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GENERAL ARTICLE

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Detection of hypoglycin A and MCPA-carnitine in equine serum and muscle tissue: optimisation and validation of a LC-MS–based method without derivatisation

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Abstract

Background: Measurement of hypoglycin A (HGA) and its toxic metabolite, methylenecyclopropylacetic acid (MCPA), in equine serum confirms a diagnosis of atypical myopathy (AM), a pasture-associated toxic rhabdomyolysis with high mortality linked to the ingestion of Acer trees plant material. Supportive diagnostic tests include plasma acyl-carnitine profiling and urine organic acid testing, but these are not specific for AM. Previously reported HGA and MCPA analytical techniques used liquid chromatography–mass spectrometry (LC-MS) with a derivatising step, but the latter prolongs testing and increases costs.

Objectives: To develop a rapid LCMS method for detection of serum and tissue HGA and MCPA that enables expedited diagnosis for horses with AM.

Study design: Analytical test validation.

Methods: Validation parameters to industry standards using as criteria precision, accuracy, linearity, reproducibility and stability in analyte-spiked samples were calculated on 9-calibration points and 3 different validation concentrations in both serum and muscle tissue.

Results: The test was successfully validated for the detection of HGA and MCPA-carnitine in equine serum and muscle. Test linearity was excellent (r^2 = .999), accuracy was very good for both analytes (93%-108%), precision did not exceed 10% coefficient of variation and reproducibility met the requirements of the Horwitz equation. Stability was unaffected by storage at a range of temperatures.

Main limitations: The spectrum of the tested analytes was limited to only two relevant analytes in favour of a quick and easy analysis. Linearity of the muscle method was not evaluated as calibration curves were not produced in this matrix.

Conclusion: We report an optimised, simplified and validated method for detection of HGA and MCPA-carnitine in equine serum and muscle suitable for rapid diagnosis of suspected AM cases. The serum-based test should also enable risk assessment of toxin exposure in cograzing horses and assessment of horses with undiagnosed myopathies, while the tissue detection test should help to confirm cases post-mortem and to determine toxin distribution, metabolism and clearance across different tissues.

KEYWORDS
horse, myopathy, rhabdomyolysis, hypoglycin, MCPA-carnitine

1 | INTRODUCTION

Hypoglycin A (HGA) (L-methylenecyclopropyl alanine) is a naturally occurring but nonproteic amino acid of certain plants that has unknown biological function:[1] the toxicity of its metabolite, methylenecyclopropylacetic acid (MCPA), has been demonstrated and studied in several animal species including human subjects and horses.[2-8] To date, HGA has only been found in trees and shrubs of the *Sapindaceae* family.[9,10] Among them, *A. pseudoplatanus* (Sycamore) and *A. negundo* (Box Elder) are pertinent to equine health due to their association with the commonly fatal disease outbreaks of atypical myopathy (AM), also known as seasonal pasture-associated myopathy.[11] Ingestion of seeds and seedlings of these trees, which contain variable amounts of HGA,[7,12-15] has been linked to development of a common, acquired, multiple acyl-CoA dehydrogenase deficiency (MADD) in horses.[7,8,16] This form of rhabdomyolysis has high mortality,[17-19] a feature that might be exacerbated by delayed identification and treatment of exposed animals.

Originally, identification of HGA intoxication relied on detection of certain acyl-carnitine and acyl-glycine metabolites in plasma or urine.[6] These compounds are metabolic intermediates that accumulate due to disruption of specific metabolic pathways. In other species, their presence is found in various related conditions that can be either acquired or genetic.[20,21] In horses, similar abnormal profiles have been identified in animals with a separate myopathy and cardiomyopathy that graze other toxic plants[22] and in a horse with a presumed genetic form of MADD.[23] Consequently, while supportive, these methods do not provide a definitive diagnosis of HGA toxicity. Instead, and more recently, several studies have measured HGA and its MCPA metabolites in serum from horses with suspected AM.[3,7,8,12]

Various groups have published analytical mass spectrometry methods for detection of HGA, MCPA metabolites and acyl-carnitines in body fluids: all of them require a derivatisation step with different chemical compounds: dansyl chloride,[24-26] butyl-ester,[7,8,27] FMOC[3] and/or aTRAQ reagent.[28] Derivatisation is commonly used in analytical chemistry to reduce selectivity issues and improve stability and detection of the analyte/s of interest,[29-31] but it increases assay costs and lengthens sample preparation time.[29] Since HGA intoxication is often fatal, development of an affordable and rapid test to allow early definitive identification of exposed individuals, initiation of supportive treatments[17] and removal of affected horses from pastures seems crucial. Only recently, Rudolph et al reported a liquid chromatography–mass spectrometry (LC-MS)-based method without derivatisation that simplified the analysis, but the assay had a relatively high limit of quantification and detection for both HGA and MCPA-carnitine, and poor recovery performance for both analytes.

Measurement of these compounds has never been attempted in tissues. Detection of toxin and metabolites should facilitate the understanding of their distribution and tissue/organ-specific metabolism, enabling study of important toxicological parameters such as bioavailability, tissue distribution and clearance rates in affected horses. Furthermore, it could be used as a rapid method to confirm the intoxication post-mortem, avoiding sometimes lengthy histopathological processes, which might lack sensitivity and specificity. Here, we describe validation of a novel method using LC-MS technology.

