Catecholamines

– stress biomarkers

Catecholamines are basic organic compounds based on a catechol molecular backbone and a side-chain amine, and they act as neurotransmitters. Epinephrine (adrenaline), norepinephrine (noradrenaline) and dopamine are the most common endogenous catecholamines, see figure 2, all produced from phenylalanine and tyrosine and can be monitored through excreted urine. Catecholamines are water-soluble and partly bound to plasma proteins, circulating in the bloodstream; therefore ideal to separate in hydrophilic interaction liquid chromatography (HILIC) mode. pKa values, LogP, and molecular weight for each analyte can be seen in table 1.
Simple sample preparation procedures using either dilution followed by filtration or protein precipitation followed by centrifugation can be applied if sample matrix is urine or plasma/serum. This application presents a stepwise method development strategy to find optimal experimental conditions for; a) UV; b) MS and c) fluorescence detection for aforementioned polar molecules useful for urine analysis. The catechol profile differ among urine and plasma/serum wherefore we also have developed a new LC-MS/MS method that allows analysis of metanephrine and normetanephrine, beside dopamine, norepinephrine and epinephrine, thus providing a solution for both saliva urine and plasma/serum analysis.

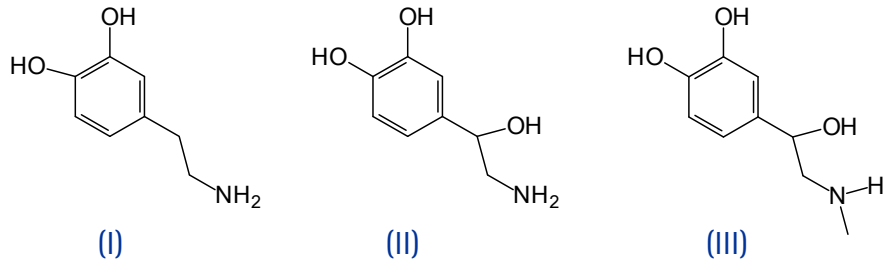


Figure 2. Chemical structure of Dopamine (I), Norepinephrine (II) and Epinephrine (III).

Compound	pKa	LogP	Molecular weight (g/mol)	Smiles*
Dopamine	8.89	-1.0	153.18	C1=CC(=C(C=C1CCN)O)O
Norepinephrine	8.4	-1.2	169.18	C1=CC(=C(C=C1C(CN)O)O)O
Epinephrine	8.55	-1.4	183.20	CNCC(C1=CC(=C(C=C1)O)O)O

* The simplified molecular-input line-entry system or SMILES is a specification in form of a line notation for describing the structure of chemical molecules using short ASCII strings. SMILES strings can be imported by most molecule editors for conversion back into two-dimensional drawings or three-dimensional models of the molecules.

Retention Prediction

Using structural information from each molecule, expressed in table 1 as their corresponding SMILES* string, retention prediction was carried out using the online SeQuant® HILIC prediction model (http://www.sequant.com/prediction). Using a ZIC®-HILIC column (100x4.6 mm, 5 µm, 200 Å) and an eluent containing 70 volume-% acetonitrile and 30 volume-% 100mM ammonium acetate buffer, pH 6.7, the model predict possible retention for dopamine and epinephrine, whereas norepinephrine would have a retention factor of 0.5. Under slightly acidic conditions (pH 5.56), but overall same conditions, the prediction model indicate less retention for norepinephrine and same possible retention for dopamine and epinephrine. An initial experiment was performed using the more beneficial neutral pH conditions but using a shorter column with only 50 mm length, see figure 3, to verify the retention prediction. Detection (UV) was set at 270 nm.

