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# Determination of free concentrations of ropivacaine and bupivacaine in plasma from neonates using small-scale equilibrium–dialysis followed by liquid chromatography-mass spectrometry

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**Abstract**

Attempts to determine a safe plasma concentration of ropivacaine and bupivacaine in neonates have not been consistent. This might be due to an underestimation of free drug in small plasma samples by currently used techniques, e.g., ultrafiltration. We describe a simple microscale equilibrium–dialysis technique for the separation of free and bound ropivacaine and bupivacaine. The free drug in the dialysate was determined using solid-phase extraction and liquid chromatography with mass spectrometry. Pentycaine was used as an internal standard and added to the dialysates prior to extraction. The method is very selective and sensitive, as no compounds other than the analyte and internal standard were observed in the resulting chromatograms at low ng/ml levels. The limit of quantitation was 2.5 ng/ml. The calibration curve was linear in the range of 2 to 1000 ng/ml. The precision of the whole procedure was 8.1% ($n=10$) and 6.5% ($n=7$) for ropivacaine and bupivacaine, respectively. The method was tested in the analysis of plasma samples taken from neonates who had received epidural injections. 

*Keywords:* Ropivacaine; Bupivacaine

## 1. Introduction

Amide-type local anaesthetics, such as bupivacaine, have been commonly used for many decades. Recently, ropivacaine [1], a new member of this group, was developed by Astra Pain Control. One of the most striking characteristics of these local anaesthetics is their high binding to plasma proteins with >90% being bound to $\alpha_1$-acid glycoprotein (AGP) [2]. Since the amount of unbound drug is considered to be related to the pharmacological effect, plasma protein binding of drugs may have significant pharmacodynamic implications. Recently it has been of major interest to determine the free plasma concentration of these drugs since there is a lack of information on the degree of binding in relation to different anaesthetic procedures. This has been carried out using high-performance liquid chromatography (HPLC) following ultrafiltration and liquid–liquid extraction [3,4]. The total and free bupivacaine concentrations in plasma samples from neonates

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have been also measured after spinal [5], epidural [6,7] and caudal [8] anaesthesia.

Techniques allowing the physical separation of bound and free drug, such as equilibrium dialysis and ultrafiltration have been extensively reviewed [9–11]. Equilibrium dialysis is often regarded as the 'reference method' for the determination of a drug–protein binding profile. Plasma protein binding of bupivacaine in pregnant women was investigated by equilibrium dialysis using $^{3}$H-labelled bupivacaine [12]. Microdialysis is the most recently developed methodology for separation of free and bound drug and is the only method which allows the in vivo determination of unbound drug concentrations in biological fluids, such as plasma [13]. Ekblom et al. [14] demonstrated that microdialysis is a valuable method for the estimation of unbound concentrations of drugs in plasma. An alternative use of this method has been investigated by Herrera et al. [15], who used microdialysis perfusion to determine the extent of drug binding to human serum proteins in vitro. This study also compared microdialysis to ultrafiltration and found that both methods determined a similar degree of binding for a number of drugs. The usefulness of commercial available filtration devices (Centrifree/Amicon and Ultrafree-CL) has been studied by a large number of investigators. They have been used in the preparation of the free concentration of many drugs, e.g., etoposide [16], methohexital [17], prilocaine [18] and sameridine [19]. However all these techniques require relatively large sample volumes.

Epidural blockade with ropivacaine or bupivacaine is being increasingly used for postoperative analgesia in neonates, but there are concerns about toxicity in this high-risk patient population. This study was initiated by the need to determine the free concentrations of ropivacaine and bupivacaine in small clinical samples (a maximum of 1 ml/kg of blood only was allowed to be taken from each baby). In the current paper the performance of a new microscale equilibrium–dialysis device from AmiKa was evaluated for the separation of free drug. A sensitive, specific and precise liquid chromatography–mass spectrometry (LC–MS) method was applied to the determination of the free drug in the dialysates. AGP is the major protein responsible for binding basic drugs and there is some concern that its levels may be low in neonates leading to reduced protein binding of basic drugs in these patients. A previously reported fluorometric assay using quinaldine red [20] was used to measure the AGP levels in plasma.