2 | MATERIALS AND METHODS

2.1 | Materials and standards

LC-MS grade solvents and high-purity analytes were used to validate the analytical method. Individual stock solutions (1 mg/mL) of HGA (Toronto Chemicals) and 3-fluorovaline (Sigma-Aldrich) (IS) were prepared by dissolving 1 mg of each compound in 1 mL of deionised water (VWR) (DI), while MCPA-carnitine (Toronto Chemicals) stock solution was prepared by dissolving 1 mg of the compound in 1 mL of methanol (VWR) (MeOH). The standard mix working solution was obtained by diluting 100 μL of HGA stock solution and 100 μL of MCPA-carnitine stock solution into 800 μL DI, while for the internal standard, 500 μL of stock solution was diluted in 500 μL DI.

For serum, heat-inactivated equine serum (Gibco) was used as a matrix-matched material to perform the validation. Serum from horses with AM and controls were obtained from samples submitted to Comparative Neuromuscular Laboratory of the Royal Veterinary College. For muscle, 100 g of long digital extensor muscle from a horse undergoing post-mortem examination for reasons unrelated to this study was taken and stored at −80°C. This material was used as a blank matrix to perform the validation. Positive AM muscle samples and controls were obtained from the same laboratory archive.

2.2 | Sample preparation

2.2.1 | Serum

Calibration curve (0.5-200 ng) and validation controls (VC) (1.8, 90 and 180 ng) stocks were produced in 10-fold excess concentration

in DI. Then, 100 µL of each were mixed with 100 µL of heated-inactivated equine serum and 50 µL of internal standard. The solution was mixed by vortex and precipitated with acetonitrile (1 mL). Samples were subsequently spun at 12 000 ***g*** for 10 minutes and the supernatant transferred to clean tubes, then evaporated at 50°C under a nitrogen stream. The pellet was reconstituted with 1 mL of 10 mmol/L heptafluorobutyric acid (HFBA) (Fisher Scientific) and mixed by vortex for 30 seconds before transfer to glass HPLC vials (VWR).

2.2.2 | Muscle

Samples prepared in Eppendorf tubes containing 50 mg of muscle, 50 µL IS, 2 steel homogenisation beads and 300 µL of MeOH were snap-frozen in dry-ice for 5 minutes before being homogenised by shaking using a Tissue-lyser II (Qiagen): 2 cycles at a frequency of 40 Hz for 1 and 2 minutes respectively. A short centrifugation cycle (10 seconds) was applied before proceeding to protein precipitation with 1.3 mL acetonitrile. Thereafter, samples were processed as described for serum. Validation controls followed the same procedure after being injected with either 100 µL of appropriate VC stock (as described for serum) or DI water for blank controls using a 29G × ½" fixed needle insulin syringe.

2.3 | LC-MS/MS

HGA and MCPA-carnitine concentrations in spiked equine serum were determined on an LCMS-8040 triple quadrupole instrument with a Nexera LC front end (Shimadzu UK, Milton Keynes) using positive-mode ESI (Figure 1). Samples were injected at 5 µL volumes onto a Phenomex 2.6µ Phenyl Hexyl 100A (4.6 × 50 mm) column at 25°C. Mobile phases consisted of 10 mmol/L ammonium formate (pH 3.0) containing 0.1% formic acid (buffer A) and a volumetric mixture of acetonitrile, methanol and propranolol 2:2:1 containing 0.1% heptafluorobutyric acid (buffer B). A gradient was delivered at 0.4 mL/min starting from 2% buffer B for 0.5 minute, followed by an increase to 50% over 3 minutes, then increase to 95% buffer B over 0.1 minute and held at 90% for 1 minutes before returning to 2% buffer B over 0.1 minute for re-equilibration for 2.3 minutes. The following optimised instrument parameters were applied for the detection of the analytes: nebulising gas at 3 L/min; drying gas at 15 L/min; heat block temperature at 400°C; desolvation line temperature at 250°C; column oven at 25°C and autosampler temperature at 10°C. The complete system was controlled by LabSolutions software (Shimadzu), version 5.65, running on a HP Prodesk computer with Windows 7 operating system.

Quantitation was determined by multiple reaction monitoring (HGA quantitation ion m/z 142.10 → 74.05, Dwell time 100 ms; Q1 Prebias at −13.0 V; Collision Energy at −9 V; and Q3 Prebias at −13.0 V. MCPA-carnitine quantitation ion m/z 256 → 85, Dwell time 100 ms; Q1 Pre-bias at −13.0 V; Collision Energy at −9 V; and Q3 Prebias at −13.0 V. FVal quantitation ion m/z 136.10 → 70.10, Dwell time 100 ms; Q1 Pre bias −13.0 V; Collision Energy at −16.0 V; and Q3 Pre-bias at −12.0 V (Figure 1).

2.4 | Optimisation of sample extraction and evaluation of matrix effect

2.4.1 | Serum

Protein precipitation is a simple technique to generate free protein samples suitable for LCMS analysis. Acetonitrile, ethanol

