Journal of Chromatography B, 1002 (2015) 92–97

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Rapid, easy analysis of urinary vanillylmandelic acid for diagnostic testing of pheochromocytoma by liquid chromatography tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 6 May 2015
Received in revised form 4 August 2015
Accepted 11 August 2015
Available online 20 August 2015

Keywords:
Vanillylmandelic acid
Pheochromocytoma
LC–MS/MS

ABSTRACT

Vanillylmandelic acid (VMA), as one of the most important catecholamine metabolites, is commonly used to aid in diagnosis of pheochromocytoma. This study develops a rapid and simple high-throughput LC–MS/MS method for the measurement of urinary VMA. Without sample pretreatment, the urine specimens were mixed with internal standard (IS) solution for direct analysis by LC–MS/MS in two minutes. VMA and VMA-d3 were detected in the multiple-reaction monitoring mode using the specific transitions m/z 197.0 → 137.0 and 200.0 → 140.0, respectively. This method was validated for consistent linearity from 1.0 to 250.0 μM with CVs ≤ 3.12%, excellent recovery, good stability and low carryover. The lowest limit of quantification (LLOQ) was 0.125 μmol/L for VMA with CV of 14.1%, and the lower limit of detection (LOD) was 0.025 μmol/L. Intra-assay and inter-assay imprecision values (CVs) for VMA were all below 2.11%. Dilution linearity was investigated with a satisfied mean accordance of 105%. Method comparison of LC–MS/MS and microcolumn chromatography in our lab was performed and the reference interval was established in agreement with that of the Mayo Clinic. All these results demonstrate that this validated LC–MS/MS approach shows improved accuracy and reproducibility compared with microcolumn chromatography. The low sample volume, simplicity, rapidity, and robustness of the method make it suitable for use as a high-throughput assay in routine clinical biochemistry laboratories.

1. Introduction

Pheochromocytoma is a rare neuroendocrine tumor arising from the chromaffin cells of the adrenal medulla and characterized by producing excessive amounts of catecholamines (CAs) and their metabolites [1,2]. For accurate analysis, non-invasive and easy urine specimen collection [3], measurement of urinary vanillylmandelic acid (VMA) is frequently used in both the clinical diagnosis and pathological study of this disease. Historically, VMA has been measured by high performance liquid chromatography coupled with electrochemical detection (HPLC–ECD) [4], microcolumn chromatography, gas chromatography-mass spectrometry (GC–MS) and immunoassay techniques [5–7]. However, HPLC–ECD method requires laborious sample preparation and extensive analytical time, and is prone to interferences. In addition, errors are easily introduced during the tedious multi-steps process of the microcolumn chromatography method. Although GC–MS possesses high identification and separation power, most analytes need extensive time-consuming derivatization to become stable, volatile and amenable to the ionization techniques, resulting in its poor practicability [8]. In recent years, liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) has been receiving much attention in clinical chemistry and replaces many of the above described HPLC–ECD, microcolumn chromatography, GC–MS and immunoassay techniques due to its high specificity, high sensitivity, high efficiency, high throughput and less laborious sample preparation [9–15]. This also applies to CAs and their

Abbreviations: CAs, catecholamines; VMA, vanillylmandelic acid; MHPG, 4-Hydroxy-3-methoxyphenylglycol; DOPAC, 3, 4-Dihydroxyphenylacetic acid; HPLC–ECD, high performance liquid chromatography coupled with electrochemical detection; GC–MS, gas chromatography-mass spectrometry; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; QC, quality control; IS, internal standard; SPE, solid phase extraction; MRM, multiple reaction monitoring; TEM, temperature; GS1, ion source gas1; GS2, ion source gas2; CUR, curtain gas; CAD, collision gas; CE, collision energy; EP, entrance potential; DP, declustering potential; CXP, collision cell exit potential; LLOQ, the lower limit of quantification; LOD, the lower limit of detection; CV, coefficient of variation; SD, standard deviation; ME, matrix effect.

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http://dx.doi.org/10.1016/j.jchromb.2015.08.013
1570-0232/

metabolites [16–21]. For VMA analysis, either derivatization process or analysis only focusing on method development appears. As a result, a rapid, easy and accurate method for VMA determination in routine clinical laboratories is urgent needed.

In our laboratory, urinary VMA determination is frequently requested and routinely performed using microcolumn chromatography. Although this method could provide satisfied results, this analysis system requires larger sample volumes and allows longer operation time. The primary purpose of developing this assay in the study was to establish a rapid, easy and stable method for VMA determination in a high-throughput clinical environment via efficient and effective use of an LC–MS/MS system, which could assist in diagnosis of neuroblastoma and pheochromocytoma.

2. Experimental

2.1. Chemicals and reagents

Vanillylmandelic acid (VMA), Homovanillic acid (HVA), 4-Hydroxy-3-methoxyphenylglycol (MHPG) hemipiperazinium salt and 3, 4-Dihydroxyphenylacetic acid (DOPAC) was obtained from Sigma–Aldich (St. Louis, MO). Stable isotopes internal standard d_3-VMA was gotten from CDN Isotopes (Pointe-Claire, Quebec, Canada). LC–MS grade water and LC–MS grade methanol were purchased form Merck KGaA (Germany). Deionized water was prepared by water purification system (ELga, England). All the chemicals and solvents were of the highest purity available from commercial sources and used without further purification.

