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Quantitative analysis of lignocaine and metabolites in equine urine and plasma by liquid chromatography–tandem mass spectrometry

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ABSTRACT

In this paper, a method for the sensitive and reproducible analysis of lignocaine and its four principal metabolites, monoethylxylidide (MEGX), glycylxylidide (GX), 3-hydroxylignocaine (3-HO-LIG), 4-hydroxylignocaine (4-HO-LIG) in equine urine and plasma samples is presented. The method uses liquid chromatography coupled to tandem mass spectrometry operating in electrospray ionisation positive ion mode (+ESI) via multiple reaction monitoring (MRM). Sample preparation involved solid-phase extraction using a mixed-mode phase. The internal standard adopted was lignocaine-d_{10}. Lignocaine and its metabolites were successfully resolved using an octadecylsilica reversed-phase column using a gradient mobile phase of acetonitrile and 0.1% (v/v) aqueous formic acid at a flow rate of 300 μL/min. Target analytes and the internal standard were determined by using the following transitions; lignocaine, 235.2 > 86.1; 3-HO-LIG and 4-HO-LIG, 251.2 > 86.1; MEGX, 207.1 > 58.1; GX, 179.1 > 122.1; and lignocaine-d_{10}, 245.2 > 96.1. Calibration curves were generated over the range 1–100 ng/mL for plasma samples and 1–1000 ng/mL for urine samples. The method was validated for instrument linearity, repeatability and detection limit (IDL), method linearity, repeatability, detection limit (MDL), quantitation limit (LOQ) and recovery. The method was successfully used to analyse both plasma and urine samples following a subcutaneous administration of lignocaine to a thoroughbred horse.

1. Introduction

Lignocaine (2-diethylamino-2′,6′-dimethylacetanilide, Fig. 1a) acts as a local anaesthetic by stabilising excitable membranes and preventing the initiation and transmission of nerve impulses. Lignocaine is used therapeutically in the veterinary setting due to its rapid onset of action and a relatively short duration of effect, lasting from 60 to 90 min [1].

Lignocaine undergoes both Phase I and Phase II metabolism to form a number of de-ethylated and hydroxylated biotransformation products [2]. A range of metabolites have been reported to be excreted including monoethylxylidide (MEGX, Fig. 1d), glycylxylidide (GX, Fig. 1e), 3-hydroxylignocaine (3-HO-LIG, Fig. 1c), 4-hydroxylignocaine (4-HO-LIG, Fig. 1b) [3]. The hydroxylated metabolites will normally be subjected to glucuronide conjugation and be excreted in this form in the urine [4]. MEGX, GX and 3-HO-LIG have been previously reported in the urine of the horse following lignocaine administration but to the best of our knowledge no report has identified 4-HO-LIG in horse urine or plasma [5–7].

Lignocaine is a banned substance in horseracing in Australia [8,9]. All race horses must be free of banned substances when presented for racing. Lignocaine is a registered therapeutic substance for use in horses and therefore concise information regarding the analytical detection times following administration is required by racing veterinarians to accurately estimate the time following administration during which a horse is not permitted to run (i.e. withdrawal time).

This paper describes a liquid chromatographic–mass spectrometric analytical procedure suitable for the quantitative determination of lignocaine and the above four metabolites in horse urine and plasma following subcutaneous administration of lignocaine. The method is applied to samples obtained from a horse following such an administration.

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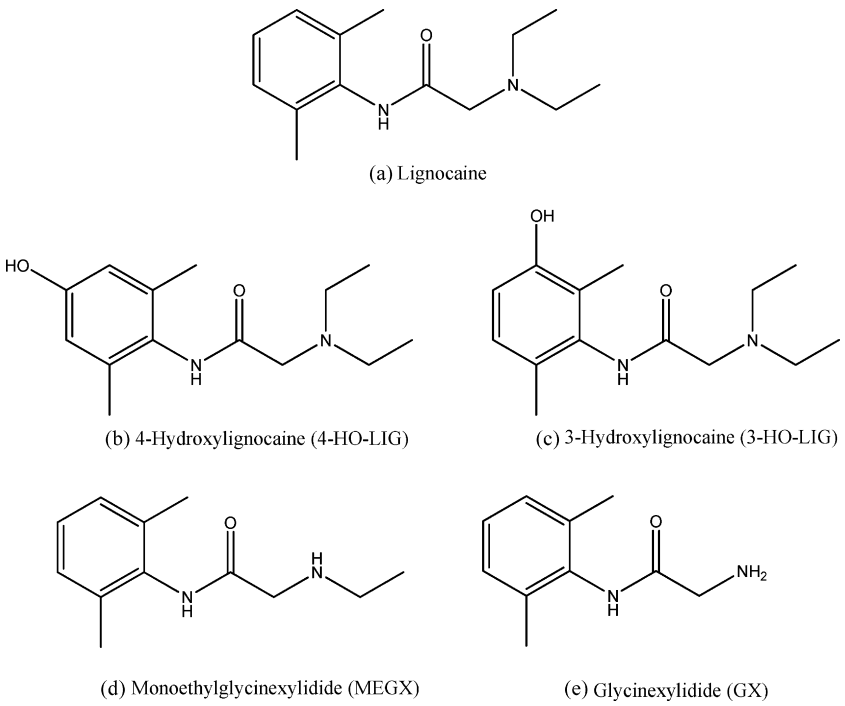

