

Simultaneous determination of lidocaine, prilocaine and the prilocaine metabolite *o*-toluidine in plasma by high-performance liquid chromatography

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Abstract

An HPLC assay is described for the measurement of prilocaine and lidocaine (components of the local anesthetic cream EMLA) as well as the prilocaine metabolite, *o*-toluidine, in plasma. The method uses UV detection, is simple, sensitive and most important, only a single 200- μ l plasma sample is needed for simultaneous analysis of prilocaine, lidocaine and *o*-toluidine with a detection limit of 4 ng/ml. The plasma, together with the internal standard (bupivacaine) is extracted with diethyl ether under alkaline conditions, followed by the extraction of the analytes from the organic phase into dilute sulphuric acid. An aliquot of the acid extract is injected onto the HPLC system and the effluent is monitored by a UV detector.

1. Introduction

In recent years the eutectic mixture of local anesthetics (EMLA) has been used successfully to alleviate pain associated with medical procedures in both children and adults [1–3]. EMLA cream penetrates intact skin without causing irritation [4]. One gram of EMLA contains 25 mg of lidocaine and 25 mg of prilocaine. Both prilocaine and lidocaine are extensively metabolized by the liver. De-ethylation of lidocaine yields aminoethylglycine xylylide (MEGX) and acetaldehyde, while prilocaine, a secondary amine, is hydrolysed by amidases to *o*-toluidine (OT) and N-tyrosylalanine.

o-Toluidine has been shown to be responsible

for methaemoglobinemia associated with doses of prilocaine exceeding 600 mg in adults [5] or 8 mg/kg in children. In newborn infants the enzyme methaemoglobin reductase is not yet fully developed, leading to increased likelihood of clinically important methaemoglobinemia. For this reason EMLA is presently not approved for this age group, pending ongoing studies on its kinetics and dynamics.

At present there are only a few studies where the plasma concentrations of prilocaine and lidocaine have been determined following the topical application of EMLA [2,3]. However, the plasma concentration of OT has never been measured. Since OT is responsible for the dose limiting toxicity of EMLA in young children, its determination is crucial for the assessment of this agent's safety.

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In this report we describe a simple, fast and sensitive high-performance liquid chromatography (HPLC) method with UV detection for the simultaneous measurement of lidocaine, prilocaine and OT in a single small plasma sample.

2. Experimental

2.1. Instrumentation and chromatographic conditions

A Shimadzu LC-6A liquid chromatograph equipped with a 5- μm Octyl 1B column 150 \times 4.6 mm I.D. (Keystone Scientific Instruments Bellefonte, PA, USA) was used at room temperature. The column effluent was monitored by a Shimadzu SPD-6AV spectrophotometer at 210 nm.

Samples were applied to the system via a Shimadzu SiL-6B autoinjector and the data were processed with a Shimadzu C-R4A chromatopack unit (Shimadzu Scientific Instruments, Columbia, MD, USA) The mobile phase was acetonitrile–0.05 M sodium phosphate (Na_2HPO_4) buffer (27:73, v/v). The pH was adjusted to 5.8 with 50% phosphoric acid. The flow-rate was 1 ml/min.

2.2. Reagents and materials

Prilocaine and lidocaine were kindly provided by Astra Pharmaceutical Products (Westborough, MA, USA). Bupivacaine was kindly supplied by Sterling-Winthrop Research Institute (Rensselaer, NY, USA). OT was purchased from Aldrich (Milwaukee, WI, USA). Anhydrous ethyl ether, acetonitrile, sodium hydroxide and sodium phosphate were purchased from Fisher Scientific (Pittsburgh, PA, USA).

2.3. Assay procedure

A 200- μl volume of plasma or standard was pipetted into a 15-ml conical centrifuge tube. A 10- μl volume of internal standard (bupivacaine 15 mg/ml) was added and mixed. One hundred

μl 2M NaOH were added, vortex-mixed briefly, and then 5 ml anhydrous ethyl ether were added. The tubes were capped, vortex-mixed for 30 s and then mixed on a rotator for 10 min. Subsequently the mixture was centrifuged at 1000 g for 5 min. Subsequently 4.5 ml of ether were transferred to a clean conical centrifuge tube and 250 μl of 0.0125 M H_2SO_4 were added. Once again the mixture was vortex-mixed for 30 s, mixed on a rotator for 10 min, centrifuged for 5 min and the top ether phase was removed and discarded. A 50- μl aliquot from the acid phase was injected onto the HPLC system.

