



Evaluation of 3-O-methyldopa as a biomarker for aromatic L-amino acid decarboxylase deficiency in 7 Brazilian cases

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ABSTRACT

Aromatic L-amino acid decarboxylase (AADCD) deficiency is an autosomal recessive neurometabolic disorder, caused by biallelic mutations in the *DDC* gene, that impairs the synthesis or metabolism of neurotransmitters leading to severe motor dysfunction. The main clinical signs are oculogyric crisis, hypotonia, hypokinesia, and dystonia. The biochemical diagnosis can be performed in cerebrospinal fluid by neurotransmitter analysis, which requires an invasive lumbar puncture, and the sample needs to be shipped frozen to a reference laboratory, usually across a country border. Measurement of AADC activity in plasma is also possible, but available in a few labs globally. 3-O-methyldopa (3-OMD) is a catabolic product of L-dopa and it is elevated in patients with AADC deficiency. The quantification of 3-OMD can be performed in dried blood spots (DBS), a sample that could be shipped at room temperature. 3-OMD levels of AADC patients and controls were quantified in DBS by liquid chromatography tandem mass spectrometry. DBS samples from 7 Brazilian patients previously diagnosed with AADC were used to validate the 3-OMD quantification as a screening procedure for this condition. All AADC patients had at least a four-fold increase of 3-OMD. Thus, 3-OMD seems to be a reliable marker for AADC, with potential use also in the newborn screening of this disease.

1. Introduction

Aromatic L-amino acid decarboxylase deficiency (AADCD, MIM#608643) is an autosomal recessive disorder caused by biallelic mutations in the *DDC* gene [1]. AADC enzyme is required for the synthesis of dopamine and serotonin [2], leading to a severe deficiency of dopamine, serotonin, norepinephrine, and epinephrine [3]. In the presence of the AADC enzyme, dopa is converted into dopamine by AADC. When AADC is deficient, L-dopa accumulates and is methylated by catechol *O*-methyltransferase, generating increased levels of 3-O-

methyldopa (3-OMD) [4–6].

The clinical signs are usually evident in the first few months of life and the most frequent symptoms are oculogyric crisis, hypotonia, hypokinesia, and dystonia. The majority of affected patients present a severe phenotype, but milder cases can occur [7–13]. The overall incidence is not well known, but a newborn screening (NBS) program in Taiwan has reported an incidence of 1:32,000 [9,14,15].

The diagnosis is based on the assay of enzyme activity in plasma and the analysis of levels of neurotransmitters in cerebrospinal fluid (CSF). Taking into account the alternative route of DOPA in the lack of AADC,

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3-OMD has been shown as a reliable marker for the diagnosis of AADC deficiency in urine, plasma, and dried blood spots (DBS) [4,14,16–18].

In the present study, we analyzed DBS samples of 7 AADC patients and 35 non-AADC controls to validate the 3-OMD quantification at our center.

2. Materials and methods

2.1. Materials

3-O-methylidopa (3-OMD) (M4255) was from Sigma-Aldrich (Saint Louis, USA). The deuterated internal standard L-3-(4-Hydroxy-3-methoxy- d_3 -phenyl)alanine (D-6782) was from C/D/N Isotopes (Quebec, Canada), formic acid (F0507) (reagent grade), and methanol (1.06018) (LC grade) were from Sigma-Aldrich (Saint Louis, USA). Sterile clear 96-well filter plates 0.45 μ M (MAIPS4510) were from Millipore-Sigma (Burlington, USA). 96-well polypropylene sample collection plates (186002643) were from Waters (Milford, USA). Working internal standard (3-OMD-D3 7.5 ng/mL) and the calibration curve (20–4380 ng/mL) were prepared as described by Chen et al., 2014 [4].

2.2. Samples

Blood was collected with EDTA by venipuncture and 150 μ L of blood was spotted onto filter paper. DBS from 7 patients with a prior diagnosis (biochemical and molecular analysis) of AADC, 35 non-AADC controls, and 22 general newborns were collected. Informed consent was obtained at each Institute for all the patients and control samples according to IRB approval. All samples were shipped (with desiccant, but without refrigeration) and stored at -20°C after arrival at the laboratory.

2.3. Sample preparation

Samples were prepared according to the method previously described by Chen et al., 2014 [4] with slight modifications. Briefly, a single 3.2 mm disc was punched into a 96-well polypropylene plate with 200 μ L of 7.5 ng/mL 3-OMD-D3 and the plate was incubated at 37°C with shaker for 3 h, as well as the spiked DBS used for the calibration curve. The supernatant was then transferred to the 0.45 μ M filter plate coupled with clean 96-well plates and centrifuged at 2000 RPM for 3 min. The filtrate was collected in the receiver plate and 1.5 μ L was injected into the liquid chromatography tandem mass spectrometer [4].

