Clinical Mass Spectrometry 4–5 (2017) 34–41

Contents lists available at ScienceDirect

Clinical Mass Spectrometry

journal homepage: www.elsevier.com/locate/clinms

Dilute, derivatise and shoot: Measurement of urinary free metanephrines and catecholamines as ethyl derivatives by LC-MSMS

Andrew G. Ellis[a,b], Philip T. Zeglinski[b], Kate E. Coleman[c], Malcolm J. Whiting[c,d,*]

[a] Clinical Pharmacology, Austin Health, Melbourne, Victoria 3084, Australia
[b] Department of Medicine, University of Melbourne, Melbourne 3000, Australia
[c] Chemical Pathology, SA Pathology, Adelaide 5000, Australia
[d] Medical Biochemistry, Flinders University, Bedford Park, South Australia 5042, Australia

ARTICLE INFO

Keywords:
Urine free metanephrines
Catecholamines
Reductive ethylation
Tandem mass spectrometry

ABSTRACT

Background: The measurement of catecholamines and their metabolites in either urine or plasma is an important diagnostic test used to exclude the presence of neuroendocrine tumours. Because of weak chromatographic retention and potential ion-suppression, reverse-phase LC-MSMS is not ideal for analysis of these polar molecules. Here, we investigate derivatisation by ethylation as an alternative approach.

Methods: A simple and rapid method involving acetaldehyde and a reducing agent was used to convert urine free metanephrines and catecholamines, and their deuterated analogues as internal standards, to mono-ethyl or diethyl- derivatives. Using an Agilent 6460 triple-quadrupole mass spectrometer, precursor and product ion mass spectra were recorded to allow comparison of multiple reaction monitoring methods for both derivatised and non-derivatised analytes under reverse-phase LC-MSMS conditions with positive electrospray ionization.

Results: Conversion of biogenic amines to less polar ethyl derivatives increased their mass and enhanced the intensity of their molecular ions and fragments. Ethylation also improved the chromatographic properties of the amines, with greater retention and elution from reverse-phase HPLC columns with a methanol or acetonitrile gradient. The signal response of tandem mass spectrometric detection was increased up to 50-fold for ethyl metanephrines compared to non-derivatised compounds. This increase allowed for the omission of solid-phase extraction of urine as a clean-up step prior to analysis. The 'dilute-derivatise-shoot' method maintained analytical performance with respect to between-run imprecision (CV < 6%) and accuracy in an external quality assurance program. Gender-related ranges for free metanephrines in early-morning spot urines, collected from adult patients, were similar using either derivatised or non-derivatised samples.

Conclusions: The LC-MSMS detection of free urine biogenic amines can be greatly enhanced by ethyl derivatisation, which is easy and rapid to perform. Advantages include improved chromatography and lower limits of quantitation, that negate the requirement for solid-phase clean-up of urine prior to analysis. A disadvantage is the potential toxicity of the derivatising agents used if they are not handled appropriately.

1. Introduction

The biochemical investigation of the neuroendocrine tumours phaeochromocytoma or paraganglioma (PPGL) involves the measurement of catecholamines and their metabolites, in particular the metanephrines [1,2]. Free metanephrines in plasma are now regarded to have the highest clinical sensitivity, compared to other analytes, in excluding a diagnosis of phaeochromocytoma, and are recommended for first-line testing [3–5]. Total (i.e., free plus conjugated) urinary metanephrines in a 24-h collection are also a recommended test [5]. Although not favored in clinical practice guidelines, urinary and plasma catecholamines are still requested in the investigation of hypertension [6,7].

For the measurement of biogenic amines, laboratories use high-pressure liquid chromatography to separate individual metanephrines and catecholamines prior to quantitation. With the increasing availability of bench-top tandem mass spectrometers (MSMS) in the clinical laboratory, these instruments are replacing electrochemical detectors to provide more specific and robust analysis of biogenic amines for all specimen types [8–12]. LC-MSMS has the advantage of profiling related analytes so that simultaneous determination of free metanephrines and free catecholamines from urine is possible [9,13]. These profiling

* Corresponding author at: Medical Biochemistry Lab 6D-219, Flinders Medical Centre, Bedford Park, South Australia 5042, Australia.
E-mail address: Malcolm.Whiting@flinders.edu.au (M.J. Whiting).

http://dx.doi.org/10.1016/j.clinms.2017.08.005
Received 8 February 2017; Received in revised form 25 August 2017; Accepted 25 August 2017
Available online 05 September 2017

methods may become increasingly useful given emerging evidence suggesting that urinary free metanephrine analysis may be the most sensitive test for screening patients for PPGL [13–15].

Chemical derivatisation has been used in quantitative mass spectrometry of small molecules to increase the sensitivity required for various applications [16]. Some specific examples for clinical purposes are the conversion of testosterone to an oxime derivative to allow the determination of the low nanomolar serum concentrations found in females and young children [17], and the derivatisation of vitamin D and its metabolites to enable measurement of 1,25-dihydroxy-vitamin D3 in serum [18]. It has been reported that ethylation-labeling of amino groups on monoamine neurotransmitters, such as noradrenaline, dopamine and serotonin, greatly increases the sensitivity of tandem mass spectrometric detection in brain micro-dialysate solutions [19]. Although biogenic amines are normally measured by LC-MSMS without derivatisation, their polar nature and low concentrations in plasma present a challenge for reverse-phase LC-MSMS, and many laboratories are using HILIC chromatography for their analysis [20,21]. In this study, we investigate the reverse-phase chromatographic and mass spectrometric properties of ethylated derivatives of metanephrines and catecholamines, and present a rapid LC-MSMS method for their direct quantitation in diluted urine that could be used in the clinical laboratory.

2. Materials and methods

2.1. Chemicals and reagents

Noradrenaline bi-tartrate, adrenaline bi-tartrate, dopamine HCl, normetanephrine HCl, 3-methoxytyramine HCl and d4-acetaldehyde were purchased from Sigma-Aldrich Australia. Metanephrine HCl was purchased from Prime Organics Inc, USA. Deuterated internal standards were purchased from CDN Isotopes via SciVac Australia and included: (±)-noradrenaline-2,5,6,α,β,β-d6 HCl, (±)-adrenaline-2,5,6,α,β,β-d6 HCl, (±)-adrenaline-d3 (N-methyl-d3), dopamine-d4 [2-(3,4-dihydroxyphenyl)ethyl-1,1,2,2-d4-amine HCl], (±)-normetanephrine-α,β,β-d3 HCl, (±)-metanephrine-d3 HCl (N-methyl-d3) and 2-(4-hydroxy-3-hydroxy-3-methoxyphenyl)ethyl-1,1,2,2-d4-amine HCl. The water used was high purity reverse osmosis, organic-filtered, 0.22 μm-filtered ('MilliQ', Millipore, Bedford, MA, USA). Methanol and acetonitrile (Mallinkrodt) were chromatography grade and ammonium acetate, formic acid, cyanoborohydride coupling buffer (Sigma-Aldrich, Australia) and acetaldehyde (BDH Chemicals, Australia) were analytical grade.