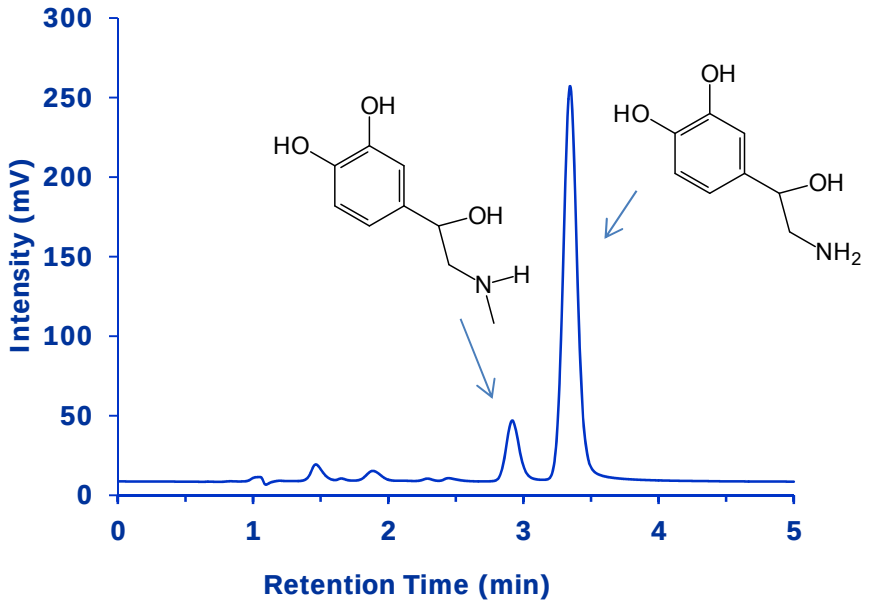


Figure 3. Separation of epinephrine and norepinephrine on a 50x4.6 mm ZIC®-HILIC column, using 70:30 (v/v) acetonitrile and ammonium acetate buffer (100 mM, pH 6.7) at a flowrate of 0.5 mL/min.

Epinephrine and norepinephrine are both well retained (retention factor around 2 for both compounds) and well resolved from each other (with a resolution factor of 2.3) under given experimental conditions. Thus it is worth pointing out that the online prediction model is only a tool to find out if a molecule has retention in HILIC mode or not. It should not be used to predict retention time. For certain molecules very accurate prediction can nonetheless be obtained. In this examples the useful information was that all molecules will have retention in HILIC mode, but this can also be seen from their LogP values.

Organic Solvent Effect in Mobile phase Composition

In order to develop a robust and cost-effective assay for different analytical purposes, buffer salt concentration and choice of organic modifier was studied. Early findings revealed that buffer salt concentration in the mobile phase could easily be lowered without compromise on separation power or selectivity (data not shown). The choice of organic modifier was thereafter tested. Acetonitrile was replaced with methanol to find more economical conditions to lower overall analysis cost for a potential quality control method. At the same time, lower detection wavelength was also tested (210 nm instead of 270 nm) in order to get higher method sensitivity.

In figure 4, acetonitrile has been replaced with methanol (but not with adjusted volume ratio in mobile phase to compensate for a methanol's higher elution strength) and a 50% reduction of buffer salt concentration. Surprisingly, more retention is seen for both analytes, but can be explained with the lower total ionic strength in the mobile phase. More expected is the loss of resolution between epinephrine and norepinephrine; from 2.3 to 1.9 due to the effect from methanol on selectivity and separation power. UV detection at 210 nm provide higher sensitivity despite methanol has much higher cut-off than acetonitrile, and being less transparent at lower wavelengths.