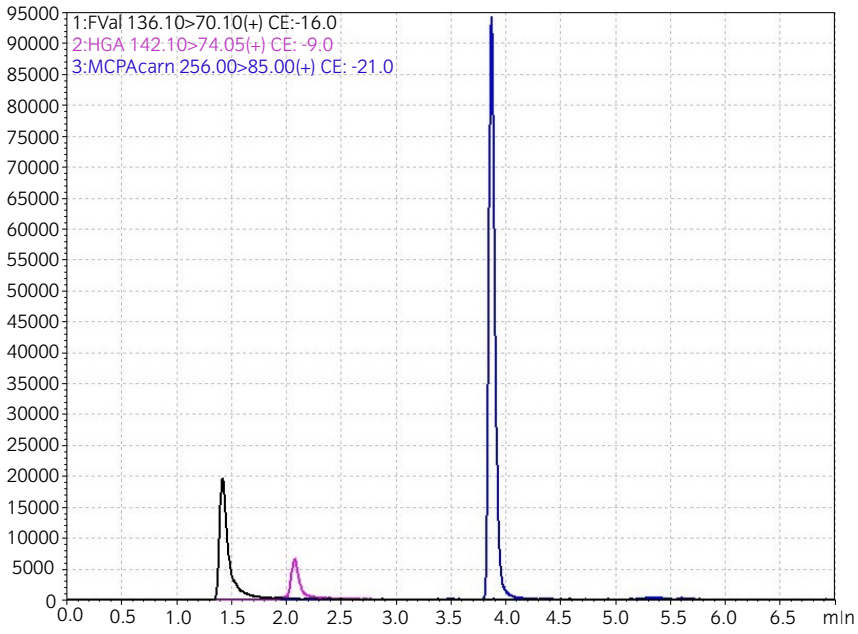

FIGURE 1 Serum Chromatogram showing the analytes of interest as detected with the Shimadzu LCMS-8040 instrument with a Phenomex 2.6µ Phenyl Hexyl 100A (4.6 × 50 mm) column in a horse with AM. Y-axis represents signal intensity and x-axis retention time for HGA (purple), MCPA-carnitine (blue) and internal standard (black). Notice the satisfactory separation between chromatograms of different analytes evaluated

and propanol were evaluated as precipitation agents by adding 1 mL of each solvent to 100 μL of heat-inactivated equine serum (VWR) spiked with 20 ng of both HGA and MCPA-carnitine, and 50 μL IS. The experiment was repeated twice, using technical replicates of each solvent. To determine matrix effect on serum analysis, technical replicates of commercial heat-inactivated serum, fresh frozen serum (AM-free serum archive from *Comparative Neuromuscular Laboratory of the Royal Veterinary College*) and deionised water were spiked with 20 ng of both HGA and MCPA-carnitine, and 50 μL IS. Additionally, serum from an AM-affected horse was prepared in 2 ways: plain (nonspiked) and spiked (+20 ng of each analytes). Finally, a set of 9 calibration standards (0.5 ng to 200 ng/mL) were prepared in both DI and heat-inactivated serum.

2.4.2 | Muscle

Extraction of both compounds, HGA and MCPA-carnitine, was performed using 3 different buffers: none (dry extraction), methanol and DNA-lysis buffer (Qiagen). Triplicate samples of fresh-frozen muscle from an AM horse were prepared for each buffer condition. The amount of buffer added and muscle weight for the first trial was based on the Tissue-lyser manufacturer's recommendations for DNA extraction in muscle. Three different weights were assessed and prepared in duplicate: 25, 50 and 100 mg. Muscle was thawed, accurately weighed and placed in an Eppendorf tube with 50 μL IS, extraction solvent (dry = none, mechanical homogenisation extraction; MeOH & lysis buffer: 25 mg = 180 μL; 50 mg = 360 μL and 100 mg = 720 μL). Results were obtained using the serum calibration curve. Each analyte concentration in muscle was calculated by dividing the result obtained by the weight (mg) of muscle analysed. The coefficient of variation in each extraction condition was then calculated using the mean values obtained for each weight.

The relative performance of mechanically homogenised only and methanol extracted muscle was further evaluated with muscle samples from another 2 AM-affected horses and 1 control horse (diagnosed with denervation muscle atrophy). Samples of 50 mg were prepared in triplicate for each extraction solvent. Two sets of triplicates were prepared as controls: 1 set was spiked with 50 ng of both HGA and MCPA-carnitine and the other set underwent only mechanical homogenisation.

2.5 | Evaluation criteria for validation

Criteria used in this work followed the National Measurement System Guidelines of the United Kingdom[32] and the European Medicines Agency[33] and are summarised below. Validation was performed over 5 days during which calibration curves and validation controls were freshly prepared 3 times on 3 different days by 2 different analysts.

2.5.1 | Accuracy

Calculated as the mean of the interday theoretical recovery at each concentration of the serum validation control (VC) samples, lower validation control (LVC), middle validation control (MVC) and upper validation control (UVC). Accuracy was considered acceptable if the recovery was between 80% and 110% of the designated HGA and MCPA-carnitine concentration.

2.5.2 | Precision

Calculated as the coefficient of variation (CV) of the intraday accuracy of serum VC samples. Precision was considered acceptable if the per cent CVs for the 3 repeated measurements were <15%, except at the LVC concentration where it could be ≤20%.

2.5.3 | Reproducibility

Calculated as the CV of the interday accuracy of serum VC samples. Reproducibility was considered acceptable if the per cent CVs did not exceed the level calculated by the Horwitz equation (Hweq) $CV = 2^{(1-0.5\ \log C)}$, where C is the concentration of the analyte as a decimal fraction.

2.5.4 | System linearity

Determined by the evaluation of 3 HGA and MCPA-carnitine standard curves prepared and analysed on 3 separate days. Each standard curve consisted of 9 standards ranging from 0.5 to 200 ng/mL equivalent concentration. For each validation run, the resulting HGA standard curve was expected to meet the following conditions:

1. The deviation of the calculated concentration of each standard should be within ±15% of the theoretical value (±20% at LLOQ).
2. Individual standards (≤1/3) could be dropped from the curve if they did not meet these criteria, although no quantitation should be extrapolated outside the range covered by the acceptable standards.

2.5.5 | The lower limit of detection (LOD)

Determined from 6 blanks according to the following formula: LOD = mean concentration response + (3 × SD).