2.4. LC-MS/MS

The mass spectrometer was a Xevo TQ-S micro from Waters (Milford, USA). Separation occurred on an LC pursuit PFP 3 μ M 100×2.0 mm column from Agilent (A3051100X020) (Palo Alto, USA) that was kept at 40°C . The method was first developed by Chen et al., 2014 [4]. The mobile phase was a gradient elution of water with 0.1% formic acid (solution A) to methanol with 0.1% formic acid (solution B). The flow rate was 0.5 mL/min, and the gradient was as follows: at 0 min. 95% solution A, 1 min. 0% solution A, 1.5 min. 0% solution A, 1.60 min. 95% solution A, and 2.20 min 95% solution A. The mass spectrometer was operated with electrospray ionization in the positive ion mode with multiple reaction monitoring (MRM). The MS/MS parameters were: source temperature of 150°C , capillary 3 kV, cone 30 V, collision (20 V and 15 V for 3-OMD and 3-OMD-D3, respectively), desolvation temperature 600°C , desolvation 1000 L/h, cone 50 L/h. Precursor and product ions (m/z) were used to quantify as follows for 3-OMD 212.2, 152.9; and 3-OMD-D3 215.1, 155.9 [4] (Sup 1). 1.5 μ L of each sample was injected with a running time of 2.20 min.

Table 1

Coefficient of variation intraday and interday.

Concentration	CV intra ¹	CV intra ²	CV inter
Std 9 (4380 ng/mL)	10.04%	8.63%	9.95%
Std 6 (547.6 ng/mL)	10.22%	8.44%	10.12%

Table 2

Distribution of AADC patients according to gender, age, 3-OMD concentrations and genotype.

ID	Gender	Age	3-OMD ng/mL	Genotype
1	Female	2.3	1441	p.Arg347Gln/Trp121Arg
2	Male	2.5	2348	p.Ser147Ile/Val60Ala
3	Male	8.11	12,302	p.Arg347Gln/Arg347Gln
4	Male	10.2	1504	p.Arg347Gln/Arg347Gln
5	Male	1.8	1587	p.Gln190Argfs*13/Leu288Pro
6	Male	19.8	1367	p.Gln190Profs*13/Arg347Gln
7	Male	10.3	2607	p.Arg347Gln/Arg347Gln

2.5. Method validation

Imprecision was calculated by replicate analysis of two different concentrations (std 9 [4380 ng/mL], and std. 6 [547.5 ng/mL]). Seven separate preparations of each dilution were measured 7 times, and the coefficient of variation (CV) was calculated as the standard deviation divided by mean \times 100. The lower limit of quantitation (LLOQ) was defined as the lowest level of the signal with an accuracy of better than 20% and the lower limit of detection (LOD) as a signal to noise ratio of <10 according to the Food and Drug Administration [19].

2.6. Statistical analysis

Sensitivity and specificity were calculated using Graphpad Prism 7.0a. Patient samples were compared to controls using student *t*-test at the level of significance of 0.05 performed using Graphpad Prism 7.0a.

3. Results

3.1. Coefficient of variation (CV), lower limit of quantitation (LLOQ), lower limit of detection (LOD), calibration curve, sensitivity, and specificity

Imprecision was calculated by seven separated replicate analysis of two different concentrations (std 9, and std. 6, respectively) (Table 1).

LLOQ was defined as 274 ng/mL and LOD was 34.2 ng/mL. The calibration curves were linear (Sup 2) with a regression coefficient (R^2) of 0.997. Sensitivity and specificity were 100%.

3.2. Patient demographics

A total of 7 patients (Table 2), 35 non AADC controls, and 22 general newborns were analyzed. All patients had AADC deficiency confirmed in plasma, with pathogenic mutations identified in the *DDC* gene (Table 2). All patients presented with severe hypotonia, oculogyric crisis, and autonomic features. Some patients also presented increased vanillylactate in organic acids.

The mean age for the AADC patients was 7.8 years of age (range: 1.8–19.8 years of age) (Fig. 1). The mean age for the non-AADC controls was 23.2 years of age (range: 1 month to 66.7 years of age) (Fig. 1).

The mean 3-OMD levels in the patient samples was 3308 ng/mL (range: 1441–12,302 ng/mL) (Fig. 2). The mean 3-OMD levels in the controls was 20 ng/mL (Fig. 2), and the mean levels for the general newborns was 98 ng/mL (range: 9–278 ng/mL) (Fig. 2). The 3-OMD levels were significantly higher in patients compared to non-AADC controls ($p < 0.0001$) and general newborns ($p = 0.0005$) (Fig. 2).