2.4. Extraction recovery

To establish the recovery of prilocaine, lidocaine and OT after extraction from plasma, drug free plasma was spiked with all three analytes to a final concentration of 20 ng/ml and 1000 ng/ml and carried through the assay procedure as described. For 100% recoveries the three analytes were spiked into empty tubes and diluted with water to final concentrations of 20 ng/ml and 1000 ng/ml. Of each preparation, 40 μl were injected directly, without extraction, onto the HPLC system.

2.5. Linearity

Drug free plasma was spiked with increasing concentrations of lidocaine, prilocaine and OT (20–1000 ng/ml) and a fixed concentration of bupivacaine. Three standard calibration curves were constructed by performing linear regression analysis of the peak-height ratios (L/bupivacaine, P/bupivacaine, OT/bupivacaine) vs. the respective analyte concentration.

2.6. Precision and sensitivity

The three analytes were added to drug free normal plasma to give concentrations of 50 ng/ml and 500 ng/ml. Precision was determined by replicate analysis ($n = 6$) of each of these plasma pools. The lower limit of quantitation was taken as the concentration of the standard giving a signal-to-noise ratio greater than two.

2.7. Preliminary pharmacokinetics of lidocaine and prilocaine in piglets

Full term male newborn piglets were premedicated with atropine, anaesthetized by inhalation of halothane, intubated and mechanically ventilated throughout the study period. The piglets were cannulated through a neck vein for venous blood sampling and a bladder catheter was placed for urine collection. On day 1 of the study 25 mg of lidocaine and 25 mg of prilocaine were administered as an intravenous bolus injection. Blood and urine samples were collected at time 0 (prior to injection), 30, 60, and 120 min after the injection, and then at 2 h intervals for a total of 8 h. These samples were analysed for prilocaine, lidocaine and OT. The entire procedure was repeated 3 days later when 1 gram of EMLA (containing 25 mg of lidocaine and 25 mg of prilocaine) was applied to the penile surfaces of the piglets. The treated area was covered with an occlusive dressing. The cream was removed after 60 min. Sampling was carried out at the same times as described above.

3. Results

A typical chromatogram of extracted plasma, containing lidocaine, prilocaine and OT is shown in Fig. 1. The chromatogram shows sharp peaks with complete baseline separation between peaks. The retention times of the analytes were as follows: prilocaine 3.32 ± 0.66 min; lidocaine: 4.90 ± 0.11 min; OT: 7.31 ± 0.17 min; bupivacaine: 9.81 ± 0.21 min; $n = 25$. The percentage recovery for each analyte is presented in Table 1. Plasma proteins do not seem to affect the extraction recoveries since the same recoveries were found when the analytes were spiked in water and then extracted.

The response was linear in the range studied (20–1000 ng/ml) for all the analytes. Typical linear regression equations were: lidocaine: $y = 0.003x - 0.014$ ($r^2 = 0.99$), prilocaine: $y = 0.003x + 0.035$ ($r^2 = 0.978$), and *o*-toluidine: $y = 0.004x - 0.032$ ($r^2 = 0.998$).

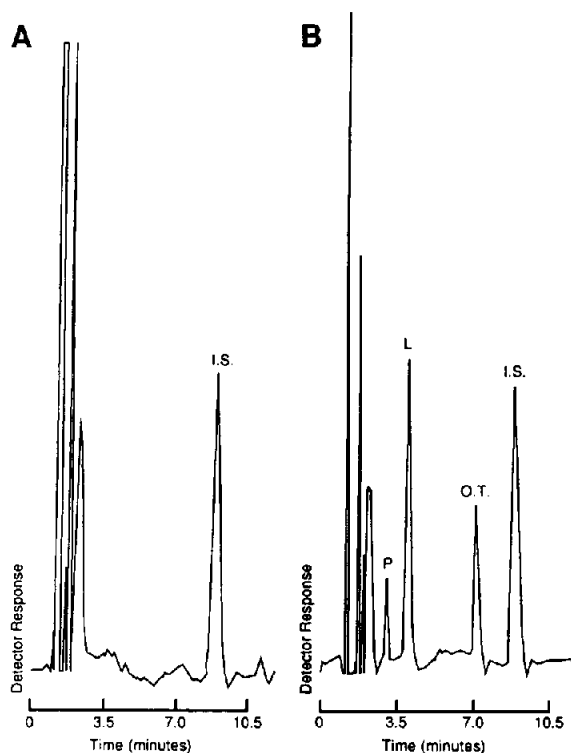


Fig. 1. HPLC elution profile for extracted plasma samples from a full term male newborn piglet. (A) Prior to medication (blank), (B) 2 h after an intravenous bolus injection of 25 mg lidocaine and 25 mg prilocaine. The concentrations of the analytes were: prilocaine: 50.8 ng/ml, lidocaine 532.0 ng/ml, *o*-toluidine: 275.3 ng/ml.

