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A novel method for simultaneous quantification of alpha-aminoadipic semialdehyde/piperideine-6-carboxylate and pipecolic acid in plasma and urine

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ABSTRACT

Objectives: Elevated levels of pipecolic acid (PA), α-aminoadipic semialdehyde (AASA) and its cyclic form Δ1-piperideine-6-carboxylate (P6C) are characteristic of pyridoxine dependent epilepsy (PDE), a rare disorder of inborn error of metabolism. Recent studies showed the effectiveness of dietary therapy in PDE patients and emphasized the importance of the assessment of these metabolites for monitoring treatment efficacy. The objective of this study was to develop a robust and sensitive method for simultaneous quantification of AASA-P6C and PA in plasma and urine.

Design and methods: Plasma and urine samples were derivatized with 3 N HCl in n-butanol (v/v) and injected onto ACQUITY BEH-C18 column. A gradient of water/methanol containing 0.1% formic acid was used for the chromatographic separation of AASA, P6C and PA. The analytes' concentrations were calculated using their calibration curves and the sum of AASA and P6C (AASA-P6C) was calculated. To evaluate the clinical utility of this test, samples from unaffected controls and patients with confirmed PDE were analyzed.

Results: The performance characteristics of the assay as well as sample stability and interferences were determined. The intra- and inter- assay CVs were ≤2.9% and ≤10.9% for AASA-P6C, and ≤3.3% and ≤12.6% for PA, respectively. Reference ranges for AASA-P6C and PA in plasma and urine were established. Comparison of values obtained from unaffected controls and PDE patients showed high clinical sensitivity and specificity of the assay.

Conclusions: This novel method for the simultaneous quantification of AASA-P6C and PA in plasma and urine can be used in a clinical laboratory setting for the diagnosis and monitoring of patients with PDE.

1. Introduction

Pyridoxine dependent epilepsy (PDE) is a rare autosomal recessive disorder (OMIM 266100) characterized by early-onset intractable seizures responsive only to high doses of Vitamin B6 (pyridoxine) [1]. PDE has been shown to be caused by impaired activity of α-aminoadipic semialdehyde dehydrogenase [2], an enzyme catalyzing one of the steps within the lysine catabolic pathway. Lysine degradation primarily takes place in the mitochondrial matrix of liver cells and begins with conjugation of lysine and α-ketoglutarate to form saccharopine, which is then converted into α-aminoadipic semialdehyde (AASA). These two reactions are catalyzed by the mitochondrial bi-functional enzyme, α-aminoadipic semialdehyde synthase. In the brain, the predominant lysine degradation pathway is through the formation of pipecolic acid (PA), which is further oxidized to Δ1-piperidine-6-carboxylate (P6C) by peroxisomal oxidase [3,4]. P6C spontaneously converts to its open-chain aldehyde form, AASA, thus bringing both upstream pathways, peroxisomal and mitochondrial, together. Further degradation of

Abbreviations: PDE, pyridoxine dependent epilepsy; PA, pipecolic acid; AASA, α-aminoadipic semialdehyde; P6C, piperideine-6-carboxylate; (PLP), pyridoxal 5′-phosphate; LC–MS/MS, liquid chromatography–mass spectrometry; AEA, allysine-ethylene acetal; (QC), quality controls; MRM, multiple reaction monitoring; LOD, limit of detection; LLOQ, lower limit of quantification.

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AASA requires the activity of α-aminoadipic semialdehyde dehydrogenase (AASA dehydrogenase) to catalyze the formation of α-aminoadipic acid from AASA.

The highly conserved *ALDH7A1* gene, known as antiquitin, encodes AASA dehydrogenase, and various pathogenic mutations in this gene have been detected in patients with PDE [2,5,6]. Impairment of AASA dehydrogenase function leads to elevations of PA, P6C and AASA in plasma, urine and CSF [2,7–10]. Once accumulated in excessive amounts, P6C reacts with the active form of vitamin B6, pyridoxal 5′-phosphate (PLP), thus sequestering it from many enzymatic reactions in which PLP serves as a cofactor, including neurotransmitter synthesis and degradation. Thus, neurological manifestations seen in PDE patients have been attributed to PLP deficiency, and, therefore, treated with large daily supplements of Vitamin B6. In addition, accumulation of AASA, P6C and PA might also have a direct neurotoxic effect as decreased levels of these PDE biomarkers have been associated with noticeable developmental progress in PDE patients [11,12].

Increases in PA, AASA and P6C are not unique to pyridoxine dependent epilepsy. Patients with peroxisomal disorders [13,14], hyperlysinuria type I [15] and chronic liver dysfunction [16,17] have been shown to have different degrees of hyperpipecolic acidemia. Moreover, normal levels of pipecolic acids have been recently reported in some cases of PDE [18]. Therefore, elevations of AASA and P6C were considered to be diagnostic biomarkers only for pyridoxine dependent epilepsy until the recent discovery of increased levels of AASA and P6C also in patients with molybdenum cofactor deficiency and isolated sulfite oxidase deficiency [19,20]. The presence of high amounts of sulfites and sulfocysteine differentiates these deficiencies from PDE.