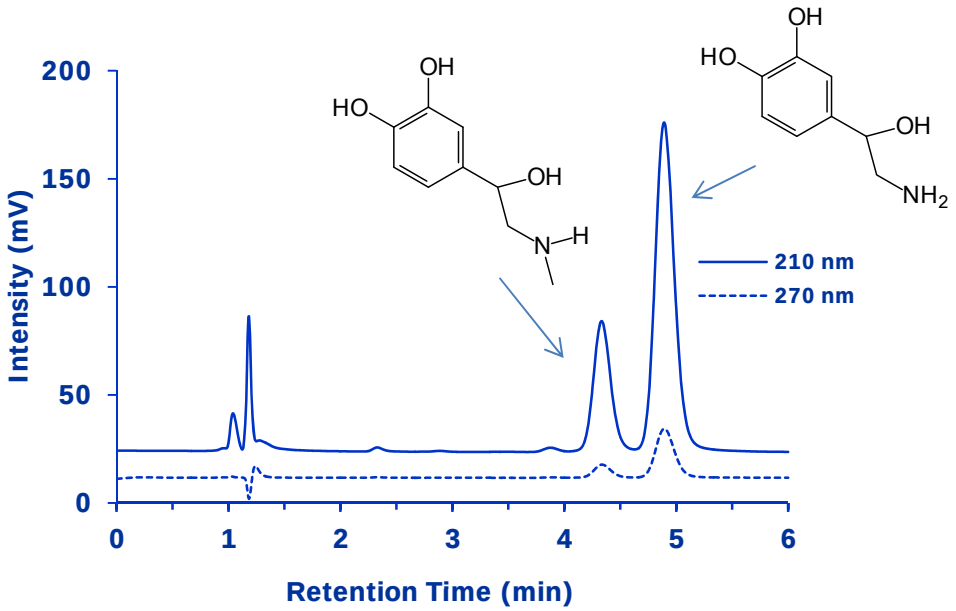


Figure 4. Separation of epinephrine and norepinephrine on a 50x4.6 mm ZIC®-HILIC column, using 70:30 (v/v) methanol and ammonium acetate buffer (50 mM, pH 6.7) at a flowrate of 0.5 mL/min.

To improve the resolution between epinephrine and norepinephrine, the mobile phase proportion of methanol was increased from 70 to 80 volume-%, and thereby also lowering overall buffer salt concentration in the mobile phase from 15 to 10 mM, see figure 5.

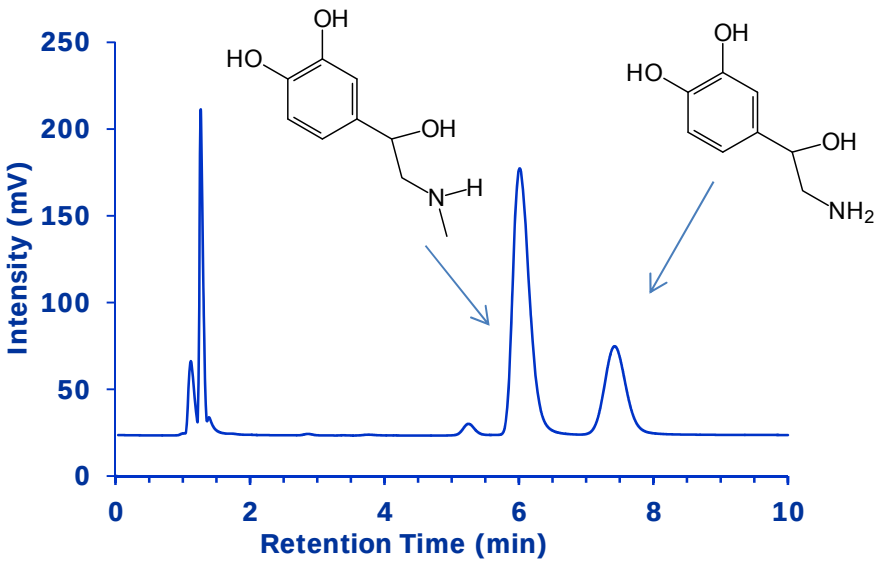


Figure 5. Separation of epinephrine and norepinephrine on a 50x4.6 mm ZIC®-HILIC column, using 80:20 (v/v) methanol and ammonium acetate buffer (50 mM, pH 6.7) at a flowrate of 0.5 mL/min.

As can bee seen in figure 5, the new experimental conditions provide almost to much retentivity to make it into an useful method, i.e. waste of chromatographic space, yet with higher resolution (2.7) between epinephrine and norepinephrine than previous chromatograms. To shorten overall run time and get maximum separation efficiency, methanol was abandoned and acetonitrile was chosen as optimal organic modifier (acetonitrile is a less viscous solvent than methanol thereby providing higher separation efficiency).

Optimal Conditions for Analysis of Epinephrine and Norepinephrine using Standard LC-UV

To obtain same resolution between epinephrine and norepinephrine it was found that a volume fraction of 75% acetonitrile was optimum, with constant total ionic strength in mobile phase of 10 mM buffer salt. To speed up the separation it was possible to increase the flow rate from 0.5 to 1.0 mL/min, see figure 6. These results are satisfactory, the method is robust, rapid and fit any standard HPLC system. The experimental conditions given in Figure 5, is thus recommended for a standard HPLC system with UV-detection for quantitation of relatively high concentrations of epinephrine and norepinephrine.

Scaling of Separation – Finding Optimal Conditions for MS Detection

To convert the application to more mass spectrometry friendly conditions, the separation in figure 5 was scaled to a 2.1 mm id column. To maximize method sensitivity and still allow a fairly large injection volume, the column length was increased from 50 to 100 mm. When scaling a separation from a 4.6 to 2.1 mm inner diameter column, the effective scaling factor is five and both flowrate and injection volume should be reduced accordingly, as can be seen in figure 7.