2.5.6 | Limits of quantitation

The lower limit of quantitation (LLOQ) and the upper limit of quantitation (ULOQ) were established for the method and defined as the

lowest and highest validated concentration that met all acceptance criteria. Accuracy and Precision was established at the LLOQ.

Calibrations curves were constructed and fitted by linear regression without weighting for HGA and forcing through zero for MCPA-carnitine.

2.5.7 | Stability of samples

Freeze/thaw stability

Three replicates of each VC level were frozen and thawed 3 times and assayed. The stability was considered successful if the mean of the freeze/thaw samples was within ±15% of the mean of freshly prepared samples at the same theoretical concentration. This parameter was determined as the percentage difference from an analysed fresh sample and calculated as follows: ((fresh sample value−stability sample value)/fresh sample value) × 100.

Short-term stability

The stability of the analytes at ambient temperature and at 4°C was evaluated over 48 hours. The short-term stability in matrix was considered adequate if the mean concentration of the stability samples was within ±15% of the mean of freshly prepared samples of the same theoretical concentration. Additionally, serum samples were evaluated while maintained for 48 hours with ice packs and/or maintained for 72 hours at both −20 and 4°C. These conditions were chosen to represent shipping and short-term storage conditions (eg over a weekend) that might occur under field conditions.

Processed samples

Re-injection stability was assessed by re-injecting a complete set of aged standards or VC samples after storage at 10°C for a minimum of 72 hours. The re-injection run was considered successful if the mean concentration of the stability samples was within ±15% of the mean of freshly prepared samples of the same theoretical concentration.

2.6 | Performance of test in samples

2.6.1 | Serum

The serum archive from the Comparative Neuromuscular Laboratory of the Royal Veterinary College was examined in order to obtain samples to evaluate the newly validated method. All samples had been stored since their arrival at the laboratory at −80°C. Selected samples were included in any of the following experiments:

1. Cases with a diagnosis of either AM (n = 7; histopathological confirmation and highly suspected based on clinical diagnosis) or control neuromuscular conditions (n = 5; polysaccharide storage myopathy (PSSM1) n = 2; recurrent exertional rhabdomyolysis (RER) n = 2 and equine motor neuron disease (EMND) n = 1).
2. Cases (n = 7) in which serum and 2 types of plasma (EDTA and heparin) had been collected simultaneously using different tubes and submitted to the laboratory.

2.6.2 | Tissue samples

The fresh-frozen archive from the Comparative Neuromuscular Laboratory of the Royal Veterinary College was searched in order to obtain samples to evaluate the newly validated method. All samples had been stored upon arrival at the laboratory in cryovials at −80°C. Samples from 3 AM horses (positive in serum testing) and 4 controls (idiopathic myopathy = 3; PSSM1 = 1) were analysed. Additionally, liver samples available from the 3 AM horses were also analysed using the new method.

2.7 | Data analysis

Statistical analysis was performed using commercial software GraphPad Prism 7.02 (Graph Pad 7.21). Recoveries for Hypoglycin A and MCPA-carnitine in serum and muscle during optimisation of extraction techniques were compared using 2-way ANOVA followed by Sidak's multiple comparison. Differences between HGA and MCPA results obtained in either serum or plasma (EDTA and Lit Hep) were assessed using one-way repeated measures ANOVA followed by Tukey test. Differences were considered statistically significantly different when $P < .05$. Validation results were evaluated following the National Measurement System Guidelines of the United Kingdom[32] and the European Medicines Agency as described previously.

3 | RESULTS

3.1 | Serum method

There was no statistically significant difference between the matrices evaluated in this experiment ($P > .5$): recoveries in heat-inactivated equine serum and equine serum (fresh-frozen) were similar to those obtained in deionised water. The percentage difference from 20 ng obtained in the spiked AM serum for both analytes was <15%, and recoveries between 88% and 105% (Figure 2). Further analysis showed that there were no significant differences in recovery between samples prepared in DI or HI serum ($P = .85$) (Table S1). Heat-inactivated serum was subsequently used for the validation.

The various protein precipitation methods did not yield statistically significant differences in recoveries of either analyte for each of the 3 solvents investigated ($P > .2$) (Table S2). Subsequently, acetonitrile was chosen as the precipitation solvent for the validation, as the pellet formed rapidly and the supernatant cleared more rapidly than with other solvents, even though after centrifugation, a clear supernatant was obtained with each of the 3 solvents.