The assay was reproducible with low intra- and inter-day variation. The accuracy and precision results are given in Table 2. The lower limit of quantitation was found to be 20 ng/ml for each analyte when 200 μ l of plasma were used. The real detection limit (signal-to-noise ratio greater than two) was found to be 4 ng/ml, *i.e.* 200 pg injected on column for each analyte.

The concentration *vs.* time curves for one of the piglets are presented in Figs. 2 and 3. Following *i.v.* administration of lidocaine and prilocaine, the highest concentrations were observed at 30 min, being 2066 ng/ml and 632 ng/ml, respectively. The highest OT level was observed also after 30 min at 242.5 ng/ml. All three analytes were measurable at each time point. The area under the curve $AUC_{0 \rightarrow 8h}$ was

Table 1
Extraction recovery

Concentration (ng/ml)	Extraction recovery (%)			
	Prilocaine	Lidocaine	<i>o</i> -Toluidine	<i>n</i>
20	98 ± 4	99 ± 3	68 ± 5	6
1000	103 ± 6	100 ± 7	75 ± 3	6

Table 2
Within-day and between-day precision of prilocaine, lidocaine, and *o*-toluidine determination

Concentration (ng/ml)	<i>n</i>	Mean concentration observed ^a (ng/ml)		
		Prilocaine	Lidocaine	<i>o</i> -Toluidine
<i>Within-day</i>				
50	6	47.3 (5.3)	53.6 (6.7)	51.0 (8.3)
500	6	541.0 (3.7)	495.5 (3.1)	476.8 (7.8)
<i>Between-day</i>				
40	6	38.8 (6.3)	42.0 (5.4)	43.2 (7.5)
200	6	202.1 (7.5)	209.6 (6.2)	189.9 (8.3)

4213 ng/ml h for lidocaine and 897 ng/ml h for prilocaine (Fig. 2).

Following administration of EMLA, the highest concentrations of lidocaine and prilocaine were observed again after 30 min and were respectively 140 ng/ml and 39 ng/ml. Detectable levels were found only at the 30 min and 60 min

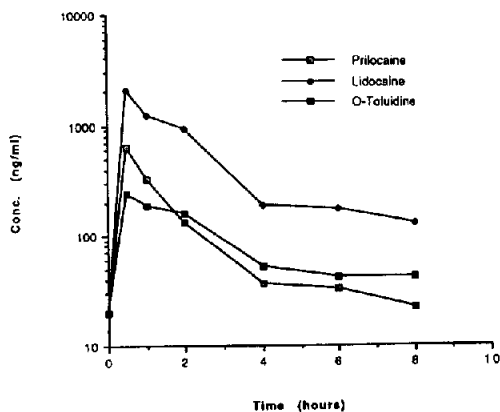


Fig. 2. Plasma drug concentration vs. time after intravenous administration of 25 mg prilocaine and 25 mg lidocaine in a full term male newborn piglet.

time points, while all other concentrations were below the limit of quantitation. OT could not be detected at any time point (Fig. 3). The bioavailability of EMLA, calculated as the ratio between the area under the curve calculated after EMLA administration and the area under the curve after i.v. administration of lidocaine and prilocaine was 3.0% for lidocaine and 5.0% for prilocaine.

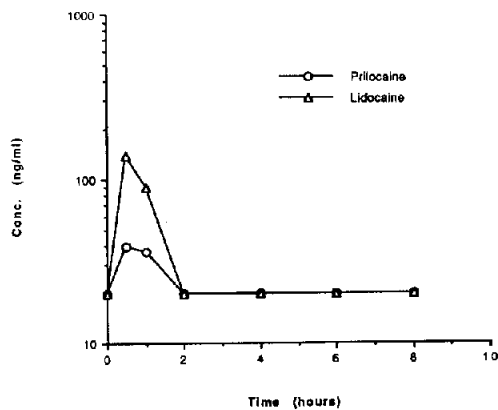


Fig. 3. Plasma drug concentration vs. time after penile application of 1 g of EMLA cream in the same piglet.

4. Discussion

The objective of this study was to develop a time- and cost-effective method for the detection of lidocaine, prilocaine and its main metabolite *o*-toluidine in plasma. While many methods have previously been described for prilocaine or lidocaine quantitation [6–9], these are expensive gas chromatographic–mass–spectrometric methods [6–8] or a mass fragmentographic method [2]. Neither of these methods are suitable for clinical use. Moreover, neither of them describes the measurement of *o*-toluidine, the prilocaine metabolite responsible for methemoglobinemia.