2.2. Preparation of calibrators, internal standards and quality controls

Standard solutions of each biogenic amine (i.e., noradrenaline, adrenaline, dopamine, normetanephrine, metanephrine and 3-methoxytyramine) were prepared as solutions of weighed material dissolved in 1% formic acid, and stored frozen at minus 70 °C.

Standard stock solutions were diluted using 1% formic acid and mixed to create a working stock of each analyte at a concentration of 10 μg/mL. Calibration standards were prepared in 1% formic acid from this working stock by serial dilution across a concentration range of 0.005–100 ng/mL.

The deuterated internal standards for each of the catecholamines (i.e., adrenaline, noradrenaline and dopamine) and the metanephrines (i.e., metanephrine, normetanephrine and 3-methoxytyramine) were prepared as 1 mg/mL methanolic stock solutions. A working stock mixed solution containing all internal standards at 1 μg/mL each (IS Mix) was prepared by dilution of the stock solutions in 1% formic acid.

Quality control samples included LyphoCheck Quantitative Urine Controls 'Normal' and 'Abnormal' (Lyphocheck, BioRad Laboratories, Australia, Cat # 376 and 377 respectively) and were prepared as per the manufacturer's instructions. Additional QC materials included pooled patient urine (aliquoted and stored frozen) used as a low QC, and surplus urine samples from an external quality assurance program of the Royal College of Pathologists of Australasia (RCPA) were utilized as high QC material. QC materials were not subjected to acid hydrolysis, so that only free urine metanephrines were measured and target concentrations were assigned in-house. External quality assurance materials for urine biogenic amines were purchased from RCPAQAP Pty Ltd (Sydney, Australia).

2.3. Collection and preparation of urine samples

Early morning spot urine samples were collected after overnight rest prior to 10 am from 198 adults (age range 23–91; 108 males, 90 females) being tested for urine albumin as part of routine care. The pH was measured (mean 5.9, range 4.5–9.0), but no stabilisers or acid were added. Urine creatinine was measured enzymatically on a Cobas c702 analyser (Roche Diagnostics, Australia). All specimens were stored frozen at minus 15 °C prior to processing by batch analysis. Samples were anonymised to the researchers at point of access and the study complied with the Declaration of Helsinki on ethical principles for medical research.

Urine free metanephrines and catecholamines were measured simultaneously following solid phase extraction (SPE) without prior derivatisation. In addition, samples were directly analysed following dilution and derivatisation without the use of SPE.

For analysis with SPE, urine (100 μL) was equilibrated with 50 μL of IS Mix. Biogenic amines were then complexed with boronate at pH 9 [9], before undergoing SPE (Versaplate Plexa, Agilent Technologies) and elution into a 96-well plate with formic acid for LC-MSMS quantitation without derivatisation.

For analysis without SPE, urine samples (50 μL) were equilibrated with 20 μL IS Mix and diluted with 1.0% formic acid to a total volume of 1.0 mL with brief vortex-mixing. Diluted urine (80 μL) was derivatised after the sequential addition of 1.0 M acetate buffer pH 5 (20 μL), 3.0 g/L cyanoborohydride buffer (40 μL), and 20% acetaldehyde solution (20 μL) as described by Ji et al. [19], followed by incubation at 36 °C for 30 min in a 96-well plate sealed with a silicon mat. The reductive amination of primary amines (i.e., noradrenaline, normetanephrine, dopamine and 3-methoxytyramine) produces diethyl-derivatives, whereas that of secondary amines (i.e., adrenaline and metanephrine) results in monoethyl-derivatives [19]. Post incubation, the plate was transferred to an autosampler held at 6 °C for analysis by LC-MSMS.

2.4. Chromatography

Chromatography was performed using Agilent 1200 Infinity HPLC modules with binary pump, autosampler and thermostatted column compartment (Agilent Technologies, Mulgrave, Australia). After SPE clean-up, non-derivatised samples were injected (20 μL) onto a Kinetex F5 column (100 mm × 3.0 mm; 2.6 μm core–shell packing, Phenomenex Australia) using a 0.3 mL/min mobile phase of 2% methanol in 0.2% formic acid for 1 min, followed by a linear 2–80% methanol gradient over 3 min, held at 80% methanol for 0.3 min and re-equilibrated to 2% methanol in 0.2% formic acid. Total run time was 6.2 min. Derivatised samples that were prepared without SPE were injected (5 μL) onto a reversed phase column (Atlantis T3 150 mm × 2.1 mm; 3 μm packing, Waters Australia) using a 0.2 mL/min flow of mobile phase delivering a linear acetonitrile gradient (4–24% over 5 min with 3 min re-equilibration) in 0.2% formic acid. Total run time was 11 min. No harmful effects of residual derivatisation agents were observed on column lifetime or performance.

2.5. Mass spectrometry

Tandem mass spectrometric detection was performed using an

Table 1
Agilent 6460 QQQ Mass Spectrometry Conditions for MRM Transitions of Non-derivatised and Ethyl-Labeled Catecholamines and Metanephrines, and their Deuterated Internal Standards. Reaction with unlabelled acetaldehyde increased mass units by 28 for mono-ethyl and 56 for di-ethyl derivatives. If d4-acetaldehyde was used, mass increases were 32 for mono-ethyl and 64 for di-ethyl derivatives.