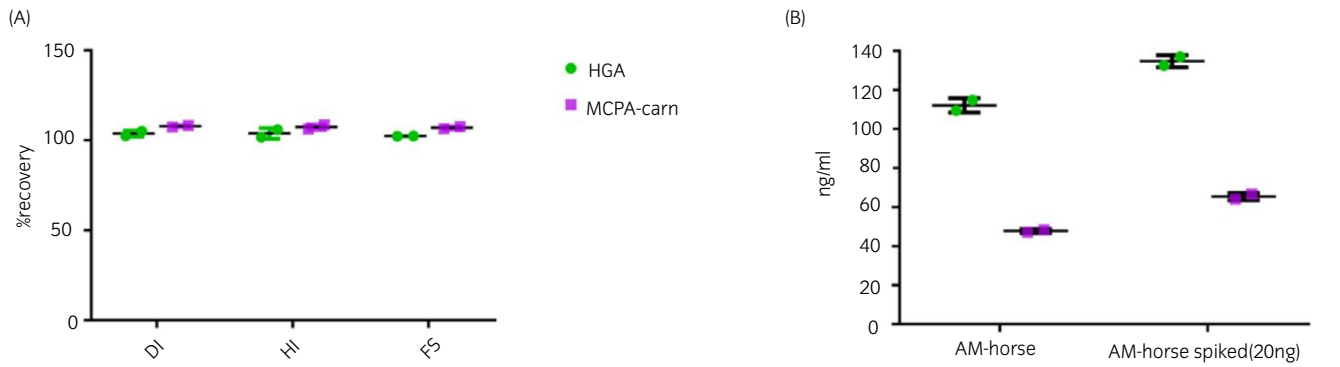

FIGURE 2 Matrix-matched sample optimisation. A, Recoveries obtained in the 3 matrices analysed (DI, deionised water; HI, heated-inactivated serum; FS, fresh-frozen serum). B, HGA and MCPA-carnitine concentration obtained in serum from 2 AM horses with and without a spike of 20 ng of each analyte. These experiments were repeated twice using technical duplicates for each condition

TABLE 1 Validation performance parameters in serum method

		LVC	MVC	UVC
		1.8 ng/mL	90 ng/mL	180 ng/mL
Validation parameters (HGA)				
Accuracy	Interday recovery (%)	97.22	95.72	93.81
Precision	Day 1 intraday CV (%)	7.95	4.9	3.87
	Day 2 intraday CV (%)	3.8	1.3	0.54
	Day 3 intraday CV (%)	4.91	3.71	3.62
Reproducibility	Horwitz equation results (%)	41.42	22.99	20.21
	Interday CV (%)	12.14	12.37	13.46
Validation parameters (MCPA-carn)				
Accuracy	Interday recovery (%)	100	98.20	101.97
Precision	Day 1 intraday CV (%)	4.91	3.47	4.16
	Day 2 intraday CV (%)	2.07	1.36	1.18
	Day 3 intraday CV (%)	9.27	2.47	2.79
Reproducibility	Horwitz equation results (%)	41.42	22.99	20.21
	Interday CV (%)	10.23	7.91	4.22

Data obtained from analysis of the results for the validation controls: Upper validation control (UVC = 180 ng/mL); middle validation control (MVC = 90 ng/mL) and lower validation control (LVC = 1.8 ng/mL). Notice that accuracy meets the validation criteria (recovery 93%-102%). Precision was below the 15%-20% coefficient of variation (CV) established and reproducibility of the method did not reach the Horwitz equation threshold.

The requirements of the validation protocol were met for the 3 concentrations evaluated (validation controls). Precision was acceptable with an intraday coefficient of variation ranging from 0.54% to 7.95% for HGA and 1.18% to 9.27% for MCPA-carnitine. Reproducibility met the criteria of the Hortwitz equation for each concentration evaluated (Table 1). System linearity was acceptable between 0.5 and 200 ng/mL (Table S3) and the limit of detection for the method was established at 0.055 ng/mL for HGA (0.024 ± 0.1 ng/mL; mean ± SD; n = 6) and 0.078 ng/mL for MCPA-carnitine (0.061 ± 0.005 ng/mL; mean ± SD; n = 6).

Short-term stability of samples was good at 4°C for 48 hours (CV < ±15%), however, samples maintained at room temperature (~18°C) for 48 hours resulted in wider variation (CV = 12%-18%) for the MCPA-carnitine analyte (Figure 3). Furthermore, no significant differences were found between samples maintained at −80°C and those kept with ice packs for 48 hours, with the percentage difference obtained with ice packs ranging from −3.55% to 9.92% at a range of concentrations. Stability of analytes was also evaluated at 72 hours, but only at a single concentration (200 ng): both analytes were stable at both −20 and at 4°C, with the percentage difference from −80°C results ranging from −10.4% to 2.55%.

Short-term stability of extracted samples was good at 10°C for 48 hours. However, by day 4, HGA was out of the acceptable range (UVC = 18.49%) with the percentage difference between results obtained on the first day and the subsequent measurements ranging from −6.38% to 4.04% for both analytes on day 2 and from 7.14% to 18.5% on day 4 (Table S4). Validation control samples were stable over 3 freeze-thaw cycles for both analytes, with the percentage difference from fresh ranging from −4.3% to 9.6%. Continuing tests have shown that the calibration curve samples are stable for

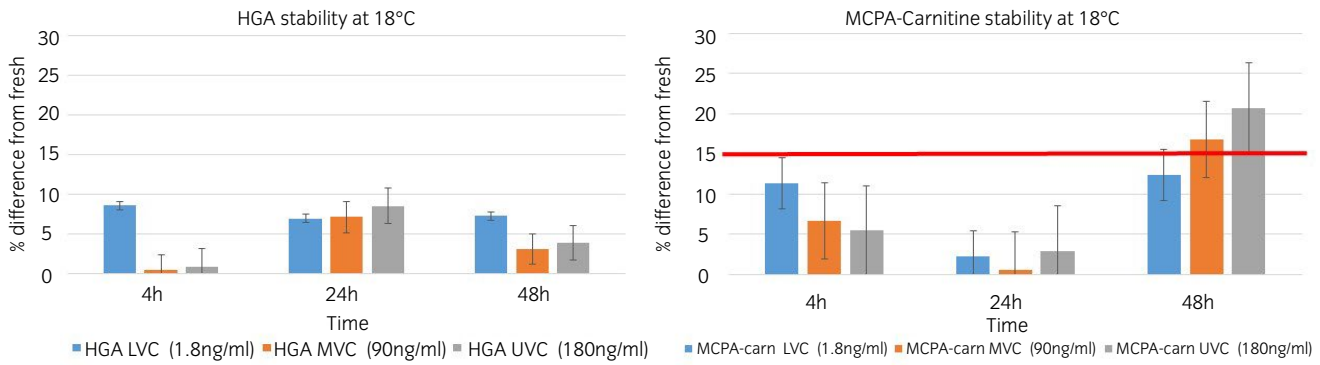

FIGURE 3 Short-term stability at room temperature of validation control samples. Figures show the coefficient of variation (CV) from fresh concentration in both HGA and MCPA-carnitine. VCs are represented as follows: blue (LVC), orange (MVC) and grey (UVC). Notice the higher coefficient of variation showed in MCPA-carnitine (MVC and UVC) at 48 h. A red line has been added to the figure to point out the benchmark of 15% CV. As a result, it was determined that MCPA-carnitine in serum was not sufficiently stable and samples should be preserved by other means