An HPLC method [10] for the detection of the local anesthetics lidocaine, mepivacaine, prilocaine, bupivacaine and etidocaine has been previously reported. However, the detection of their metabolites is not described. Using as starting material 1 ml of plasma, the detection limit for each anesthetic analyzed was 30 ng/ml. By modifying both the extraction procedure and the chromatographic conditions (mobile phase, choice of column), we were able to detect in one single 200- μ l plasma sample prilocaine, lidocaine and *o*-toluidine.

While EMLA cream has been shown to reduce procedural pain [10], its potential adverse effects in neonates necessitates in-depth studies of its pharmacokinetics and pharmacodynamics.

Methaemoglobinemia can be a life threatening complication of local anesthesia in some infants [11], due to immaturity of the methaemoglobin reductase enzyme in the first 3 months of life. The therapeutic dose of EMLA in infants between 6–12 months of age is recommended to be no greater than 2 g applied for no longer than 4 h. Currently EMLA is not recommended for use in infants under the age of 6 months [12]. The dose of EMLA used in the newborn piglets was 1 g which is comparable to the amount that may be used in human newborns undergoing circumcision. The mechanism for methaemoglobin production is by metabolism of prilocaine to *o*-toluidine and subsequent oxidation of haemoglobin to superoxide anions, hydroperoxy radicals, hydrogen peroxide or intermediate peroxhaemoglobin complexes [13]. No correlation

has been found between the maximum prilocaine concentration and maximum methaemoglobin values [14]. Since OT has never been quantitated in plasma, a direct correlation between OT concentration and methaemoglobin formation could not be assessed. Due to the limitations in obtaining blood samples from neonates, there is a need for a simple and accurate method for quantifying prilocaine and its OT metabolite in plasma. Our preliminary studies in piglets show that, following intravenous administration of lidocaine and prilocaine, there was considerable formation of OT, while when EMLA was applied topically OT levels were marginal with minimal increase in methaemoglobin over baseline in either case.

The ability to use only a single 200- μ l plasma sample for the simultaneous determination of lidocaine, prilocaine and OT will likely speed up the study of the toxicokinetics of EMLA in young infants. Measurement of systemic exposure to prilocaine in terms of the area under the concentration–time curve is crucial for calculating the formation and elimination of the toxic OT metabolites. The excellent between- and within-day precisions of this assay and its high sensitivity are likely to make it useful for establishing EMLA therapy in young children, ultimately leading to an improvement in the management of procedural pain in this age group.

5. Acknowledgement

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6. References

- [1] B. Hallen, P. Carlsson and A. Uppfeldt, *Brit. J. Anaesthesia*, 57 (1985) 326.
- [2] I.E. Malmros, T. Nilsen and S. Lillieborg, *Acta. Derm. Venereol.*, 70 (1990) 227.
- [3] G. Engberg, K. Danielson, S. Henneberg and A. Nilsson, *Acta. Anaesthesiol. Scand.*, 31 (1987) 624.
- [4] H. Evens, O. Van Dardel, L. Juhlin, L. Ohlsen and E. Vinnars, *Anaesthesia*, 57 (1985) 997.

- [5] J.P. Cano, M. Charbit-Bues and T. Rochaix, *Cahiers D'Anesthesiologie*, 33 (1985) 501.
- [6] R.L. Nation, E.J. Triggs and M. Selig, *J. Chromatogr.*, 116 (1976) 188.
- [7] N. Benowitz and M. Rowland, *Anesthesiology*, 39 (1973) 639.
- [8] R.L. Nation, G.W. Peng and W.L. Chion, *J. Chromatogr.*, 162 (1979) 466.
- [9] H.A. Adams, J. Biscopig, K. Ludolf, A. Borgmann, B. Bechmann-Mand and G. Hempelman, *Regional Anaesthesia*, 12 (1989) 53.
- [10] C. Wohlstedt, H. Kollberg, C. Moller and A. Uppfeldt, *Lancet*, ii (1984) 106.
- [11] S. Mandel, *JAMA*, 261 (1989) 702.
- [12] EMLA[®] cream, *Astra Product Monograph*, 1990.
- [13] M. Heljm, B. Ragnarsson and P. Wistrand, *Biochem. Pharm.*, 21 (1972) 2825.
- [14] A. Nilsson, S. Engberg, S. Henneberg, K. Danielson and C.H. DeVerdier, *Brit. J. Anesthesia*, 64 (1990) 72.