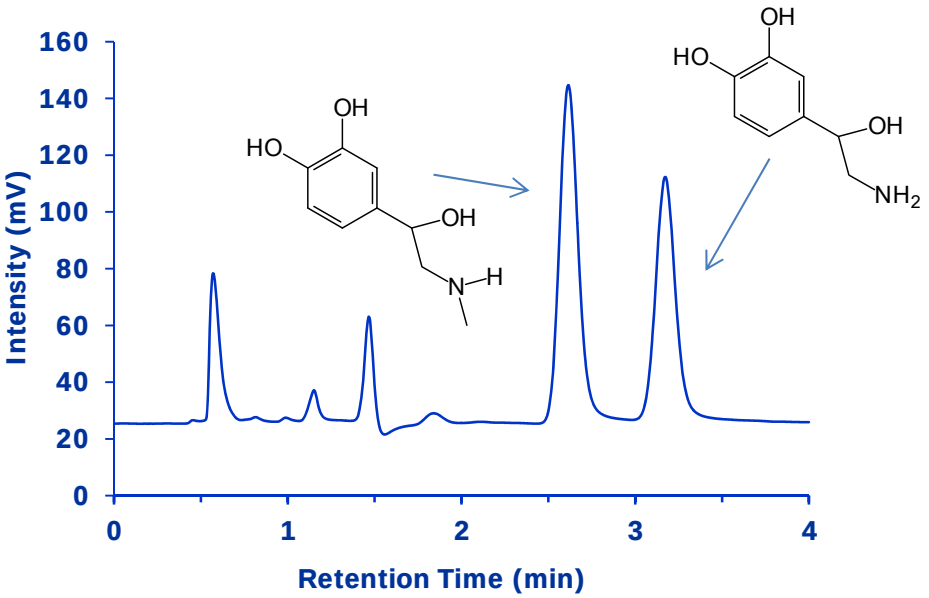


Figure 6. Separation of 25 ppm of each epinephrine and norepinephrine on a 50x4.6 mm ZIC®-HILIC column, using 75:25 (v/v) acetonitrile and ammonium acetate buffer (40 mM, pH 6.7; total ionic strength 10 mM) at a flowrate of 1.0 mL/min. Injection volume is 10 µL.

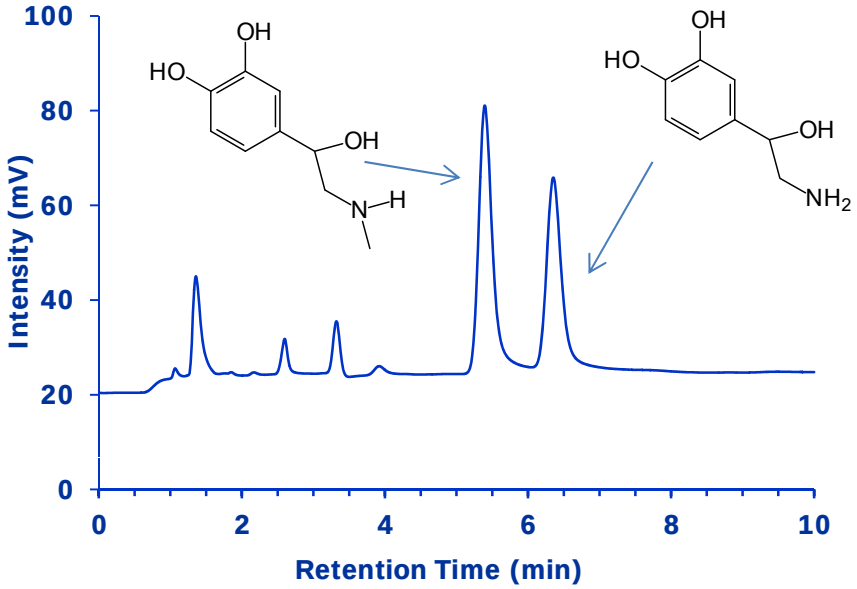


Figure 7. Separation of 25 ppm of each epinephrine and norepinephrine on a 100x2.1 mm ZIC®-HILIC column, using 75:25 (v/v) acetonitrile and ammonium acetate buffer (40 mM, pH 6.7; total ionic strength 10 mM) at a flowrate of 0.2 mL/min. Injection volume is 2 µL.

