Chapter 5

Automated mass spectrometric analysis of Urinary Free Catecholamines Using On-Line Solid Phase Extraction

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Submitted for publication

Abstract

Background: Analysis of catecholamines (epinephrine, norepinephrine and dopamine) in plasma and urine is used for diagnosis and treatment of catecholamine-producing pheochromocytoma. Current analytical techniques for catecholamine quantification are laborious, time consuming and technically demanding. Aim was to develop an automated on-line solid-phase extraction method coupled to high performance liquid chromatography-tandem mass spectrometry (XLC-MS/MS) for the quantification of free catecholamines in urine.

Methods: Five µL urine equivalent was pre-purified by automated on-line solid-phase extraction, using phenylboronic acid (PBA) complexation. Reversed phase (pentafluorophenylpropyl column) chromatography was applied. Mass spectrometric detection was operated in multiple reaction monitoring mode using a quadrupole tandem mass spectrometer with positive electrospray ionisation. Urinary reference intervals were set in 24h-urine collections of 120 healthy subjects. XLC-MS/MS was compared with liquid-chromatography with electrochemical detection (HPLC-ECD).

Results: Total run-time was 14 min. Intra- and inter-assay analytical variation were <10%. Linearity was excellent (R2>0.99). Quantification limits were 1.47 nmol/L, 15.8 nmol/L and 11.7 nmol/L for epinephrine, norepinephrine and dopamine, respectively. XLC-MS/MS correlated well with HPLC-ECD (correlation coefficient >0.98). Reference intervals were 1-10, 10-50 and 60-225 µmol/mol creatinine for epinephrine, norepinephrine and dopamine, respectively.

Conclusions: Advantages of the XLC-MS/MS catecholamine method include its high analytical performance by selective PBA affinity and high specificity and sensitivity by unique MS/MS fragmentation.

Introduction

Analysis of urinary catecholamines (i.e. epinephrine, norepinephrine and dopamine) is used for the diagnosis pheochromocytoma and paraganglioma which are catecholamine-producing tumors. Traditionally, biochemical diagnosis of pheochromocytoma is based on the presence of catecholamines and/or their metabolites in urine and plasma. Although the measurement of free metanephrines (3-O-methylated catecholamine-metabolites) in plasma or urine is recommended[1-5], urinary metanephrines in combination with measurements of catecholamines are commonly applied[4,5]. This means that there is still a place for measurements of urinary catecholamines in clinical chemistry. However, rather complex analytical methods are required for these compounds, since catecholamines are present at the nanomolar concentration range and are susceptible to oxidation. Therefore, sensitive and selective methods are required to determine their precise concentrations. Traditionally, high performance liquid chromatography (HPLC) is applied with electrochemical or fluorometric detection[6,7]. Usually, analysis times are long, mainly caused by extensive and technically demanding sample preparation with ion-pair or derivatisation reagents. In addition, these methods are susceptible to interferences caused by co-eluting compounds, which complicates the interpretation of results[8].

In the last decade liquid chromatography with tandem mass spectrometric detection (LC-MS/MS) methods have been developed[9-11] for analyzing free catecholamines in urine with high sensitivity and specificity. Still, laborious sample preparation is required, usually performed with activated alumina, cation-exchange adsorbents or phenylboronic acid (PBA) complexation[12]. These principles do enable automated on-line analysis of urine samples[13]. Currently, on-line coupling of these catecholamine sample preparation principles directly to LC-MS/MS has not been shown. Our aim was to develop an automated on-line sample preparation LC-MS/MS method for the quantification of free catecholamines in urine in order to replace the current laborious and time-consuming conventional HPLC method.

Materials and Methods

Chemicals and Reagents

HPLC-grade methanol was obtained from Rathburn Chemicals Ltd. (Walkerburn, Scotland, UK); ammonium formate 99.995+%, ammonium hydroxide and acetic acid from Sigma-Aldrich Ltd. (Steinheim, Germany); formic acid 98% to 100% ultrapure from BDH Laboratory Supplies (Poole, UK); urine preservatives hydrochloric acid, ascorbic acid and EDTA were acquired from Merck KGaA (Darmstadt, Germany), as was ammonium chloride (p.a. quality).