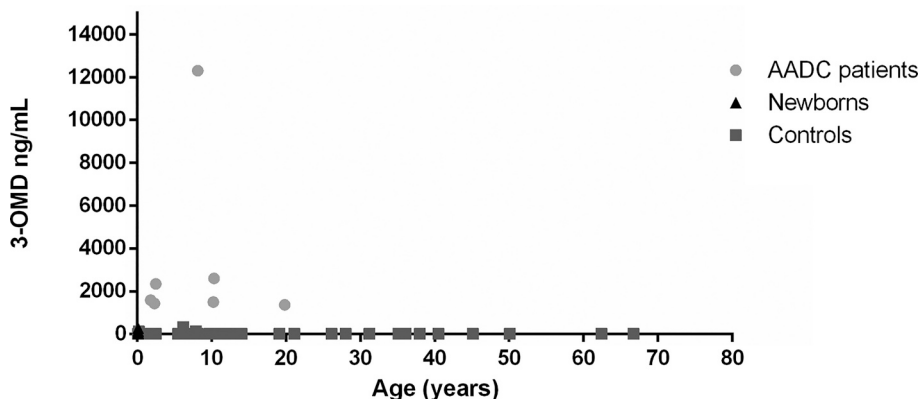


Fig. 1. Distribution of 3-OMD levels in AADC patients, general newborns and non-AADC controls according to age.

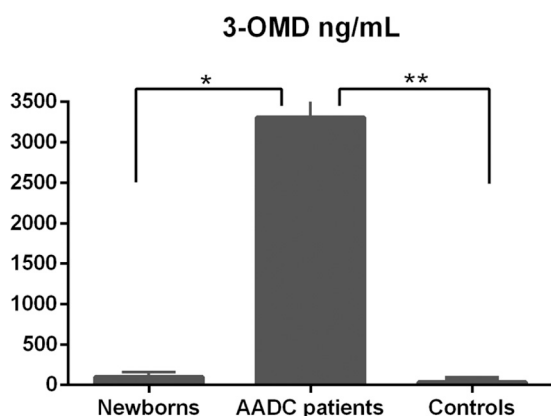


Fig. 2. Average levels of 3-OMD in AADC patients, general newborns and non-AADC controls. The levels of 3-OMD were significantly higher compared to newborns* ($p = 0.0005$) and to controls ** ($p < 0.0001$).

The most frequent genotype was p.Arg347Gln/Arg347Gln found in three patients (Table 2). It is important to mention that patient 3 was under levodopa treatment which lead to an increase in the 3-OMD levels (12,302 ng/mL) compared to the other patients (1504 ng/mL, and 2607 ng/mL, respectively) (Table 2).

4. Discussion

3-OMD has been shown as a reliable and stable marker for the identification of AADC patients [4,14,17,18,20]. It is also applicable to several sample types such as urine [17], plasma [16], and DBS [4,14,18]. In addition to being a convenient sample for the screening of patients at risk. In large countries like Brazil, it is highly valuable to set up methods using DBS because it is a much easier sample to ship and store. Another advantage of this validated method is its applicability to the newborn screening of AADC [4,14,18] which can provide an early diagnosis and a more realistic disease incidence, not influenced by underdiagnosis and misdiagnosis.

Several clinical trials are currently being performed for the development of a specific treatment for AADC, including gene therapy [9,21,22]. If these protocols demonstrate safety and efficacy, they could be approved, which will be a major step for the inclusion of AADC among the diseases recommended for newborn screening, since it is technically feasible as demonstrated by the validation of the current method [4,14]. It is also important to mention that with the identification of younger patients with very heterogeneous genotypes, new gene therapy guidelines will be needed in order to decide when and how to treat patients with milder disease phenotypes (once gene therapy studies

have only included patients with severe phenotypes).

We should note that 3-OMD levels can also be increased in pyridoxine 5-phosphate oxidase (PNPO) deficiency [6], in vitamin B₆ biosynthesis defects [14], and thus the differential diagnosis should be performed (with further biochemical and/or molecular genetics methods). It is important to mention that newborns with PNPO present with seizures, which is uncommon in newborns with AADC.

In our center, we have developed an algorithm for the high-risk screening of AADC with the quantification of 3-OMD in DBS followed by DNA extraction from the same DBS to perform molecular analysis by next-generation sequencing of a customized gene panel, including the *DDC* and the *PNPO* genes. In samples with high 3-OMD levels and less than two pathogenic variants identified in the *DDC* gene, plasma will be collected for the assay of the activity of the AADC enzyme. It is also important to ask the mothers of the infants included in the NBS if any medication that could increase the levels of 3-OMD was taken.

No false negatives or PNPO samples were identified in our validation study, but we had a limited sample size (only 7 AADC patients). As more positive samples are identified, more precise cutoffs will be established.

5. Conclusion

We have validated a very sensitive and fast method for the quantification of 3-OMD that can shorten the long diagnostic odyssey faced by patients with AADC in a less invasive manner than having a lumbar puncture performed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ymgmr.2021.100744>.

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