Determination of lidocaine and its two *N*-desethylated metabolites in dog and horse plasma by high-performance liquid chromatography combined with electrospray ionization tandem mass spectrometry

A. Maes [a,*], L. Weiland [b], C. Sandersen [c], F. Gasthuys [b], P. De Backer [a], S. Croubels [a]

[a] *Department of Pharmacology, Toxicology, Biochemistry and Organ Physiology, Faculty of Veterinary Medicine,*
Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium
[b] *Department of Surgery and Anaesthesiology of Domestic Animals, Faculty of Veterinary Medicine, Ghent University,*
Salisburylaan 133, B-9820 Merelbeke, Belgium
[c] *Département de Sciences Cliniques, ULg-Faculté de Médecine Vétérinaire, Blvd. de Colonster 20, Bât. B42, B-4000 Liège, Belgium*

Abstract

A sensitive method for the quantification of lidocaine and its metabolites, monoethylglycinexylidide (MEGX) and glycinexylidide (GX), in animal plasma using high-performance liquid chromatography combined with electrospray ionization mass spectrometry is described. The sample preparation includes a liquid–liquid extraction with methyl *tert*-butylmethyl ether after addition of 2 M sodium hydroxide. Ethylmethylglycinexylidide (EMGX) is used as an internal standard. For chromatographic separation, an ODS Hypersil column was used. Isocratic elution was achieved with 0.01 M ammonium acetate and acetonitrile as mobile phases. Good linearity was observed in the range of 2.5–1000 ng ml^{-1} for lidocaine in both dog and horse plasma. For MEGX, linear calibration curves were obtained in the range of 5–1000 ng ml^{-1} and 20–1000 ng ml^{-1} for dog and horse plasma, respectively. In dog and horse plasma good linearity was observed in the range of 200–1500 ng ml^{-1} for GX. The limit of quantification (LOQ) in dog plasma for lidocaine, MEGX and GX was set at 2.5 ng ml^{-1}, 20 ng ml^{-1} and 200 ng ml^{-1}, respectively. For horse plasma a limit of quantification of 2.5 ng ml^{-1}, 5 ng ml^{-1} and 200 ng ml^{-1} was achieved for lidocaine, MEGX and GX, respectively. In dog plasma, the limit of detection (LOD) was found to be 0.8 ng ml^{-1}, 2.3 ng ml^{-1} and 55 ng ml^{-1} for lidocaine, MEGX and GX, respectively. In horse plasma the LOD's found for lidocaine, MEGX and GX, were 1.1 ng ml^{-1}, 0.5 ng ml^{-1} and 13 ng ml^{-1}, respectively. The method was shown to be of use in pharmacokinetic studies after application of a transdermal patch in dogs and after an intravenous infusion in horses.

1. Introduction

Lidocaine is an aminoethylamide local anaesthetic drug. The clinical application of intravenous lidocaine for the treatment of acute postoperative pain in people and animals was reported more than 40 years ago [1,2]. Besides the use as local anaesthetic drug, lidocaine is also indicated for short-term emergency control of ventricular arrythmias [3]. The drug is extensively used topically for treatment of postherpetic neuralgia and other peripheral pain syndromes in men.

Transdermal drug delivery systems offer thereby specific benefits, such as almost no side effects which may be induced by systemic uptake. Since transdermal systems have minimal systemic absorption, it is expected that plasma concentrations are very low. Until now, only one study has been performed about the use of a transdermal lidocaine patch in animals (dogs) [4].

The two main de-ethylated metabolites of lidocaine are monoethylglycinexylidide (MEGX) and glycinexylidide (GX) [5]. These two metabolites have similar pharmacological and toxicological actions, being however less potent than lidocaine. In order to study the pharmacokinetic properties of lidocaine and its two metabolites in plasma, there is a need for highly sensitive and specific methods, especially after, e.g. transder-

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* Corresponding author. Tel.: +32 9 264 73 24; fax: +32 9 264 74 97.
E-mail address: an.maes@ugent.be (A. Maes).

mal drug delivery where low plasma concentrations can be expected.

A few procedures for the determination of lidocaine and metabolites in plasma using high-performance liquid chromatography with UV detection have been described [6–10]. The limit of quantification (LOQ) ranged from $50 \, \mathrm{ng \, ml^{-1}}$ to $800 \, \mathrm{ng \, ml^{-1}}$. Fukuda et al. [6,7] only mentioned the limit of detection (LOD), which ranged from $10 \, \mathrm{ng \, ml^{-1}}$ to $20 \, \mathrm{ng \, ml^{-1}}$.

With the introduction of LC–MS, very low LOQ's for lidocaine in human plasma, ranging from $0.2 \, \mathrm{ng \, ml^{-1}}$ to $2.5 \, \mathrm{ng \, ml^{-1}}$, have been achieved [11–13]. However, only one of these methods was developed to determine lidocaine in animal (rat) plasma, but without simultaneous quantification of its metabolites [11]. To our knowledge, the method published by Abdel-Rehim et al. was the first LC–MS/MS method reported for the simultaneous determination of lidocaine and its metabolites in human plasma and urine. For the sample clean-up an ultrafiltration step was performed and a triple quadrupole MS instrument was used for detection. The LOQ's described were $0.4 \, \mathrm{ng \, ml^{-1}}$ for lidocaine, MEGX and GX [14].