Fig. 1. Structures of lignocaine and its four principle equine metabolites.

2. Materials and methods

2.1. Materials

All reagents used were analytical grade or better. Water was treated by reverse osmosis (RO) and additionally by activated carbon, ion exchange filters and UV irradiation. Acetonitrile used for the preparation of the mobile phase was HPLC grade (Lab-Scan, Lomb Scientific, Taren Point, NSW, Australia). The solvents used in sample extraction, ethyl acetate, acetone, chloroform (Lab-Scan, Lomb Scientific Pty Ltd., Taren Point, NSW, Australia), and methanol, ethanol, dichloromethane, and n-hexane (Merck, Labtek Pty Ltd., Brendale, QLD, Australia) were analytical grade or better. Acetic acid, glacial, was from Univar (Crown Scientific, Minto, NSW, Australia). β-Glucuronidase/aryl sulfatase enzyme solution from *Helix pomatia* was supplied by Roche Diagnostics Australia Pty Ltd., Castle Hill, NSW, Australia.

The analytical standards, lignocaine, 2,6-xylidine (Sigma–Aldrich Pty Ltd., Castle Hill, NSW, Australia), monoethylglycine xylidide (MEGX) and glycine xylidide (GX) (AstraZeneca Australia, North Ryde, NSW, Australia) were supplied as undiluted material. The analytical standards, 3-hydroxylignocaine (3-HO-LIG) and 4-hydroxylignocaine (4-HO-LIG) (Neogen Corporation, Lexington, KY, USA, 1 mg/mL certified solutions) were used as supplied. Lignocaine-d_{10} (CDN Isotopes, Pointe-Claire, Quebec, Canada) was used as the internal standard.

Stock solutions (1 mg/mL) of lignocaine, lignocaine-d_{10}, MEGX and GX were prepared by dissolving 25.0 mg of the solid material in ethanol and diluting to 25 mL. A mixed working solution (10 μg/mL) was prepared by diluting 500 μL of the stock solutions of lignocaine, MEGX, GX, 3-HO-LIG and 4-HO-LIG to 50 mL with water. The working internal standard solution was prepared by diluting the stock solution of lignocaine-d_{10} to a concentration of 10 μg/mL with water. All solutions were stored at 4 °C while not in use. Working solutions were discarded after 7 days.

Spiked plasma and urine calibration standards were prepared by adding mixed working solution to drug-free urine and plasma. Plasma calibration standards ranged from 1 to 100 ng/mL and urine calibration standards ranged from 1 to 1000 ng/mL. The calibration standards were prepared daily and run with each batch of samples.

2.2. Drug administration and sample collection

The drug administration study was carried out on a 4-year-old thoroughbred female horse. A thorough veterinary examination was conducted prior to the administration to ensure the horse was in good health and condition and the horse was weighed (470 kg). Baseline samples of urine and plasma were collected immediately prior to the administration. Lignocaine (200 mg, Lignocaine 20 Injection, Ilium Veterinary Products, Smithfield, Australia) was administered subcutaneously using a 21 gauge needle. The horse had free access to food and water at all times.

Post-administration blood samples were collected from the left jugular vein into 10 mL evacuated lithium-heparinised tubes (Beckton-Dickinson, Rutherford, NJ, USA). Blood samples were collected at 15 and 30 min during the first hour then at 2, 3, 5 and 7 h post-administration. The blood samples were centrifuged at 3000 rpm for 10 min and the plasma transferred to plastic screw capped tubes. The plasma samples were stored at −20 °C until analysis.

Urine samples were collected using a stainless steel long handled sampling pan. The horse was conditioned to urinate on encouragement. Urine samples were collected at 3.5, 5, 8, 24.33, 27, 31.25 and 47.25 h post-administration. The urine samples were stored at −20 °C until analysis.

