Mobile phase B: 0,1% formic acid in acetonitrile				Mobile phase A: 0,1% formic acid in Milli-Q water Mobile phase B: 0,1% formic acid in acetonitrile			
Flow rate: 0,7 ml/min Mobile phase start: 90/10 A/B Column back pressure at start: 230 bar				Flow rate: 0,4 ml/min Mobile phase start: 95/5 A/B Column back pressure at start: 170 bar			
Gradient				Gradient			
Time (min)	Mobile Phase A (%)	Mobile Phase B (%)	Flow rate (ml/min)	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)	Flow rate (ml/min)
0,00	90	10	0,7	0,00	95	5	0,4
				0,20	95	5	0,4
0,25	90	10	0,7	2,00	50	50	0,4
2,00	10	90	0,7	2,50	10	90	0,4
2,10	90	10	0,7	2,80	10	90	0,4
3,00	90	10	0,7	3,00	95	5	0,5
				4,50	95	5	0,5

Quantification of Buprenorphine and Nonbuprenorphine with UHPLC-MS/MS



Quantification of Tramadol and N-Desmethyl-Tramadol with UHPLC-MS/MS



Conclusion

Purospher® STAR RP-18e, 2 µm is designed to meet the demands for modern UHPLC reversed-phase separation with respect to stability, durability and performance. It complements the well- established 5 µm and 3 µm stationary phases of the Purospher® STAR family providing a broad selectivity profile, extraordinary pH stability and high separation efficiency. Due to the smaller dimensions of the columns, chromatographic runs can be performed much faster and the solvent consumption is dramatically reduced. Identification and quantification of Buprenorphine, Tramadol and its metabolites can be done in few minutes.

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Compound	No	LogP	Molecular weight (g/mol)
Metanephrine	I	-1.0	197.23
Normetanephrine	II	-0.9	183.20
Dopamine	III	-1.0	153.18
Norepinephrine	IV	-1.2	169.18
Epinephrine	V	-1.4	183.20

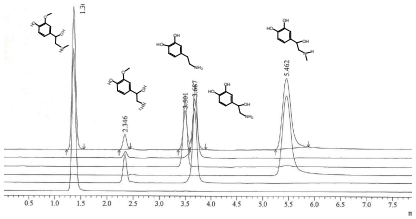
Separation of Metanephrine, Normetanephrine, Dopamine, Epinephrine, and Norepinephrine with HILIC-MS

In the human body, the most abundant catecholamines are epinephrine (adrenaline), norepinephrine (noradrenaline) and dopamine, but the catecholamine profiles in blood and urine sample differ. In urine it is common to monitor metanephrine and normetanephrine. Thus often separate methods for catecholamines are used for plasma/serum and urine samples. Endogeneous molecules in bodyfluids are, in fact, mostly of a hydrophilic/polar character and as such ideal for HILIC mode separation. As seen in Figure 1b) the urine sample contain numerous entities (molecules) having retention on a bonded zwitterionic HILIC column. The vast amount of eluting peaks from a urine sample calls for more selective detection technique to efficate clinical routine analysis.

We aim at developing a method for catecholamine screening useful for any type of samples. Below a chromatogram is shown for the simultaneous separation/quantitation of Metanephrine (I), Normetanephrine (II), Dopamine (III), Epinephrine (IV), and Norepinephrine (V) using the new SeQuant® bonded zwitterionic stationary phase, ZIC®-cHILIC and single-stage mass spectrometric detection using a Shimadzu LC-2010 EV system. This separation is currently being transferred to an LC-MS/MS platform and where final method development and method validation will be performed. This poster illustrate the possibility with using HILIC to separate all clinically relevant catecholamines in same run.

Experiment

Metanephrine, Normetanephrine, Dopamine, Epinephrine and Norepinephrine		
Instrument: Shimadzu LC-MS 2010 EV SIM Mode (positive ESI): m/z 193 (Norepinephrine: MH+ + Na), 195 (Dopamine: MH+ + Na and H20), 225 (Epinephrine and Normetanephrine: MH+ + Na and H20) and 239 (Metanephrine: MH+ + Na and H20)		
HPLC: Shimadzu LC-20AD equipped with a CBM-20 controller and SIL-20AC autosampler		
Column: SeQuant® ZIC®-cHILIC (3 µm, 100 A) 100x2.1 mm (PN 1.50658.0001) Column oven (CTO-20AC) temperature: 50 degrees Celsius		
Mobile phase A: Acetonitrile and ammonium formate (50 mM, pH 6.3) 90:10 v/v Mobile phase B: Acetonitrile and ammonium formate (25 mM, pH 6.3) 80:20 v/v		
Flow rate: 1,0 ml/min Mobile phase start: 100 % A Column back pressure at start: 120 bar		
Injection volume: 30 µL		
Sample: 200 ppb (200 ng/mL) each of Metanephrine (I), Normetanephrine (II), Dopamine (III), Epinephrine (IV) and Norepinephrine (V) diluted in mobile phase A		
Gradient		
Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0,00	100	0
7.00	20	80
7,10	20	80
8.00	20	80
8.10	100	0
10,00	100	0



Conclusion

Development of liquid chromatographic – mass spectrometric (LC-MS/LC-MS/MS) assays for use in clinical routine laboratories has been a significant trend over the past few years, and where HILIC is being regarded as an important tool for the separation of endogenous molecules in biological samples. In this poster we highlight the importance of choosing the right chromatographic separation mode for the molecules/probes of interest. In our opinion, the hydrophilicity/hydrophobicity scale should be explored more, and the choice of column should be a compromise between retentivity, selectivity and easy of coupling with "right" detection technique in order to achieve robust and sensitive methods with good reproducibility and robustness.

References to other methods with bonded zwitterionic HILIC columns developed by EMD Millipore:

1. Analysis of Methylmalonic acid (MMA) Quantification of Methylmalonic Acid in Human Plasma with Hydrophilic Interaction Liquid Chromatography Separation and Mass Spectrometric Detection; P-A Lalan, P. Appelblad, J. Schiewietz, Clin. Chem., 54 (2008) 2028
2. Analysis of Ethyl glucuronide (EtG) and Ethylsulfate (EtS): "Direct Determination of Ethyl Glucuronide and Ethyl Sulfate in Postmortem Urine Specimens Using Hydrophilic Interaction Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry , A.J. Al-Asmari, R.A. Anderson, P. Appelblad, J. Anal. Tox. 34 (2010) 361-372

www.emdmillipore.com/chromatography

Application support: chromatography@merckgroup.com