Biochemical diagnosis of aromatic-L-amino acid decarboxylase deficiency (AADCD) by assay of AADC activity in plasma using liquid chromatography/tandem mass spectrometry

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ABSTRACT

Aromatic l-amino acid decarboxylase (AADC, EC 4.1.1.28) deficiency is a rare genetic disorder characterized by developmental delay, oculogyric crises, autonomic dysfunction and other problems, caused by biallelic mutations in the *DDC* gene leading to deficient activity of aromatic l-amino acid decarboxylase, an enzyme involved in the formation of important neurotransmitters, such as dopamine and serotonin. A clinical development program of gene therapy for AADC deficiency is ongoing. An important step for the success of this therapy is the early and precise identification of the affected individuals, but it has been estimated that around 90% of the cases remain undiagnosed. The availability measurement of the AADC activity is mandatory for an accurate biochemical diagnosis. Based on these statements, our objectives were to develop a liquid chromatography tandem mass spectrometry (LC-MS/MS) method suitable for the determination of the AADC activity, and to evaluate its capacity to confirm the deficiency of AADC in potential patients in Brazil. The AADC activities were measured in plasma samples of seven AADC deficient patients and 35 healthy controls, after enzymatic reaction and LC-MS/MS analysis of dopamine, the main reaction product. The results obtained showed clear discrimination between confirmed AADC deficient patients and healthy controls. The method presented here could be incorporated in the IEM laboratories for confirmation of the diagnosis of when a suspicion of AADC deficiency is present due to clinical signs and/or abnormal biomarkers, including when an increased level of 3-*O*-methyldopa (3-OMD) is found in dried blood spots (DBS) samples from high-risk patients or from newborn screening programs.

Table 1
Clinical signs and genotype of the AADCD patients studied.

Patient number	Gender	Age (years)	Genotype
1	Female	3.8	p.Arg347Gln/Trp121Arg
2	Male	3	p.Ser147Ile/Val60Ala
3	Male	10.1	p.Arg347Gln/Arg347Gln
4	Male	11.5	p.Arg347Gln/Arg347Gln
5	Male	2.7	p.Gln190Argfs*13/Leu288Pro
6	Female	1.4	c.1055del:p.(Pro352Hisfs*9
			c.1410c > G:p.(Asp470Glu)
7	Female	4.7	c.1040G > A; p.Arg347Gln

1. Background

Aromatic l-amino acid decarboxylase deficiency (AADCD) is a rare and severe genetic disorder caused by biallelic mutations in the *DDC* gene, leading to decreased activity of aromatic l-amino acid decarboxylase (AADC), an enzyme involved in the formation of important neurotransmitters, such as dopamine and serotonin [1]. Clinical signs of the disease usually include development delay, oculogyric crises, and autonomic disfunction, among other features. Vanyllactate could be increased in the urine organic acids profile. A clinical development program of gene therapy for AADCD is ongoing. It is of consensus that an important step for the success of this therapy is the early and precise identification of the affected individuals [2].

Useful methods in the diagnostic process are the sequencing of the *DDC* and the measurement of specific metabolites in the cerebrospinal fluid, which is not widely available. In 2014, Chen et al. [3] reported a novel liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay to measure the levels of 3-*O*-methyldopa (3-OMD) in dried blood spots (DBS) samples, a biomarker which was found to be increased in patients with AADCD. Later, this assay was further validated by Kubaski et al. [4]. However, as 3-OMD levels are not 100% specific to AADCD, thus the diagnosis should be ultimately based on the quantification of the AADC activity.

Currently, AADC enzyme assay is based on the enrichment of the natural AADC substrates, L-Dopa or 5-hydroxy-tryptophan, followed by the analysis of the reaction products, dopamine or serotonin, by HPLC [5–7]. Nevertheless, there are several reports related to the determination of biogenic amines by LC-MS/MS [8–10], being this alternative more suitable for a precise and sensitive characterization and measurement of the reaction products of the AADC activity. Based on these statements, our objectives were to develop a confirmatory method based on LC-MS/MS to measure the AADC enzyme activity, in order to identify the deficiency of AADC in suspected patients, and have them eligible for the proper disease management with the measures available.

2. Material and methods

2.1. Samples

Plasma samples were collected from AADCD patients and shipped to the Medical Genetics Service from Hospital de Clínicas de Porto Alegre (HCPA) with dry ice. All patients had *DDC* gene sequencing that confirmed the presence of pathogenic variants of AADCD. The mean age of the patients was 4.7 years of age, with a range of 1.4 to 11.5 years (Table 1). Exclusion criteria were samples improperly collected, transported, or stored, as this could lead to enzyme degradation. All tests were performed within the diagnostic activities of the IEM Brazil Network. Plasma samples of the seven AADCD patients were compared with plasma samples from 35 healthy controls, with mean of age of 22 years of age, range of 2 months to 63 years. All samples were stored at −20 °C before the analysis. Ethical approval was granted by the Research Ethics Committee of HCPA (protocol # 2006–0351).