Epinephrine, norepinephrine and dopamine-HCl were obtained from Sigma-Aldrich Ltd. Epinephrine-N-methyl-d3, norepinephrine-2,5,6,α,β,β-d6 and dopamine-1,1,2,2,-d4-HCl were from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada); Reagent-grade water, obtained from a Barnstead system, was used throughout the study procedure. All chemicals and solvents were of analytical reagent grade.

INSTRUMENTS

A Spark Holland Symbiosis® on-line solid phase extraction (SPE) system (Spark Holland, Emmen, the Netherlands) was used for all analyses. The system consists of a temperature-controlled autosampler (temperature maintained at 10 °C), a SPE controller unit (automated cartridge exchanger or ACE), a solvent delivery unit (2 high-pressure dispensers), and an HPLC pump, as shown previously[14-16]. The ACE module contains 2 connectable 6-way valves and a SPE cartridge-exchange module. The high-pressure dispensers provide SPE cartridges with solvents for conditioning, equilibration, sample application, and cleanup. The integrated HPLC pump was a binary high-pressure gradient pump. Column temperature was controlled with a Mistral Column Oven (Spark Holland). Detection was performed with a Xevo® TQ tandem mass spectrometer equipped with a Z Spray® ion source operated in positive electrospray ionization mode (Waters, Milford, MA). All aspects of system operation and data acquisition were controlled using MassLynx v4.1 software with automated data processing using the TargetLynx Application Manager (Waters).

SAMPLE PREPARATION

A catecholamine mix working solution was diluted with ascorbic acid (400 mg/L) from separate stock solutions (8.5-60 mg/mL) in methanol on the day of analysis. Stock solutions were stored at -20 °C for a maximum of 6 months. Deuterated internal standard stock and mix working solution were treated the same way. Urinary calibrators were prepared by addition of working solution mix in the concentration ranges 0-1400 nmol/L for epinephrine, 0-5000 nmol/L for norepinephrine and 0-9600 nmol/L for dopamine. All solutions were prepared in blank urine with ascorbic acid solution (400 mg/L) added as preservative. Low, medium and high quality-control samples were made by spiking and dilution of urine from a healthy control. Urine samples were stored at –20°C until analysis. Blank urine without the presence of catecholamines was obtained by oxidation of regular urine under alkaline conditions at 100 °C followed by acidification to pH 4. Fifty µL of urine (acidified with HCl to pH 4 and containing the preservatives ascorbic acid and EDTA (1:1 w/w), added prior to collection) was mixed directly in an autosampler vial with 100 µL internal standard solution (final concentration 12.3 nmol/L for epinephrine, 66.6 nmol/L for

norepinephrine and 90.5 nmol/L for dopamine). Urine samples were diluted with ascorbic acid solution (400 mg/L) to reach a final volume of 1 mL. 100 µL sample was injected into the XLC-MS/MS system. This injection volume was equivalent to 5 µL of urine.

ON-LINE SPE

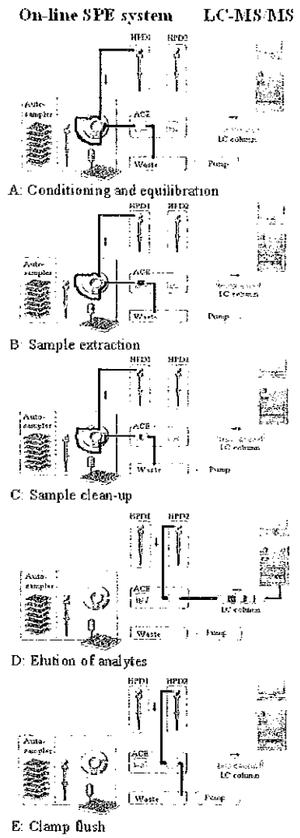
Sample clean-up took place by on-line SPE, following the technique as described previously[14-16]. Bond Elut® PBA (phenyl boronic acid; Varian Inc. Palo Alto, CA) 10 by 2 mm SPE cartridges (Spark Holland) were used for sample extraction. The Symbiosis® system was designed to proceed automatically through a series of programmable routines (Fig.1) during which the SPE cartridge is conditioned (Fig. 1A), loaded (with 200 mM ammonium chloride buffer pH 8, Fig. 1B), washed (with methanol and water, Fig. 1C), and eluted (250 µL 100 mM ammonium formate pH 3, Fig 1D). Solvents were delivered by two high pressure dispensers. The eluted fraction was directly mixed with the chromatographic mobile phase and transferred to the analytical column (Fig 1D). Processing of subsequent samples was carried out in parallel and cartridges and tubing were flushed and regenerated (Fig. 1E).