## 2. Experimental

### 2.1. Chemicals and reagents

Potassium phosphate dibasic (A.C.S. reagent), potassium phosphate monobasic (A.C.S. reagent), ammonium hydroxide (30%, A.C.S. reagent), human AGP, human serum albumin (HSA), γ-globulin (GB), 5-α-androstane-3,17-dione (androstanedione) and quinaldine red (QR) were obtained from Sigma–Aldrich (Dorset, UK). Water (HiPerSolv), methanol (HiPerSolv), sodium dihydrogenorthophosphate 1-hydrate (AnalaR), disodium hydrogenphosphate (AnalaR) and ammonium acetate (G.P. reagent) were purchased from BDH (Poole, UK). Pentycaine (batch No. 0A 420/10), bupivacaine hydrochloride (batch No. 28784-2) and ropivacaine hydrochloride monohydrate (batch No. 201/94) were donated by Astra (Södertälje, Sweden). Drug-free plasma, donated by healthy subjects was obtained from the Blood Donor Centre (Glasgow, UK) and stored at −20°C.

### 2.2. Microscale equilibrium–dialysis

Prior to dialysis plasma samples and dialysis buffer were temperature equilibrated at 37°C for 1 h. Separation of free drug was done by microscale equilibrium–dialysis using an equilibrium dialyser (SE0200, AmiKa, Columbia, USA). The sample and buffer compartment were separated by a regenerated cellulose membrane [molecular mass cut-off (MWCO) 10 000; A100S, AmiKa] and filled with 100 μl patient or control plasma (spiked with 0.5 μg/ml ropivacaine or bupivacaine) and 0.2 *M* potassium phosphate buffer, pH 7.4, respectively. Experiments were carried out overnight at 37°C and under constant shaking (Clifton shaking bath NE-28CE, Nickel Electro, UK).

An 80-μl volume of dialysate was spiked with 10 μl of internal standard (pentycaine solution: 0.5 μg/ml in 0.2 *M* potassium phosphate buffer, pH 3.0)

and buffered with 920 μl 0.2 *M* potassium phosphate buffer, pH 3.0. The sample solutions were then further processed by solid-phase extraction (SPE).

### 2.3. Extraction

Isolute SCX (100 mg; 1 ml) cartridges (International Sorbent Technology, Hengoed, UK) were placed on a vacuum manifold and conditioned with 1 ml of methanol, 1 ml of water and 1 ml of 0.2 *M* potassium phosphate buffer, pH 3.0. After applying the sample solutions the cartridges were washed with 1 ml of water and 1 ml of methanol. The retained analytes were then eluted with 1 ml 3% ammoniacal methanol into a 1-ml V-vial, evaporated to dryness at 90°C and reconstituted in 100 μl mobile phase. Prior to analysis by LC–MS samples were transferred to a 200-μl insert of a HPLC autosampler vial and 20 μl was injected into the LC–MS system.

### 2.4. LC–MS analysis

The liquid chromatographic system consisted of SpectraSYSTEM P4000 gradient pump (Thermo Separation Products) and a SpectraSYSTEM AS3000 autosampler (Thermo Separation Products), equipped with a Type 7010-150 Rheodyne injection valve (20-μl loop). The chromatographic separation was performed in the isocratic mode with a Luna $C_{18}$ (2) column (75×4.6 mm I.D., 3 μm particle size, Phenomenex, UK). The mobile phase consisted of acetonitrile–50 m*M* ammonium acetate buffer, pH 7.0 (60:40, v/v). The flow-rate was 0.8 ml/min and all the eluent was introduced into the instrument. Data were acquired and processed with Xcalibur software (ThermoQuest FINNIGAN). The MS system (Automass Multi with an electrospray interface, ThermoQuest FINNIGAN) was operated with a capillary voltage of 2.5 kV, a cone voltage of 30 V and a drying gas temperature of 500°C. Nitrogen gas was used as nebuliser and sheath gas. Interface variables, such as voltages, lenses and gas heating were checked daily during direct infusion of a 0.1 μg/ml solution of target analytes and, if need be, slightly modified to obtain optimum sensitivity. The selected ion monitoring (SIM) mode was used and following ions ($[M+H]^+$) were monitored: *m/z* 275 for ropivacaine, *m/z* 289 for bupivacaine and *m/z* 303 for pentycaine.