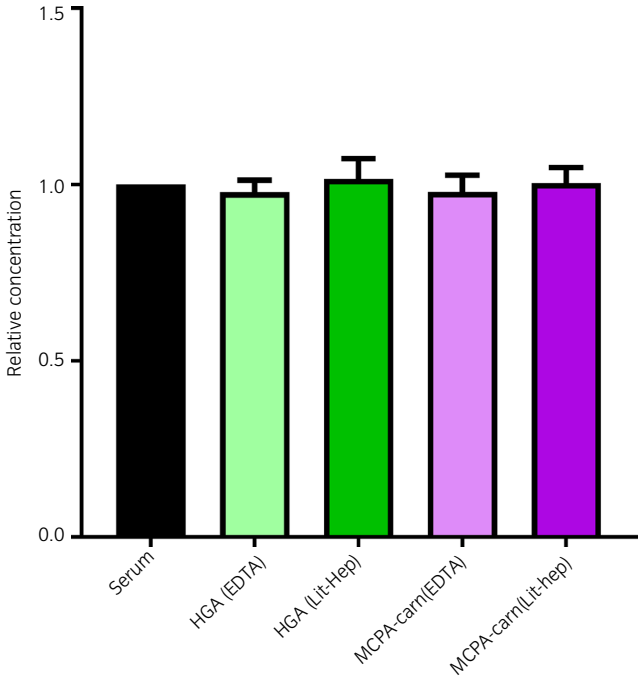

FIGURE 4 Comparison of HGA and MCPA-carnitine in serum and plasma. Bars show summary of mean results (±SE) obtained from 7 horses in which blood samples were obtained simultaneously in plain and EDTA and lithium heparin tubes, normalised to the serum result for each animal. No significant differences were found

up to 2 months after 5 freeze/thaw cycles. Analysis of the calibration curve from day 1 on days 16, 40 and 57 showed no significant differences between time points ($P > .3$) (Figure S5).

There were no statistically significant differences in HGA and MCPA-carnitine concentrations obtained when samples from the same animals were analysed as serum or as either EDTA- or lithium-heparin plasma: both analytes had CV < 15% across matrices (Figure 4). Evaluation of test performance in clinical cases and controls yielded satisfactory results (Table 2). HGA and MCPA-carnitine were detected in all serum samples analysed from both histopathologically confirmed and highly suspected (based on clinical presentation and biochemistry) AM cases, whereas the analytes were undetectable in horses with other neuromuscular conditions.

3.2 | Detection of hypoglycin A and MCPA-carnitine in muscle

Extraction of both HGA and MCPA-carnitine was achieved with all extraction solvents, however, lysis buffer was disregarded as the CV among the samples exceeded 20% for both analytes (HGA CV = 22.19%; MCPA-carnitine CV = 46.25%). Mechanical homogenisation extraction only also showed high CV for MCPA-carnitine (28.8%), while CVs for both analytes were below 15% in the methanol extraction (Figure 5A). Further evaluation of methanol and mechanical homogenisation confirmed significant differences for the MCPA-carnitine analyte ($P = .001$), in which matrix enhancement seemed to occur (% recovery methanol = 107.9 ± 0.06; % recovery mechanical homogenisation = 127 ± 6.9) (Figure 5B). Therefore, methanol was used as extraction buffer in the validation work.

The requirements of the validation protocol were met for the 3 concentrations evaluated (VC). Precision was acceptable with an intraday coefficient of variation ranging from 0.85% to 5.29% for HGA and 1.18% to 9.27% for MCPA-carnitine. Reproducibility met the criteria of the Hortwitz equation for each concentration evaluated (Table 3). Linearity was not assessed in muscle as calibration curves from serum were used for this validation. As a result, limits of quantitation were based on the same range for the serum method. However, the limit of detection was established at 0.1 pg/mg for HGA (0.11 ± 0.03 pg/mg; mean ± SD) and 0.5 pg/mg for MCPA-carnitine (0.48 ± 0.13 ng/mL; mean ± SD) after analysis of 9 muscle blank samples. The analytes were stable in muscle samples upon 4 freeze-thaw cycles when kept at −20°C (Table S6). Performance of the test in samples available at the laboratory archive showed that horses with other neuromuscular diagnoses confirmed by histopathology did not have HGA and MCPA-carnitine in their muscles, while AM horses did (Table S7). HGA was detected in muscle, liver and serum in all AM affected

TABLE 2 HGA and MCPA-carnitine results obtained in serum from horses in which AM was confirmed by histopathology (HP) or was highly suspected (HS) based on clinical presentation and biochemistry

Horse ID	HGA (ng/mL)	MCPA-carn (ng/mL)
HP 1	666.86	102.26
HP 2	426.56	30.95
HP 3	281	57.12
HP 4	450.47	180.73
HS 1	489.27	15.2
HS 2	33.51	38.63
HS 3	83.97	24.65
HS 4	678	14.37
RER 1	<LOD	<LOD
RER2	<LOD	<LOD
PSSM1-A	<LOD	<LOD
PSSM1-B	<LOD	<LOD
EMND	<LOD	<LOD

Additionally, results obtained from 5 control horses are shown: recurrent exertional rhabdomyolysis (RER); Polysaccharide-storage myopathy (PSSM1); Equine motor neuron disease (EMND).

horses; in contrast, MCPA-carnitine was detected in serum and muscle of all AM horses but was below the limit of quantification in liver from 1 horse and below limit of detection in the other 2 horses (Table S8).

