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Quantification of hypoglycin A as butyl ester

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ABSTRACT

L-α-amino-methylenecyclopropyl propionic acid (Hypoglycin A, HGA) has been found to be the toxic compound in fruits of the *Sapindaceae* family causing acute intoxication when ingested as food or feed. Clinical symptoms are consistent with acquired multiple acyl-CoA dehydrogenase deficiency (MADD). Ultra performance liquid chromatography-tandem mass spectrometry was used to measure HGA after butylation. Sample volumes were 10 μL for serum and 20 μL for urine. Internal standard for HGA was d3-leucine, samples were plotted on a 7-point linear calibration curve. Coefficients of variation were <15% at 0.01 μmol HGA/L and ≤4.1% at 10 μmol/L. R^2 values for linearity were ≥0.995.

In order to quantify non-metabolized HGA together with some of its metabolites plus a spectrum of acyl glycines and acyl carnitines typical for acquired MADD in one single analysis HGA measurement was integrated into a method which we previously developed for metabolites of HGA and acyl conjugates.

The new method is suitable for biochemical diagnosis of Ackee fruit poisoning or atypical myopathy in horses and for forensic purposes in cases of suspected HGA poisoning.

1. Introduction

1.1. Background

Hypoglycin A (L-α-amino-methylenecyclopropyl propionic acid, HGA) is the plant toxin recently shown to cause atypical myopathy (AM), also called seasonal pasture myopathy, in grazing horses ingesting maple seeds [1–4]. HGA was first isolated by Hassall and Reyle [5] and has for long been known as the toxic compound causing Jamaican vomiting disease by consumption of fruits of the Ackee tree [6–8]. Maple trees which belong to the genus *Acer* as well as the Ackee tree (genus *Blighia*) are members of the *Sapindaceae* family in the *Sapindales* order of plants.

A first attempt to quantitatively measure HGA in blood and plasma was undertaken by Fincham, however, the method, although precise and reproducible, was not sensitive enough to find HGA in blood samples obtained from 3 children admitted to hospital for symptoms suggestive of Ackee fruit poisoning [9]. It was the introduction of mass spectrometry into amino acid analysis that has recently greatly increased the sensitivity of HGA measurements. Carlier et al. [10] identified HGA after dansyl derivatization by ultra-high performance liquid chromatography-high resolution tandem mass spectrometry. The substance was extracted from 250 μL of whole blood on HILIC cartridges. The detection limit was 2.45 nmol/L. In two serum samples of horses suffering from atypical myopathy they found 3.16 and 0.62 μmol/L.

Bochnia et al. [3] applied a method based on an amino acid analysis published by Ziegler et al. [11]. Derivatisation reagent was 9-fluorenylmethoxycarbonyl chloride. The resulting amino acid derivatives were purified by solid phase extraction and separated by reversed phase HPLC, subsequently quantified by LC-ESI–MS/MS using multiple reaction monitoring. The lowest concentration found in horse serum was 0.26 μmol/L. Another very sensitive amino acid method recently published by Boemer et al. [12] made use of derivatization with a commercially available aTRAQ® kit, quantification of HGA was then done on a tandem mass spectrometer. The authors describe linearity in the range of 0.085–50.0 μmol/L.

1.2. Objective of study

The objective of our present study was to evaluate quantitative measurement of HGA as a butyl ester by UPLC–MS/MS. The aim was to add HGA as an additional analyte to a method we established for the diagnosis of acquired multiple acyl carnitine dehydrogenase deficiency (MADD) due to HGA intoxication [13]. This would

Abbreviations: AM, atypical myopathy in horses; HGA, hypoglycin A (L-α-amino-methylenecyclopropyl propionic acid); MADD, multiple acyl carnitine dehydrogenase deficiency; MCPA, methylenecyclopropylacetic acid.

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allow us to measure HGA and its metabolites plus acylcarnitines and acylglycines simultaneously after only one simple extraction and derivatisation step.

2. Material and methods

2.1. Reagents

Authentic hypoglycin A (Toronto Research Chemicals, Toronto, Canada) was used as reference. Internal standard for HGA measurement was d3-leucine (H. ten Brink, Academisch Medisch Center (AMC), Amsterdam, The Netherlands). Methanol, acetonitrile, formic acid and deionised water were commercial products of highest quality made for liquid chromatography and mass spectrometry (Biosolve BV, Valkenswaard, The Netherlands). Butanolic HCl (3N) was available as highest analytical quality (Fluka, Deisenhofen, Germany).

2.2. Preparation of internal standard and HGA calibrators

A stock solution of d3-leucine was prepared in methanol and stored at −20 °C. The final concentration of d3-leucine in methanol used for the extraction of samples was 0.134 μmol/l. For the preparation of HGA calibrators serum and urine (prediluted to 7600 μmol/L creatinine) were spiked with HGA stock solution to give concentrations of 0, 0.01, 0.03, 0.06, 0.1, 1.0, 10 μmol/L HGA.