### 2.5. Calibration of the method for the determination of free drug in dialysate

Stock solutions of ropivacaine, bupivacaine and pentycaine at a concentration level of 1 mg/ml were prepared in methanol and stored in at 4°C. A linear range was established over the ranges 0.5–1000 ng/ml (12 calibration points) 2–1000 ng/ml (10 calibration points) for bupivacaine and ropivacaine, respectively. Thereafter free ropivacaine and bupivacaine in the plasma dialysate was determined by single-point calibration. Three aliquots of a standard solution (80 μl containing 4 ng of ropivacaine or bupivacaine and 10 ng of pentycaine in water) were assayed at the same time as each batch of dialysates. The patient samples were quantified against the mean peak area ratio obtained for these samples.

### 2.6. Accuracy and precision of the determination of free drug in dialysates

Quality control (QC) plasma samples (2.0, 0.5 and 0.2 μg/ml of total drug) were prepared by adding ropivacaine or bupivacaine to pooled blank plasma. A volume of plasma (40 ml) was spiked with 80, 20 or 8 μl ropivacaine or bupivacaine (1 mg/ml of free base in methanol). Twenty aliquots of 0.5 ml and 30 aliquots of 1.0 ml were stored frozen at −20°C. Five aliquots (100 μl) of the QC sample containing 0.5 μg/ml of drug were run on different days to calculate within- and between-run precision. Additional data were achieved by analysing the dialysates obtained from 100 μl aliquots of 0.5 μg/ml QC samples in duplicate or triplicate at the same time as the patient samples.

The adsorption of drug to the cellulose membrane of the microdialysis device was studied at a concentration of 0.1 μg/ml (10 ng/100 μl in 0.2 *M* potassium phosphate, pH 7.4). After dialysis overnight the concentrations of drug were determined in the dialysate and retentate and the relative recovery calculated according to Eq. (1).

The recovery from the SPE procedure was obtained by extracting replicates of QC plasma samples (without internal standard and using exact volumes)

while the references were prepared from unextracted aqueous solutions. The internal standard was added to the plasma extracts in the reconstitution step.

### 2.7. Determination of total drug concentration in plasma

The concentration of total ropivacaine or bupivacaine in plasma was also determined by SPE and LC–MS. Plasma samples (20 μl) were spiked with 10 μl of pentycaine solution (5 μg/ml in 0.2 *M* potassium phosphate buffer, pH 3.0) and buffered by adding 980 μl of 0.2 *M* potassium phosphate buffer, pH 3.0 prior to SPE. The quantification of total drug in each batch of patient samples was carried out by analysing three replicates of QC plasma spiked with bupivacaine or ropivacaine at a concentration of 0.5 μg/ml (10 ng/20 μl aliquot) at the same time as the patient plasma samples. The precision for the determination of the total drug was determined by analysis of plasma spiked with 0.2, 0.5 and 2.0 μg/ml of the drugs, in order to allow for the wider variation in total levels compared with the free levels, on four occasions.

### 2.8. Calculations

The ropivacaine/bupivacaine concentrations were measured in the retentate ($C_r$) and in the dialysate ($C_d$). The relative recovery was estimated according to Eq. (1):

$$\text{Relative recovery} = C_r + C_d / C_s \quad (1)$$

where $C_s$: total starting concentration.