	Compound	Precursor Ions	Product Ions	Dwell Time	Fragmentor Potential	Collision Energy	Cell Accel Voltage
		[m/z]	[m/z]	[msec]	[V]	[V]	[V]
Non-derivatised	Noradrenaline	170, 152*	152, 107	30	65, 100	20, 15	5
	Adrenaline	184	166, 107	30	70	8, 24	5
Gas Temp 325 °C	Dopamine	154	137, 91	30	75	8, 28	3
Gas Flow 5 L/min	Normetanephrine	166*	134, 106	30	105	16, 20	3
Nebulizer 35 psi	Metanephrine	180*	165, 148	30	120	16	5
Sheath Gas Temp 375 °C	3-MT	151*	119, 91	30	135	12, 20	3
Sheath Gas flow 12 L/min	*Internal Standards*						
Capillary 3000 V	D6-Noradrenaline	176	158	30	65	4	5
	D6-Adrenaline	190	172, 112	30	70	8, 24	5
	D4-Dopamine	158	141	30	75	8	3
	D3-Normetanephrine	169	109	30	105	20	3
	D3-Metanephrine	183	168	30	120	16	5
	D4-3MT	155	95	30	135	24	3
Derivatised [reductive amination with acetaldehyde]	Noradrenaline - diethyl	226	208, 179	20	90	10, 20	2
	Adrenaline - monoethyl	212	194, 150	20	70	18, 25	4
	Dopamine - diethyl	210	137, 119	20	100	15, 25	2
Gas Temp 300 °C	Normetanephrine - diethyl	240	222, 207	20	80	8, 20	2
Gas Flow 9 L/min	Metanephrine - monoethyl	226, 208*	208, 193	20	90, 140	10, 18	2
Nebulizer 60 psi	3-MT - diethyl	224	151, 119	20	100	12, 25	5
Sheath Gas Temp 400 °C	*Internal Standards*						
Sheath Gas flow 12 L/min	D6-Noradrenaline - diethyl	232	214, 185	20	90	10, 20	2
Capillary 3000 V	D3-Adrenaline - monoethyl	215	197, 153	20	70	18	4
	D4-Dopamine - diethyl	214	141, 123	20	100	15, 25	2
	D3-Normetanephrine - diethyl	243	225, 210	20	80	8, 20	2
	D3-Metanephrine - monoethyl	229, 211*	211, 196	20	80, 140	10, 18	2
	D4-3MT - diethyl	228	155, 123	20	100	12, 25	5

* In-source fragmentation with loss of water.

Agilent 6460 series instrument with jet stream technology (Agilent Technologies, Australia). Electrospray ionization was used in positive ion mode at unit mass resolution. Optimized detector settings for voltages, gas temperatures and flows are listed in Table 1. The ion source was regularly cleaned, and the check and auto-tune features of the MassHunter software were used to maintain mass accuracy of the instrument over time with electrospray tuning mix (Agilent Technologies, Santa Clara, USA).

2.6. Data analysis

Data were stored in Microsoft Excel spreadsheets and statistical analyses carried out using Analyse-It (Version 2.22). Comparisons of means were performed using the *t*-test, and ranges were determined as 95th percentile ranges of log-transformed data distributions. Scatter plots were examined by non-parametric Passing-Bablok regression analysis.

3. Results

3.1. Mass spectrometer tuning for non-derivatised and derivatised biogenic amines

Optimized multiple reaction monitoring (MRM) parameters for monitoring non-derivatised catecholamines and metanephrines, and their ethyl-derivatives formed by reaction, either with unlabeled acetaldehyde or D4-acetaldehyde, are shown in Table 1 for the Agilent 6460 triple quadrupole mass spectrometer. Also shown are the same parameters for their deuterated internal standards. In their original publication, Ji et al. [19] compared different aldehydes for their performance characteristics in the assay of monamine neurotransmitters in rat brain micro dialysates. By using formaldehyde or acetaldehyde with one, three or four isotopic atoms, derivatives of different masses were produced and assessed for possible interference peaks and low background noise. Although they selected D4-acetaldehyde as the ethyl donor, unlabelled acetaldehyde is cheaper and more readily available, and was used for the majority of the present work.

From Table 1, there are two features of the mass spectrometric properties of biogenic amines that should be noted. First, the loss of water occurs readily and can be induced in-source in the production of precursor ions, or in the collision cell at low energy in the formation of product ions. Although not highly specific, MRM transitions involving water loss do give rise to intense product ions which can be useful for sensitive quantitation, provided that they are free from interferences. Second, isobars exist that require chromatographic separation of adrenaline and normetanephrine when not derivatised (e.g., 184 > 166). The ethylation of these biogenic amines with acetaldehyde removes this isobaric pair, but introduces a new pair with noradrenaline and metanephrine (226 > 208). This does not occur if D4-acetaldehyde is used, due to the formation of diethyl and monoethyl-derivatives with different masses, and thus offers a theoretical advantage for the labelled reagent.

3.2. Chromatographic profiles of non-derivatised and derivatised biogenic amines

The HPLC profiles of catecholamine and metanephrine internal standards on a core–shell reverse-phase column promoted for polar molecule retention (Phenomenex Kinetex F5) are shown in Fig. 1, with elution using a methanol gradient. Before derivatisation, all 6 analytes were well separated under the conditions used and the order of elution between 1.6 and 4.0 min was: noradrenaline, adrenaline,

