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Determination of plasma pipecolic acid by an easy and rapid liquid chromatography–tandem mass spectrometry method

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A B S T R A C T

Pipecolic acid (PA) is an important biochemical marker for the diagnosis of peroxisomal disorders. PA is also a factor responsible for hepatic encephalopathy and a possible biomarker for pyridoxine-dependent seizures. We developed an easy and rapid PA quantification method, by high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS), requiring no derivatization and applicable to small sample volumes. Plasma (100 μl) is extracted with 500 μl acetonitrile (ACN) containing 2 μmol/l [$^2H_5$]-phenylalanine as internal standard, vortexed and centrifuged. The supernatant is analyzed by HPLC-MS/MS in positive-ion mode using multiple reaction monitoring scan type. HPLC column is a Luna HILIC (150 × 3.0 mm; 3 μ 200A): Buffer A: ammonium formate 5 mmol/l; Buffer B: ACN/$H_2$0 90:10 containing ammonium formate 5 mmol/l. PA retention time is 4.86 min.

Recovery was 93.8%, linearity was assessed between 0.05 and 50 μmol/l ($R^2 = 0.998$), lower limit of detection was 0.010 μmol/l and lower limit of quantification was 0.050 μmol/l. Coefficient of variation was 3.2% intra-assay and 3.4% inter-assay, respectively.

Clinical validation was obtained by comparing PA plasma values from 5 patients affected by peroxisomal disorders (mean, 23.38 μmol/l; range, 11.20–37.1 μmol/l) to 24 ages related healthy subjects (mean, 1.711 μmol/l; range, 0.517–3.580 μmol/l).

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1. Introduction

Pipecolic acid (PA: piperidine-2-carboxylic acid) is a cyclic secondary imino acid including D- and L-enantiomers. It derives from one of the two pathways of lysine metabolism, merging at the level of α-aminoadipic acid semialdehyde (AASA) [1,2,3]. In the brain, the conversion of lysine to acetoacetyl CoA is predominant via L-pipecolic acid, while in the liver and in most other vertebrate tissues, lysine is metabolized via saccharopine. The exogenous source of pipecolic acid comes instead from D-lysine catabolism by intestinal bacteria and to a minor part from plant source [4].

L-Pipecolic acid is accumulated in biological fluids of patients with peroxisomal disorders, including hyperpipecolic acidemia, Zellweger syndrome, neonatal adrenoleukodystrophy or infantile Refsum disease [5].

L-Pipecolic acid is also involved in the pathway of α-aminobutyric acid (GABA) receptor agonist: it was found that both D-PA and L-PA were moderately increase in patients with liver cirrhosis and in patients with chronic hepatic encephalopathy. Although L-PA remained the predominantly circulating form, D-PA was proportionally higher in liver disease patients than in healthy individuals [4].

Finally, elevated PA is a secondary biomarker for pyridoxine-dependent seizure disorder, a recessive disorder characterized by seizures in neonates or infants up to 3 years of age which respond to pharmacological doses or pyridoxine [6].

Plasma levels of PA consist of high levels of L-enantiomers and low levels of D-enantiomers (about 2%) in normal subjects [7,8]. For this reason, we only focused on the analysis and quantification of the L-form in plasma.

Abbreviations: PA, pipecolic acid; ACN, acetonitrile; HPLC-MS/MS, liquid chromatography–tandem mass spectrometry; [$^2H_5$]-Phe, [$^2H_5$]-phenylalanine; AASA, α-aminoadipic acid semialdehyde; GABA, α-aminobutyric acid; GC-MS, gas chromatography–mass spectrometry; DP, declustering potential; CXP, collision cell exit potential; CE, collision energy; LOD, limit of detection; LLOQ, lower limit of quantification; S/N, signal-to-noise ratio; SD, standard deviation; RT, retention time; MRM, multiple reaction monitoring; NA, nipecotic acid; INA, isonipecotic acid.

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Initially, total PA (D-PA and L-PA) was determined by ion-exchange chromatography on an amino acid analyzer or by HPLC using the acid ninhydrin method [9]. Several more sensitive single-ion monitoring gas chromatography–mass spectrometry (GC-MS) methods have been published for the determination of total PA in plasma and urine, usually involving solid-phase extraction followed by derivatization and analysis on an achiral GC column [10,11]. The analysis of the derivative compounds by electron-capture negative-ion GC-MS or GC with electron-capture detection had also allowed the analysis of PA in cerebrospinal fluid, where the concentrations are extremely low [12,13].

Subsequently, several GC-MS or LC-MS/MS methods were published, with or without derivatization, analyzing PA as individual enantiomers using a chiral column [7,8,14]. In this study, we present an easy and rapid liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) method for the quantification of PA in plasma requiring no derivatization, simple sample preparation and a short analysis time.

2. Materials and methods

2.1. Reagents

L-PA, nipecotic acid (NA), isonipecotic acid (INA) and ammonium formate were purchased from Sigma-Aldrich (Steinheim, Germany). [$^2H_5$]-Phenylalanine ([$^2H_5$]-Phe) was purchased from Cambridge Isotopes. HPLC grade acetonitrile (ACN) and water were purchased from Romil Ltd. (The Source Convent Drive Waterbeach Cambridge, United Kingdom). Quality control samples were from ERNDIM.

