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Preanalytical validation and reference values for a mass spectrometric assay of serum vanillylmandelic acid for screening of catecholamine secreting neuroendocrine tumors

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ABSTRACT

Background: Urinary vanillylmandelic acid (VMA) is used to diagnose and monitor catecholamine secreting neuroendocrine tumors (NETs). We developed and validated a new liquid chromatography tandem mass spectrometry (LC–MS/MS) assay for determination of serum VMA.
Methods: We used serum samples from healthy volunteers (n = 314) and patients suspected for NET (n = 36). Deuterated VMA as an internal standard was added to samples before solid phase extraction (SPE) and LC–MS/MS analysis. We studied the effects of sample storage, sampling device and a meal on serum VMA and metanephrine concentrations. Diurnal variation and age-dependent reference intervals were established. The diagnostic performance was compared with a urinary HPLC assay for VMA and metanephrines and a serum metanephrine LC–MS/MS assay.
Results: Serum VMA is stable at least for one day at +4 °C, seven days at room temperature and 98 days at −20 °C. Type of sampling device was not critical, but elevated serum VMA occurs after a meal ($p = 0.031$). Serum VMA increased with age. Therefore, we suggest clinical cut-off values of 62 nmol/L, 80 nmol/L and 108 nmol/L for age groups 18–50 yrs, 51–70 yrs and >70 yrs, respectively. Comparison between a urinary VMA HPLC assay and serum VMA LC–MS/MS assay showed good correlation.
Conclusions: Our LC–MS/MS assay is fast and sensitive and suits well for use in a clinical laboratory. Compared to 24-h urine collection our serum assay enables well controlled sampling and convenient preanalytical steps.

1. Introduction

Pheochromocytomas (PCCs) are rare chromaffin cell tumors located in the medulla of the adrenal gland. At least 30% of PCCs are known to be hereditary and occur most often during young-adult to mid-adult life [1]. The prevalence is estimated to be around 0.05% in the general population [2]. Catecholamine secreting tumors occurring outside of the adrenal gland are called paragangliomas [3]. Neuroblastomas are the most common catecholamine secreting malignant extracranial tumors of childhood [4]. The most typical symptoms of the catecholamine secreting NETs are headache, hypertension, perspiration and palpitations. The symptoms are caused by overproduction of catecholamines in the tumor [5].

Measurement of catecholamines and/or their metabolites in urine or plasma is the most common laboratory tests used for diagnosis of NETs. Urinary catecholamine metabolite vanillylmandelic acid (VMA) is the routine test for the diagnosis and monitoring of NETs (especially neuroblastoma), but urinary or plasma metanephrine assay is generally used side by side with it [6,7]. VMA in urine has been analyzed by HPLC, immunoassay and gas or liquid chromatography mass spectrometry [8–10]. However, 24-hour urine collection is time-consuming and prone to errors during the collection. Urinary VMA HPLC and immunoassays may suffer from analytical interferences (e.g. drugs and metabolites) [11].

Plasma or serum VMA assays with GC–MS after derivatization have been reported earlier [12]. In a routine clinical laboratory more straightforward methods are preferred. To our knowledge, there are only few reports describing quantification of serum VMA levels by LC–MS/MS. In the assay of Cai et al. [13] plasma VMA is measured by LC–MS/MS after a dansylation procedure. Fang et al. [14] described an LC–MS/MS assay without the use of an internal standard (IS). Recently, Sadilkova et al. [15] published an UPLC–MS/MS method for serum VMA.

Neuroblastomas occur mostly in childhood and 24-h urine collection especially from children is a challenge. Therefore, normalization of random urinary specimen VMA against creatinine is a generally accepted procedure for pediatric samples. To avoid this, we developed and validated a straightforward LC–MS/MS method for quantification of serum VMA with deuterated IS. We compared our LC–MS/MS assay

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with a urinary VMA and metanephrine HPLC and with a serum metanephrine LC–MS/MS assays. We also established reference intervals and studied pre-analytical factors such as sample stability, sampling device, postprandial effect, the effect of dopamine-containing foods and diurnal variation of serum VMA and metanephrines.

2. Materials and methods

2.1. Reagents and materials

Stock solutions (10 μmol/L, 2 mg/L) of VMA (Sigma) and deuterium labeled VMA-D_3 IS (Medical Isotopes Inc. Pelham, NH, USA, www.medicalisotopes.com) were prepared in water. Working solutions of VMA (10–1000 nmol/L) in water and IS (1000 nmol/L) in 0.2% formic acid were freshly prepared from stock solutions. Plate extraction manifold and Oasis® MAX μElution plates (96-wells, Waters, Milford, MA, USA) were used for sample purification. MS-grade methanol, MS-grade acetonitrile (ACN), formic acid, ammonium hydroxide and ammonium formate were purchased from Fluka. All reagents were of the highest analytical grade.