The purpose of our study was to develop an LC–MS/MS method using an ion trap instrument for the simultaneous determination of lidocaine and its metabolites MEGX and GX in dog and horse plasma.

2. Experimental

2.1. Biological samples

The dog plasma samples originated from six beagle dogs that were treated with a lidocaine patch containing 5% of lidocaine (700 mg/patch, Neurodol® Tissugel; IBSA, Lugano, Switzerland) [4]. The horse plasma samples originated from six horses that received an intravenous infusion of lidocaine, at a loading dose of $0.13 \, \mathrm{mg \, kg^{-1} \, min^{-1}}$ for 10 min and a maintenance dose of $0.05 \, \mathrm{mg \, kg^{-1} \, min^{-1}}$ for 110 min.

2.2. Chemicals and standards

Lidocaine, its metabolites monoethylglycinexylidide (MEGX) and glycinexylidide (GX), and the internal standard ethylmethylglycinexylidide (EMGX) were donated by AstraZeneca (Södertälje, Sweden). The structures of these analytes are shown in Fig. 1. HPLC grade water, methanol and acetonitrile were obtained from Acros (Geel, Belgium). Ammonium acetate and *tert*-butylmethyl ether were delivered by Sigma–Aldrich (Bornem, Belgium). Sodium hydroxide was purchased from Merck (Brussels, Belgium).

2.3. Instruments and methods

The LC–MS/MS analyses were performed using a Surveyor LC system consisting of a quaternary pump with vacuum degasser, an autosampler with cooling device and a LCQ Advantage® ion trap mass spectrometer equipped with an ESI source (all from Thermo Finnigan, San Jose, USA), run by XCALIBUR software (version 1.3).

For chromatographic separation, an ODS Hypersil column ($5 \, \mu\mathrm{m}$, $100 \, \mathrm{mm} \times 3.0 \, \mathrm{mm}$ i.d.) from Thermo Finnigan with a guard column of the same type ($5 \, \mu\mathrm{m}$, $10 \, \mathrm{mm} \times 3.0 \, \mathrm{mm}$ i.d.) was used. An isocratic run of 6 min was performed with a mobile phase of 0.01 M ammonium acetate in water/acetonitrile (40/60, v/v) at a flow rate of $0.2 \, \mathrm{ml \, min^{-1}}$. After each isocratic run the column was washed with 100% acetonitrile during 2 min and reconditioned for 10 min with the same mobile phase as the isocratic run.

Operating conditions for the ESI source, used in the positive ionization mode, were optimized by constantly adding a mixture of lidocaine, its metabolites and EMGX, each at a concentration of $10 \, \mu\mathrm{g \, ml^{-1}}$ in water/methanol (50/50, v/v), to the HPLC flow by a syringe pump via a T connector in the infusion mode. The following tune parameters were obtained for optimal lidocaine detection: spray voltage, 4.5 kV; sheath gas flow-rate, 60 (arbitrary units); auxiliary gas flow-rate, 10 (arbitrary units); capillary voltage, 3.0 V; capillary temperature, 230 °C; tube lens offset, 30 V; octapole 1 offset, −1.5 V; lens voltage, −7.0 V; octapole 2 offset, −6.0 V and octapole r.f. amplitude, $400 \, \mathrm{V_{p\text{-}p}}$. These tune parameters were also suitable for detection of GX, MEGX and EMGX, given the structural similarity between these components. The optimal collision energy in the MS/MS mode, corresponding to nearly 100% fragmentation of the protonated molecular ion of lidocaine (m/z 235 for M_r of 234.34), was found to be 1.9 V. For MEGX (m/z 207 for M_r of 206.29) the optimal collision energy was determined at 1.6 V and at 1.8 V for GX (m/z 179 for M_r of 178.24). Under these conditions, most abundant product ions at m/z 86, 58 and 122 were obtained for lidocaine, MEGX and GX, respectively. The optimal collision energy for EMGX (m/z 221 for M_r of 220.32) was determined at 1.7 V and m/z 72 was found to be the most abundant product ion. Quantification was effected with the LCQuan software, using the above-mentioned product ions.

