Analysis of catecholamines in urine by unique LC/MS suitable ion-pairing chromatography

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ABSTRACT

The catecholamines, epinephrine (E) and norepinephrine (NE) are small polar, hydrophilic molecules, posing significant challenges to liquid chromatography − tandem mass spectrometry (LC–MS/MS) method development. Specifically, these compounds show little retention on conventional reversed-phase liquid chromatography columns. This work presents development and validation of an LC–MS/MS method for determining catecholamines in urine, based on a new approach to ion-pairing chromatography (IPC), in which the ion-pairing reagent (IPR), 1-Heptane Sulfonic Acid (HSA), is added to the extracted samples instead of the mobile phases. A Hamilton STARlet workstation carried out the solid phase extraction of urine samples. The extracted samples were diluted with 60 mmol/L HSA and injected on a Kinetex core-shell biphenyl column with conventional LC–MS/MS suitable mobile phases. Chromatographic separation of E and NE was achieved successfully with very stable retention times (RT). In 484 injections, the RTs were steady with a CV of less than ± 4%. Furthermore, HSA was separated from E and NE, allowing HSA to be diverted to waste instead of entering the mass spectrometer ion chamber. The method was validated with good analytical performance, and even though the analysis for urinary catecholamines is increasingly being replaced by plasma free metanephrines in diagnosing pheochromocytomas, this work represents the application of a new analytical technique that can be transferred to other small polar molecules, that are difficult to chromatograph on traditional reversed phase columns.

1. Introduction

Catecholamines are small very polar molecules and are therefore poorly retained on reversed-phase C18 columns. This causes short retention times and difficulty in separating epinephrine (E) and norepinephrine (NE) from each other and other isobaric compounds, such as normetanephrine, present in the matrix. In addition, the small polar compounds elute in solvent with very low organic content (maximum 5%), which can lead to poor ionization in the mass spectrometer. Thus, E and NE elution too close to the solvent front will hamper the sensitivity of the method, and increase the risk of ion suppression caused by other compounds. Accordingly, several methods have been published where NE elutes at, or very close to, the solvent front using reversed-phase chromatography [1–3]. When taking the dead volume of the columns and solvent flow into account, the retention factor of NE and E in these methods range from 0 to 0.8

which, in our experience, leads to reproducibility problems in daily routine use.

Several approaches have been used to overcome these difficulties, for example pentafluorophenylpropyl chromatography (PFP), hydrophilic interaction chromatography (HILIC) and ion-pair chromatography (IPC) [3–5]. These approaches do, however, also have their limitations. PFP column chemistry does not provide adequately reproducible retention times and suffers from short column lifetime. HILIC has some challenges in robustness and poor peak shapes. For instance compounds with more easily ionisable groups, such as E and NE, can form complex interactions with the column chemistry, and the methods are therefore sensitive to small changes in conditions [6].

In traditional IPC, an ion-pair reagent (IPR) is added to the mobile phases and the agent will continuously be introduced into the mass spectrometer. This can contaminate the mass spectrometer, resulting in signal reduction and time consuming cleaning procedures [7]. Further-

more the ion-pair reagents can cause severe ion suppression [8,9]. In a recent development, a new approach to IPC has been established that does not require the addition of the IPRs into the mobile phases [10]. Instead, the IPRs are added into the sample prior to injection on the liquid chromatography column. For both traditional IPC and the new approach the retention mechanisms are complex, with the formation of an electrical double layer, ion-pair formation and ion-exchange as critical parameters [11]. Thus, the retention behavior of the analytes is similar, and the same factors impact the retention. For example, increasing the IPR alkyl-chain length/hydrophobicity leads to an increase in retention, and increasing the concentration of the IPR leads to an increase in retention up to a threshold value above which is stays steady or decreases [10,11].

Therefore, the most important characteristic of the new technique is using the concentration of the IPR that will form an adequate electrical double layer at the head of the LC column. Furthermore, the IPR molecules must form ion-pairs that are neutral and will be retained by a hydrophobic stationary phase.

The goal of this work was to develop 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.

2. Material and methods

2.1. Reagents and chemicals

(−)-Epinephrine (+) bitartrate salt, DL-Noradrenaline hydrochloride, DL-norepinephrine 2,5,6,α,β,β-d6 hydrochloride, formic acid, Trizma®base, ethylenediaminetetraacetic acid disodium salt (EDTA) and 1-heptane sulfonic acid were purchased from Sigma Aldrich. Ammonia solution 25% and hydrochloric acid were obtained from Merck, acetonitrile and methanol from Th.Geyer and DL-epinephrine α,α,β-d3, acetic acid, ClinCal® calibrator, Lyphochek® Quantitative Urine Quality Control 1 and 2 from Cambridge Isotope Laboratories, VWR, Recipe and Bio-Rad Laboratories, respectively.