Measurements of AASA and P6C as well as PA in plasma and urine remain the first-line of testing to identify patients with PDE, followed by the analysis of *ALDH7A1* gene. The levels of these neurotoxic compounds in bodily fluids are also monitored to assess treatment efficacy [11,21–24]. Thus far, few liquid chromatography–mass spectrometry (LC–MS/MS)-based methods for AASA, P6C and PA have been reported (Table S1): the levels of these analytes have been simultaneously measured in plasma [8] while urinary concentrations of AASA and P6C have been determined in two separate LC–MS/MS assays performed under very different extraction and instrument conditions [2,10,25].

Here, we describe a sensitive and reliable method for simultaneous quantification of AASA-P6C and PA in plasma and urine samples. The performance characteristics of the assay as well as the reference ranges in plasma and urine were established. The clinical sensitivity and specificity was determined by analyzing samples from several patients confirmed to have PDE; therefore, this method is suitable for the diagnosis and monitoring of patients with PDE.

2. Material and methods

2.1. Reagents

The following reagents were purchased: hydrochloric acid, 3N in n-butanol (Regis Technologies, Inc.), methanol and acetonitrile (VWR Scientific), de-lipidated plasma (SeraCare life science), ASSURANCE™ Interference Test Kit (Sun Diagnostics), formic acid, pipecolic acid and 5-sulfosalicylic acid dihydrate (Sigma), Amberlyst® 15 dry resin (Fluka), allysine-ethylene acetal (Chiralix), and d9-Pipecolic acid (CDN Isotopes).

2.2. AASA-P6C synthesis

Lacking commercially available standards, the AASA-P6C reference material (a mix of AASA and P6C) was synthesized from allysine-ethylene acetal (AEA) using Amberlyst® 15 bead according to published procedures [2,6,26]. After elution of the synthesized compounds with 25% of ammonium hydroxide, the solution was dried under N2 flow at room temperature and stored at −80 °C. To confirm full conversion of AEA to AASA-P6C, equivalent concentrations of the starting material and the synthesized compounds were analyzed by ion exchange chromatography on a Biochrom HPLC system using conditions as described [27]. Only traces of AEA (<5%) were detected in the synthesized AASA-P6C samples. The efficiency of the conversion of AEA to AASA-P6C was further confirmed by direct flow injection on a Waters Xevo UPLC–MS/MS system.

Reproducibility of AASA-P6C synthesis was evaluated by comparing batches of material synthesized on three different days in triplicate. Equal amounts of synthesized material from the different batches were injected on a Biochrom HPLC system, and the peak areas of AASA and P6C for the different batches were compared. The results of this study showed an intra- and inter-synthesis variability of less than 10% (data not shown). The final concentration of AASA-P6C in reference material was calculated based on the assumption of 100% of synthesis efficiency.

2.3. Calibration and controls

Six non-zero calibrators were prepared at concentrations of 1–200 μmol/L for AASA-P6C and 0.5–100 μmol/L for PA in de-lipidated plasma, and at 2–1000 μmol/L for AASA-P6C and 0.2–100 μmol/L for PA in urine. To determine the relative quantities of AASA and P6C in the reference material, plasma and urine samples spiked with the reference material were injected on a Biochrom HPLC system. Comparison of AASA and P6C peak areas in these samples showed ~1:2 ratio of AASA to P6C in plasma and ~1:3 ratio of AASA to P6C in urine; these ratios were used to assign the concentrations of AASA and P6C in the calibrators prepared in de-lipidated plasma and urine, respectively.

Three quality controls (QC) were prepared by spiking normal plasma with AASA-P6C and PA standards at low (10 μmol/L), medium (50 μmol/L) and high (200 μmol/L) concentrations. Three QCs were prepared in urine at low (10 μmol/L AASA-P6C and PA), medium (100 μmol/L AASA-P6C, 50 μmol/L PA) and high (500 μmol/L AASA-P6C, 100 μmol/L PA) concentrations.

2.4. Plasma and urine specimens

Reference ranges for plasma (n = 185) and urine (n = 288) were established using samples submitted to our laboratory. Only samples with normal findings on routine biochemical genetic tests were used to establish reference ranges. Reference intervals were determined using EP evaluator (David G. Rhoads Associates, Inc) by non-parametric analysis and represent the central 95% (2.5–97.5%) of the populations. A Student's *t*-test (2-tailed) was used to test differences in AASA-P6C and PA concentrations between various age groups. P-value < 0.05 was considered to be significant. Plasma (n = 13) and urine samples (n = 7) from four PDE patients were used for the clinical validation of the assay. All patients were on daily Vitamin B6 supplements at the time of testing. Two patients also received dietary therapy (lysine restriction diet and arginine supplements).

All samples were de-identified and used according to a protocol approved by the IRB of the University of Utah. The samples were stored at −80 °C until analysis.