Same retention factor and same resolution is nonetheless obtained in both figure 6 and 7 (as expected for a scalable and robust stationary phase). The only differences is that the peak height is lower and the overall retention time is doubled for the narrow inner diameter column.

The experimental conditions given in figure 6 provide robust conditions for LC-MS quantitation of epinephrine and norepinephrine. However, if more resolution among the two model analytes is desired or wanted, it is possible to change the pore size of the material instead of further adjustment of mobile phase composition. All separations in figure 3-7 are generated on columns with a stationary phase having a 200 Angstrom pore size. Changing to stationary phase with a smaller pore size, the surface area will be larger, hence more retention will be attained. In figure 8, all experimental parameters are same as in figure 7, but where the stationary phase is changed to contain both a smaller particle size (from 5 to 3.5 µm) and pore size (from 200 to 100 A). This resulted in increase in both overall retention and resolution.

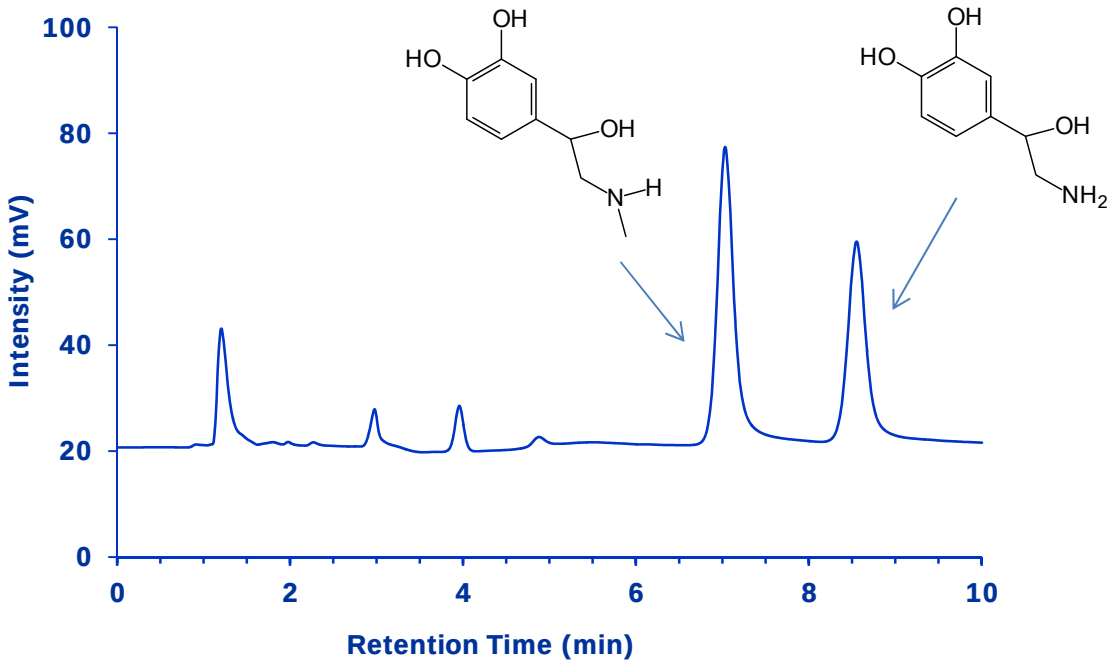
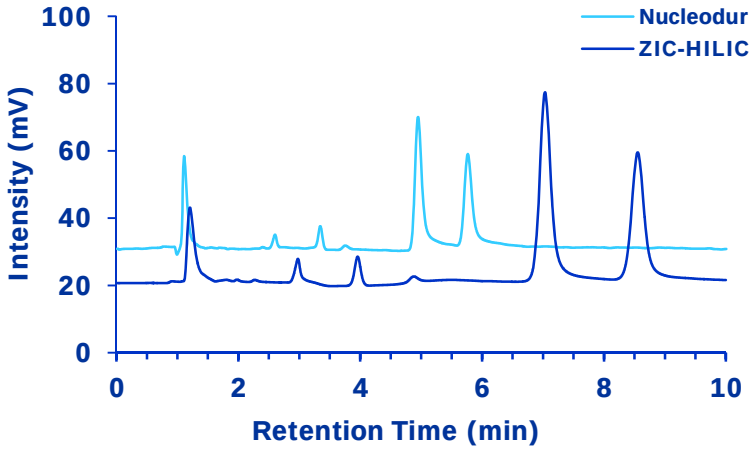