LIQUID CHROMATOGRAPHY

Chromatographic separation was achieved by using an Allure PFP (pentafluorophenyl) propyl column (particle size 5 µm, 4.6 mm internal diameter by 150 mm; Restek, Bellevonte, PA). A gradient flow starting with 80% of 25 mmol/L ammonium formate in water adjusted to pH 3.0 with formic acid (A) and 20 % of methanol (B) and a flow rate of 0.50 mL/min was applied to the chromatographic column (Table 1). Gradients applied were linear. Column temperature was kept at 25 °C.

MASS SPECTROMETRY

The mass spectrometer was directly coupled to the chromatographic column. In positive electrospray ionization mode epinephrine, norepinephrine and dopamine were protonated to produce ions at the form $[M+H]^+$. Upon collision-induced dissociation (CID) with argon gas, these precursor ions produced characteristic product ions of [M+H-H2O] (Table 2). Other mass transitions monitored were used as qualifiers (Table 2), following EU directive 2002/657/EC.



A: Conditioning and equilibration
A1: New cartridge — From tray to left clamp
A2: Conditioning
A3: Equilibration

B: Sample extraction
B1: Injection — Start loopfill with sample
B2: Extraction — Sample loading using 200 mM ammonium chloride pH8

C: Sample clean-up
C1: Wash cartridge — Methanol/Water (90/10)
C2: Wash cartridge — Methanol/Water (10/90)

D: Elution of analytes
D1: Transfer cartridge — From left to right clamp
D2: Elution — Elution of analytes from cartridge To LC column using HPD2
D3: New cartridge — From tray to left clamp

E: Clamp flush
E1-E5: Cartridge wash and re-equilibration
E6: Move cartridge — From right clamp to tray

Figure 1. Schematic representation of the on-line solid phase extraction.
A: conditioning and activation of the SPE cartridge in the left clamp. B: sample extraction after filling of the autosampler loop. C: sample cleanup. D: right clamp elution of analytes, mixture with the chromatographic liquids, chromatographic separation and mass spectrometric detection. E: cartridge and clamp cleanup.
Abbreviations: SPE: solid phase extraction; HPD: high-pressure dispenser: LC: liquid chromatography; ACE: automatic cartridge exchanger, MS/MS: tandem mass spectrometry.

Table 1. Gradient elution scheme liquid chromatography

Time (mm:ss)	Flow (mL/min)	Solvent A (%)	Solvent B (%)
00:01	0.50	90	10
00:02*	0.10	90	10
00:36*	0.10	90	10
00:37	0.50	90	10
06:00	0.50	40	60
11:30	0.50	40	60
12:00	0.50	90	10
14:00	0.50	90	10

Solvent A: 25 mM ammonium formate pH 3; Solvent B: Acetonitrile
* Total column flow is 0.50 mL/min due to mixing with elution solvent.

URINE SAMPLES

For method-comparison studies, 59 urine samples from the routine catecholamine analysis with conventional HPLC were analyzed with XLC-MS/MS and Deming regression analysis was applied. Concentrations ranged from 6.5-1500, 57-2200 and 170-9000 nmol/L for epinephrine, norepinephrine and dopamine, respectively. Reference intervals for urinary free catecholamines were obtained from the analysis of 24 h urine collections of 120 healthy subjects, participating in the LifeLines study[17]. LifeLines is a multi-disciplinary prospective population-based cohort study examining in a unique three-generation design the health and health-related behaviour of 165,000 persons living in the North East region of The Netherlands. It employs a broad range of investigative procedures in assessing the biomedical, socio-demographic, behavioural, physical and psychological factors which contribute to the health and disease of the general population, with a special focus on multimorbidity. In addition, the LifeLines project comprises a number of cross-sectional sub-studies which investigate specific age-related conditions. These include investigations into metabolic and hormonal diseases, including obesity, cardiovascular and renal diseases, pulmonary diseases and allergy, cognitive function and depression, and musculoskeletal conditions. Reference intervals were calculated using EP EvaluatorTM software[18] as recommended by the Clinical and Laboratory Standards Institute.