2.3. Sample preparation

Urine samples, spiked urine calibration standards and blank urine samples (1 mL) were adjusted to pH 5–5.5 with dilute HCl and/or dilute ammonia solution as required and diluted with water (4 mL). β-Glucuronidase/aryl sulfatase enzyme solution (*H. pomatia*, 1000 units, 100 μL) and lignocaine-d_{10} (10 μL of 10 μg/mL working solution) were added and the samples were mixed thoroughly by vortex. Samples were incubated overnight at 37 °C. Ammonium sulfate (solid, 1 g) was added and the samples mixed

Table 1
Method validation parameters for lignocaine quantitation method.

Parameter		Lignocaine	MEGX	GX	3-HO-LIG	4-HO-LIG
Instrument	Correlation (R^2)	0.999	0.999	0.999	0.998	0.998
	Fit	Quadratic	Quadratic	Quadratic	Quadratic	Quadratic
	Repeatability (%RSD)	<5	<5	<5	<5	<5
	Detection limit (IDL, ng/mL)	0.1	0.2	0.3	0.04	0.06
Method (Urine Matrix)	Correlation (R^2)	>0.999	>0.999	>0.999	>0.999	>0.999
	Fit	Quadratic	Quadratic	Quadratic	Quadratic	Quadratic
	Repeatability (%RSD)	<10	<10	<10	<10	<10
	Detection limit (MDL, ng/mL)	0.4	0.2	0.3	0.5	0.5
	Quantitation limit (LOQ, ng/mL)	1.1	0.8	0.9	1.7	1.5
	Recovery (%) (Ave over conc. range)	105	104	108	94	93
	Accuracy (±%) (Ave over conc. range)	6.9	9.5	14.1	14.5	13.2
Method (Plasma Matrix)	Correlation (R^2)	>0.999	>0.999	>0.999	>0.999	>0.999
	Fit	Quadratic	Quadratic	Quadratic	Quadratic	Quadratic
	Repeatability (%RSD)	<10	<10	<10	<10	<10
	Detection limit (MDL, ng/mL)	0.4	0.2	0.3	0.6	0.5
	Quantitation limit (LOQ, ng/mL)	1.2	0.6	0.9	1.8	1.5
	Recovery (%) (Ave over conc. range)	111	99	77	93	91
	Accuracy (±%) (Ave over conc. range)	10.7	8.0	19.6	13.2	14.2

by rotation for 5 min. The samples were centrifuged at 3600 rpm for 20 min and surface particulates removed by aspiration. The samples were decanted into disposable extraction tubes.

Plasma samples, spiked plasma calibration standards and positive control plasma samples (1 mL) were diluted with pH 6 0.1 M potassium dihydrogen orthophosphate buffer (2 mL) and water (4 mL) and lignocaine-d_{10} was added. The samples were mixed thoroughly by vortex. The samples were centrifuged at 3600 rpm for 20 min and surface particulates removed by aspiration. The samples were decanted into disposable extraction tubes.

2.4. Sample extraction

All samples were extracted by solid-phase extraction using customised Gilson ASPEC GX-274 instrumentation. SPE cartridges (CSDAU503, United Chemical Technologies, Inc., PA, USA) were prepared with methanol (2 mL), water (2 mL) and pH 6 0.1 M phosphate buffer (2 mL). The samples were loaded onto the cartridges and washed with water (2 mL), pH 6 0.1 M phosphate buffer (2 mL) and 1 M acetic acid (2 mL). The cartridge was dried with nitrogen for 5 min and then washed with chloroform/acetone (3:1 v/v, 5 mL) and methanol (2 mL). The cartridge was dried with nitrogen for 5 min and the target analytes eluted with ethyl acetate/dichloromethane/isopropanol/ammonium hydroxide solution (5:4:1 v/v/v + 4%, 5 mL). The extract was evaporated to dryness under a controlled flow of nitrogen and reconstituted with water/acetonitrile (70:30 v/v, 100 μL).

2.5. Instrumental analysis

Chromatography was carried out on a Shimadzu Prominence UPLC System (Shimadzu Scientific Instruments (Oceania) Pty Ltd., Rydalmere, NSW, Australia) using a Polaris C18-A 2.1 mm × 150 mm 5 μm analytical column (Varian Inc., Mulgrave, VIC, Australia) with a C18 2.1 mm × 4 mm 5 μm guard column (Phenomonex Australia Pty Ltd., Lane Cove, NSW, Australia). The mobile phase used comprised a mixture of acetonitrile and formic acid (aqueous 0.1% v/v) at 300 μL/min, and the composition was varied from 10 to 100% acetonitrile over a 9 min period. Sample analysis was performed on a 4000QTRAP mass spectrometer (Applied Biosystems, Mulgrave, VIC, Australia) using electrospray ionisation (ESI – Turbo V) in positive ion mode. Target analytes and the internal standard were determined by multiple reaction monitoring (MRM) of the following transitions; lignocaine, 235.2 > 86.1; 3-HO-LIG and 4-HO-LIG, 251.2 > 86.1; MEGX, 207.1 > 58.1; GX, 179.1 > 122.1; and lignocaine-d_{10}, 245.2 > 96.1. The response for each standard was calculated as a ratio of the target analyte peak area to the internal standard peak area. Standard responses were plotted against their respective concentrations to provide calibration curves.