Figure 8. Separation of 25 ppm of each epinephrine and norepinephrine on a 100x2.1 mm ZIC®-HILIC column with 3.5 micron particle size and 100 Å pore size, using 75:25 (v/v) acetonitrile and ammonium acetate buffer (40 mM, pH 6.7; total ionic strength 10 mM) at a flowrate of 0.2 mL/min. Injection volume is 2 µL.

Analysis of Catecholamines Using other Zwitterionic HILIC Columns

To investigate how this separation work on other HILIC columns in general and on other bonded zwitterionic HILIC stationary phases more specifically. Two columns were chosen; one 100x4.6 mm id Nucleodur HILIC column with 3 micron particles having 100 Ångstrom (Å) pore size, and one 100x4.6 mm id Shiseido PC-HILIC with 5 micron particles also having 100 Angstrom pore size. Neither of these columns were commecially available in 2.1 mm id format when this study was carried out, hence a slight discrepancy in data.



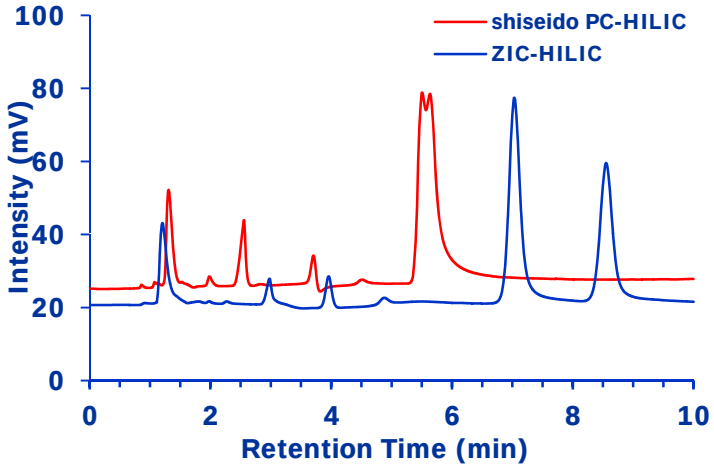


Figure 9 and 10. Separation of 25 ppm of each epinephrine and norepinephrine on a 100x2.1 mm ZIC®-HILIC column with 3.5 micron particle size and 100 Å pore size (dark blue trace), on a 100x2.1 Nucleodur HILIC column with 3.5 micron particle size and 100 A pore size (light blue trace), and on a 100x4.6 mm id Shiseido PC-HILIC with 5 micron particles also having 100 Angstrom pore size (red trace) using 75:25 (v/v) acetonitrile and ammonium acetate buffer (40 mM, pH 6.7; total ionic strength 10 mM) at a flowrate of 0.2 mL/min (ZIC-HILIC) or 1.0 mL/min (Nucleodur and Shiseido PC-HILIC) and. injection volume was 2 µL (ZIC-HILIC) or 10 µL (Nucleodur and Shiseido PC-HILIC).

Analysis of Catecholamines in Urine with HILIC and Fluorescence Detection

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. Of this reason, there is a vast interest in developing new assays at hospitals and laboratories. 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 a viable alternative.

Catecholamines such as dopamine, epinephrine and norepinephrine are hydrophilic compounds, and therefore very suitable for HILIC, but they also have molecular backbones, see figure1, that would allow use of fluorescence (FL) detection. HILIC combined with FL detection can provide high sensitivity due to use of high percentage of organic solvent in mobile phase, and where the potential impurity contribution from water and reagent sources can be reduced compared with a classical reversed phase (RP) – fluorescence detection method. Obvious drawback with both RP and HILIC-FL is that positive identification as obtained with mass spectrometric detection is nonetheless not possible. For laboratories and hospitals with narrow or no budgets for expensive instruments HILIC-FL can represent a more economical approach. In figure 11, a chromatogram is shown for the separation of a standard solution containing dopamine, epinephrine and norepinephrine, and figure 12 show applicability to human urine samples diluted 100 times with mobile phase as only sample preparation method.