2.4. Extraction method

2.4.1. Calibration curve and quality control samples

Stock solutions of lidocaine, MEGX, GX and EMGX of $100 \, \mu\mathrm{g \, ml^{-1}}$ were prepared in methanol and stored at −20 °C. The stock solutions of lidocaine, MEGX and GX were combined and diluted with methanol/water (50/50, v/v) to obtain working solutions containing $0.025 \, \mu\mathrm{g \, ml^{-1}}$, $0.05 \, \mu\mathrm{g \, ml^{-1}}$, $0.2 \, \mu\mathrm{g \, ml^{-1}}$, $0.5 \, \mu\mathrm{g \, ml^{-1}}$, $1.0 \, \mu\mathrm{g \, ml^{-1}}$, $2.5 \, \mu\mathrm{g \, ml^{-1}}$, $5.0 \, \mu\mathrm{g \, ml^{-1}}$ and $10 \, \mu\mathrm{g \, ml^{-1}}$ lidocaine, MEGX and GX. By adding $100 \, \mu\mathrm{l}$ of these working solutions or $100 \, \mu\mathrm{l}$ methanol/water (50/50, v/v) to $900 \, \mu\mathrm{l}$ of plasma, lidocaine, MEGX and GX concentrations of $2.5 \, \mathrm{ng \, ml^{-1}}$, $5 \, \mathrm{ng \, ml^{-1}}$, $20 \, \mathrm{ng \, ml^{-1}}$, $50 \, \mathrm{ng \, ml^{-1}}$, $100 \, \mathrm{ng \, ml^{-1}}$, $250 \, \mathrm{ng \, ml^{-1}}$, $500 \, \mathrm{ng \, ml^{-1}}$, $1000 \, \mathrm{ng \, ml^{-1}}$ and $0 \, \mathrm{ng \, ml^{-1}}$ in plasma, respectively, were obtained. All the working solutions were stored in a refrigerator (2–8 °C). The stock solution of the internal standard was also diluted in methanol/water (50/50, v/v) to a final concentration of $10 \, \mu\mathrm{g \, ml^{-1}}$. Quality control (QC) samples were prepared in a similar way using the working solution of $0.5 \, \mu\mathrm{g \, ml^{-1}}$ and $2.5 \, \mu\mathrm{g \, ml^{-1}}$, yielding a concentration of $50 \, \mathrm{ng \, ml^{-1}}$ and $250 \, \mathrm{ng \, ml^{-1}}$ of each analyte. For the analysis of
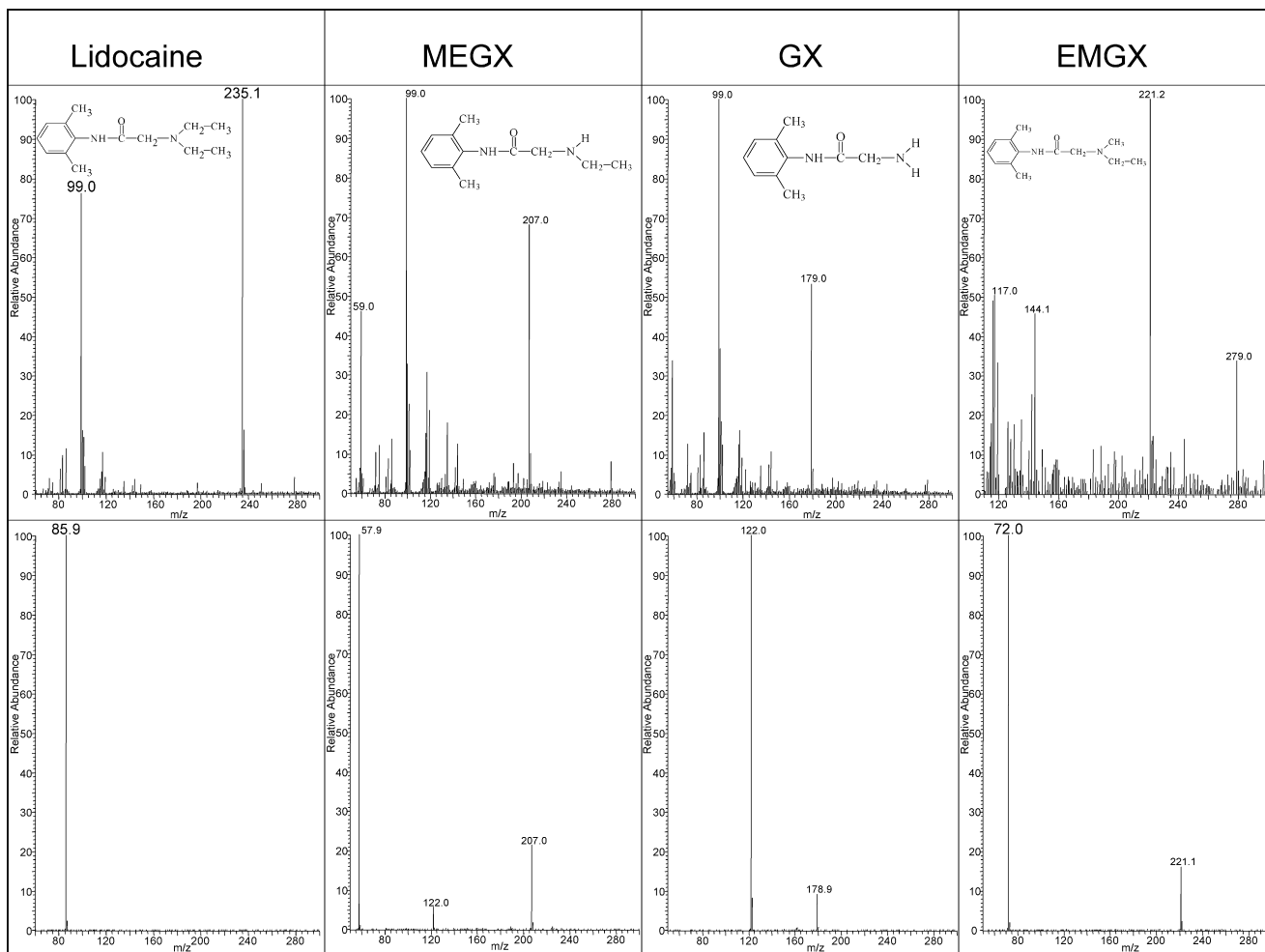
Fig. 1. Structure, MS and MS/MS spectra of lidocaine, MEGX, GX and EMGX, obtained after direct infusion of standard solutions of $10\,\mu g\,ml^{-1}$ of lidocaine, MEGX, GX and EMGX (ESI positive mode, collision energy in MS/MS = 1.9 V, 1.8 V, 1.6 V and 1.7 V for lidocaine, MEGX, GX and EMGX, respectively).

dog plasma, QC samples containing only $250\,ng\,ml^{-1}$ of each analyte were prepared.

