Journal of Neuroscience Methods 184 (2009) 136–141

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

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

Simultaneous determination of alpha-aminoadipic semialdehyde, piperideine-6-carboxylate and pipecolic acid by LC–MS/MS for pyridoxine-dependent seizures and folinic acid-responsive seizures

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ARTICLE INFO

Article history:
Received 20 May 2009
Received in revised form 13 July 2009
Accepted 15 July 2009

Keywords:
Pyridoxine-dependent seizures
α-Aminoadipic semialdehyde
Pipecolic acid
Antiquitin
δ^1-Piperideine-6-carboxylate
Folinic acid-responsive seizures
ALDH7A1

ABSTRACT

Pyridoxine-dependent seizures (PDS) is an autosomal recessive disorder characterized by seizures presenting in neonates or infants up to 3 years of age which respond to pharmacological doses of pyridoxine. Alpha-aminoadipic semialdehyde dehydrogenase (antiquitin) deficiency was identified as an underlying defect in PDS characterized by accumulation of alpha-aminoadipic semialdehyde (α-AASA) as a specific marker and recently folinic acid-responsive seizures (FRS) were found to be allelic to PDS as the putative mutations were identified in the antiquitin gene (*ALDH7A1*). α-AASA is known to be in reversible equilibrium with its cyclic Shiff base, δ^1-piperideine-6-carboxylate (P6C). Pipecolic acid (PA) is another biomarker often elevated but is not specific to PDS. Here, we developed the liquid chromatography–mass spectrometry (LC–MS/MS) method to determine the analytes of α-AASA, P6C and PA simultaneously in plasma and validated the assay using samples from confirmed cases. This approach eliminates the extra time and expense of running multiple assays and provides valuable information for the rapid diagnosis and treatment of patients with PDS and FRS which potentially could lead to a better outcome with improved quality of life. The stability study showed that α-AASA and P6C were unstable even at −20 °C. A careful sample handling with immediate freezing and testing is required for reliable result.

1. Introduction

Patients with pyridoxine-dependent seizures (PDS) classically present with neonatal seizures. Less commonly, later-onset cases present through the second to third year of life. Seizures in these patients are unresponsive to conventional anticonvulsant therapy but can be controlled with pyridoxine monotherapy (Baxter, 2003; Been et al., 2005; Gospe, 2006; Surtees and Wolf, 2007). PDS is a rare autosomal recessive disease with an estimated incidence between 1:100,000 and 1:750,000 (Baxter, 2003; Plecko et al., 2005; Gospe, 2006; Bok et al., 2007), although this may be an underestimate. PDS is caused by a deficiency of α-aminoadipic semialdehyde dehydrogenase (antiquitin), an enzyme that functions within the cerebral lysine catabolism pathway, leading to an accumulation of α-aminoadipic semialdehyde (α-AASA) that is in reversible equilibrium with its cyclic Shiff base, δ^1-piperideine-6-carboxylate (P6C) (Mills et al., 2006). P6C inactivates pyridoxal-5′-phosphate (PLP), the active form of pyridoxine, by a Knoevenagel condensation reaction, leading to secondary PLP deficiency. As PLP is a co-factor of various enzymes in the central nervous system, seizures in PDS are most probably due to a perturbation in the metabolism of amino acids and neurotransmitters such as glutamate, gamma-aminobutyric acid (GABA), dopamine, and serotonin. Oral pyridoxine supplementation resolves the seizures and must be administered for the rest of patient's life. Elevated pipecolic acid (PA) is another biomarker for PDS disorder, but is non-specific as it could also be elevated in peroxisomal disorders and liver disease (Peduto et al., 2004). Recently, folinic acid-responsive seizures (FRS) were found to be allelic to PDS with the mutations being demonstrated in the antiquitin gene (*ALDH7A1*) (Gallagher et al., 2009).

Determination of α-AASA in PDS patients by LC–MS/MS method were previously published (Mills et al., 2006; Bok et al., 2007; Plecko et al., 2007; Salomons et al., 2007) but with limited methodological information. Quantification of PA by isotope dilution GC/MS is

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doi:10.1016/j.jneumeth.2009.07.019

routinely performed in reference laboratories as a single analyte assay; however, there is little information available regarding the measurement of P6C in PDS. Our goal was to develop a fast and time efficient LC–MS/MS method for simultaneous detection of α-AASA, P6C, and PA for a rapid diagnosis, and subsequent treatment and monitoring of patients with PDS and FRS disorders.

2. Methods

2.1. Materials

HPLC grade water, pentane and ammonium hydroxide Plus were obtained from Fisher Scientific (Pennsylvania, USA). Acetonitrile (ACN) LC–MS Chromasolv® and formic acid (FA) were from Riedel-de Haen (Germany). Acetone Chromasolv® Plus was from Sigma–Aldrich (Missouri, USA), Amberlyst® 15 Dry Resin (Amberlyst) was purchased from Fluka (Pennsylvania, USA), and fluorenylmethoxycarbonyl chloride (FMOC-chloride) was from Advanced ChemTech (Kentucky, USA). PA was from Acros Organics (Belgium), allysine-ethylene acetal (AEA) was purchased from Chiralix (The Netherlands). d_9-Pipecolic acid (d_9-PA) and d_3-α-aminoadipic acid (d_3-α-AAA) were from CDN (Quebec, Canada). Bovine serum was obtained from Invitrogen Corporation (California, USA).