4 | DISCUSSION

We describe new methodology for reliable quantification of HGA and MCPA-carnitine in equine serum, plasma and muscle and lower the previously reported limits of quantitation.[10,34,35] This novel method also avoids use of a derivatising agent thereby reducing sample analysis time (to under 2 hours) and costs.

Chemical derivatisation is a useful technique that increases the sensitivity of some analytical methods, when lower detection limits are desired or when the chemical characteristics of the analyte are a constraint.[29-31] However, derivatisation can lead to uncontrollable reaction recovery and the necessary subsequent purification procedures can reduce the method's reliability.[29] The current work reveals that detection of HGA and MCPA-carnitine in serum and muscle do not require derivatisation to produce reliable, reproducible and sensitive results. Indeed, we achieved a significantly lower limit of quantitation for both analytes when compared with a recent published method.[35] Moreover, the recoveries obtained in serum by Rudolph et al method (around 20% for both analytes) were strikingly low when compared with the method presented in this work (97%-100%).[35] Perhaps the use of a different chromatographic column might account for the previously reported results: ion suppression or poor ionisation of analytes might occur when samples are not sufficiently clean, affecting the recovery process.[32] Besides, the current assay allows detection of both compounds simultaneously while previous methods required the use of 2 different techniques and/or analyses to detect HGA and MCPA-carnitine in serum/plasma.[3,36] Additionally, no selectivity issues of the method were detected as shown in the optimisation section by testing of both blank and spiked samples in both equine serum and deionised water as well as in samples from true affected horses.

A rapid, accurate and affordable test for plant-derived toxins in horses should enable rapid confirmation of cases and/or detection of exposed animals, therefore, prompting need for veterinary treatment in affected horses, which has been associated with improved survival,[17] and/or early establishment of preventive measures in

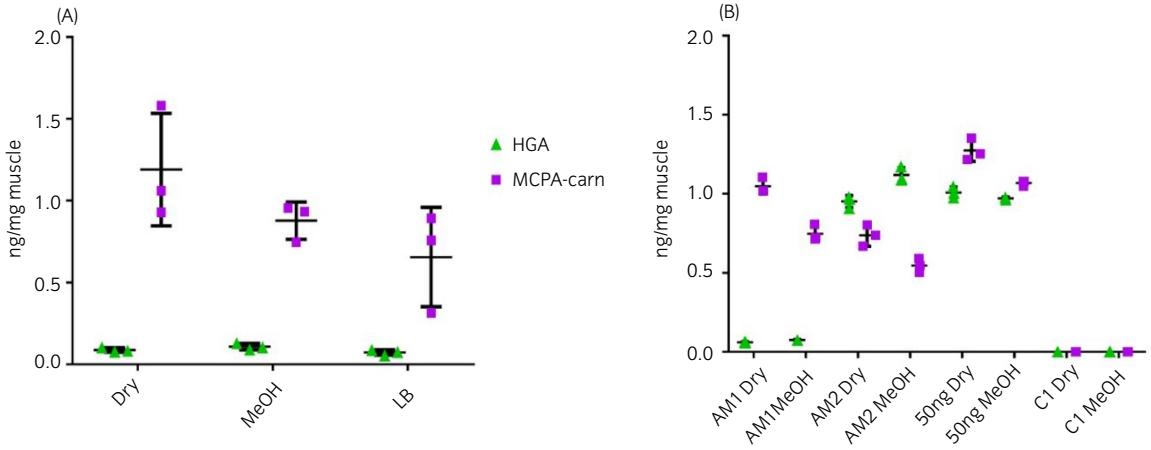

FIGURE 5 Optimisation of HGA and MCPA-carnitine extraction from muscle samples. A, Mechanical homogenisation (only) extraction and 2 solvents were evaluated in muscle from an AM horse: mechanical extraction (dry); methanol = MeOH and DNA-extraction buffer = LB. All data points plotted (n = 3 technical replicates for each condition). CVs among values obtained with LB were above 15% for both analytes and therefore disregarded as extraction buffer. Mechanical homogenisation also presented CV = 28.8% for MCPA-carnitine. B, Comparison of methanol and dry extraction using muscle from 2 AM horses, 1 unaffected horse spiked with 50 ng of both analytes and control muscle. Mechanical extraction produced significantly higher extraction values for MCPA-carnitine denoting some degree of matrix enhancement (recovery = 127%); it was therefore disregarded as the extraction method