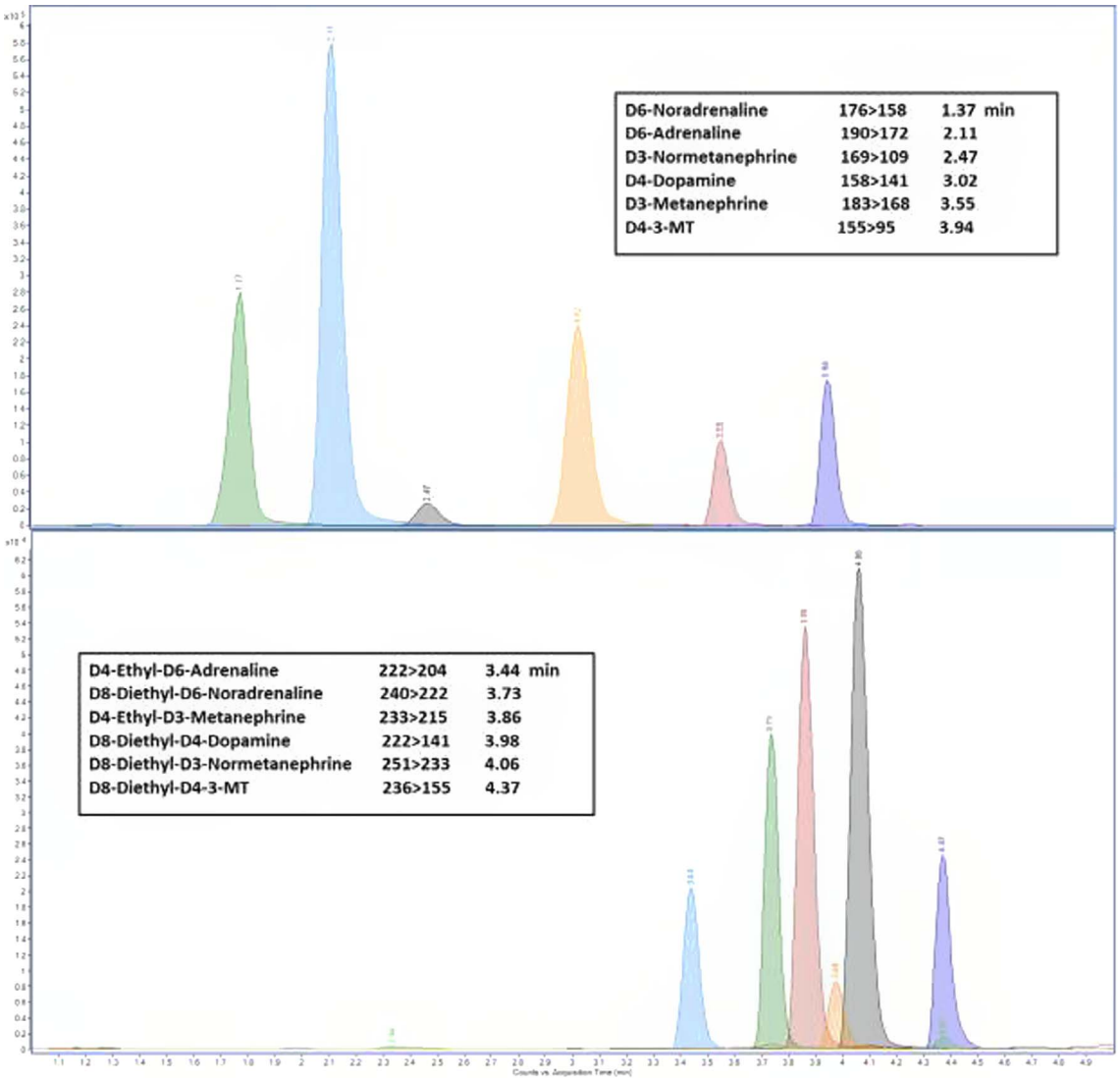

Fig. 1. Chromatography profile of catecholamines and metanephrines before (upper panel) and after (lower panel) reductive amination to form mono-ethyl and di-ethyl derivatives. Mixtures of 6 amines were injected onto a Kinetex F5 core–shell column (100 × 3 mm) at a flow rate of 300 uL/min and a 2–80% methanol gradient was used to elute compounds, which were identified by specific MRM ion transitions, as indicated.

normetanephrine, dopamine, metanephrine and 3-MT. Due to their polar nature, noradrenaline and adrenaline were poorly retained on the column, even with the starting aqueous mobile phase of 0.2% formic acid containing only 2% methanol.

After conversion to ethyl derivatives with deuterated acetaldehyde, both catecholamine and metanephrine internal standards showed much greater retention on the column, as shown in Fig. 1. With the same methanol elution gradient (2–80%) as used for non-derivatised amines, the order of elution between 3.3 and 4.6 min was altered to: adrenaline, noradrenaline, metanephrine, dopamine, normetanephrine and 3-MT. The change in retention order was because the primary amines formed less polar di-ethyl derivatives, whereas secondary amines formed mono-ethyl derivatives with relatively less affinity for the HPLC stationary phase. Under the HPLC gradient conditions used, di-ethyl derivatisation increased the retention times of noradrenaline and normetanephrine by 2.3 and 1.6 min, respectively, whereas mono-ethyl derivatisation only increased retention times for adrenaline and metanephrine by 1.3 and 0.3 min, respectively. Nevertheless, all ethyl derivatives were retained on the HPLC column under starting mobile phase conditions of 2% methanol, and could be eluted as sharp peaks by increasing the methanol concentration in the mobile phase. Similar improved retention and separation using the ethyl derivatives was obtained using a Waters Atlantis T3 column (150 × 2.1 mm, 3 μm packing) and an acetonitrile gradient (4–24% over 5 min plus 3 min re-equilibration), with elution occurring in the same order and a run time of 9 min.

As well as showing improved chromatographic properties, the derivatised biogenic amines were detected with much greater sensitivity by the tandem mass spectrometer. For example, with the mass transitions shown in Fig. 1, peak heights per pmol of analyte injected onto the HPLC column for normetanephrine, metanephrine and 3-MT were 50, 13 and 4 times higher, respectively, after ethylation, with similar low levels of background noise. This large increase in signal-to-noise ratio is not unlike that found in the original report where ethylation derivatisation was used in the analysis of monoamine neurotransmitters in rat brain micro-dialysate solutions [19].

3.3. *Free metanephrines in spot urines measured before and after conversion to ethyl derivatives*

Due to an interest in early-morning spot urines as alternative specimens to 24-h acidified urine collections, and free instead of total (i.e., free plus conjugated) metanephrines as alternative analytes for PPGL screening, a group of 198 spot urines were analysed from patients without known PPGL. The urine sample pH range was between 4.5 and

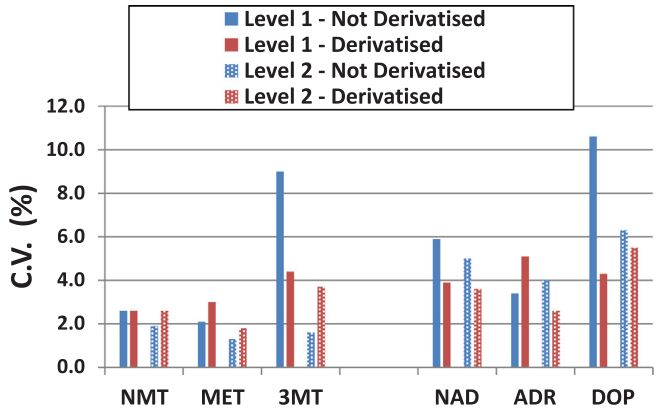

Fig. 2. Inter-run imprecision (% C.V.) in the measurement of urine free metanephrines and catecholamines with (red bars) and without (blue bars) derivatisation at two QC levels. Concentrations in nmol/L in level 1 (solid bars) and level 2 (dotted bars) QC materials were normetanephrine (NMT) 1350, 7300; metanephrine (MET) 370, 2870; 3-methoxytyramine (3MT) 76, 2620; noradrenaline (NAD) 230, 1070; adrenaline (ADR) 90, 530; dopamine (DOP) 600, 3380.

9.0, with 94% of the urines having a pH < 7. To avoid any possible hydrolysis of conjugated metanephrines during urine storage, no acid preservative was used.

The urines in this reference group were first subjected to biogenic amine profiling by LC-MSMS of non-derivatised samples after SPE clean-up of boronate complexes using a previously validated method [9]. An example of ion chromatograms obtained from a patient urine is shown in Supplementary Fig. 1. A commercial urine quality control material was used at two levels to monitor assay performance, and the between-run assay imprecision is shown in Fig. 2. For non-derivatised samples, the coefficients of variation for free metanephrines were less than those for free catecholamines, but all were < 6% except for 3-MT and dopamine in level 1 QC material.