2.4.2. Sample preparation

A $1000\,\mu l$ plasma sample was transferred into an extraction tube. All plasma samples, including calibration standards and quality control samples, were spiked with $100\,\mu l$ of the diluted internal standard ($10\,\mu g\,ml^{-1}$), except for the blanks. To all samples $250\,\mu l$ of 2 M sodium hydroxide was added. After vortex mixing for 15 s, 5 ml *tert*-butylmetyl ether was added. The tubes were placed on a horizontal roller and rotated for 10 min. The samples were then centrifugated at $1800 \times g$ for 10 min. The organic layer was transferred in another extraction tube and evaporated at $40\,°C$ under a gentle nitrogen stream. The dry residue was dissolved in $250\,\mu l$ of 0.01 M ammonium acetate/acetonitrile (50/50, v/v). An aliquot of $100\,\mu l$ was injected.

3. Validation procedure

The proposed method for the quantitative determination of lidocaine and metabolites was validated by a set of parameters which are in compliance with the recommendations as defined by the EC [15–18]:

1. Linearity: determined on calibration curves using spiked blank plasma samples (for levels, see Section 2.4.1). Peak area ratios between lidocaine, MEGX or GX and EMGX were plotted against their concentration and a linear regression was performed. The acceptance criterion was a correlation coefficient $r \geq 0.99$ and a goodness-of-fit coefficient $g \leq 20\%$ for lidocaine and MEGX and $g \leq 10\%$ for GX.
2. Trueness: determined by analyzing six independently spiked blank plasma samples at the same spike level. For dog plasma these levels were $20\,ng\,ml^{-1}$ and $500\,ng\,ml^{-1}$ for lidocaine, $50\,ng\,ml^{-1}$ and $100\,ng\,ml^{-1}$ for MEGX and $500\,ng\,ml^{-1}$ and $1000\,ng\,ml^{-1}$ for GX. In horse plasma spike levels of $5\,ng\,ml^{-1}$ and $25\,ng\,ml^{-1}$ for lidocaine, $25\,ng\,ml^{-1}$ and $500\,ng\,ml^{-1}$ for MEGX and $250\,ng\,ml^{-1}$ and $500\,ng\,ml^{-1}$ for GX were used. The trueness, expressed as the difference between the mean found concentration and the spiked concentration, should be in the range of -30% to $+10\%$ for levels $\leq 10\,ng\,ml^{-1}$ and -20% to $+10\%$ for levels $>10\,ng\,ml^{-1}$.
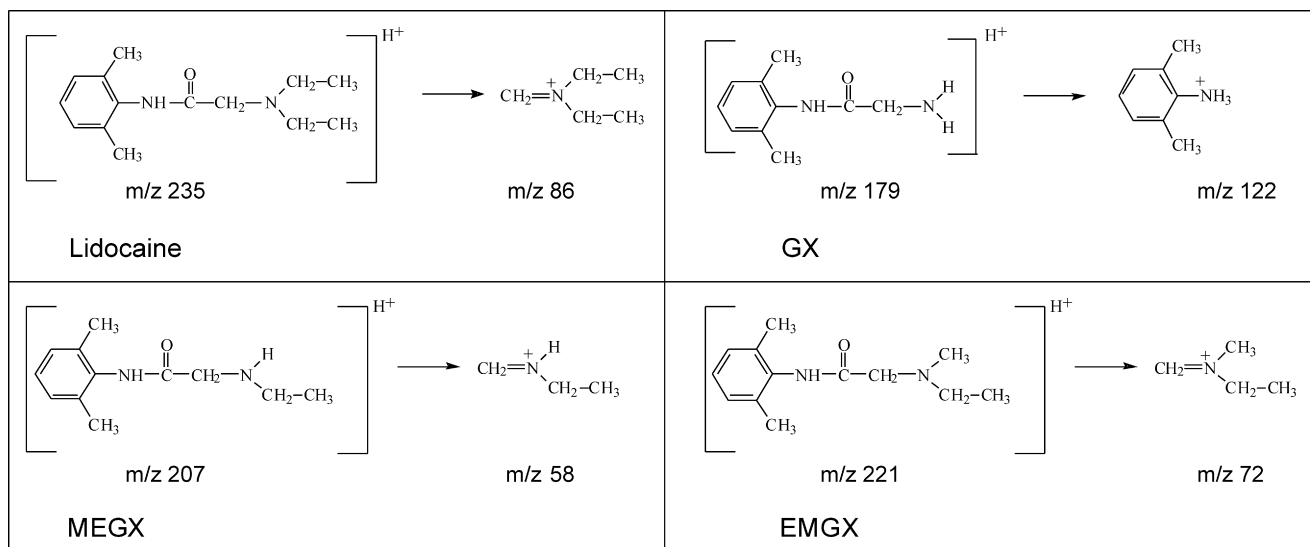
Fig. 2. Possible fragmentation scheme for lidocaine, MEGX, GX and EMGX based on the data published by Dal Bo et al. [13].
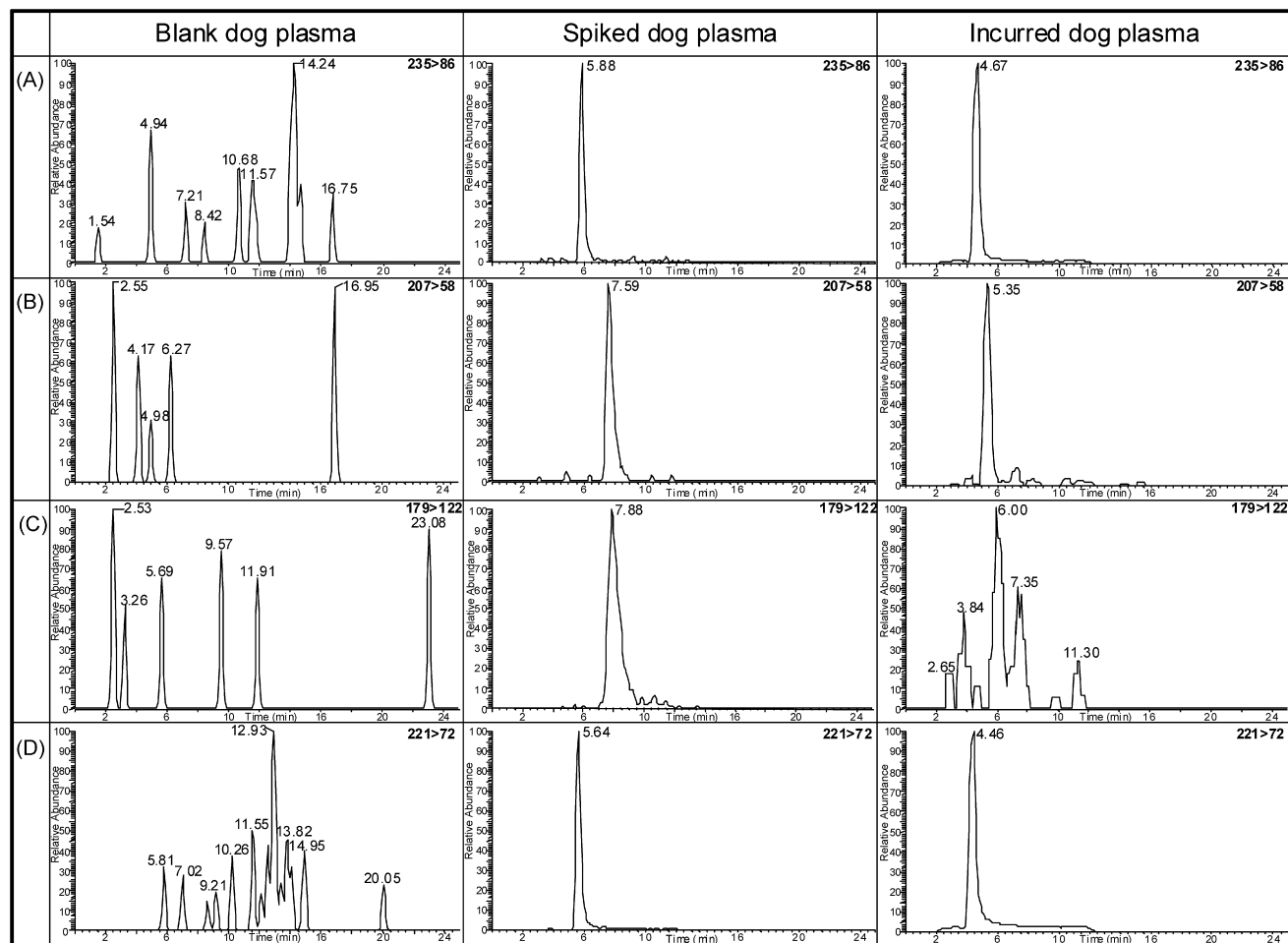

