Journal of Chromatography B, 1002 (2015) 63–70

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Validation of an assay for quantification of free normetanephrine, metanephrine and methoxytyramine in plasma by high performance liquid chromatography with coulometric detection: Comparison of peak-area vs. peak-height measurements

Dawid Nieć, Paweł K. Kunicki *

Department of Clinical Biochemistry, Institute of Cardiology, Alpejska 42, 04-628 Warsaw, Poland

ARTICLE INFO

Article history:
Received 22 December 2014
Received in revised form 9 June 2015
Accepted 3 July 2015
Available online 29 July 2015

Keywords:
High performance liquid chromatography
Coulometric detection
Plasma
Normetanephrine
Metanephrine
Methoxytyramine

ABSTRACT

Background: Measurements of plasma concentrations of free normetanephrine (NMN), metanephrine (MN) and methoxytyramine (MTY) constitute the most diagnostically accurate screening test for pheochromocytomas and paragangliomas. The aim of this article is to present the results from a validation of an analytical method utilizing high performance liquid chromatography with coulometric detection (HPLC-CD) for quantifying plasma free NMN, MN and MTY. Additionally, peak integration by height and area and the use of one calibration curve for all batches or individual calibration curve for each batch of samples was explored as to determine the optimal approach with regard to accuracy and precision.
Methods: The method was validated using charcoal stripped plasma spiked with solutions of NMN, MN, MTY and internal standard (4-hydroxy-3-methoxybenzylamine) with the exception of selectivity which was evaluated by analysis of real plasma samples. Calibration curve performance, accuracy, precision and recovery were determined following both peak-area and peak-height measurements and the obtained results were compared. The most accurate and precise method of calibration was evaluated by analyzing quality control samples at three concentration levels in 30 analytical runs.
Results: The detector response was linear over the entire tested concentration range from 10 to 2000 pg/mL with $R^2 \geq 0.9988$. The LLOQ was 10 pg/mL for each analyte of interest. To improve accuracy for measurements at low concentrations, a weighted (1/amount) linear regression model was employed, which resulted in inaccuracies of −2.48 to 9.78% and 0.22 to 7.81% following peak-area and peak-height integration, respectively. The imprecisions ranged from 1.07 to 15.45% and from 0.70 to 11.65% for peak-area and peak-height measurements, respectively. The optimal approach to calibration was the one utilizing an individual calibration curve for each batch of samples and peak-height measurements. It was characterized by inaccuracies ranging from −3.39 to +3.27% and imprecisions from 2.17 to 13.57%.
Conclusions: The established HPLC-CD method enables accurate and precise measurements of plasma free NMN, MN and MTY with reasonable selectivity. Preparing calibration curve based on peak-height measurements for each batch of samples yields optimal accuracy and precision.

1. Introduction

Plasma free (unconjugated) metanephrines, normetanephrine (NMN) and metanephrine (MN), and methoxytyramine (MTY) are 3-*O*-methylated metabolites of respective catecholamines: noradrenaline, adrenaline and dopamine, the hormones produced in excess by pheochromocytomas and thoracic, abdominal or pelvic paragangliomas which are the tumors arising from, respectively, intra-adrenal (80–85% of patients) and extra-adrenal sympathetic (10–20% of patients) chromaffin tissue [1]. The tumoral secretion of adrenaline, noradrenaline and dopamine can lead to potentially lethal cardiovascular complications [1], hence prompt and reliable diagnosis is a matter of the utmost importance. Determination of free metanephrines in plasma or fractionated metanephrines in urine is recommended tests for initial screening for pheochromocytomas and paragangliomas [1,2]. Nevertheless, when implemented correctly, measurements of plasma free NMN, MN and MTY offer superior diagnostic accuracy among all alternative biochemical tests measuring either precursor catecholamines or other

* Corresponding author at: Clinical Pharmacology Unit, Department of Clinical Biochemistry, Institute of Cardiology, Alpejska 42, 04-628 Warsaw, Poland.
E-mail address: p.kunicki@ikard.pl (P.K. Kunicki).

http://dx.doi.org/10.1016/j.jchromb.2015.07.004
1570-0232/

catecholamine metabolites [2–5]. Currently, measurements of plasma free metanephrines are performed using following analytical techniques: high performance liquid chromatography with coulometric detection (HPLC-CD) [6,7], liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) [8–14] and immunoassay methods [15,16].

Data analysis in chromatography involves measurements of peak sizes which can be accomplished by the determination of either area or height of the peak. Although analysts most widely use peak-area measurements, peak height is the parameter that should preferably be considered for quantitation of analytes of interest in trace analyses where inadequate resolution is often an issue [17]. Since the contribution from overlapping peak to peak of interest is smaller for height than for area, peak height measurement results in less integration error and thus in a better accuracy [17,18].

This paper describes the validation of the HPLC-CD method adopted with minor modifications from methodology originally developed by Lenders et al. [6] and discusses different approaches to peak integration and method calibration with regard to accuracy and precision of measurements.