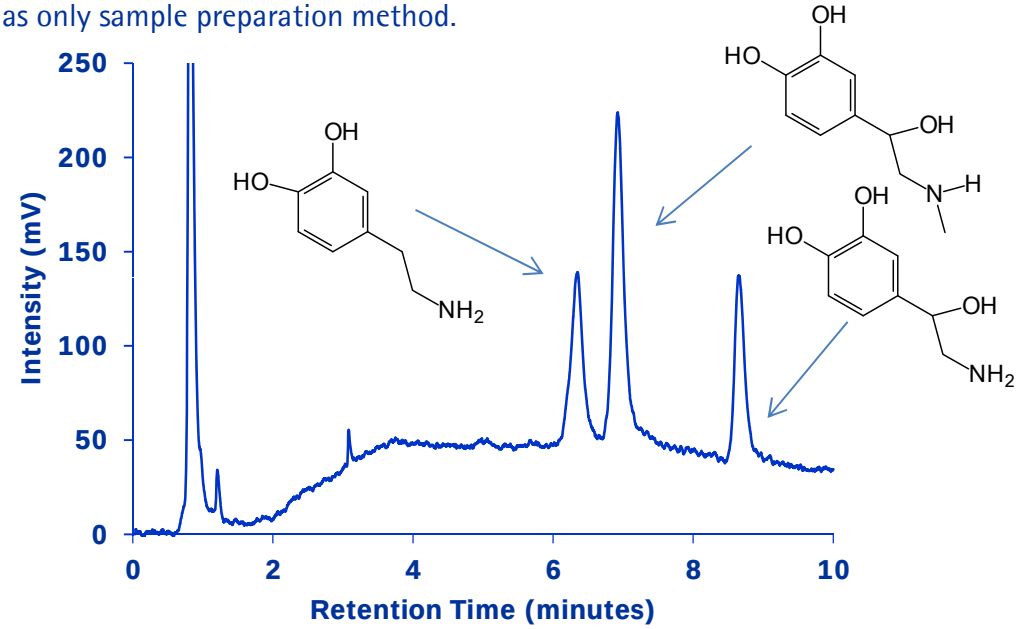


Figure 11. Separation of 50 ppb dopamine and epinephrine, and 75 ppb norepinephrine on a 150x4.6 mm ZIC®-HILIC column with 3.5 micron particle size and 100 A pore size, using gradient elution with acetonitrile and ammonium formate buffer at a flowrate of 2.0 mL/min. See experimental section for complete experimental details.

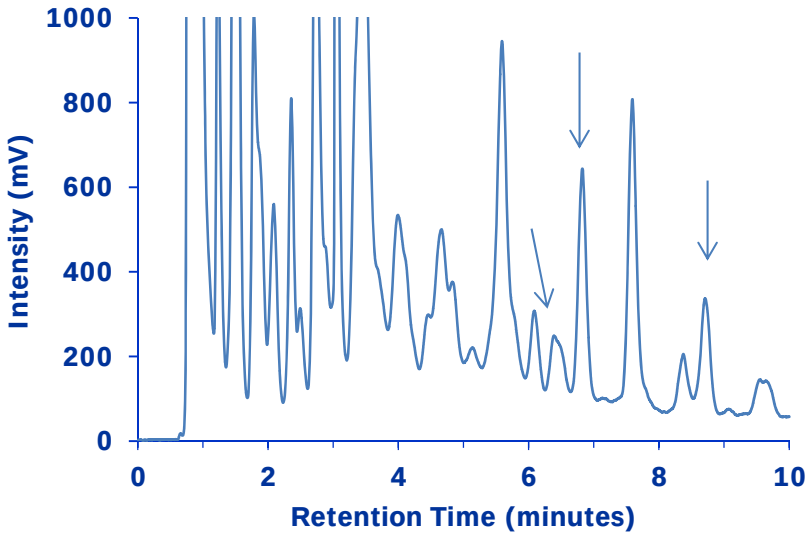


Figure 12. Analysis of a human urine sample (stored in cooler 24 hours) diluted 100X in mobile phase A on a 150x4.6 mm ZIC®-HILIC column with 3.5 micron particle size and 100 Å pore size, using gradient elution with acetonitrile and ammonium formate buffer at a flowrate of 2.0 mL/min. See experimental section for complete experimental details.