The concentration of ropivacaine in 90 μl dialysate is equal to the concentration of free ropivacaine in 100 μl of plasma, because the plasma consists of ca. 10% protein and only 90% water. Therefore a conversion factor of 0.9 was applied and the concentration of free ropivacaine or bupivacaine was calculated according to Eq. (2):

$$c\ (\text{ng/ml plasma}) = c\ (\text{ng/ml dialysate}) \cdot 0.9/\text{relative recovery} \quad (2)$$

The free fraction was calculated as (Eq. (3)):

$$\text{free fraction} = [\text{free concentration (ng/ml)}/\text{total concentration (ng/ml)}] \cdot 100 \quad (3)$$

### 2.9. Clinical samples

With ethics approval and parental consent, neonates undergoing epidural analgesia were recruited to the study. Anaesthesia was induced by a single injection. The Yorkhill Ethics Committee only allowed a maximum of 1 ml/kg of venous blood for the purposes of research. Therefore up to three blood samples of 1 ml (giving up to 0.5 ml plasma) were taken from each baby at three different times and stored at −20°C. The free and total plasma drug concentrations of these samples were determined according to the procedure described above. The patient samples were analysed by single determination, but duplicate or triplicate of QC samples (0.5 μg/ml) were included in each assay.

### 2.10. Measurement of AGP levels

The concentration of AGP was measured by fluorometric determination. The previously developed method [20] was slightly modified to enable the use of smaller sample volumes. The AGP standard curve (0, 50, 100, 150 and 200 mg/100 ml) was prepared by adjusting appropriate volumes of 1/15 *M* sodium phosphate buffer, pH 7.4 (50–100 μl) with the AGP solution (2 mg/ml; 0–50 μl) to 100 μl. Then 25 μl of HSA solution (80 mg/ml) and 25 μl of γ-GB solution (34 mg/ml) were added to the 100 μl of standard solutions, which were then diluted with 2.9 ml of 15 μ*M* androstanedione solution. Finally 100 μl of 1.5 m*M* QR solution were added to the mixture and the fluorescence intensity ($F$) was measured at 600 nm with a excitation wavelength 470 nm. For the analysis of samples 100 μl of plasma were used and instead of 2.9 ml of 15 μ*M* androstanedione solution, 2.95 ml of 1/15 *M* sodium phosphate buffer, pH 7.4 was added.

## 3. Results and discussion

Arvidsson and Eklund [5] evaluated the use of ultrafiltration for determining the free concentration

of ropivacaine and bupivacaine in blood. Ultrafiltration is generally restricted to assay of 10% of the total sample volume, to avoid mass action effects from concentration of the protein. Thus, ultrafiltration was not the method of first choice for our small sample volumes of less than 0.5 ml (often as low as 0.2 ml). Using microscale equilibrium–dialysis the sample could be incubated in a constant-temperature water-bath for precise temperature control. Unlike ultrafiltration, microscale equilibrium–dialysis does not disturb the drug binding equilibrium, because the sample volume is not changed and therefore the protein concentration remains constant. Problems of non-specific adsorption are also less severe with microscale equilibrium–dialysis relative to ultrafiltration. The new microscale equilibrium–dialysis system introduced by AmiKa is very suitable for the dialysis of small volumes, which can be as low as 10 μl.

The sample and the buffer compartment were separated by a regenerated cellulose membrane, which reduces the binding of lipophilic drugs, such as bupivacaine and ropivacaine. The non-specific binding of the drug to the dialysis membrane was studied in the absence of protein. Although loss of the analyte by adsorption to the membrane occurred during the dialysis, the concentration of free drug could be determined correctly by dividing the measured concentration by the relative recovery of 0.82.

The time required to attain equilibrium during dialysis studies is often considered to limit the usefulness of the method. The equilibrium time depends on the molecular mass cut off of the membrane and the molecular mass of the drug and can be 1–2 h to overnight. In the present study a stable dialysate concentration ($C_d$) was reached within the first 3 h of dialysis, but for convenience reasons the samples were dialysed overnight in subsequent assays. The manufacturer of these dialysis devices had also recommended that the dialysis should be done overnight to get the best results.

Drug–protein binding is generally characterised by a reversible equilibrium and is affected by physio–chemical parameters, such as pH, temperature and adsorption. Beauvoir et al. [5] reported an increase of the free fraction of ropivacaine from 6.7 to 8.7% when the temperature was increased from 32 to 40°C. During preliminary work we did not observe a large change of the free fraction when the temperature was raised from 22 to 37°C. However, for the purposes of the study the temperature used for dialysis was set to the 'physiological' value of 37°C.