2.2. Chemical and reagents

Ultrapure water was obtained from the Milli-Q system from Millipore (Bedford, USA). LC-MS grade methanol, ultrapure formic acid, pyridoxal-5-phosphate, dopamine hydrochloride, dopamine-1,1,2,2-d4 hydrochloride, and 3,4-dihydroxy-*L*-phenylalanine (L-dopa) were purchased from Sigma-Aldrich (Saint Louis, USA), and HPLC grade acetonitrile was purchased from JT Baker® (Radnor, USA). A calibration curve with 5, 25, 50, 75, and 100 nanomoles/mL of dopamine was prepared, and D4-dopamine was used as a surrogate standard.

2.3. AADC enzyme assay

Assay reagents include sodium phosphate buffer 167 mM pH 7, pyridoxal-5-phosphate 0.7 mM, dopamine, dopamine-d4 50nM, and L-dopa 20 mM. The procedure was performed according to Heales [7], replacing perchloric acid with acetonitrile for the protein precipitation step. Briefly, 100 μL of plasma, 50 μL of pyridoxal phosphate, and 300 μL of phosphate buffer were incubated for 2 h at 37 °C in a dry bath with a shaker (600 RPM). After this time, 50 μL of L-dopa 20 mM was added and further incubated at 37 °C for 90 min. After incubation, 500 μL of acetonitrile was added to stop the reaction. Then, 20 μL of dopamine-d4 50 nM was added to the sample followed by centrifugation at 14,000 RPM for 10 min. The supernatants were kept at 4 °C until injection into the LC-MS/MS. The level of activity of AADC was related to the amount of dopamine (in nanomoles/min/L) produced after the addition of L-dopa in the enzyme assay.

2.4. Dopamine measurement by LC-MS/MS

The measurement of dopamine was performed on an ACQUITY UPLC H Class PLUS coupled to a triple quadrupole mass spectrometer Xevo TQ-S micro from Waters (Milford, USA). Separation occurred on an UPLC BEH C18 1.7uM (2.1 × 50 mm) from Waters (186002350). The mobile phase was a gradient elution of water with 0.1% formic acid (A) to acetonitrile with 0.1% formic acid (B). The flow rate was 0.4 mL/min, and the gradient was as follows: at 0 min. 99.5% of A, 1 min. 99.5% of A, 1.01 min. 5% of A, 2 min. 5% of A, and 2.1 min. 99.5% of A, and 3 min 99.5% of A. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode with quantification transition of 154.1/137.1, and confirmation of 154.1/191.1 and 154.1/91 for dopamine, and single reaction monitoring of 158.1/141.1 for D4-dopamine, with a general collision energy of 7 V. The ionization source was an electrospray operated in positive mode and temperature of 150 °C, capillary of 3.50 kV, cone 27 V, dessolvation temperature 650 °C, dessolvation 950 L/h, and cone gas of 150 L/h. 1uL of each sample was injected with a running time of 3 min.