Experimental conditions for Urine Analysis with SeQuant® ZIC®-HILIC

Column: SeQuant® ZIC®-HILIC (5µm, 200Å) 150 x 4.6 mm (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

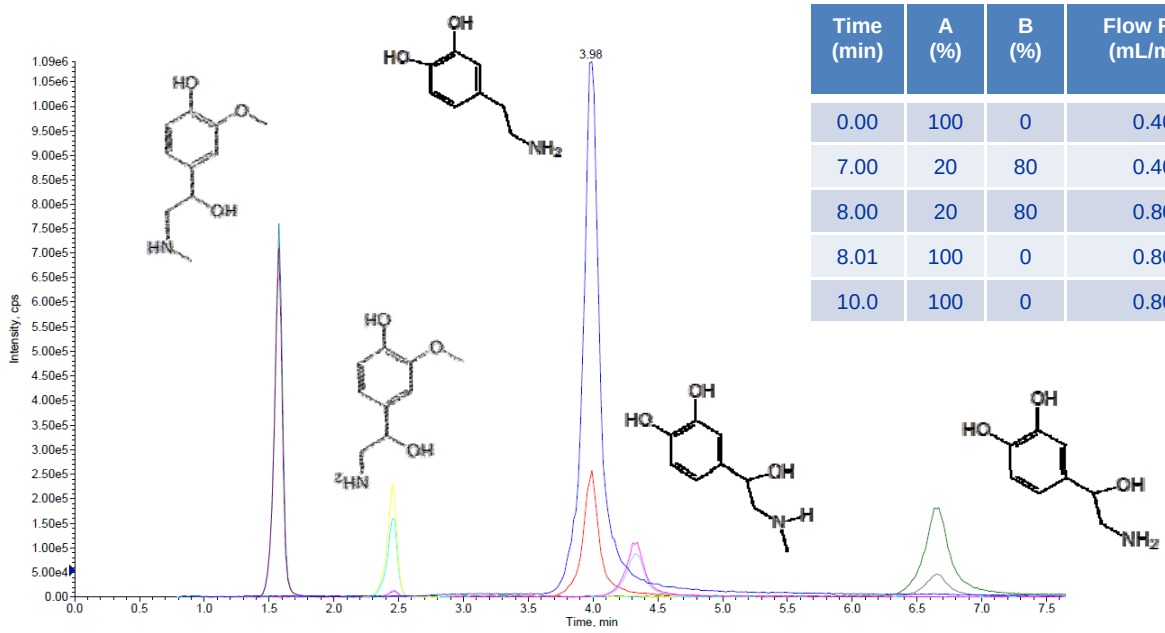
Sample preparation:
1. Pipette 100 µL urine and diluted to 10 mL with mobile phase A.
2. Fill autosampler vials with sample and perform direct injection.

Catecholamines in Urine and Plasma - MS/MS

SeQuant® ZIC®-cHILIC

Chromatographic Conditions

Column:	SeQuant® ZIC®-cHILIC (3µm, 100Å) PEEK 100 × 2.1 mm	(1.50658.0001)
Injection:	30 µL in mobile phase	
Detection:	LC-MS/MS, positive ESI mode	
Flow Rate:	1.0 mL/min	
Mobile Phase:	A: Acetonitrile and ammonium formate (25 mM, pH 6.3) 90:10 v/v B: Acetonitrile and ammonium formate (25 mM, pH 6.3) 80:20 v/v	
Temperature:	50 °C	
Sample:	20 ppb of each, metanephrine, normetanephrine, dopamine, epinephrine and norepinephrine in mobile phase.	



Time (min)	A (%)	B (%)	Flow Rate (mL/min)
0.00	100	0	0.40
7.00	20	80	0.40
8.00	20	80	0.80
8.01	100	0	0.80
10.0	100	0	0.80

Chromatographic Data

No.	Compound	Retention Time (min)	m/z	
1	Void volume	0.5	-	
2	Metanephrine	1.6	198.1→165.0	198.1→148.0
3	Normetanephrine	2.5	184.1→149.1	184.1→134.1
4	Dopamine	4.0	154.0→137.1	154.0→119.1
5	Epinephrine	4.3	184.0→151.0	184.0→135.1
6	Norepinephrine	6.7	170.0→152.1	154.0→135.2