TABLE 3 Validation performance parameters in muscle method

		LVC	MVC	UVC
		0.036 ng/mg	1.8 ng/mg	3.6 ng/mg
Validation parameters (HGA)				
Accuracy	Interday recovery (%)	99.56	102.28	97.09
Precision	Day 1 intraday CV (%)	7.02	4.73	2.44
	Day 2 intraday CV (%)	4.80	5.29	2.21
	Day 3 intraday CV (%)	4.90	1.61	0.85
Reproducibility	Horwitz equation results (%)	41.42	22.99	20.21
	Interday CV (%)	2.94	1.32	5.56
Validation parameters (MCPA-carn)				
Accuracy	Interday recovery (%)	100.75	107.74	104.06
Precision	Day 1 intraday CV (%)	7.33	4.17	4.74
	Day 2 intraday CV (%)	2.07	1.36	1.18
	Day 3 intraday CV (%)	0.58	1.97	1.17
Reproducibility	Horwitz equation results (%)	41.42	22.99	20.21
	Interday CV (%)	0.90	1.10	2.1

Data obtained from analysis of the results for the validation controls: Upper validation control (UVC = 3.6 ng/mg); middle validation control (MVC = 1.8 ng/mg) and lower validation control (LVC = 0.036 ng/mg). Notice that accuracy meets the validation criteria (recovery 97%-108%). Precision was below the 15%-20%, maximal pre-assigned coefficient of variation (CV) and reproducibility of the method did not reach the Horwitz equation threshold.

horses at risk. Furthermore, this is the first report of detection of HGA and MCPA-carnitine in tissues, which is of paramount importance to understand HGA metabolism and tissue-specific detoxification pathways in intoxicated animals and might be applicable to assessment of horses post-mortem. As a result, this method should enable a more accurate characterisation of bioavailability, pharmacokinetics and clearance of HGA in horses by testing both serum and tissue. We anticipate its use in other species of relevance such as human subjects[24,25] or ruminants.[37]

Specific calibration curves in muscle were not performed during the validation of the muscle method. Although this might have been preferred in terms of matrix-matched standards, the good recoveries obtained using the serum calibration curves emphasise the validity of the technique; furthermore, this methodology allows analysis of both types of samples (serum and tissues) simultaneously. Biochemical composition of both biological matrices is relatively similar, since water and proteins are the major components of both, although the latter is considerably higher in muscle.[38] Whether excess protein content has an impact on the quantitation range is unknown at this stage, however, it seems unlikely due to the good recoveries obtained in spiked samples.

Early studies established that the main targets of HGA metabolites are medium-chain and short-chain acyl-CoA dehydrogenases[39,40] and suggested MCPA-glycine as the primary detoxification metabolite.[41,42] As a result, detection of acyl-conjugates (metabolic intermediates produced in large quantities due to defective beta-oxidation pathways) has been used traditionally to confirm multiple acyl-dehydrogenase defects (MADD) and HGA poisoning.[6,40,43] However, evidence of toxic precursor and toxic metabolites seems necessary to differentiate HGA-associated acquired MADD from genetic forms of the disorder, or other toxicities.[22,23] Previous studies showed that MCPA-carnitine is detectable in serum when certain acyl-carnitines, (particularly short and medium species) are also present, and it has always been associated with clinically relevant AM disease.[3,8,44] Therefore, concomitant measurement of plasma acyl-carnitine concentrations seems unnecessary to confirm cases of AM, although it might be helpful when establishing a prognosis.[36]

MCPA-carnitine seems to be the most prevalent HGA metabolite in equine serum while MCPA-glycine is usually very low or undetectable, although is present at higher concentrations in urine.[3,7,35,44] Furthermore, MCPA-glycine and HGA are commonly excreted in urine of healthy equines grazing areas affected by AM,[3] suggesting that HGA can be successfully eliminated in urine as a free molecule and/or via glycine conjugation to some extent without associated clinical signs in horses and human subjects.[3,45]

We could not detect MCPA-carnitine in liver tissue of AM-affected horses, which might be related to low metabolism of HGA in this tissue. This finding might account for the disparity of tissue involvement seen among horses, human subjects or rats: in horses, HGA intoxication is primarily associated with a myopathy, whereas in human subjects and rats, a hepatopathy is the most significant problem.[46-48] In these latter species, HGA might be detoxified by glycine-N-acylase enzymes within liver and kidneys.[42,49] The lack of

relevant concentrations of MCPA-glycine in serum[3] and absence of MCPA-carnitine in tissue samples might then suggest relatively low hepatic metabolism of HGA in horses. Confirmation of this finding requires analysis of additional samples and might be facilitated by in vitro (cell culture) studies.

In conclusion, this work describes the successful validation of an improved and novel LC-MS method for detection of HGA and MCPA-carnitine in horses that does not require the use of chemical derivatisation to produce accurate and reproducible results. This method should enable affordable, robust and reliable methods to facilitate future research into metabolism and pharmacokinetics of HGA to help explain risk factors and equine-to-equine variation in morbidity and mortality. Practically, the method is suitable for rapid confirmation of HGA intoxication when establishing a diagnosis of AM.

ETHICAL ANIMAL RESEARCH

Study was conducted with institutional ethical approval (URN 2017 1708).

OWNER INFORMED CONSENT

Owners gave consent for their animals' inclusion in the study.

DATA ACCESSIBILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

S. González-Medina, C. Hyde and R. Piercy contributed to study design. S. González-Medina, C. Hyde and I. Lovera contributed to study execution. All authors contributed to data analysis and interpretation. S. González-Medina, C. Hyde and R. Piercy contributed to preparation of the manuscript. All authors gave their final approval of the manuscript. S. González-Medina had full access to all the data in the manuscript and takes responsibility for the integrity of the data and accuracy of the data analysis.

CONFLICT OF INTEREST

The Comparative Neuromuscular Diseases Laboratory receives payment for measurement of serum HGA and MCPA-carnitine and proceeds to contribute to ongoing laboratory research.

PEER REVIEW

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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