2. Materials and methods

2.1. Chemicals

DL-Normetanephrine hydrochloride, DL-metanephrine hydrochloride, 3-methoxytyramine hydrochloride, 4-hydroxy-3-methoxybenzylamine hydrochloride (used as internal standard, IS), 1-octanesulfonic acid sodium salt, ammonium phosphate monobasic and activated charcoal (20/40 mesh) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Sodium dihydrogen orthophosphate monohydrate (NaH_2PO_4), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), ammonium acetate, HPLC-gradient grade methanol, HPLC-gradient grade acetonitrile, ammonia solution min. 25%, potassium hydroxide 2 M in methanol, orthophosphoric acid 85% (H_3PO_4) were obtained from VWR (Radnor, PA, USA). Acetic acid 99.5–99.9% was from POCH (Gliwice, Poland). Ultrapure (deionized) water was produced and filtered (0.22 μm) in-house using Direct-Q® 3 UV-R water purification system from Millipore Corporation (Billerica, MA, USA). Human plasma was obtained from Regional Centre of Blood Donation and Blood Therapy (Warsaw, Poland).

2.2. Instrumentation

Chromatography and detection of analytes of interest were performed using UltiMate 3000 high performance liquid chromatography system and Coulochem III detector (both from Thermo Fisher Scientific Inc., Waltham, MA, USA). The former consisted of ISO-3100BM pump, WPS-3000TPL autosampler and TCC-3000SD column compartment. The detector was equipped with model 5021 conditioning cell and model 5011A analytical cell (Thermo Fisher Scientific Inc.).

Sample preparation apparatus included a solid phase extraction unit consisting of Vac Elut SPS 24 vacuum manifold (Agilent Technologies, Santa Clara, CA, USA), Bond Elut LRC-AccuCAT 200 mg bed weight solid phase extraction columns (Agilent Technologies) and a vacuum pump model MZ2CNT + 2AK (Vacuubrand Inc., Essex, CT, USA), centrifuges: model 5424 and 5702R from Eppendorf (Hamburg, Germany) and a sample lyophilizing unit consisting of SC250EXP SpeedVac concentrator, RVT4104 refrigerated vapour trap and VLP200 vacuum pump (all from Thermo Fisher Scientific Inc.).

2.3. Chromatographic and detection conditions

The separation of NMN, MN, MTY and IS was achieved on Luna® HPLC column (C18 with TMS endcapping, 4.6 mm × 150 mm, 3 μm, 100 Å) protected by SecurityGuard™ cartridge (C18, 4 mm × 3 mm), both from Phenomenex, Torrance, CA, USA.

The mobile phase consisted of: NaH_2PO_4 (100 mM), octanesulfonic acid (0.65 mM), EDTA (0.027 mM), acetonitrile (6.7%). The pH was adjusted to 3.35 with H_3PO_4. The flow rate was set at 1 mL/min. Prior to injection the samples were kept at 4 °C in the autosampler. The temperature of the column compartment was maintained at 33 °C.

Following optimization, the electrode of the conditioning cell was set at a potential of 330 mV and the first and the second electrodes of the analytical cell were set at potentials of 75 mV and −275 mV, respectively.

2.4. Solutions and calibration

Stock solutions of NMN, MN and MTY were prepared by dissolving appropriate amounts of their hydrochloric salts in 0.2 M acetic acid and were subsequently aliquoted to polypropylene tubes for storage at −80 °C for further use. Before sample preparation, stock solutions of NMN, MN and MTY were thawed and diluted with 0.2 M acetic acid to give 7 concentration levels of calibration standards (10, 20, 50, 100, 400, 1000 and 2000 pg in 100 μL of 0.2 M acetic acid) and 3 levels (low – LQC, medium – MQC and high – HQC) of quality control solution (30, 150, 1500 pg in 100 μL of 0.2 M acetic acid for NMN and MN; 15, 75, 1500 pg in 100 μL of 0.2 M acetic acid for MTY). The lowest and the highest concentrations of calibration standards were regarded as the lowest limit of quantification (LLOQ) and the upper limit of quantification (ULOQ).

Working solution of IS was prepared by dissolving a proper amount of HMBA in 0.2 M acetic acid, so as to obtain a concentration of 1000 pg in 20 μL of 0.2 M acetic acid, and subsequently aliquoted to polypropylene tubes for storage at −80 °C for further use.

Removing endogenous NMN, MN and MTY from plasma (obtained from the blood bank) was accomplished by mixing the pooled plasma with the activated charcoal in the span of 60 min at room temperature.

Calibration standard or quality control solution and IS were added to charcoal stripped plasma prior to the extraction procedure.

2.5. Sample preparation

1 mL of charcoal stripped plasma was transferred to 5 mL polystyrene test tube into which: 1 mL of deionized water, 20 μL of working solution of IS and 100 μL of calibration standard or 100 μL of quality control solution or 100 μL of pure 0.2 M acetic acid (blank sample) were initially added. The mixture was then vortex mixed and subsequently centrifuged at 4 °C.

In order to activate the stationary phase of the extraction column, it was washed with 5 mL of a mixture of methanol, deionized water and ammonia (30:9:1, v/v/v) followed by 2 mL of a solution of potassium hydroxide in methanol (89.2 mM). Then, the column was rinsed with 2 mL of deionized water. During those three steps of sorbent conditioning, vacuum applied to the manifold chamber was of −68 mbar. Subsequently, 2 mL of diluted plasma already processed as described above was loaded onto the column and passed through the sorbent with no suction applied. To remove unwanted impurities retained along with the analytes of interest, the column was then rinsed with 2 mL of 10 mM acetic acid-methanol (9:1, v/v), followed by 2 mL of 10 mM ammonium