2.5. Validation of analytical procedures

The validation was performed to fit the method purpose, based on parameters proposed by ANVISA Resolution RDC N. 27/2021, and Food and Drug Administration [11,12], in terms of selectivity, considering the retention time and peak area of analytes added to the tested sample, and calculating ion ration for dopamine in samples and calibration curves; linearity was evaluated by calculating the correlation coefficient (R^2), intercept, and slope of the regression line at five concentration levels ranging from 5 to 100 ng/ml; precision, calculated by replicate analysis of three different concentrations (5, 25, and 75 ng/mL) in the same day for intra-assay, and in two different days for inter-assay tests. In this case, three separate preparations of each dilution were measured 5 times. Duplicate samples from 7 healthy controls were also assayed to verify intra and inter-assay variations. The coefficient of variation (CV) was calculated as the standard deviation divided by mean x 100. The lower limit of quantitation (LLOQ) was defined as the lowest level of the signal with a signal-to-noise ratio above 2:1, and a lower limit of
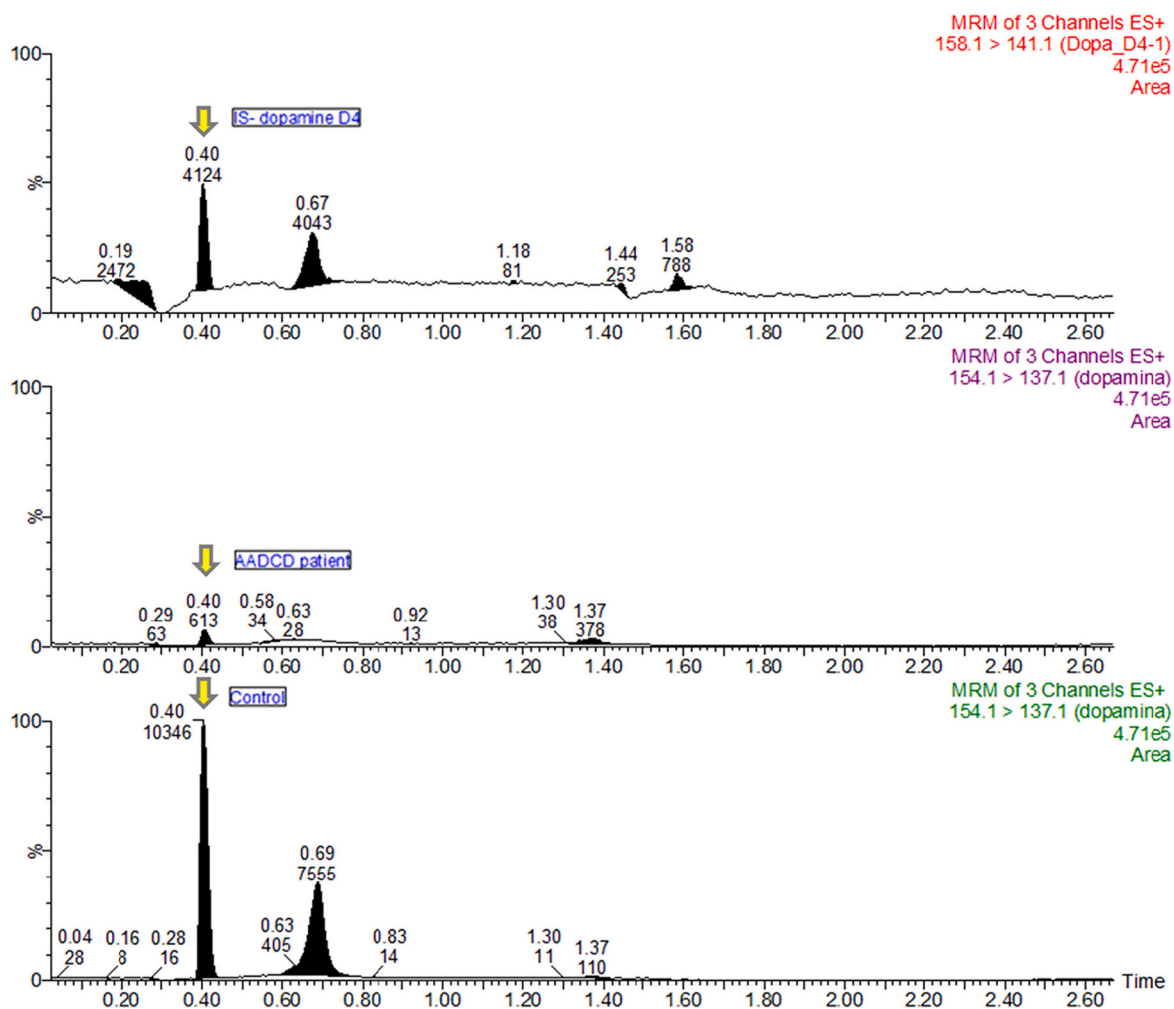
Fig. 1. Peak area of dopamine in an AADCD patient and healthy control. The retention time is 0.40 min as indicated by the retention time in the internal standard (S-dopamine-D4). The peak area in the AADCD patient is 613 (AADC enzyme activity of 0 nmol/min/L, and a healthy subject has a peak area of 10,346 (AADC activity of 48.24 nmol/min/L).

detection (LLOD) as a signal-to-noise ratio above 10.

2.6. Statistical analysis

Statistical analyses were performed using GraphPad Prism v.8.0. Mann-Whitney test was used to evaluate differences between the AADC enzyme activities of healthy controls and patients at the levels of significance of 0.05.