A number of calibration curves revealed correlation coefficients in excess of 0.990 and were linear over a concentration range of 0.5–1000 ng/ml and 2–1000 ng/ml for bupivacaine and ropivacaine, respectively. The quantification of free and total drug in plasma samples was then based on a single-point calibration and the measurement of a triplicate of calibration standards at a concentration level of 50 ng/ml resulted in an acceptable degree of within-run precision (0.9 to 10.0%, $n=34$). The mean extraction recovery of total bupivacaine (ropivacaine) was 54% (52%) at 0.2 μg/ml, 58% (54%) at 0.5 μg/ml and 57% (52%) at 2.0 μg/ml.

To evaluate the reproducibility of the determination of the total drug, three QC samples at the concentration levels of 0.2, 0.5 and 2.0 μg/ml were analysed immediately after spiking and on three additional occasions. The results of the accuracy and precision (within- and between-run) are given in Tables 1 and 2. The determination of free drug in QC samples (containing total drug at 0.5 μg/ml) resulted in slightly higher relative standard deviations (RSDs) (Table 3). They were calculated from the mean of each set of replicates and ranged from 0.8 to 14.3%. Validation guidelines [21] indicate, that for bioanalytical methods an RSD of ±15% is appropriate, except at the quantification limit where ±20% is acceptable. Most of our quality control samples were within these limits but three were just outside. However, the method was regarded as fit for purpose since the study was looking for levels of unbound drug 2–3-times those of the normal range.

The limit of detection (signal-to-noise ratio of 3) of the LC–MS method was 2 ng/ml and 0.5 ng/ml for ropivacaine and bupivacaine, respectively. However, the limit of quantification (the lowest concentration that can be measured with acceptable accuracy and precision) was 2.5 ng/ml. The method proved to be sensitive enough to quantify accurately the 'free drug' concentration of the lowest QC sample (concentration of total drug: 0.2 μg/ml giving a concentration of free drug in processed sample of approx. 3 ng/ml). Figs. 1 and 2 show SIM

Table 1
Accuracy and within- and between-run precision of the determination of the total ropivacaine in plasma

| Spiked concentration (μg/ml) | Measured concentration (μg/ml) | RSD (%) | Accuracy (%) | *n* |
|---|---|---|---|---|
| *Within-run* | | | | |
| 0.200 | 0.214 | 1.5 | 107 | 3 |
| 0.200 | 0.226 | 10.2 | 113 | 3 |
| 0.200 | 0.232 | 6.0 | 116 | 3 |
| 0.200 | 0.227 | 4.9 | 114 | 3 |
| 0.500 | 0.503 | 2.6 | 101 | 3 |
| 0.500 | 0.543 | 5.8 | 109 | 3 |
| 0.500 | 0.522 | 4.6 | 104 | 3 |
| 0.500 | 0.588 | 3.5 | 118 | 3 |
| 2.000 | 1.679 | 3.0 | 84 | 3 |
| 2.000 | 1.879 | 5.2 | 94 | 3 |
| 2.000 | 1.926 | 4.3 | 96 | 3 |
| 2.000 | 1.925 | 9.4 | 96 | 3 |
| *Between-run* | | | | |
| 0.200 | 0.225 | 2.9 | | 4 |
| 0.500 | 0.539 | 5.9 | | 4 |
| 2.000 | 1.852 | 5.5 | | 4 |

chromatograms of plasma samples obtained from neonates after administration of bupivacaine or ropivacaine, there were no interfering peaks in blank plasma (Figs. 3 and 4).

