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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

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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).

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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