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ORIGINAL RESEARCH

Simultaneous determination of ropivacaine and antipyrine by high performance liquid chromatography and its application to the *in vitro* transplacental study

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ABSTRACT: A simple and reliable RP-HPLC method has been developed for the simultaneous determination of ropivacaine and antipyrine in perfusate samples. Samples were analyzed on an ODS column with UV detection at 210 nm after liquid–liquid extraction. The mobile phase consisted of potassium dihydrogenphosphate (25 mM, adjusted to pH 5.0 with phosphoric acid)–acetonitrile (79:21, v/v). The method has been validated to be precise, accurate and linear. It has been applied to the investigation of placental transfer of ropivacaine via a dually perfused cotyledon model of human placenta *in vitro*.

KEYWORDS: ropivacaine; antipyrine; placental transfer

INTRODUCTION

Ropivacaine, the first amide-type local anesthetic agent supplied as pure optical isomer, has been widely clinically used. It has been demonstrated that ropivacaine has a more favorable clinical safety profile and less toxicity to the cardiac system, compared with other anesthetics. In addition, it can sensitively block sensory fibers, while less motor fiber block occurs. This makes ropivacaine an attractive local anesthetic for obstetric use.

There have been major concerns regarding fetal exposure to maternal medications as the number of drugs used in pregnancy continues to increase. It is necessary to perform placental transfer studies of drugs to determine their safety for use during pregnancy, since some drugs may reach high concentrations in fetal circulations and produce side effects.

A dually perfused modle of human placental single cyledon has been usually used in transplacental study since 1972 (Schneider *et al*., 1972). Antipyrine was selected as an ideal marker of placental transfer as it is of moderate solubility and does not bind to plasma proteins. Antipyrine has been determined either spectrophotometrically or by high performance liquid chromatography in some reported transplacental studies (Heikkine *et al*., 2002; Johnson *et al*., 1999). The simultaneous analysis of ropivacaine and antipyrine has not been reported. We reported a validated simultaneous assay of ropivacaine and antipyrine in perfusate samples by high performance liquid chromatography with UV detection in the present study. Using this simple method, a transplacental study of ropivacaine has been performed via an open model (single pass mode) of perfused Chinese human placenta.

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Abbreviations used: KRB, Krebs–Ringer buffer.

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EXPERIMENTAL

Chemicals and reagents. Ropivacaine hydrochloride was supplied as Naropin® (2.0 mg/mL) ampoules obtained by AstraZeneca (NSW, Sweden), which contained normal saline in isotonic concentrations with no other substances. Antipyrine was purchased from Sigma–Aldrich (Dorset, UK), and 4-acetylaniline (internal standard, purity >98.0%) was obtained from Shanghai Chemical Reagents Co. (Shanghai, China).

Potassium dihydrogenphosphate, phosphoric acid (85%) and ethyl acetate of analytical grade were all purchased from Shanghai Chemical Reagents Co. (Shanghai, China). Acetonitrile of HPLC grade was supplied by TEDIA (Fairfield, USA). Krebs–Ringer buffer (KRB) solution (NaCl 119.0 mM, KCl 4.8 mM, $CaCl_2{\cdot}2H_2O$ 2.6 mM, KH_2PO_4 1.2 mM, $MgSO_4{\cdot}7H_2O$ 1.2 mM and $NaHCO_3$ 25.0 mM) was prepared in the laboratory.

HPLC analysis. All chromatographic analysis were performed on an Agilent 1100 HPLC system (Wilmington, DE, USA) equipped with a G1322A online degasser (Japan), a G1311A QuatPump system (German), a G1316A COLCOM column box (German), a G1314A variable wavelength UV detector (Japan) set at 210 nm, a Rheodyne 7725i manual sample injector (USA), and an Agilent chemstation for LC 3D. The separation was performed on a Diamonsil® C_{18} column (150 ×

4.6 mm i.d., 5 μm), and the column temperature was set at 30°C. The mobile phase consisted of 25 mmol/L potassium dihydrogenphosphate–acetonitrile (79:21, v/v), which contained 20 mmol/L triethylamine and was adjusted to pH 5.0 with phosphoric acid. The flow rate was set at 1.0 mL/min.

Sample extraction. To 1 mL perfusate sample placed in 15 mL glass tube, 100 μL of I.S. solution (2.5 μg/mL) and 0.5 mL of 0.2 mol/L sodium carbonate–ammonia water buffer solution (pH = 11.0) were added. The samples were extracted with 6 mL of ethyl acetate for 2 min on a vertex mixer. After centrifuged at 2500 ***g*** for 10 min, the organic layers were transferred into another clean tube and evaporated to dryness under a nitrogen stream in a water bath of 50°C. The residues were dissolved in 100 μL mixture of methanol and water (1:1, v/v). Aliquots of 20 μL were injected into the HPLC system.