The performance of the method was tested by analysing replicates of human plasma control samples which were spiked with drug to give a total concentration in the same range as that of the

Table 2
Accuracy and within- and between-run precision of the determination of the total bupivacaine in plasma

| Spiked concentration (μg/ml) | Measured concentration (μg/ml) | RSD (%) | Accuracy (%) | *n* |
|---|---|---|---|---|
| *Within-run* | | | | |
| 0.200 | 0.202 | 3.2 | 101 | 3 |
| 0.200 | 0.203 | 5.9 | 102 | 2 |
| 0.200 | 0.212 | 2.4 | 106 | 3 |
| 0.200 | 0.219 | 8.9 | 110 | 3 |
| 0.500 | 0.539 | 3.2 | 108 | 3 |
| 0.500 | 0.564 | 2.0 | 113 | 3 |
| 0.500 | 0.553 | 5.5 | 111 | 3 |
| 0.500 | 0.572 | 13.8 | 114 | 3 |
| 2.000 | 1.981 | 3.3 | 99 | 3 |
| 2.000 | 1.957 | 0.7 | 98 | 3 |
| 2.000 | 1.809 | 6.9 | 90 | 3 |
| 2.000 | 1.974 | 10.2 | 99 | 3 |
| *Between-run* | | | | |
| 0.200 | 0.209 | 3.3 | | 4 |
| 0.500 | 0.557 | 2.2 | | 4 |
| 2.000 | 1.942 | 2.6 | | 4 |

Table 3
Within- and between-run precision of the determination of free drug in QC samples containing 0.5 μg/ml of total drug[a]

| Occasion | Ropivacaine | | | Bupivacaine | | |
|---|---|---|---|---|---|---|
| | Level of free drug (ng/ml) | RSD (%) | *n* | Level of free drug (ng/ml) | RSD (%) | *n* |
| Within-run | | | | | | |
| 1 | 15.47 | 14.3 | 5 | 16.30 | 9.6 | 5 |
| 2 | 19.00 | 11.4 | 5 | 13.87 | 8.5 | 5 |
| 3 | 18.48 | 8.1 | 5 | 15.07 | 10.2 | 5 |
| 4 | 20.62 | 8.2 | 3 | 19.71 | 12.3 | 5 |
| 5 | 18.65 | 5.9 | 3 | 15.50 | 1.5 | 2 |
| 6 | 18.49 | 10.7 | 3 | 15.59 | 2.9 | 2 |
| 7 | 20.50 | 7.0 | 3 | 14.38 | 0.8 | 2 |
| 8 | 18.06 | 3.4 | 3 | | | |
| 9 | 22.81 | 3.6 | 3 | | | |
| 10 | 28.64 | 8.0 | 3 | | | |
| Between-run | Mean 20.07 | 16.9 | | Mean 15.77 | 11.2 | |

[a] AGP levels in the control plasma are shown in Table 5.

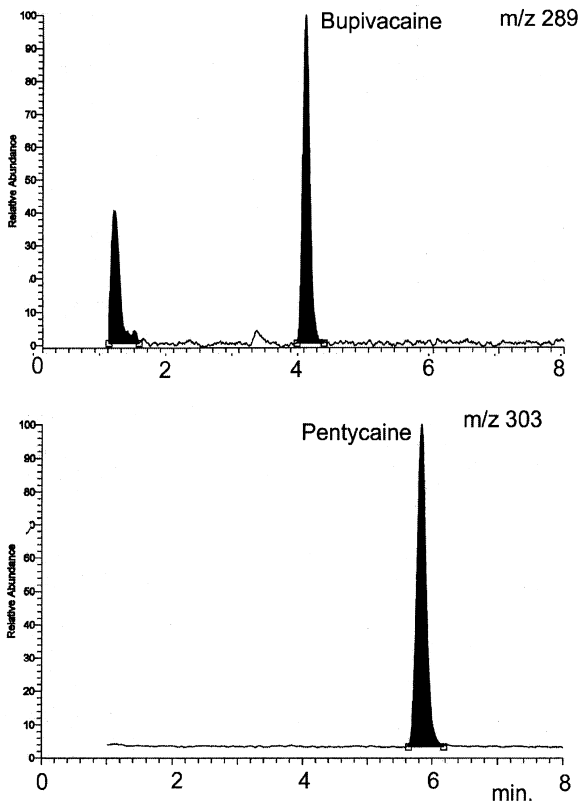


Fig. 1. SIM chromatogram of dialysate (80 μl) of plasma from a patient after administration of bupivacaine (dialysate spiked with 5 ng of pentycaine, concentration of free bupivacaine: 12.7 ng/ml of plasma).