In a study of the excretion of free biogenic amines using 24-h urine collections, males were found to excrete significantly higher amounts per day than females [13]. For the present study, biogenic amine concentrations were converted to μmol/mol creatinine to take into account variability in spot urine concentrations. Ranges obtained for free urine metanephrines are summarised in Table 2a when analysed without derivatisation. Although median urine concentrations of biogenic amines (nmol/L) in males tended to be higher than in females, after normalisation for urine creatinine, ranges for females were higher for females than males, especially for normetanephrine and 3-methoxytyramine. Normalised urine concentrations of free metanephrines also appeared to be higher in adults over 60 compared to adults under 60 years old, but this difference did not reach statistical significance in this group. Ranges for females were consistently wider than for males and reached higher upper limits of normal, due principally to the lower creatinine concentrations in female urine.

The early-morning adult spot urine samples were also analysed for free biogenic amine concentrations using a 'dilute, derivatise and shoot' approach. As described in *Materials and Methods*, diluted non-acidified urines plus internal standards were subjected to ethylation with unlabeled acetaldehyde in sealed 96-well plates, which were then placed onto the LC-MSMS autosampler for direct injection and analysis. Under these conditions, derivatised samples were stable for at least 72 h (data not shown). An example of ion chromatograms obtained from a patient urine is shown in Supplementary Fig. 2.

From the analysis of the same urine QC material as used for non-derivatised samples, between-run imprecision data are included in Fig. 2. The coefficients of variation (CV) for free normetanephrine and metanephrine were < 3% for derivatised samples, while values were higher at < 6% for noradrenaline and adrenaline. When compared to non-derivatised samples, the imprecision data were similar, except for level 1 free 3-MT and dopamine, with CV around 10% being reduced to below 5% by derivatisation.

LC-MSMS analysis of diluted and derivatised urines produced similar concentrations of free biogenic amines to SPE-treated, non-derivatised urines, as shown in the 6 scatter plots in Fig. 3. The slopes of Passing-Bablok regression lines were close to 1.00 for all 6 analytes with few outliers, which did not affect the non-parametric statistical analysis. The most scatter was observed for free adrenaline, but this analyte had the lowest concentration with all urine samples < 120 nmol/L, and most samples < 30 nmol/L.

Free metanephrine concentrations in derivatised spot urines are shown in Table 2b for males and females. While the mean free normetanephrine concentration in females was no longer significantly higher than for males, the overall ranges of results were similar for concentrations estimated from derivatised and non-derivatised urine samples, with no statistically significant differences between the two methods.

Ion suppression effects were assessed by the comparison of the peak areas of ethylated internal standards in derivatised patient urine samples with peak areas from non-matrix standard injections. The mean recoveries were 76%, 68% and 67% for normetanephrine, metanephrine and 3-methoxytyramine respectively (n = 200 and CV < 10% for each analyte) and for catecholamines were greater than 78%.

3.4. Comparative method performance in an EQA program

An external quality assurance (EQA) program of the RCPA provided more evidence that the two LC-MSMS methods for profiling biogenic amines in urine produced similar results. This EQA program releases 12 urine samples over 6 months with random duplicates to allow imprecision to be calculated as SD, along with the bias from target concentrations. In Fig. 4, method performance is expressed as total error (2 SD + bias) divided by the allowable limit of performance as set by the RCPA. A ratio less than 1.0 is considered desirable for each analyte in this EQA program. For all 6 biogenic amines, the ratios were less than 1.5 with most less than 1.0, and were similar between non-derivatised and derivatised samples. Urine noradrenaline had the worst performance value, and total error exceeded its allowable limit of performance by both methods. Urine metanephrines for EQA samples were analysed after an acid hydrolysis step, since the target concentrations provided in the EQA program were for total (i.e., free plus conjugated) urine metanephrine concentrations.

Table 2
Urine free metanephrine concentrations according to gender, as determined from analysis of a group of 198 early morning spot urines, before and after derivatisation. Concentrations expressed as μmol/mol creatinine are shown as means and 95th percentile ranges of normal distributions of log-transformed data. P values represent the statistical significance levels of differences between means for males and females using the *t*-test.

	Males (n = 108)		Females (n = 90)		M vs F
	Mean	Range	Mean	Range	*P*
a. Not Derivatised					
Normetanephrine	10	4–20	14	5–31	*0.0003*
Metanephrine	6	2–14	7	2–20	*0.08*
3-Methoxytyramine	14	6–28	21	8–42	*0.0003*
b. Derivatised					
Normetanephrine	9	3–21	11	4–26	*0.078*
Metanephrine	5	2–13	6	1–18	*0.37*
3-Methoxytyramine	14	6–29	19	9–37	*0.0001*

4. Discussion

The present work describes the use of ethylated derivatives for the LC-MSMS analysis of biogenic amines in urine in the clinical laboratory,