Method validation. Calibration curves were prepared with drug-free perfusate spiked with ropivacaine, antipyrine and 4-acetylaniline (nine standards in duplicate) to cover the concentration ranges of 10–1000 ng/mL ropivacaine and 0.1–10 μg/mL antipyrine with the internal standard at the fixed concentration of 250 ng/mL. Calibration curves were obtained by plotting drug concentrations against the peak–area ratios of ropivacaine/I.S. or antipyrine/I.S. The concentrations of unknown samples (ropivacaine or antipyrine) were determined using the linear regression analysis (unweighed) of the calibration standards.

The accuracy of the analytical method was determined by comparing the concentrations of ropivacaine and antipyrine found from drug-free perfusate spiked with ropivacaine and antipyrine to the added concentrations of ropivacaine (25, 250 and 1000 ng/mL) and antipyrine (0.25, 2.5 and 10 μg/mL), five replicates at every concentration.

The extraction recovery of antipyrine in perfusate was determined at concentrations of 0.25, 2.5 and 10 μg/mL, and that of ropivacaine at concentrations of 25, 250 and 1000 ng/mL by comparing the peak area obtained after extraction to those obtained by the direct injection of standard aqueous solutions.

The inter-day precisions of the method were evaluated by analyzing drug-free perfusate, to which ropivacaine had been added at concentrations of 25, 250 and 1000 ng/mL and antipyrine at concentrations of 0.25, 2.5 and 10 μg/mL, every in five replicates. Intra-day precisions were evaluated on five different days at concentrations as mentioned above.

Placental transfer study. After Institutional Review Board approval and informed, written consent, six normal term human placentas were obtained immediately after vaginal or cesarean deliveries from healthy mothers, who had had uncomplicated prenatal courses. These freshly obtained placentas were perfused immediately via the umbilical artery using cold (4°C) heparinized 0.9% NaCl solution. Transplacental study of ropivacaine was performed on an open (single pass mode) perfusion system, as described in the literature (Johnson *et al*., 1999). The perfusion was performed with 250 mL drug-free plasma in maternal side and 100 mL KRB solution with 4 g/100mL albumin in the fetal circuit. The flow rates of the pefusate in the maternal and fetal sides were 15 and 2 mL/min, respectively. The perfusate of both sides was held at a pH value of 7.4, equilibrated with 95% O_2 and 5% CO_2 and maintained at 37°C throughout the perfusion.

After a stabilization period of 30 min, the maternal perfusate was replaced by drug-free human plasma spiked with ropivacaine hydrochloride (1 μg/mL) and antipyrine (10 μg/mL). Samples of perfusate 2 mL were collected from the fetal and maternal circuits at 15 and 30 min and 1.0, 1.5 and 2.0 h after adding the drugs, and frozen at –20°C until analysis.

Calculations. Transplacental transfer of ropivacaine and antipyrine were evaluated as transplacental clearance, which can be calculated as follows:

$$Cl = \frac{C_{fv} \cdot Q_f}{C_{ma}}$$

where Q_f is the flow rate of the fetal outflow; C_{fv} and C_{ma} are the venous concentration in the fetal outflow and the arterial concentration in the maternal inflow, respectively.

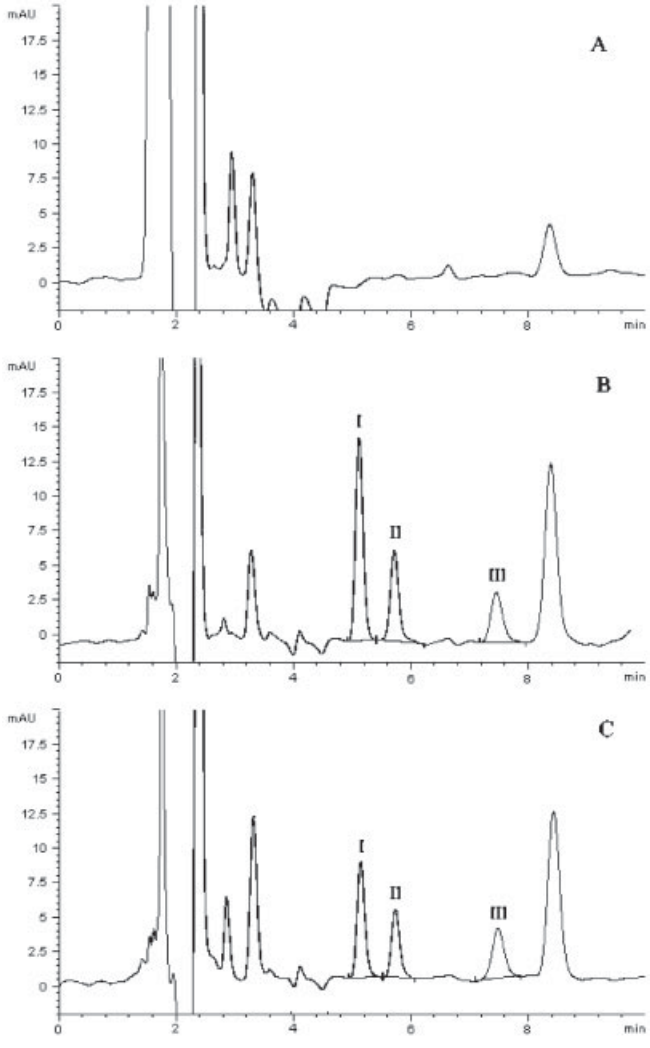

Figure 1. Chromatograms of (A) drug-free plasma, (B) plasma spiked with ropivacaine, antipyrine and 4-acetylaniline, (C) perfusate sample: I = antipyrine; II = 4-acetylaniline (I.S.); III = ropivacaine.

