A novel Approach to Catecholamine LC-MS/MS Analysis

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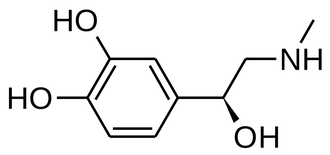
Headlines

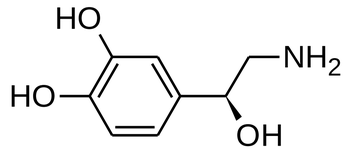
- ***LC-MS/MS analysis of urine catecholamines***
- ***Automatic sample preparation, analysis and transfer of results to LIMS***
- ***Ion-pairing chromatography – getting the benefits without the disadvantages***

Background

Catecholamines are small, very polar molecules that are poorly retained on reversed-phase C18 columns.



Epinephrine



Norepinephrine

Short retention leads to:

- Difficulties in separating epinephrine (E) and norepinephrine (NE) from other isobaric compounds present in the matrix.
- Poor sensitivity due to increased risk of ion suppression and poor ionization in the mass spectrometer because of the low organic content in the mobile phase.

Several approaches have been used to overcome these difficulties, but they all suffer from drawbacks:

- Hydrophilic interaction chromatography (HILIC)
  - Severe challenges in robustness and poor peak shapes.
- Ion-pairing chromatography (IPC)
  - The ion-pairing reagent (IPR) is continuously introduced into the mass spectrometer resulting in signal reduction and contamination of the mass spectrometer.
- Pentafluorophenyl (PFP)
  - Suffers in our experience from reproducibility problems and short column life.

A new approach to IPC does not require the addition of the IPRs into the mobile phases. Instead, the IPRs are added into the sample prior to injection on the liquid chromatography column.

We present here an automated liquid chromatography – tandem mass spectrometry (LC-MS/MS) method for selective analysis of E and NE in urine samples using the new IPC technique with 1-heptane sulfonate (HSA) as IPR.

Method

Apparatus: Hamilton STARlet workstation and Waters Acquity UPLC with Xevo TQ-S tandem mass spectrometer.

Sample preparation: Urine catecholamines were extracted by using either a Sep-Pak Alumina B, 100 mg 96-well extraction plate (Waters) or a Strata Alumina-N, 100 mg 96-well extraction plate (Phenomenex), with d6-norepinephrine hydrochloride (d6-NE) and d3-epinephrine (d3-E) as internal standard. The eluate from the extraction was diluted with 1000 µL HSA solution.

The sample list for mass spectrometry was generated by the Hamilton robot and imported into Masslynx software that controls the LC-MS/MS system.

LC-MS/MS:

LC column: Phenomenex Kinetex Biphenyl, 100 x 2.1 mm, 2.6 µm
Mobile phase A: 0.1% formic acid in water
Mobile phase B: 0.1% formic acid in methanol
Ten µL diluted sample was injected at a flowrate of 0.5 mL/min.

ESI positive MRM conditions			
	Precursor ion m/z	Collision energy (eV)	Product ion (m/z)
Epinephrine (E)	165.86	17	107.11
d3-Epinephrine (d3-E)	168.86	17	110.11
Norepinephrine (NE)	151.92	15	106.96
d6-Norepinephrine (d6-NE)	157.92	15	109.96
Heptane sulfonate (HSA)	180.11	17	81.00

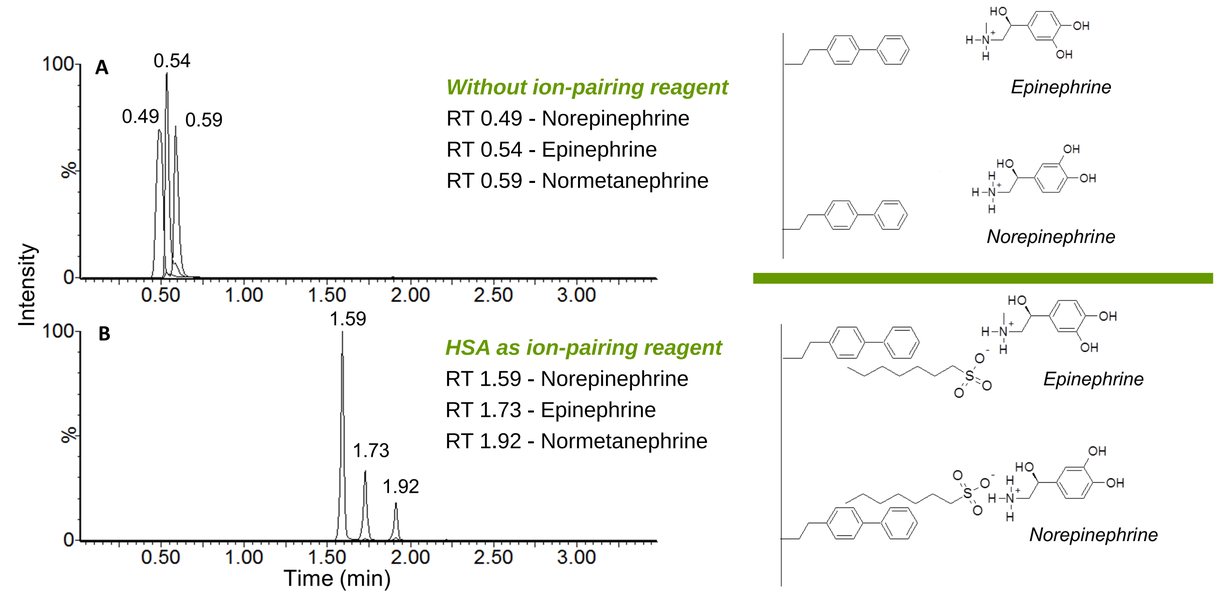
Results are, after inspection of chromatograms, transferred automatically to the LIMS system (BCC).