Table 2. Mass spectrometric parameters

Precursor m/z	Product m/z	Dwell time	Cone voltage	Collision energy
MRM Quantifiers				
184.15 (E)	166.10	0.105 sec	12 V	12 eV
169.15 (E-d3)	107.00	0.105 sec	32 V	18 eV
170.25 (NE)	152.00	0.105 sec	12 V	8 eV
176.25 (NE-d6)	158.00	0.105 sec	12 V	8 eV
154.15 (DA)	137.00	0.105 sec	17 V	17 eV
158.15 (DA-d4)	141.00	0.105 sec	17 V	17 eV
MRM Qualifiers				
184.15 (E)	166.10	0.105 sec	12 V	12 eV
187.15 (E-d3)	169.10	0.105 sec	12 V	12 eV
166.15 (E)	107.00	0.105 sec	32 V	18 eV
169.15 (E-d3)	107.00	0.105 sec	32 V	18 eV
152.15 (NE)	107.00	0.105 sec	25 V	18 eV
158.15 (NE-d6)	111.00	0.105 sec	25 V	18 eV
137.10 (DA)	91.00	0.105 sec	35 V	35 eV
141.10 (DA-d4)	95.00	0.105 sec	35 V	35 eV

Abbreviations: MRM: multiple reaction monitoring mode. m/z: mass to charge ratio. E: epinephrine, NE: norepinephrine, DA: dopamine, d3: 3 times deuterated compound. d6: 6 times deuterated compound. d4: 4 times deuterated compound.

Results

Chromatography

Total cycle time per sample, including extraction, was 14 min. Epinephrine and its deuterated internal standard (E-d3) co-eluted after 7.3 min, norepinephrine and NE-d6 after 6.3 min and dopamine and DA-d4 after 8.7 min (Fig. 2). The identity of the compound was confirmed by the specific mass spectrum of an aqueous standard and a urine sample from a healthy subject (data not shown). No ion suppression was observed.

Detection limits

The limit of quantification (S/N 10:1) was 1.47 nmol/L for epinephrine, 15.8 nmol/L for norepinephrine and 11.7 nmol/L for dopamine with CVs of 9.0%, 6.2% and 11.8%, respectively (n=20).

Linearity and precision

Inter-assay linearity (n=18) obtained over a concentration ranges from 1.5-1500 nmol/L for epinephrine, 16-5000 nmol/L for norepinephrine and 12-9600 nmol/L for dopamine in oxidized blank urine was excellent. The mean slope was 0.0271 for epinephrine, 0.0016 for norepinephrine and 0.0008 for dopamine, with no significant intercept and correlation coefficients of 0.99. Aqueous calibration curves gave comparable slopes. Intra-assay precision was determined by replicate analyses in a single run at three concentrations (n=20). Inter-assay was determined by analysis of three concentrations over 4 weeks (n=18). For low concentrations intra-assay CVs were <10%. For medium and high concentrations intra-assay CVs were <2%. Inter-assay CVs were <10% for low concentrations and <5% for medium and high concentrations. Samples with high catecholamine concentrations exceeding the calibration ranges can be diluted up to 50 times, which results in comparable outcomes. Cartridges can be reused 2 times with consistent results and without occurrence of carry-over (< 0.1%).