2.2. Samples

Serum samples were obtained from healthy volunteers participating in the Nordic Reference Interval Project (NORIP) [16] and from our laboratory staff (165 women and 149 men). The samples were kept at −80 °C until analysis. The subjects were divided into age groups 18–30, 31–50, 51–70 and over 70 yrs according to gender for determination of reference intervals for VMA and metanephrines. Each group comprised 10–17 and 32–49 samples, respectively. We also used 24-h urine samples from healthy volunteers (n = 8). Informed consent was obtained from all healthy individuals. The patients (n = 36) were referred to the outpatient Department of Endocrinology at the Helsinki University Central Hospital between 2010 and 2013 because of clinical symptoms and suspicion of NETs. Eight of them were diagnosed to have neuroblastoma (4 before treatment at progressive stage and 4 remission after treatment), 1 paraganglioma and 1 adrenal adenoma (Table 1). Patient samples were divided into two groups according to age (<16 yrs and ≥16 yrs). The study of dopamine-containing foods was approved by the ethical committee of Helsinki University Central Hospital, Finland.

2.3. Sample and calibrator preparation

Serum samples and calibrators (200 μL) were pipetted into a 96-well plate. One hundred microliter of IS working solution in 0.2% formic acid was added into the wells. MAX μElution plate was conditioned with 200 μL of methanol and with 200 μL of water. Samples and standards with IS were transferred into the μElution wells followed by washing with 200 μL of 5% ammonium hydroxide and 200 μL of water. VMA was then eluted with 150 μL of a solution containing 3% 100 mmol/L ammonium formate pH 2.2 and 97% ACN and injected directly to LC–MS/MS.

Table 1
Characteristics of the patients.

	<16 yrs	≥16 yrs
Sex, M/F	9/8	14/5
Age, average (range)	6 yrs 3 mon (1 mon–15 yrs 6 mon)	54 years (16–80)
Tumor category, n		
Neuroblastoma	8	0
Paraganglioma	0	1
Adenoma	0	1
Clinical status, n		
Remission	4	0
progressive disease	4	2
healthy	9	17

2.4. LC–MS/MS

The instrumentation consisted of an Agilent 1200 liquid chromatograph (Agilent Technologies, Santa Clara, CA, USA) and a 4000 QTRAP mass spectrometer (AB Sciex, Toronto, Canada) equipped with a Turbo-V electrospray ion source. The assay was performed in a gradient mode with a total run time of 10 min. We used an Atlantis HILIC 50 × 2.10 mm 2.6 μ column (Waters) and a flow rate of 400 μL/min. The elution buffers were (A) ACN and (B) 100 mmol/L ammonium formate pH 2.2. The gradient was as follows: 0 min, 3% B; 1.2 min, 60% B; 3.2 min, 3% B; and 10 min, 3% B. Multiple reaction monitoring (MRM) mode was used with the following transitions: VMA m/z 196.9 → 136.6 and IS m/z 199.9 → 139.5. Ionization was carried out in negative mode with the ion source spray voltage set at −4500 V and temperature at 500 °C. The gas settings were curtain gas 20 L/min, nebulizer gas 50 L/min, heater gas 50 L/min and collision gas setting 4. Data were acquired and processed with the Analyst Software (Ver. 1.5, AB Sciex).

2.5. Validation of the method

Eleven calibrators (1.25–10,000 nmol/L) were prepared by serial dilution and measured on three different days to calculate the linearity of the method. The calibration curves were derived using $1/x^2$ weighted linear least-squares regression by the quantitation option of the Analyst 1.5 software (AB Sciex). The accuracy (relative error, RE%) and the precision (coefficient of variation, CV%) were calculated. The limit of detection (LOD) was determined as the average signal + 3 SD of ten zero samples (mobile phase). Limit of quantitation (LOQ) and linear range were defined as the lowest concentration and range, respectively, that could be measured with an inaccuracy and imprecision <20%.

As a low level QA sample we used pooled serum from healthy donors. A high level QA sample was prepared by supplementing pooled serum with 400 nmol/L VMA. The intra- and inter-assay variations were calculated from the QA samples in a single run (n = 14) and on separate days (n = 14), respectively. The recovery of added VMA was determined using three serum samples with and without supplementation of 200 nmol/L and 500 nmol/L of VMA.

The matrix effect was studied with three serum samples containing 60–77 nmol/L VMA. The samples were extracted in duplicates. After extraction one of the duplicates was supplemented with 200 nmol/L of VMA before LC–MS/MS analysis. The same amount of VMA was added to the mobile phase. The signal suppression/enhancement was calculated from the peak areas.

2.6. Preanalytical validation

To study the stability of serum VMA, freshly drawn serum samples from healthy individuals (n = 9) were divided into aliquots and stored at room temperature, +4 °C and −20 °C for various time periods. Samples stored at room temperature and +4 °C were assayed on days 0, 1, 2, 3, 4 and 7. Samples stored at −20 °C were assayed on days 0, 7, 14, 21, 35, 56 and 98 without thawing and after repeated freezing and thawing up to five times at 1–2 week intervals. Samples were allowed to reach room temperature before analysis and then returned to the original storage temperature for repeated assay of the same aliquot. We considered serum VMA stable if the concentration changed <15% [17].

Blood samples from 18 healthy volunteers were collected into plain serum tubes, serum catalyzator tubes (CAT), serum gel tubes (SST™ II Advance, all from BD Vacutainer, Plymouth, UK) and lithium-heparin tubes (Venosafe 60 USP U Lithium Heparin, Terumo, Leuven, Belgium) to compare the effect of the sampling device. The effect of a breakfast meal was studied in 23 volunteers. Blood samples were collected before

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