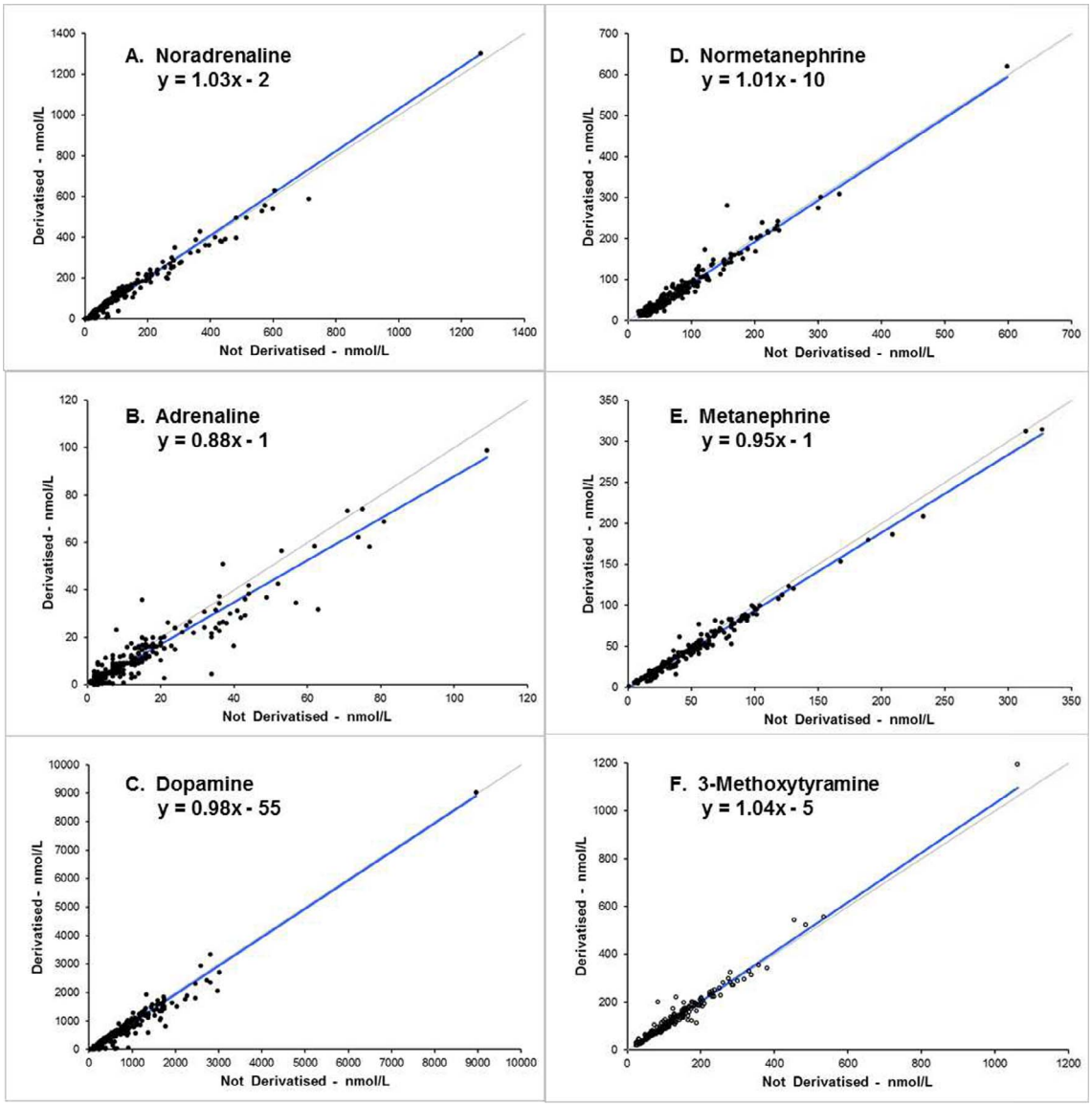

Fig. 3. Scatter plots for free urine catecholamine and metanephrine concentrations, as measured in nmol/L in 198 early-morning spot urines by LC-MSMS with (y-axis) and without (x-axis) derivatisation. Non-parametric Passing-Bablok regression equations for lines of fit are shown for each of the 6 biogenic amines, and were not influenced by the outlier urine with high concentrations of biogenic amines and creatinine.

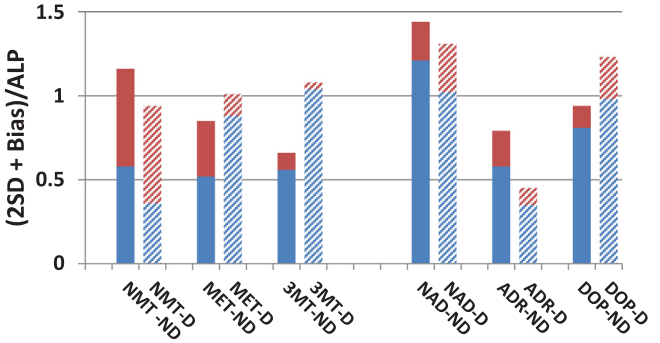

Fig. 4. Performance summary for cycle 63 of the urine biogenic amines external quality assurance program of RCPAQAP. Comparative performance in the analysis of 12 urine samples over 6 months is shown for normetanephrine (NMT), metanephrine (MET), 3-methoxytyramine (3MT), noradrenaline (NAD), adrenaline (ADR) and dopamine (DOP) without (ND-solid bars) and with (D-striped bars) derivatisation. The 2 SD of the analyses is represented by blue bars and the bias fraction by red bars. The sum of the 2SD and bias components are divided by the allowable limits of performance (ALP) set for each analyte by the RCPAQAP as an indicator of relative performance.

in particular for rapid profiling of free metanephrines and catecholamines in diluted spot urine samples. Derivatisation of these analytes improved their reverse-phase chromatographic performance and decreased their limits of quantitation, which in turn allowed the replacement of multi-step SPE sample preparation with a simple 'dilute, derivatise and shoot' approach.

Our work illustrates that, while not common in LC-MSMS, derivatisation can provide benefits to offset the work required for this additional procedural step. The general principles of derivatisation and reactions according to functional chemical groups have been reviewed by Xu et al. [16]. For primary and secondary amine groups, acylation reagents have been used to form amide derivatives. However, in our work, a simple and rapid reductive ethylation procedure, which was first described by Ji et al. [19] to measure monoamine neurotransmitters in rat brain dialysates, has been applied. These authors further exploited the use of these stable ethyl derivatives in the development of a method for measuring plasma catecholamines [22,23], and brief reports by others, including our group, have appeared describing their use to enhance measurement of plasma free metanephrines [24] and urine biogenic amines [25].

In clinical chemistry, a major reason to consider a derivatisation step in LC-MSMS is to increase measurement sensitivity for analytes that are present in plasma at low concentrations. For example, Kushnir et al. [17] converted testosterone in plasma collected from women and children to an oxime derivative and found a lower limit of quantification of 35 pmol/L. This limit was reduced a further 10-fold with a novel quaternary amino-oxy (QAO) reagent that is available commercially as Amplifex Keto reagent [26]. 1,25-Dihydroxy-vitamin D is another low-concentration analyte that has been measured by LC-MSMS following sample derivatisation, particularly with 1,2,4-triazoline-3,5-dione (TAD)-based reagents. In this case, another commercially-developed reagent, Amplifex Diene, was found to significantly enhance ionization, and lower the limits of detection in plasma to 5 pmol/L [27]. Increased detection sensitivity may vary widely between analytes and derivatives, which can be explained by improved mass fragmentation patterns, better ionization efficiency in the electrospray source and reduced matrix interference following derivatisation. For metanephrines, an increased detection sensitivity of nearly 100-fold has been reported for normetanephrine [19], and between 6- and 34-fold for free metanephrines [24], as is also found in the present work.