Fig. 3. Extracted-ion chromatogram of lidocaine (A), MEGX (B), GX (C) and EMGX (D) for a blank dog plasma sample (left), a blank dog plasma sample spiked at $50 \, ng \, ml^{-1}$ of lidocaine and MEGX and at $500 \, ng \, ml^{-1}$ and $1000 \, ng \, ml^{-1}$ for GX and EMGX, respectively (middle), and an incurred dog plasma sample at $25.1 \, ng \, ml^{-1}$ and $5.9 \, ng \, ml^{-1}$ of lidocaine and MEGX, respectively (right). No GX was detected.
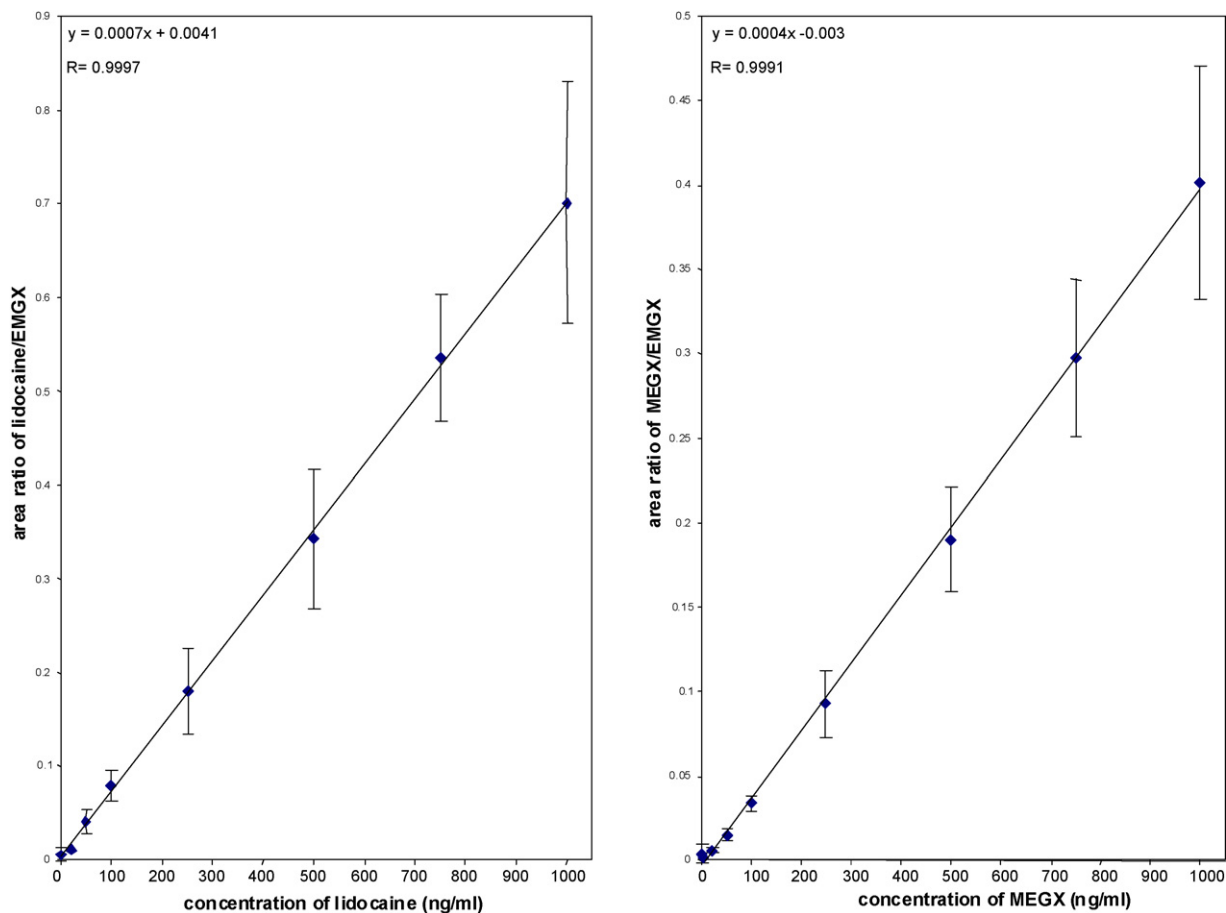
Fig. 4. Calibration curve of lidocaine (left) and MEGX (right) in dog plasma represented as the mean of six calibration curves constructed over a period of 144 days, each individual calibration curve being a new set of extractions.