3. Results

3.1. Method validation

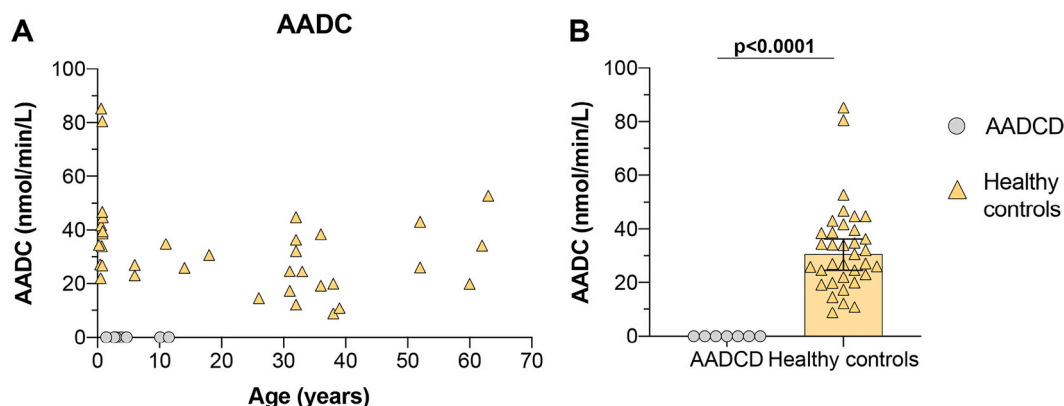According to the above referenced ANVISA and FDA validation

Fig. 2. Levels of AADC activity in AADCD patients and healthy controls depicted by age (A) and group (B).

guidelines, precision must have a CV <15%, calibration curves with a correlation coefficient above 0.99, LLOD and LLOQ above at least the ratios of 2:1 and 10:1, respectively. The proposed method presented here shows a good agreement with the mentioned validation guidances. Precision, in terms of intra-assay and inter-assay variations, is shown in the supplementary materials, Tables 1S–3S. LLOQ was defined as 5 ng/ mL and LLOD was 0.25 ng/mL. The calibration curves were linear with a regression coefficient (R^2) above 0.999, with a regression equation of y $= 0.034\times + 0.101$ (Fig. 1, supplementary materials). Method selectivity was considered satisfactory, with no interfering compounds observed for dopamine transitions and its internal standard after additions of reagents in all samples analyzed. Acetonitrile, instead of perchloric acid, was used to stop the enzyme reaction, and to avoid possible ion suppression in the LC-MS/MS system [13].

3.2. Dopamine concentration and AADC activity

Chromatographic data shows that all the patients had lower peak areas for dopamine compared to healthy controls. Fig. 1 shows the dopamine peak area in a healthy control compared to an AADCD patient. The healthy control has a peak area of 10,346 for dopamine representing an AADC activity of 48.24 nmol/min/L, and the AADC patient has a peak area of 613 with an undetectable AADC activity.

The mean level AADC activities after the L-Dopa enrichment in the enzyme assay was undetectable in the seven AADCD patients, and the activities in the 35 healthy controls were 32.66 ± 14.60 nmoL/min/L (range: 8.8 to 85.2). Fig. 2 shows the high level of difference in the activities of AADC in both groups ($p < 0.0001$, 2-tailed Mann-Whitney test).

4. Discussion

The AADC activity was measured in plasma samples followed by UPLC-MSMS analysis of dopamine, the main reaction product of the enzyme assay. The data obtained showed clear discrimination between confirmed AADCD patients and healthy controls. These results are in accordance to previous reports, with slight differences [5–7]. To our knowledge, there are no reports in the bibliography concerning the quantification of AADC activity using LC-MS/MS to measure the final product of the reaction.

When compared with the original HPLC-based enzyme assays, the use of LC-MS/MS is highly recommended since this technology improves the correct identification and quantification of the reaction products, preventing possible false positive or negative results. Moreover, the use of this methodology allow the evaluation of other metabolites related to the AADC activity in the same assay, just by adding their correspondent MRM parameters in the LC-MS/MS program.

The enzyme assay here presented could be incorporated into the IEMs laboratories for diagnostic confirmation of AADC deficiency. The assay should be performed when a clinical suspicion of AADCD is present, or as a confirmatory procedure, after the detection of increased levels of 3-OMD in DBS from newborn or high-risk screening.

As a next step, we envision the measurement of AADC activity assay in DBS samples. This, coupled with the 3-OMD determination in the same DBS, would aid early and precise detection of AADCD, and its immediate enrollment in the therapy programs. Moreover, samples of neonatal screening programs could be assayed for retrospective diagnosis, or as confirmatory diagnosis in the same sample after a detection of elevated 3-OMD [14–16].

Finally, the combined enzyme assay with UPLC-MS/MS presented here could be the base for other enzyme studies concerning the metabolism of the biogenic amines.

Data availability

The data that has been used is confidential.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi. org/10.1016/j.ymgmr.2022.100888.

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