Stock solution of α-AASA (1 mmol/L) and its cyclic Shiff base P6C (3 mmol/L), in equilibrium when in solution, was obtained by complete conversion of 4 mmol/L AEA solution (Rumbero et al., 1995; Mills et al., 2006). 5 mg AEA was deblocked with 15 mg Amberlyst in 1 mL water by mixing for 10 min. Solution was filtered. Remaining Amberlyst beads were washed with 0.5 mL 25% ammonia solution and transferred onto the same filter. The washing and filtering step was repeated twice with 1 mL water each. Deblocking efficiency of AEA by Amberlyst-15 into AASA and P6C in reaction mixture was checked by flow injection into mass spectrometer. Spectra of a mixture before and after reaction were compared. There was no AEA left after the conversion. The obtained solution was further diluted with 3.11 mL water to get total of 6.61 mL of α-AASA/P6C stock solution. Comparison of MS intensities of both compounds in aqueous solution showed 1:3 ratio of α-AASA to P6C. This result was reproducible as tested by multiple injections during several stock preparations. We used this ratio to approximate AASA/P6C concentrations based on assumption that the ionization efficiency of AASA and P6C are close. Molecular ion intensities measured by flow injection into a mass spectrometer showed that amount and ratio of both compounds in stock solution stored at 4 °C were stable for at least 7 days. PA stock solution of 7.74 mmol/L (1 g/L) was prepared in methanol and stable at −20 °C for at least 6 months. We also tested possible oxidation of AASA into α-aminoadipic acid (α-AAA) right after reaction and in regular time increments later. We did not find any evidence of α-AAA present in reaction mixture.

2.2. Standard and control samples

Standards were prepared in bovine serum by spiking both α-AASA/P6C and PA stock solutions to add α-AASA concentrations of 0.1, 0.2, 1, 5, and 10 μmol/L, P6C concentrations of 0.3, 0.6, 3, 15, and 30 μmol/L, and PA concentrations of 0.5, 1, 5, 25, and 50 μmol/L. Different stock solutions of the same concentrations were used for quality controls preparation in bovine serum. Final concentration added in three quality control (QC) samples was 0.5, 2, and 8 μmol/L of AASA, 1.5, 6, and 24 μmol/L of P6C, and 2.5, 10, and 40 μmol/L of PA. Calibrators and QC samples were stored at −80 °C up to 2 months.

2.3. Patient samples

Blood specimens from five previously described patients with neonatal-onset PDS with heterozygous mutations in *ALDH7A1* were obtained at the time of routine diagnostic testing (Bennett et al., 2009). The patient's age at the time of the clinical diagnosis of PDS, the patient's current age at the time of blood sampling, the current pyridoxine dose, and the *ALDH7A1* mutations are listed in Table 1. Immediately after the blood draw, the specimens were anonymized, and the plasma was frozen and then stored at −80 °C.

2.4. Sample preparations

All calibrators, QCs, and patient samples (heparinized plasma) were processed as follows. 100 μL of sample was precipitated with 200 μL of ACN containing stable isotope labeled internal standards (IS; 2.9 μmol/L of d_9-PA, and 12.2 μmol/L of d_3-α-AAA), vortex-mixed and centrifuged. 200 μL of supernatant was combined with 50 μL of borate buffer (0.4 mol/L, pH 8.5) and 20 μL of 100 mmol/L FMOC-Cl in acetone; these conditions were further optimized from a previously published protocol (Einarsson et al., 1983). The mixture was stirred for 11 min to form FMOC-derivatives. The reaction

Table 1
Patient's results and reference range.

Samples[a]	α-AASA (μmol/L)	P6C (μmol/L)	PA (μmol/L)	Mutation	Age at time of clinical PDS diagnosis (months)	Current age (years)	Current daily dose of pyridoxine
Patient A	7.0	28.4	2.1	750g > a, 109 −9t > g 109 −15t > c	10	11	450 mg 13.7 mg/kg/day
Patient B	3.0	20.3	3.9	750g > a, 109 −9t > g 109 −15t > c	Neonate[b]	3	250 mg 14.9 mg/kg/day
Patient C	3.9	15.5	7.3	750g > a, 1121 InsA	2	7	450 mg 18.2 mg/kg/day
Patient D	0.9	4.5	6.9	c.[del −1-3, IVS6)], 1195g > c (E399Q)	1	4	500 mg 26.9 mg/kg/day
Patient E	5.8	3	15.9	1195g > c, 1429g > c	Neonate	5	400 mg 18 mg/kg/day
Normal, <1 week (n = 5)	<0.3	0.6–2.6	0.6–2.6				
Normal, >1 week (n = 20)	<0.2	0.1–1.7	0.1–3.0				

ND: not determined.

[a] Clinical features of these five cases have been described previously (Bennett et al., 2009).

[b] Younger sibling of Patient A.