3. Precision: expressed as the relative standard deviation (RSD, %), being the ratio between the standard deviation (SD) and the mean found concentration. For the within-day precision, the RSD should be lower than the values calculated according to two-thirds of the Horwitz equation: $RSD_{max} = 2/3 \times 2^{(1-0.5 \log C)}$, with C being the concentration at which the sample is fortified. It is determined using the same samples as for the trueness.

   The between-day precision was evaluated using samples with the same spike levels, but prepared and analyzed on different days. Dog plasma samples were fortified with $50 \, ng \, ml^{-1}$ and $250 \, ng \, ml^{-1}$. For horse plasma only levels at $50 \, ng \, ml^{-1}$ were used. The RSD should be lower than the $RSD_{max} = 2^{(1-0.5 \log C)}$.

4. Limit of quantification (LOQ): determined as the lowest concentration for which the method is validated with a trueness and precision that fall within the ranges recommended by the EU.

5. Limit of detection (LOD): determined as the lowest measured content from which it is possible to deduce the presence of the analyte with reasonable statistical certainty, using the criterion of a signal-to-noise (S/N) ratio of 3/1.

6. Ion suppression: for the study of the ion suppression a post-column infusion technique was used. A blank plasma sample

was injected onto the LC–MS instrument. A standard solution containing lidocaine, MEGX, GX and EMGX was continuously infused through a T-coupling device into the LC eluate. This allowed to visualize sections in the chromatogram where ion suppression occurs.

4. Results and discussion

4.1. Mass spectrometry

The structures of lidocaine, MEGX, GX and EMGX are shown in Fig. 1, together with their MS and MS/MS traces, obtained after direct infusion of a standard solution of $10 \, \mu g \, ml^{-1}$ in the ESI source operating in positive ion mode. In negative mode no signal was obtained. All publications found for the determination of lidocaine with LC–MS/MS also used the ESI source in positive mode [11–14]. In the MS mode the precursor ion for lidocaine, MEGX, GX and EMGX is the protonated molecular ion $[M+H]^+$ at m/z 235, 207, 179 and 221, respectively. In the MS/MS mode the most intense product ion is at m/z 86, 58, 122 and 72 for lidocaine, MEGX, GX and EMGX, respectively. A possible fragmentation scheme is presented in Fig. 2, based on the data for lidocaine published by Dal Bo et al. [13].

4.2. Sample preparation and chromatography

The chromatographic and extraction method was based on the method of Dal Bo et al. [13]. For chromatographic separation several columns were evaluated, i.e. Nucleosil C_{18} (Varian, 100 mm × 3.0 mm), ODS Persuit (Varian, 100 mm × 3.0 mm) and PRLP-S (Polymer Laboratories, 150 mm × 2.1 mm). These columns all gave broad peaks with poor peak shapes. The Hypersil ODS column (Thermo Finnigan, 100 mm × 3.0 mm) was chosen because acceptable peak shapes and retention times for all four components were obtained.