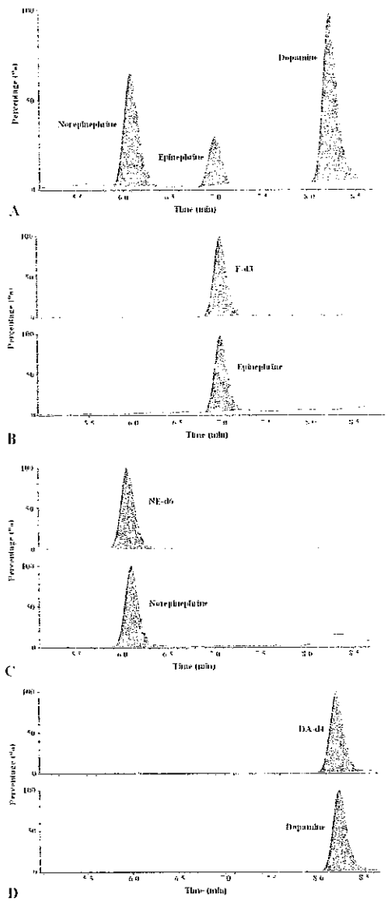


Fig. 2: Total ion current mass chromatograms and mass specific chromatograms of catecholamines and their deuterated internal standards in obtained in multiple reaction monitoring (MRM) mode by XLC-MS/MS for a urine sample from a healthy subject.
A: Total ion current mass chromatograms of all MRM transitions used. Retention times are 7.0, 6.1 and 8.2 min for epinephrine, norepinephrine and dopamine, respectively; B: Mass specific chromatograms of epinephrine and its deuterated internal standard E-d3 (m/z 184→166 and 169→107, respectively); C: Mass specific chromatograms of norepinephrine and its deuterated internal standard NE-d6 (m/z 170→152 and 176→158, respectively); D: Mass specific chromatogram of dopamine and its deuterated internal standard DA-d4 (m/z 154→137 and 158→141, respectively).
Retention time is indicated in min. Peak abundance is normalized to percentages relative to the highest peak in the chromatogram.

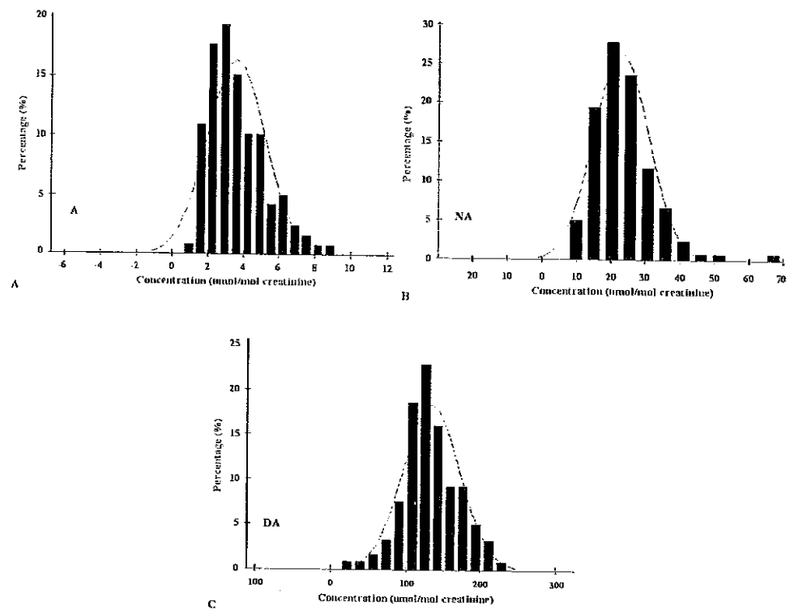


Fig. 3: Distribution of urinary catecholamine concentrations in reference samples from 120 healthy individuals in µmol/mol creatinine. The distributions of individual catecholamine concentrations are shown in histograms with a mean concentrations of 3.6, 23.5, 133.8 µmol/mol creatinine for epinephrine, norepinephrine and dopamine respectively. The black line shows a normal distribution which is in correspondence with the distribution of the individual serotonin concentrations. A: Epinephrine, reference interval 0-10 µmol/mol creatinine; B: Norepinephrine, reference interval 0-50 µmol/mol creatinine; C: Dopamine, reference interval 0-250 µmol/mol creatinine.

STABILITY

Urine samples, containing conservatives as described above, were found to be stable during 3 freeze-thaw cycles. In addition, isotope-diluted samples containing ascorbic acid were stable up to 9 days at 4-10 °C in the dark (autosampler).