2.2. Preparation of standard solutions

Stock solutions 10 mmol/l of PA and internal standard [$^2H_5$]-Phe were prepared in water and stored at −80 °C. The 10 μmol/l daily internal standard solution [$^2H_5$]-Phe was prepared in ACN by scalar dilution of the 10 mmol/l stock solution.

2.3. Sample treatment procedure

One-hundred microliters of plasma and 500 μl ACN containing internal standard [$^2H_5$]-Phe (2 μmol/l) were added to a microfuge tube. The tube was then vortexed vigorously for 30 s and centrifuged at 10000 g for 5 min. Two hundred microliters of the clear supernatant was transferred to the wells of a 96 well-plates microplate. The microplate was covered with protective sheets to prevent solvent evaporation. Twenty microliters of extract was injected into the mass spectrometer.

2.4. Liquid chromatography–mass spectrometry

Chromatography was performed on an Agilent series 1200 pump and autosampler (Agilent technologies Inc., Wilmington, DE, USA). The column for chromatographic separation was a 150 × 3.00 mm 200A 3 μ Luna HILIC column (Phenomenex, Castel Maggiore, Italy). The mobile phase A was H_2O containing 5 mmol/l ammonium formate and the mobile phase B was ACN/H_2O (90%/10%) containing 5 mmol/l ammonium formate. Flow rate was 400 μl/min. The column was maintained at room temperature. Twenty microliters of sample were injected onto the column. Chromatographic separation of metabolites was obtained with gradient elution. Mobile phase B changed from 95% to 55% in 5 min and then remained at 55% for 2 min and finally initial condition had restored in 4 minutes. The total run time was 11 min.

Tandem mass spectrometry experiments were carried out on an API3200 triple quadrupole mass spectrometer (Applied Biosystems-MDS Sciex, Toronto, Canada), equipped with a Turbo Ion Spray Source operating in positive ion mode with a needle potential of 5500 V, and the source temperature was 500 °C. Declustering potential (DP), collision cell exit potential (CXP) and collision energy (CE) were optimized by direct infusion at flow rate 10 μl/min of each analyte in the mass spectrometer. The resulting DP was 32 eV, and optimal CE and CXP were found at 43 eV and 3 eV for PA and [$^2H_5$]-Phe, respectively. The following transitions were monitored in positive-ion mode using multiple reaction monitoring scan type: m/z 130.10 > 84.20 and 130.10 > 56.20 for PA and 171.20 > 125.10 for [$^2H_5$]-Phe.

2.5. Standard curves for quantification

Serial dilutions of 10 mmol/l PA stock solution were used to obtain calibration points (50 μmol/l, 30 μmol/l, 20 μmol/l, 10 μmol/l, 1 μmol/l, 0.5 μmol/l, 0.1 μmol/l and 0.05 μmol/l). Calibration curve points were treated and analyzed as plasma samples.

LC-MS/MS-based assays can also be affected by matrix ion suppression effects related to variation in ionization response due to matrix components coeluting with the analyte. We thus evaluated the suppression coefficient for PA by calculating the ratio of the average peak area response in spiked plasma sample to the average peak area response in ACN. The suppression coefficient of PA was 0.82, indicating acceptable matrix effects in plasma.

The slopes of the calibration curves were shown to be nearly identical in ACN and plasma, indicating that ACN was a suitable surrogate matrix (Table 1). The intercept of the plasma calibration curve was larger than zero due to the endogenous PA concentrations, whereas the intercept of the ACN calibration curve was close to zero.

The acquired data were processed using the Analyst® version 1.4.2 software (Applied Biosystems-Sciex), including option for chromatographic and spectral interpretation and for quantitative generation information. Calibration curves were constructed with the Analyst Quantification program using a linear least-square regression non-weighted analysis.

The lower limit of detection (LLOD) was determined by progressive dilutions of calibrator solutions for each analyte, and it was considered as the lowest concentration with a signal-to-noise ratio (S/N) of at least 3, as indicated by the Analyst software. The lower limit of quantification (LLOQ) was determined by preparing calibrator solutions with decreasing concentration of each analyte and was considered at the lowest concentration with an S/N of at least 10.

2.6. Sample collection of patients and controls

For reference values, 24 plasma samples, were obtained from healthy subjects (12 females and 12 males; age: 1 month to 35 years). Five plasma samples were obtained from patients affected by peroxisomal disorders: 4 with peroxisome biogenesis disorders (PBD) and 1 with adult Refsum disease. Controls and patient blood sample were collected after obtaining informed consent. The samples were treated as described in sample treatment procedure. Quality Control and External Quality Assessment samples from ERNDIM (European Research Network for Evaluation and Improvement of Screening, Diagnosis, and Treatment of Inherited Disorders of Metabolism) were analyzed in each batch of ours to confirm the normal range of values and the abnormal levels found for the peroxisomal patients. The results obtained were within the 95% confidence interval.

Table 1
Characteristics of calibration regression line data prepared in ACN and in plasma.

Analyte	Matrix	Slope	Intercept	R^2
PA	ACN	0.181	0.042	0.998
	Plasma	0.172	0.434	0.996

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