For the extraction method the organic layer of the samples was not frozen as described by Dal Bo et al., but was centrifugated longer and at a higher g-force. This was sufficient to obtain a good separation between the organic layer and plasma. Using this method, the organic layer was directly evaporated to dryness instead of performing a back-extraction using 17 mM H_3PO_4 solution. By evaporating the organic layer, the dry residue could by redissolved in the mobile phase, which was essential to obtain optimal chromatography. This method is straightforward and less time consuming since no back-extraction was performed.

Fig. 3 shows the extracted-ion chromatograms of lidocaine, MEGX, GX and EMGX for a blank dog plasma sample, a blank dog plasma sample fortified at 50 ng ml^{-1} for lidocaine and MEGX and at 500 ng ml^{-1} and 1000 ng ml^{-1} for GX and EMGX, respectively, and an incurred dog plasma sample containing 25 ng ml^{-1} and 5.9 ng ml^{-1} of lidocaine and MEGX, respectively. No GX was detected.

The blank plasma sample shown in Fig. 3 demonstrates that there are no interferences of endogenous components at the elution time zones of all four components.

4.3. Method validation

For the calibration curves good linearity was observed up to 1000 ng ml^{-1} for both dog and horse plasma. The goodness-of-fit coefficients (g) of the individual calibration curves were all <10.94% and the correlation coefficients (r) all >0.9938. In Fig. 4 the calibration curve of lidocaine and MEGX in dog plasma is presented as the mean of six calibration curves made over a period of 144 days, each calibration curve originating from a new set of extractions.

The results of the trueness and precision evaluation are summarized in Table 1. The trueness fell within the range of −30% and +10% for concentrations ≤10 ng ml^{-1} and within −20% and +10% for levels >10 ng ml^{-1}, testifying the good trueness

Table 1
Results of the trueness, within-day and between-day precision evaluation experiments for dog and horse plasma

Dog plasma	Lidocaine (ng ml^{-1})			MEGX (ng ml^{-1})			GX (ng ml^{-1})		
	2.5	20	500	20	50	100	200	500	1000
Within-day (n)	6	6	6	6	6	6	6	6	6
Average	2.7	18.7	488.3	20.8	52.7	102.2	209.3	436.0	914.2
RSD (%)	25.7	5.5	9.3	6.6	9.2	6.2	7.6	4.5	6.2
RSD$_{max}$ (%)	26.3	19.2	11.8	19.2	16.7	15.1	13.6	11.8	10.7
Trueness (%)	+7.8	−6.7	−2.3	+3.9	+5.5	+2.2	+4.7	−12.8	−8.6

Dog plasma	Lidocaine (ng ml^{-1})		MEGX (ng ml^{-1})	
	50	250	50	250
Between-day (n)	12	12	10	9
Average	51.0	246.2	51.3	222.5
RSD (%)	5.9	8.2	5.7	11.0
RSD$_{max}$ (%)	25.1	19.7	25.1	19.7
Trueness (%)	+1.9	−1.5	+2.6	−11.0

Horse plasma	Lidocaine (ng ml^{-1})			MEGX (ng ml^{-1})			GX (ng ml^{-1})		
	2.5	5.0	25	5.0	25	500	200	250	500
Within-day (n)	6	6	6	6	6	6	6	6	4
Average	2.4	5.1	25.0	5.1	24.6	470.6	191.7	273.2	486.9
RSD (%)	8.3	7.4	6.4	11.3	8.1	11.3	11.3	1.8	9.0
RSD$_{max}$ (%)	26.3	23.7	18.6	23.7	18.6	11.8	13.6	13.1	11.8
Trueness (%)	−4.9	1.5	−0.1	2.8	−1.7	−5.9	−4.1	+9.3	−2.6