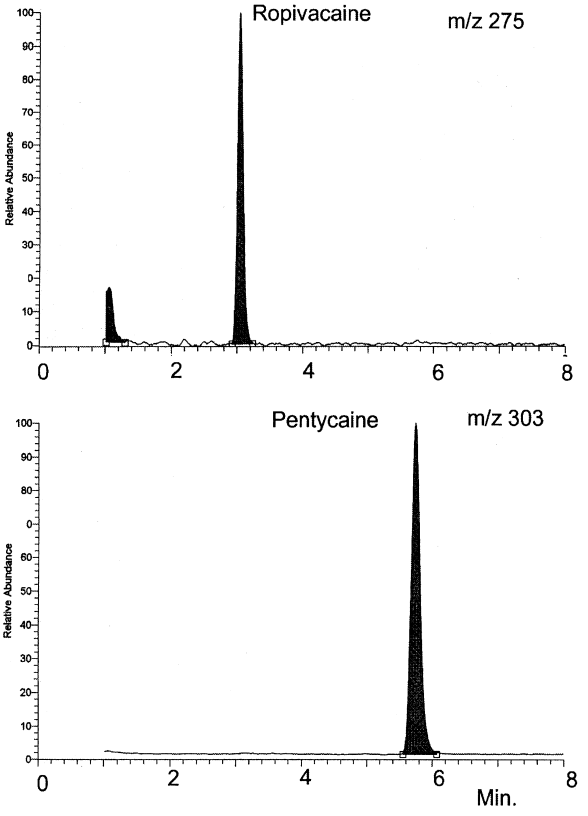


Fig. 2. SIM chromatogram of dialysate (80 μl) of plasma from a patient after administration of ropivacaine (dialysate spiked with 5 ng of pentycaine, concentration of free ropivacaine: 17.7 ng/ml of plasma).

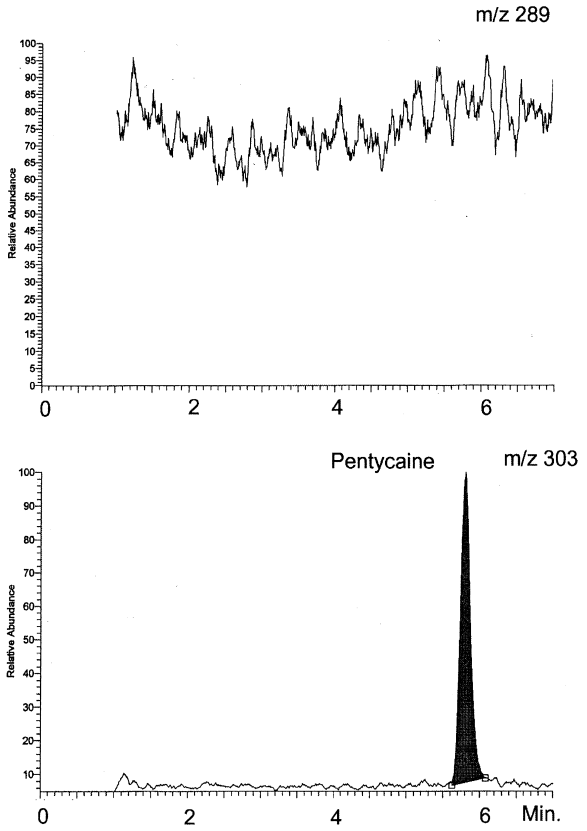


Fig. 3. Dialysate from blank plasma spiked with 10 ng of pentycaine showing SIM traces for pentycaine and bupivacaine.