While detection sensitivity is not of crucial importance in analysis of the relatively high concentrations of catecholamines and metanephrines present in human urine, the use of ethyl derivatives allows their direct measurement in diluted samples without the need for SPE clean-up of urine. Of the methods published to date that profile non-derivatised urinary catecholamines and metanephrines by LC-MSMS, all use SPE, either in reverse-phase mode of di-phenylboronate complexes at pH 9 [9,28], or in weak cation-exchange format at pH 6.5 [13,29]. Ethylation allows a 'dilute, derivatise and shoot' approach for the measurement of free urinary biogenic amines. This method, when compared to offline SPE approaches, reduces the sample preparation time, the number of overall steps involved, the solvent and sample volumes used, as well as the cost and risk of errors associated with the sample preparation. It is consistent with the requirements of modern sample preparation techniques associated with bioanalytical LC-MSMS, as identified by Nováková [30]. However, care must be taken to work in a fume hood or use sealed containers, because sodium cyanoborohydride is a highly toxic chemical that will produce hydrogen cyanide gas if exposed to strong acid.

In the case of urinary biogenic amines, derivatisation also has chromatographic benefits. By increasing analyte hydrophobicity, chromatographic retention and separation using reverse-phase HPLC conditions is markedly improved. This approach is preferable to using HILIC chromatography for polar molecules, as analyte separation can be difficult on HILIC columns and interferences due to isobars or cross-talk of ions in the mass spectrometer have been reported [31,32].

Ion suppression effects were evident when evaluated by assessment of the recoveries of isotope-labeled internal standards. The mean recoveries for metanephrines of around 70% with a CV < 10% were considered reasonable for patient urines that contained varying levels of normetanephrine, metanephrine and 3-methoxytyramine and other matrix components. Despite signal suppression, the use of co-eluting labelled internal standards for each analyte compensated for these effects with respect to quantitation accuracy. Additional inherent features of the method that were designed to reduce ion suppression included the extensive dilution of the sample prior to LC-MSMS analysis and the use of low injection volume.

When screening for the neuroendocrine tumours phaeochromocytoma and paraganglioma, the expert-recommended tests are plasma free metanephrines, or total (i.e., free plus conjugated) metanephrine excretion from a 24-h urine collection [5,33]. Nevertheless, it has been recognised that in practice many blood and urine specimens are not collected under proper conditions, thus limiting the utility of these tests. A suggestion has been made that free metanephrines in early-morning spot urine may be the best test in the future from the perspective of both the patient and the laboratory [33]. With this in mind, ranges for free metanephrine concentrations in adult spot urines have been determined in the present study: first, as non-derivatised samples after SPE clean-up, and then as ethyl derivatives without preliminary SPE. Because there was very good agreement between the LC-MSMS analysis of both types of sample, the concentration ranges in the reference group urines were not significantly different. There are limited data available in adults to compare the ranges for free urine metanephrines in spot samples when expressed as μmol/mol creatinine, but similar values have been published recently for normetanephrine and metanephrine [28]. To assist in the diagnosis of neuroblastoma in children, age-related decision limits have been published for free urine catecholamines, metanephrines and their metabolites measured by HPLC with electrochemical detection [34]. The adult ranges determined in the present study should be of value in evaluating the diagnostic performance of early-morning spot urine free metanephrines in screening for phaeochromocytoma and paraganglioma. For simultaneous measurement of free catecholamines and metanephrines, spot urines should be collected with citric acid to produce a pH between 4 and 5, or be frozen immediately, since these conditions have been shown to stabilise free biogenic amines prior to LC-MSMS analysis [35].

Declaration of conflicting interests

The authors declare no conflict of interest in the work described.

Author contributorship

Development and validation of the method: MW, AE and PZ; Performed the experiments: PZ and KC; Performed analysis of the data: AE and MW; Wrote, revised and approved the final version of the manuscript: all authors.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.clinms.2017.08.005.

References

[1] R.T. Peaston, C. Weinkove, Measurement of catecholamines and their metabolites, Ann. Clin. Biochem. 41 (2004) 17–38.
[2] A.M. Sawka, R. Jaeschke, R.J. Singh, W.F. Young, A comparison of biochemical tests for phaeochromocytoma: measurement of fractionated plasma metanephrines compared with the combination of 24-hour urinary metanephrines and catecholamines, J. Clin. Endocrinol. Metab. 88 (2003) 553–558.
[3] J.W. Lenders, K. Pacak, M.M. Walther, W.M. Linehan, M. Mannelli, P. Friberg, H.R. Keiser, D.S. Goldstein, G. Eisenhofer, Biochemical diagnosis of phaeochromocytoma: which test is best? JAMA 287 (2002) 1427–1434.
[4] P.E. Hickman, M. Leong, J. Chang, S.R. Wilson, B. McWhinney, Plasma free metanephrines are superior to urine and plasma catecholamines and urine catecholamine metabolites for the investigation of phaeochromocytoma, Pathology 41 (2009) 173–177.
[5] J.W. Lenders, Q.Y. Duh, G. Eisenhofer, A.P. Gimenez-Roqueplo, S.K. Grebe, M.H. Murad, M. Naruse, K. Pacak, W.F. Young, Phaeochromocytoma and paraganglioma: an endocrine society clinical practice guideline, J. Clin. Endocrinol. Metab. 99 (2014) 1915–1942.
[6] R.T. Peaston, S. Ball, Biochemical detection of phaeochromocytoma: why are we continuing to ignore the evidence? Ann. Clin. Biochem. 45 (2008) 6–10.
[7] G.R. Lee, P.C. Johnston, A.B. Atkinson, D. McKillop, P. Auld, S.J. Hunter, A comparison of plasma-free metanephrines with plasma catecholamines in the investigation of suspected pheochromocytoma, J. Hypertens. 29 (2011) 2422–2428.
[8] W.H. de Jong, E.G. de Vries, B.H. Wolffenbuttel, I.P. Kema, Automated mass spectrometric analysis of urinary free catecholamines using on-line solid phase extraction, J. Chromatogr. B 878 (2010) 1506–1512.
[9] M.J. Whiting, Simultaneous measurement of urinary metanephrines and catecholamines by liquid chromatography with tandem mass spectrometric detection, Ann. Clin. Biochem. 46 (2009) 129–136.
[10] J. Gabler, A. Miller, S. Wang, A simple liquid chromatography-tandem mass spectrometry method for measuring metanephrine and normetanephrine in urine, Clin. Chem. Lab. Med. 49 (2011) 1213–1216.
[11] V.M. Carvalho, The coming of age of liquid chromatography coupled to tandem mass spectrometry in the endocrinology laboratory, J. Chromatogr. B 883 (2012) 50–58.
[12] G. Eisenhofer, M. Peitzsch, B.C. McWhinney, Impact of LC-MS/MS on the laboratory