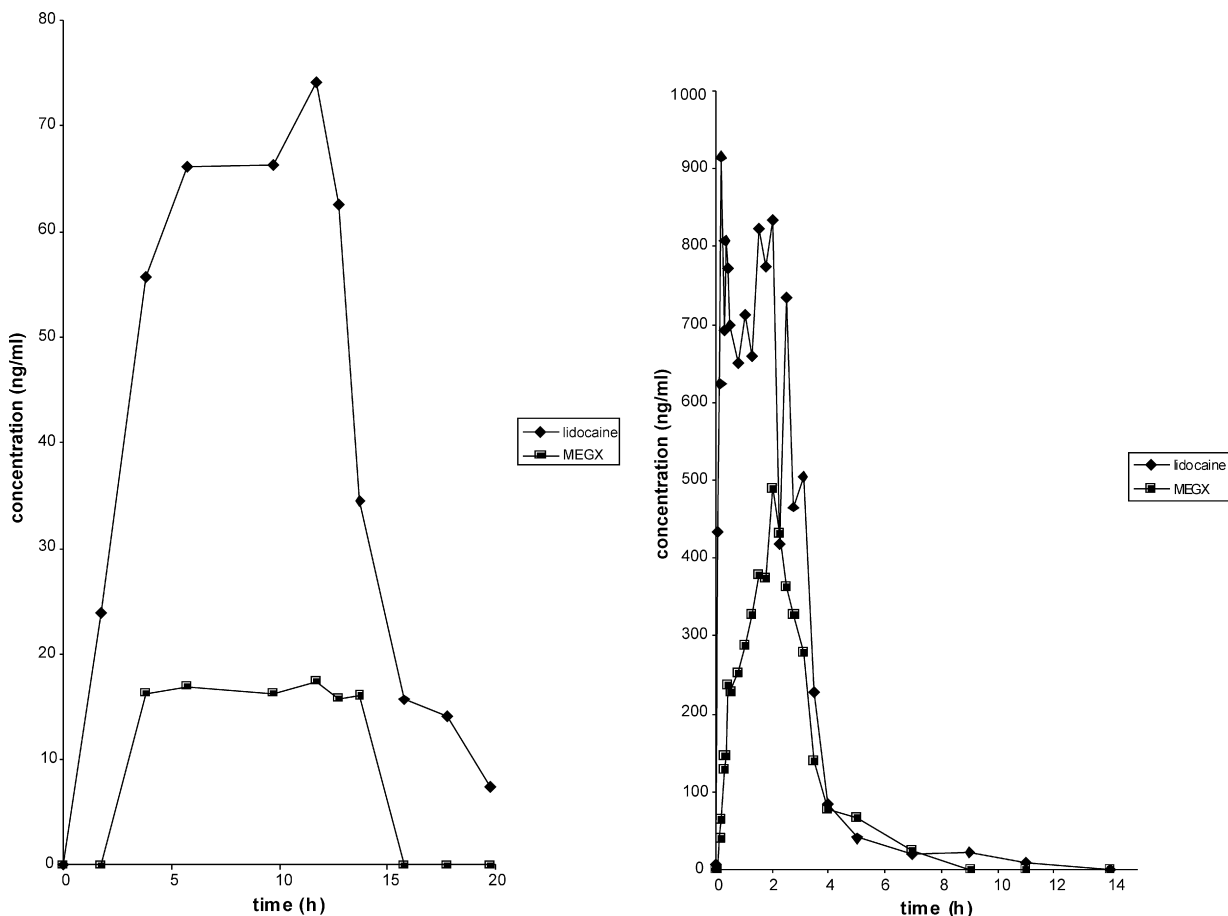| Horse plasma	Lidocaine (ng ml^{-1})	MEGX (ng ml^{-1})
	50	50
Between-day (n)	10	7
Average	50.5	49.9
RSD (%)	9.2	10.2
RSD$_{max}$ (%)	25.1	25.1
Trueness (%)	0.9	−0.3

Fig. 5. Plasma concentrations of lidocaine (♦) and MEGX (■) in a dog treated with a single lidocaine patch 5% for 12 h (left) and in a horse that received an intravenous infusion (right).

of the method. The precision also fell within the maximum RSD values.

The LOQ was established by analyzing six blank plasma samples, which were spiked with lidocaine at a level of 2.5 ng ml^{-1} for both dog and horse plasma, with MEGX at a level of 5 ng ml^{-1} and 20 ng ml^{-1} for horse and dog plasma, respectively, and with GX at 200 ng ml^{-1} for both dog and horse plasma. The results are summarized in Table 1. Since these levels could be quantified fulfilling the criteria for trueness and precision, they were set as the LOQ for lidocaine, MEGX and GX, respectively.

The mean S/N ratio for the six LOQ samples was used to calculate the LOD, which was 0.8 ng ml^{-1}, 2.3 ng ml^{-1} and 55 ng ml^{-1} for lidocaine, MEGX and GX, respectively, in dog plasma. For horse plasma the calculated LOD was 1.1 ng ml^{-1}, 0.5 ng ml^{-1} and 13 ng ml^{-1} for lidocaine, MEGX and GX, respectively.

No ion suppression was noticed at the elution time zones of all four analytes in either dog or horse plasma.

4.4. Analysis of biological samples

The above described method for the quantitation of lidocaine and metabolites in dog plasma was used in a pharmacokinetic study [4]. A single lidocaine patch 5% was applied for 12 h to

six dogs. Lidocaine concentrations varied from 2.8 ng ml^{-1} to 155.4 ng ml^{-1}.

Another pharmacokinetic study was performed in six horses after intravenous infusion. Plasma concentrations ranged from 4.6 ng ml^{-1} to 1956.3 ng ml^{-1} and from 10.8 ng ml^{-1} to 490.6 ng ml^{-1} for lidocaine and MEGX, respectively, in samples collected between 1 min and 14 h after start of the administration.

A representative plasma concentration versus time profile in one dog and one horse is shown in Fig. 5.

Moreover, to demonstrate further the practicability and applicability of the LC–MS/MS method, the following data can be mentioned. The total number of unknown incurred dog plasma and horse plasma samples was 168 and 189, respectively. The number of spiked samples analyzed was 72 and 24 for dog and horse plasma, respectively. All samples were analyzed using the same HPLC column.

5. Conclusion

The method described in this paper for the quantitation of lidocaine, MEGX and GX is a fast procedure. The minimal sample preparation, namely a liquid–liquid extraction, allows the extraction of many samples a day. This method was successfully applied for the analysis of dog and horse

plasma during various pharmacokinetic studies. The LOQ's of the method made it possible to quantify low plasma concentrations, which is especially of use after transdermal drug delivery and to characterize the terminal elimination phase of the compounds.

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