2.6. Method validation

The method was validated as per the accredited Racing Science Centre Method Validation Procedure Ver. 2.3. This procedure is based on the guidelines outlined in the Eurachem Guide [10], International Laboratory Accreditation Cooperation (ILAC) G7 [11], Australian Standard AS ISO/IEC 17025:2005 [12], and NATA Technical Note 17 [13]. The following parameters were determined; instrument linearity, repeatability and detection limit (IDL), method linearity, repeatability, detection limit (MDL), quantitation limit (LOQ), accuracy and recovery.

3. Results and discussion

3.1. Detection and chromatography

Lignocaine and its hydroxylated (3-HO-LIG, 4-HO-LIG) and de-ethylated metabolites (MEGX, GX) were readily detected as chromatographically resolved peaks using the above procedure in positive ion mode ESI-MRM for both plasma and urine samples. The composite chromatograms (generated by adding the individual lignocaine and metabolite MRM responses) for plasma and urine samples, spikes and their respective blanks are shown in Fig. 2. No detectable interferences were observed on any of the chromatograms demonstrating good method specificity.

This method was unable to detect the theoretical mass ions of the following potential lignocaine metabolites; (3- or 4-)-hydroxymonoethylglycinexylidide, (3- or 4-)-hydroxyglycinexylidide, 4-hydroxy-2,6-xylidine. There was evidence of the 2,6-xylidine in urine samples but its measurement reproducibility was poor and the data has consequently not been reported. This method was unable to detect any contribution to non-deuterated lignocaine from the lignocaine-d_{10} internal standard.

3.2. Validation and calibration

The calibration curves were generated over the range 1–100 ng/mL for plasma samples and urine calibration standards ranged from 1 to 1000 ng/mL. A quadratic expression of the form