2.2. Solutions

The method was calibrated by the single level ClinCal® calibrator with 0.686 μmol/L NE and 0.159 μmol/L E. Two urinary controls, Lyphochek® Quantitative Urine Quality Control 1 and 2 were used as internal controls. Calibrator and controls were prepared according to the manufacturers' manual, and stored at −20 °C.

Stock solutions of internal standards DL-epinephrine α,α,β-d3 (d3-E) and DL-norepinephrine 2,5,6,α,β,β-d6 hydrochloride (d6-NE) were prepared in 0.1 mol/L hydrochloric acid, and diluted with 0.1 mol/L hydrochloric acid to a working internal standard (IS) solution containing 1.5 μmol/L d6-NE and 0.5 μmol/L d3-E.

Individual stock solutions of E and NE were prepared in 0.2 mol/L acetic acid, and diluted with water to a working solution of 0.1 mmol/L for both analytes. This working solution was used to prepare eight spiked samples from 0 to 2 μmol/L in urine from one healthy volunteer.

2.3. Sample extraction procedure

Using a Hamilton STARlet workstation, 350 μL of urine was mixed with 175 μL IS solution and 560 μL buffer solution (1 mol/L Tris and 0.05 mol/L EDTA in water) in a 96-well microtiter plate. A Sep-Pak Alumina B, 100 mg 96- well extraction plate (Waters) was used for the extraction. Each well was conditioned, first with 1000 μL acetonitrile, then with 1000 μL aqueous 0.05% ammonia and, last with 1000 μL buffer solution. Next, 950 μL of sample was loaded on the Alumina plate followed by one 800 μL wash with buffer solution, and three washes with water.

The samples were eluted with 550 μL elution solution (2.5% formic

Table 1
Mass spectrometric parameters.

	Precursor ion m/z	Collision energy (eV)	Product ion (m/z)
E	165.86	17	107.11
d3-E	168.86	17	110.11
NE	151.92	15	106.96
d6-NE	157.92	15	109.96
HSA	180.11	17	81.00

Multiple reaction monitoring conditions for analytes, epinephrine (E) and norepinephrine (NE) and their respective internal standards DL-epinephrine α,α,β-d3 (d3-E) and DL-norepinephrine 2,5,6,α,β,β-d6 hydrochloride (d6-NE) and the ion-pair reagent 1-heptane sulfonic acid (HSA).

acid and 4% water in acetonitrile), and diluted with 1000 μL HSA solution (60 mmol/L HSA in aqueous 0.1% formic acid).

The Sep-Pak Alumina from Waters was custom made to obtain the plate format. To increase the reliability of supply we made a comparison to Strata Alumina-N, 100 mg 96-well extraction plate from Phenomenex, also custom made.

2.4. LC–MS/MS conditions

The analyses were conducted on a Waters Acquity ultra performance liquid chromatograph (UPLC) with Xevo TQ-S tandem mass spectrometer operated in electrospray positive mode. The chromatographic separation was achieved with a Phenomenex Kinetex Biphenyl (100 × 2.1 mm, 2.6 μm) column. The mobile phases consisted of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in methanol (mobile phase B). Gradient elution was as follows: 0-0.5 min 95% A, 5% B; 0.5–3 min linear gradient to 72% A, 28% B; 3–4.5 min 5% A, 95% B; 4.5–6 min equilibrate with 95% A, 5% B. Ten μL diluted sample was injected at a flowrate of 0.5 mL/min. The multiple reaction monitoring (MRM) transitions for E, NE and their respective internal standards can be seen in Table 1.

2.5. Method validation

To investigate the linearity of the assay in the clinically relevant concentration range, urine from one healthy volunteer was spiked with 0, 0.1, 0.2, 0.4, 0.8, 1.2 and 2 μmol/L of both E and NE and analyzed on 3 separate occasions over an 11 week period. Data was analyzed using least squares linear regression on GraphPad Prism 7 (GraphPad Software).

The intermediate precision was calculated from repeated measurements of two urinary controls in the normal and abnormal range, respectively. The measurements were performed on different days in 31 and 28 separate analysis batches.

To determine the limit of quantification (LOQ), the assay precision was investigated on pooled patient samples with low E and NE concentrations.

The carryover of the assay was assessed by sequential injections of the highest standard and a blank sample.

Quantitative matrix effects were investigated by comparing the mean response of IS added to pre-extracted samples from 8 different patients with the mean response of 8 samples where the same amount of IS was added to the HSA solution. The IS response for the samples in HSA solution represents the relative 100% response value and ion suppression was calculated as the decrease in IS response for the pre-extracted sample. Furthermore, qualitative matrix effects were examined by post column infusion of 1 μmol/L E and 4 μmol/L NE at a flowrate of 10 μL/min, while analyzing extracted patient samples with low E and NE levels.