2.2. Standard solutions

Stock VMA solution (6.0 mM) was prepared by dissolving pure VMA in 50% methanol-water. Stock internal standard (d_3-VMA) was prepared at a concentration of 45 μM in 50% methanol -water. Working internal standard (IS) was prepared by adding 300 μL stock d_3-VMA solution into 10 mL flask and make up to 10 mL with 50 % methanol. Stock and working standards were stable for 1 y at −80 °C.

2.3. Calibration standards and quality controls

Calibration standards at concentration of 1.0, 2.0, 10.0, 50.0, 100.0 and 250.0 μM to approximate the expected biological concentrations were prepared by dilution of the stock standard solutions with VMA-free urine matrix. This VMA-depleted urine matrix was created by pooling excess human urine, passing aliquots through the anion exchange resin (AusBio, Laboratories Co., Ltd.), and repooling the urine, when VMA were absorbed onto the sorbent. An aliquot of the eluent was collected and then analyzed to ensure that no VMA could be detected. Aliquots of calibrator solutions were stored at −80 °C. These solutions were stable for at least one year.

Three levels of quality control (QC) material were prepared from separately made working solutions and were spiked into the pooled urine obtained as above, which was first verified to have no interferences at a final volume of 10 mL. The concentrations of VMA in low, median and high QC materials were 6.0, 12.0 and 60.0 μM, respectively. Aliquots of the QC materials were stored at −80 °C and could be stable for one year.

2.4. Sample collection and preparation

For adult subjects, 24-h urine were collected in bottles and preserved by concentrated hydrochloric acid (HCl). The total volume was measured, and 1-mL aliquots were stored at −80 °C until analysis. The urine specimens were centrifuged at 10,000 g for 2 min to remove sediments prior to the analysis. Then, 50 μL of each patient samples, standards or controls was transferred to a tube and mixed with 450 μL of IS working solution. The mixed samples were stirred for 1 min before direct injection.

2.5. LC–MS/MS conditions

All analyses were performed on a Shimadzu LC-30AT (Shimadzu, Japan) coupled to a Q-trap 4500 triple quadrupole /linearity ion trap mass spectrometer (Applied Biosystems, Foster City, USA). The HPLC of VMA was carried out on a PFP column (100 × 2.0 mm, Luna, 3 μm PFP, 100 Å pore size, Phenomenex, Torrance, CA, USA), protected by a Phenomenex PFP security guard column (4.0 × 3.0 mm). The flow rate was 0.2 mL/min. Mobile phase consisted of water (solvent A) and methanol (solvent B). The elution was performed by 40% B and total analysis time was 2 min. The column temperature was 40 °C and the sample injection volume was 5.0 μL. Between injections, autosampler syringe was washed once with 50% methanol.

For MS analysis, the instrument was operated in negative-ion mode and acquisition was achieved in the multiple reaction monitoring (MRM) scanning mode. Analyte precursor ions were fragmented by collision with neutral gas molecules to selected product ions. The transitions of the precursor ions to the product ions were 197.0 > 137.0 m/z for VMA and 200.0 > 140.0 m/z for VMA-d_3.The conditions of the MS were as follows: ion-spray voltage (IS), −4500 kV; temperature (TEM), 5500 °C; ion source gas1 (GS1), 40.0 psi; ion source gas2 (GS2), 50.0 psi; curtain gas (CUR), 30.0 psi; collision gas (CAD), medium; collision energy (CE), −30.0 V; entrance potential (EP), −10.0 V; declustering potential (DP), −60.0 V for VMA, −50.0 V for VMA-d_3; collision Cell Exit potential (CXP), −7.0 V for VMA, −6.0 V for VMA-d_3.

2.6. Assay validation

2.6.1. Linearity and limit of quantification

To assess the linearity of the assay, five replicates at each of the six concentration of VMA were analyzed (1.0–250.0 μM). The lower limit of quantification (LLOQ), defined as the lowest concentration that gives a signal-to-noise >10, was determined by assaying low concentration solutions (1.0, 0.5, 0.25, 0.125, 0.025 and 0.005 μM) near the detection limit of the assay for 15 times over three days and this value was defined as the concentration at which the inter-assay coefficient of variation (CV) was 20%. The lower limit of detection (LOD) represents the absolute limit of detection that produced a signal-to-noise >3.

2.6.2. Imprecision

Three control materials at concentration of 6.0, 12.0 and 60.0 μM were used to estimate imprecision. Duplicate of each control were assayed over twenty days to evaluate the within-run, between-day and total imprecision. Total CVs were calculated as the square root of the sum of squared within-run and between-day CVs.

2.6.3. Recovery

Recovery was performed by spiking a known amount of VMA (5.0, 25.0 and 50.0 μM) into the human samples. All measurements were performed in five times and the recovery was calculated as [(final concentration-initial concentration)/added concentration].

2.6.4. Carryover

Carryover was evaluated as follows: low and high concentration samples (1.0, 100.0 and 200.0 μM) were injected in a sequence that allows for the determination of the mean and standard deviation (SD) for low concentration following a low sample, and low concentration following a high sample. The injection sequence was 1.0, 1.0,