Calibrators and internal controls: The analysis was calibrated by using single level ClinCal® calibrator from Recipe, and the two urinary controls, Lyphochek® Quantitative Urine Quality Control 1 and 2 from BioRad were used as internal controls. Furthermore, for validation a 7 point spiked urine calibration was used.

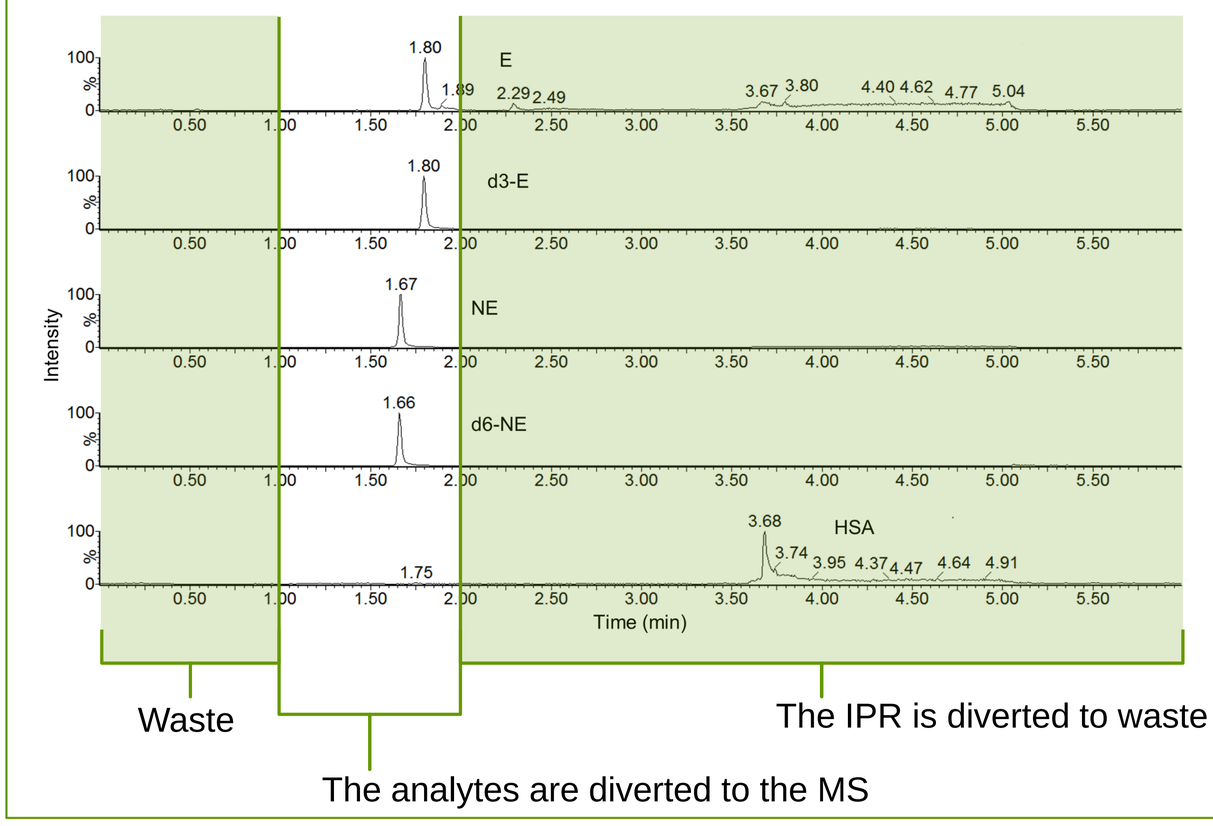
Chromatograms

The IPR added to the sample effectively increases the retention times of the analytes E and NE. At the same time there is a good separation between the analytes and the IPR, enabling diverting HSA to waste instead of into the ionization source.

Increased retention times when the IPR is added



Chromatogram for patient sample containing 0.17 µmol/L E and 0.24 µmol/L NE.



Method validation

Linearity: Least squares linear regression shows that the calibration curve was linear up to a concentration of 2.0 µmol/L for both analytes.

Intermediate precision: Intermediate precision for the normal (n=31) and abnormal (n=28) control samples were below 7.5 % for both E and NE.

Limit of quantification: A sample with 0.005 µmol/L of both analytes was analyzed with a CV of less than 9 % (n=7).

Matrix effects: Matrix effect experiments showed a 10% (CV=3.6%) lower response for pre-extracted samples with added internal standard than for internal standard in HSA solution, indicating slight ion suppression.

Carryover: No carryover was observed in blank samples when injected after the highest calibrator (2.0 µmol/L).

Patient sample comparison: Patient samples were analyzed by the new LC-MS/MS method and compared with results from the same samples analyzed by our previous routine LC-MS/MS method, using the same sample preparation and MS detection, but with traditional IPC with 3 mmol/L heptafluorobutyric acid. Deming regression analysis of the E and NE measurements showed good correlation between the two methods. Bland-Altman plots showed that the new LC-MS/MS method for NE has a slight bias of -0.024 µmol/L with a standard deviation of 0.031 µmol/L. This bias is not seen for E.

Conclusion

The method described here is to our knowledge the first example of a quantitative LC-MS/MS method in routine use in a diagnostic laboratory, based on a new approach to IPC where the IPR is added to the extracted samples and not the mobile phases. This overcomes the disadvantages of traditional IPC and our method displays very good performance characteristics. The IPR added to the sample effectively increases the retention times of the analytes E and NE in a consistent and robust manner.