Interplacental variability in lobule size and flow was diminished by expressing data as the ropivacaine/antipyrine clearance ratio (clearance index, *CI*), which was calculated based on the trasplacental clearance of ropivacaine ($Cl_{ropivacaine}$) and antipyrine ($Cl_{antipyrine}$) as follows:

$$CI = \frac{Cl_{ropivacaine}}{Cl_{antipyrine}}$$

RESULTS AND DISCUSSION

Chromatographic analysis

Different reversed-phase columns have been employed in order to obtain a rapid simultaneous analysis of ropivacaine and antipyrine. A C_{18} column was finally chosen since both drugs have a proper retention time. The eluent's pH influenced the retention of ropivacaine greatly, while it did little to antipyrine. A proper resolution was achieved with mobile phase at pH 5. Under the described chromatographic conditions, good resolution of antipyrine, 4-acetylaniline (an internal standard) and ropivacaine was achieved, and no endogenous substances interfered with their detection. Typical chromatograms of extracts from drug-free perfusate, perfusate spiked with ropivacaine, antipyrine and 4-acetylaniline, and a perfusate sample are shown in Fig. 1.

A linear relationship was found between the peak area ratio of ropivacaine to 4-acetylaniline and ropivacaine concentration in perfusate over the range 10–1000 ng/mL with a correlation efficient of 0.998 (n = 9). It confirmed the equation y = 0.005596c – 0.01607, where y refers to the area ratio of ropivacaine to 4-acetylaniline. The calibration curve for antipyrine in perfusate was also linear over the concentration range 0.1–10 μg/mL, with a correlation coefficient of 0.9991 (n = 9). Its linear regression equation was y = 5.466c – 0.2936, where y refers to the area ratio of antipyrine to 4-acetylaniline.

The extraction recovery of antipyrine varied greatly with the change of extraction solvent. A maximum mean recovery of about 60% (n = 3) was obtained with ethyl acetate as the solvent. Its extraction yields were all below 50% with other solvents, such as diethyl ether, dichloromethane, mixture of hexane and *iso*-propanol. The mean extraction recovery (n = 3) of ropivacaine was between 83 and 94%, and not significantly influenced by the change of extraction solvents.

The inter-day (n = 5) and intra-day (n = 5) studies showed an acceptable precision and accuracy of the method, as shown in Table 1.

Transplacental study

Both ropivacaine and antipyrine can cross the human placenta. After 2 h perfusion, mean ropivacaine concentration decreased from 886 to 535 ng/mL in the maternal side and increased gradually from 91 to 163 ng/mL in the fetal side; mean antipyrine concentration decreased from 4.85 to 2.68 μg/mL in the maternal side and increased gradually from 0.51 to 1.06 μg/mL in the fetal side. At the end of 2 h perfusion, clearance ($Cl_{m\rightarrow f}$) of ropivacaine and antipyrine was 0.56 ± 0.16 and 0.75 ± 0.20 mL/min, respectively. The clearance index (CI) of ropivacaine was 0.75 ± 0.17 (n = 6), which agrees with previously reported data (Johnson *et al.*, 1999). No

Table 1. The precision and accuracy of plasma ropivacaine and antipyrine determined by HPLC

Concentration added	Concentration detected (mean ± SD)	RSD (%)	Accuracy (%)
Inter-day (n = 5)			
Ropivacaine (ng/mL)			
25	25.7 ± 2.2	8.6	102.8
250	233 ± 17	7.3	93.2
1000	991 ± 31	3.1	99.1
Antipyrine (μg/mL)			
0.25	0.240 ± 0.018	7.5	96.0
2.5	2.47 ± 0.07	2.8	98.8
10	10.3 ± 0.6	5.8	103.2
Intra-day (n = 5)			
Ropivacaine (ng/mL)			
25	28.1 ± 3.0	10.7	112.4
250	238 ± 13	5.5	95.2
1000	942 ± 59	6.3	94.2
Antipyrine (μg/mL)			
0.25	0.234 ± 0.023	9.8	93.6
2.5	2.25 ± 0.20	8.9	90.0
10	9.34 ± 0.57	6.1	93.4

significant differences were observed between the mean ropivacaine and antipyrine clearances ($p > 0.05$).

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