2.3. Preparation of samples

For the evaluation of HGA measurement urine and serum of a healthy horse were used. The samples were collected for diagnostic workup unrelated to atypical myopathy. HGA was added to achieve appropriate concentrations. Urine was prediluted to give a creatinine concentration of 7600 μmol/L. Linearity and precision were determined in serially diluted samples of HGA prepared in urine and serum. We used the same concentrations as described for the calibration curve.

Serum (10 μL) or diluted urine (20 μL) were each extracted with 300 μL methanolic internal standard solution, vortexed and centrifuged for 10 min at RCF 17,000. Of the clear supernatant 200 μL were transferred to a microtiter plate and dried at 65 °C under a gentle stream of nitrogen. The residue was treated with 50 μL 3 N butanolic HCl for 15 min at 65 °C and dried again at 65 °C. The dried material was resolved in 200 μL methanol/water (80:20 vol/vol). This solution was further diluted 1:5 with water. 90 μL were transferred to a 384 microtiter plate, centrifuged at RCF 17 000 and then used for UPLC–MS/MS analysis. A total of 100 min was needed to prepare a series of 20 samples.

2.4. UPLC–MS/MS

The method used for the quantification of HGA was exactly the same as recently described for acyl conjugates [13]. In brief: Analysis was done on a Xevo UPLC–MS/MS system (Waters, Eschborn, Germany). 5 μL of the sample solution were injected onto an Acquity UPLC BEH C18 1.7 μm, 2.1 × 50 mm column (Waters). We used a gradient composed of acetonitrile/water modified by 0.1% formic acid and 0.01% trifluoroacetic acid.

The analyzed transitions [*m*/*z*] were 198.1 > 73.9 for the butyl ester of HGA and 191.0 > 89.0 for butylated d3 leucine. The butyl esters were detected in ESI positive mode by multiple reaction monitoring (MRM) mode. A ratio was calculated from the signals obtained for HGA and the internal standard d3-leucine, for diagnostic samples the ratios were plotted on the 7-point linear calibration curve described above.

Table 1

HGA μmol/L	Urine CV%		Serum CV%	
	Intraday	interday	intraday	interday
0.01	14.8	14.9	12.9	14.1
0.03	9.0	13.7	14.4	14.2
0.06	9.2	11.4	10.7	12.0
0.10	9.1	9.8	7.5	9.2
1.00	3.8	5.2	5.9	5.1
10.00	3.4	4.9	4.0	4.8

Evaluation of imprecision of HGA quantification in urine and serum of a healthy horse spiked with HGA.
CV%: coefficient of variation in percent (8 measurements each).
Table 2

2.5. Integration of HGA measurement into the analysis of a spectrum of acyl conjugates

In order to demonstrate the compatibility of the new method for HGA quantification with our previously published method [13] we added d3-octanoyl, d7-butyryl, and d9-isovaleryl carnitines, and d3-valeryl and d3-hexanoyl glycines as internal standards and measured 12 different parameters in urine and serum taken from a horse presenting with acute signs of severe myopathy and clinically diagnosed to have AM.

3. Results

3.1. Method performance

Using the method described by van Eeckhaut et al. [14] no matrix effect on quantitative results was observed during the time window given by the chromatographic separation of HGA. There was a background signal at HGA 198.1 > 73.9 which was < 50% of the signal produced by 0.01 μmol/L HGA.

Data for precision of HGA quantification in urine and serum are given in Table 1. The CV was below 15% in the range of concentrations tested from 0.01 to 10.0 μmol/L HGA. As shown in Fig. 1 linearity of quantification was excellent in the same range of concentrations, coefficient of determination (r^2 values) being 0.998 for serum and 0.9986 for urine. The line does not run through the origin indicating that there is a low non-specific signal. This non-specific signal at the retention time of HGA is obvious in the blank material (Fig. 2). At the lowest level of spiked HGA there is still a shoulder visible in serum which may influence the quantitative result. Using a CV below 15%, which is a convenient basis for calculating the lower limit of quantification (LLOQ) this might be defined at 0.01 μmol/L. However applying the calculation published by Carlier et al. [10], a method closer to diagnostic laboratory practice (mean plus 10 times standard deviation of 10 independent negative samples), the LLOQ is 0.034 μmol/L.

3.2. Inclusion of HGA quantification in acyl conjugate profiling

Intergration of HGA as an additional parameter into the already established method [13] resulted in a clear chromatographic separation of HGA from the other acyl carnitines and glycines measured. The hands on time and instrumental time of 14 min needed for acyl carnitines and acyl glycines alone was not extended. Table 2 shows urine and serum samples of a horse diseased with atypical myopathy due to ingestion of maple seeds.

4. Discussion

First measurements of HGA were done 60 years ago. Demonstration of the presence of the toxic compound in food that had been