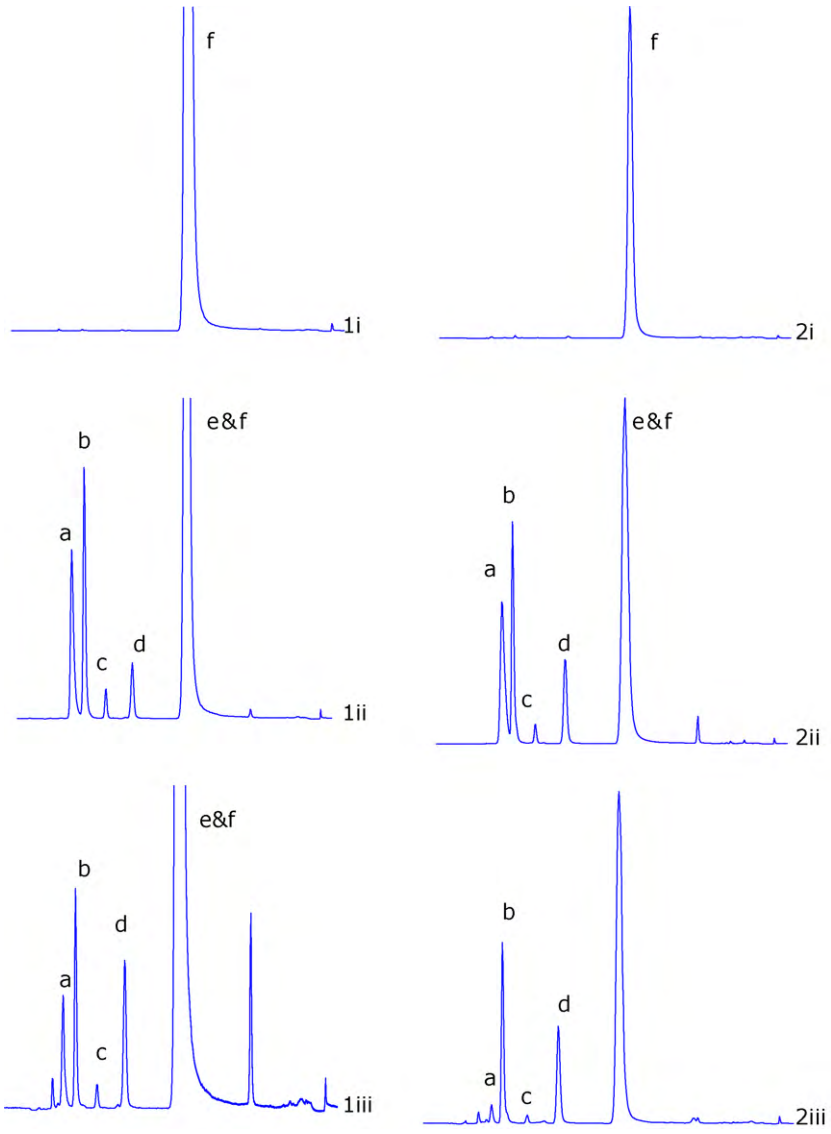

Fig. 2. Chromatograms of lignocaine and metabolites analysis in (1i) plasma blank, (1ii) plasma spike, (1iii) plasma sample 30 min post-administration, (2i) urine blank, (2ii) urine spike and (2iii) urine sample 2 h post-administration. Compound identification: (a) 4-HO-LIG, (b) 3-HO-LIG, (c) GX, (d) MEGX, (e) lignocaine and (f) lignocaine-d_{10}.

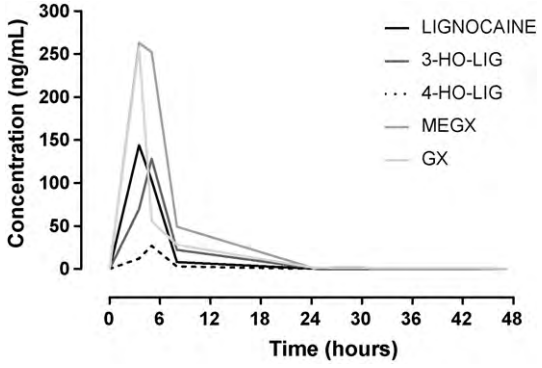

Fig. 3. Urinary concentrations per times post-administration of subcutaneously administered lignocaine.

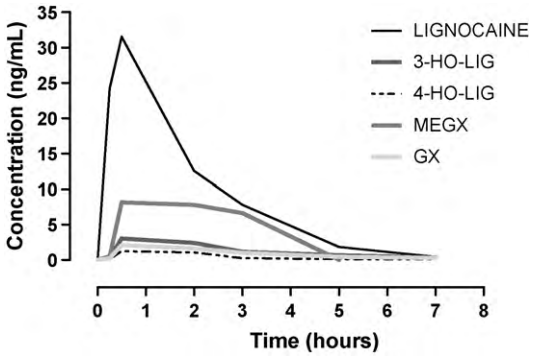

Fig. 4. Plasma concentrations per times post-administration of subcutaneously administered lignocaine.

$y = a + bx + cx^2$ was fitted to the calibration data and a correlation coefficient (R^2) of 0.99 or greater was obtained for lignocaine and all metabolites in both plasma and urine samples.

Repeatability or variability for replicate samples was better than 95% for all target analytes; the instrument detection limit ranged from 0.05 to 0.3 ng/mL (dependent on analyte); the method detection limits ranged from 0.1 to 0.6 ng/mL for all target analytes in both plasma and urine matrices; the limit of quantitation ranged from 0.3 to 1.8 ng/mL. The recovery of the target analytes ranged from 77 to 111%. The validation parameters are itemised in Table 1.

3.3. Plasma and urine concentration–time profiles (lignocaine and metabolites)

The described method was successfully applied to plasma and urine samples obtained from a horse following subcutaneous lignocaine administration. The results for lignocaine and the four metabolites are shown in Figs. 3 and 4. The concentrations of the metabolites observed in plasma were, in order, MEGX, 3-HO-LIG, GX, with smaller concentrations of 4-HO-LIG being seen over the sampling times. In urine, the concentrations of the metabolites were, in order, MEGX, GX, 3-HO-LIG and 4-HO-LIG.

4. Conclusions

The above method has been demonstrated to be sensitive, specific and robust and capable of generating reproducible data when used in clinical analyses for equine samples. The use of lignocaine-d_{10} as the internal standard proved to be very successful.

We are confident that this method is fit for purpose for the routine quantitative analysis of equine samples containing lignocaine and metabolites to meet the requirements of the racing industry. To the best of our knowledge, this is also the first report of plasma and urinary concentrations of 4-HO-LIG following lignocaine administration.

Acknowledgements

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