diagnosis of catecholamine-producing tumours, Trends Anal. Chem. 84 (2016) 106–116.
[13] M. Peitzsch, D. Pelzel, S. Glockner, A. Prejbisz, M. Fassnacht, F. Beuschlein, A. Januszewicz, G. Siegert, G. Eisenhofer, Simultaneous liquid chromatography tandem mass spectrometric determination of urinary free metanephrines and catecholamines, with comparisons of free and deconjugated metabolites, Clin. Chim. Acta 418 (2013) 50–58.
[14] J.G. Boyle, D.F. Davidson, C.G. Perry, J.M.C. Connell, Comparison of diagnostic accuracy of urinary free metanephrines, vanillyl mandelic acid, and catecholamines, and plasma catecholamines for diagnosis of phaeochromocytoma, J. Clin. Endocrinol. Metab. 92 (2007) 4602–4608.
[15] D.F. Davidson, N. Bradshaw, C.G. Perry, R. Lindsay, E.M. Freel, Urinary free (unconjugated) metadrenalines in different hereditary forms of catecholamine-secreting phaeochromocytoma/paraganglioma, Ann. Clin. Biochem. 49 (2012) 486–490.
[16] F. Xu, L. Zou, Y. Liu, Z. Zhang, C.N. Ong, Enhancement of the capabilities of liquid chromatography-mass spectrometry with derivatization: general principles and applications, Mass Spec. Rev. 30 (2011) 1143–1172.
[17] M.M. Kushnir, A.L. Rockwood, W.L. Roberts, E.G. Pattison, A.M. Bunker, R.L. Fitzgerald, A.W. Meikle, Performance characteristics of a novel tandem mass spectrometry assay for serum testosterone, Clin. Chem. 52 (2006) 120–128.
[18] X. Duan, B. Weinstock-Guttman, H. Wang, E. Bang, J. Li, M. Ramanathan, J. Qu, Ultrasensitive quantification of serum vitamin D metabolites using selective solid-phase extraction coupled to microflow liquid chromatography and isotope-dilution mass spectrometry, Anal. Chem. 82 (2010) 2488–2497.
[19] C. Ji, W. Li, X. Ren, A.F. El-Kattan, R. Kozak, S. Fountain, C. Lepsy, Diethylation labelling combined with UPLC/MS/MS for simultaneous determination of a panel of monoamine neurotransmitters in rat prefrontal cortex micro-dialysates, Anal. Chem. 80 (2008) 9195–9203.
[20] W.H. de Jong, K.S. Graham, J.C. van der Molen, J.P. Links, M.R. Morris, H.A. Ross, E.G. de Vries, I.P. Kema, Plasma free metanephrine measurement using automated online solid-phase extraction HPLC tandem mass spectrometry, Clin. Chem. 53 (2007) 1684–1693.
[21] R.T. Peaston, K.S. Graham, E. Chambers, J.C. van der Molen, S. Ball, Performance of plasma free metanephrines measured by liquid chromatography-tandem mass spectrometry in the diagnosis of phaeochromocytoma, Clin. Chim. Acta 411 (2010) 546–552.
[22] C. Ji, J. Walton, Y. Su, M. Tella, Simultaneous determination of plasma epinephrine and norepinephrine using an integrated strategy of a fully automated protein precipitation technique, reductive ethylation labeling and UPLC-MS/MS, Anal. Chim. Acta 670 (2010) 84–91.
[23] G. Zhang, Y. Zhang, C. Ji, T. McDonald, J. Walton, E.A. Groeber, R.C. Steenwyk, Z. Lin, Ultra- sensitive measurement of endogenous epinephrine and norepinephrine in human plasma by semi-automated SPE-LC-MS/MS, J. Chromatogr. B 895–896 (2012) 186–190.
[24] M.J. Whiting, Measurement of biogenic amines and their metabolites by tandem mass spectrometry (MS/MS), Clin. Chim. Acta 411 (2010) 899.
[25] J. Palaty, A comprehensive LC/MS assay for biogenic amines in urine, Clin. Biochem. 47 (2014) 1141–1142.
[26] M. Star-Weinstock, B.L. Williamson, S. Dey, S. Pillai, S. Purkayastha, LC-ESI-MS/MS analysis of testosterone at sub-picogram levels using a novel derivatization reagent, Anal. Chem. 84 (2012) 9310–9317.
[27] C.J. Hedman, D.A. Wiebe, S. Dey, J. Plath, J.W. Kemnitz, T.E. Ziegler, Development of a sensitive LC/MS/MS method for vitamin D metabolites: 1,25-Dihydroxy-vitamin D2 & 3 measurement using a novel derivatization agent, J. Chromatogr. B 953–4 (2014) 62–67.
[28] X. Li, S. Li, G. Kellermann, Pre-analytical and analytical validations and clinical applications of a miniaturized, simple and cost-effective solid phase extraction combined with LC-MS/MS for the simultaneous determination of catecholamines and metanephrines in spot urine samples, Talanta 159 (2016) 238–247.
[29] H.I. Woo, J.S. Yang, H.J. Oh, Y.Y. Cho, J.H. Kim, H.D. Park, S.Y. Lee, A simple and rapid analytical method based on solid-phase extraction and liquid chromatography–tandem mass spectrometry for the simultaneous determination of free catecholamines and metanephrines in urine and its application to routine clinical analysis, Clin. Biochem. 49 (2016) 573–579.
[30] L. Nováková, Challenges in the development of bioanalytical liquid chromatography–mass spectrometry method with emphasis on fast analysis, J. Chromatogr. A 1292 (2013) 25–37.
[31] J.M. Twentyman, K.W. Cradic, R.J. Singh, S. Grebe, Ionic cross-talk can lead to over-estimation of 3-methoxytyramine during quantification of metanephrines by mass spectrometry, Clin. Chem. 58 (2012) 1156–1158.
[32] M.J. Wright, R.L. Thomas, P.E. Stanford, A.R. Horvath, Multiple reaction monitoring with multistage fragmentation (MRM3) detection enhances selectivity for LC-MS/MS analysis of plasma free metanephrines, Clin. Chem. 61 (2015) 505–513.
[33] G. Eisenhofer, M. Peitzsch, Laboratory evaluation of pheochromocytoma and paraganglioma, Clin. Chem. 60 (2014) 1486–1499.
[34] D.F. Davidson, P.J. Hammond, D. Murphy, R. Carachi, Age-related medical decision limits for urinary free (unconjugated) metadrenalines, catecholamines and metabolites in random urine specimens from children, Ann. Clin. Biochem. 48 (2011) 358–366.
[35] M. Peitzsch, D. Pelzel, P. Lattke, G. Siegert, G. Eisenhofer, Preservation of urine free catecholamines and their free O-methylated metabolites with citric acid as an alternative to hydrochloric acid for LC-MS/MS-based analyses, Clin. Chem. Lab. Med. 54 (2016) 37–43.