URINE SAMPLES

The new XLC-MS/MS method correlated with the former HPLC with a correlation coefficient of 0.99 for epinephrine, 0.98 for norepinephrine and dopamine. The regression equations for the XLC-MS/MS method (x) and the HPLC method (y) had slopes of 1.15, 1.00 and 0.86 for epinephrine, norepinephrine and dopamine, respectively.

The distribution of urinary dopamine concentrations in the 120 healthy subjects for determination of the reference limits was normal and dopamine reference intervals were calculated with EP evaluator in parametric manner (Fig. 3A) according to CLSI C28-A2[23]. Concentrations of norepinephrine and epinephrine were logarithmically transformed in order to achieve normal distribution and these reference intervals were therefore calculated in a transformed parametric manner (Fig. 3B&C). Reference intervals in urine were 1-10, 10-50 and 60-225 μmol/mol creatinine for epinephrine, norepinephrine and dopamine, respectively.

Discussion

This study shows that physiological concentrations of catecholamines can be measured reproducibly and accurately in urine without extensive manual sample preparation and relatively short chromatographic time using automated on-line XLC-MS/MS. Importantly, due to tandem mass spectrometric detection chance of analytical interferences is theoretically diminished, which is another major drawback of conventional HPLC methods[11]. Catecholamines are biogenic amines characterized by an amino group in the alkalic side chain together with a phenolic catechol group (i.e. hydroxyl groups at 3- and 4-positions). The use of PBA-based extraction specific for catechol-containing compounds such as these catecholamines has been reported to be more effective than the commonly used SPE techniques as cation-exchange and activated alumina[20,21] for sample clean-up. The specificity of PBA-base extraction is based on the affinity of catecholamines for cartridge-bound PBA. In alkaline conditions (pH>8) formation of the reactive phenylboronate ion [PhB(OH)2-] is promoted. Compounds containing a catechol-group (cis-diols) covalently bind to the boronate ion on the cartridge by releasing a water molecule. The required alkaline conditions (pH>8) for formation of the reactive phenylboronate ion do not cause oxidation of the catecholamines, since this pH is only applied during extraction. The catecholamines are eluted from the PBA-catecholamine complex by applying an acidic solution[11], which is compatible with LC-MS/MS. On-line extraction with PBA-cartridges has not been performed previously for quantification of catecholamines in urine samples, although such method has been described for brain tissues [22].

Chromatographic separation of the three catecholamines is usually performed by reversed-phase LC with the use of expensive ion-pairing reagents[7,23,24]. Because of the highly polar nature of these biogenic amines such methods are required to achieve sufficient retention and separation. However, chromatographic runs are laborious while coupling to MS/MS is not possible due to incompatibility of the ion-pairing reagents with the electrospray ionization, resulting in signal suppression and MS contamination. Thus, for the

development of an XLC-MS/MS method other approaches of reversed phase chromatography are desired. The solution was found in a PFP propyl column, known to increase retention for compounds with electrophilic properties, as the catecholamines that are protonated on the amine-group under acidic conditions. In order to achieve retention of the catecholamines and especially NE, high percentages of aqueous mobile phase were required. Although the mass spectrometer prefers high organic contents for ionization, the sensitivity of the method showed to be excellent.

The chromatographic analysis time of the developed XLC-MS/MS method is relatively long in order to achieve complete separation of the three catecholamines and interfering compounds. In comparison with the previously used HPLC-ECD method at our laboratory, analysis time is greatly reduced from 8 hr sample preparation for a small sample batch and 30 min chromatography per sample to 14 min total analysis time per sample. Application of ultra performance liquid chromatography (UPLC) will most likely decrease chromatographic time further[25,26]. However, the on-line combination of UPLC with applied automated SPE system is not available yet.

Conclusions

We present a method for the selective automated analysis of free catecholamines in urine that uses on-line SPE coupled to LC-MS/MS. The advantages of the method include its high selectivity by the PBA affinity of catecholamines and the high specificity and sensitivity by unique MS/MS fragmentation of catecholamines, resulting in less interferences. Automation substantially reduces total analysis time leading to high throughput. This method therefore has several advantages over other methods for the analysis of catecholamines reported previously for the diagnosis and treatment of neuroendocrine disorders, especially pheochromocytoma.

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