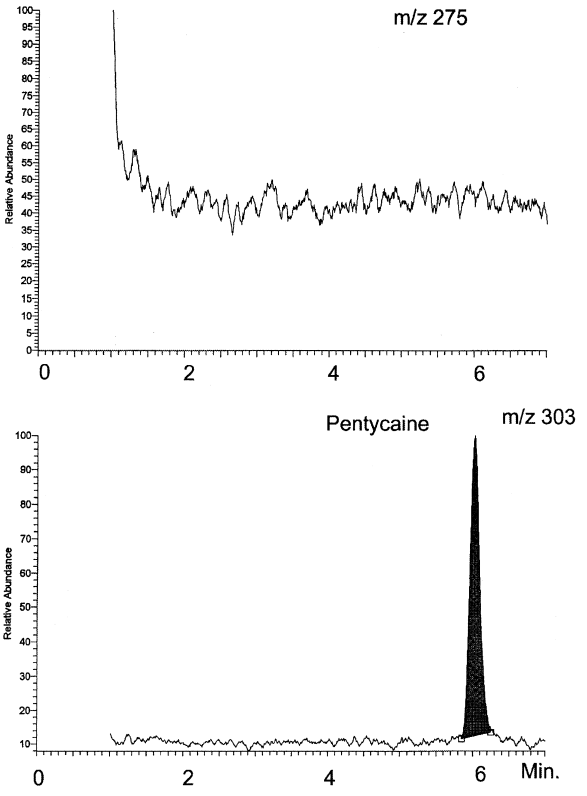


Fig. 4. Dialysate from blank plasma spiked with 10 ng of pentycaine showing SIM traces for pentycaine and ropivacaine.

unknown samples. The mean free and total ropivacaine/bupivacaine concentrations and the resulting free fraction are given in Table 3. The mean free fraction was 3.4 and 2.9% for ropivacaine and bupivacaine, respectively. A study investigating the pharmacokinetics of ropivacaine in women receiving continuous epidural infusions found protein binding of approx. 96% [22]. Our findings are also close to the observations of Veering et al. [23] where the binding of bupivacaine was determined by equilibrium dialysis with a Dianorm dialysis system and gave a mean free fraction of 5%.

Once the method had been shown to perform with acceptable precision, the analysis of patient samples was carried out by a single determination without the need for duplicate analysis. Analysis of quality control samples in duplicate or triplicate alongside the patient samples monitored the performance of each assay. All the data from the patient samples could be accepted and the results from five patients are summarised in Table 4. The results from all the patients in the study will be reported elsewhere. The levels of unbound drug ranged from 1.9 to 5.6% and 3.3 to 5.0% for bupivacaine and ropivacaine, respectively. The results were in correlation with those reported by Luz et al. [7] who measured free and total plasma concentrations of bupivacaine in 14 infants and children aged 6 days to 9 years after 8 h of continuous epidural anaesthesia. They found a significantly higher free fraction of drug in neonates (4.8–4.9%) in comparison with infants (2.9–3.9%) and children (1.2–4%). No data are available on the free fraction of ropivacaine in infants.

A higher free fraction of ropivacaine and bupivacaine in neonates is associated with a lower concentration of AGP in plasma. In this study a simple fluorometric method [20] was used to mea-

Table 4
Total (and free) concentrations of bupivacaine and ropivacaine in plasma from neonates

| Patient | Drug | Protocol | Total drug, ng/ml (% free) | | | |
|---|---|---|---|---|---|---|
| | | | 15 min | 30 min | 45 min | 60 min |
| 1 | Bupivacaine | Single injection | | 508 (2.5) | 647 (1.9) | 527 (2.4) |
| 2 | Bupivacaine | Single injection | | | 636 (2.0) | |
| 3 | Bupivacaine | Single injection | | | | 366 (5.6) |
| 4 | Ropivacaine | Single injection | 493 (3.6) | 612 (5.0) | | |
| 5 | Ropivacaine | Single injection | | | | 855 (3.3) |

sure the AGP levels of the patient samples. Although AGP concentrations were below the limit of detection of the method it was readily apparent that they were well below the normal range of 40–100 mg/100 ml (Table 5). Similar results have been found by Larsson et al. [6] while investigating AGP levels in neonates after continuous epidural infusion of bupivacaine. The AGP level was 19 mg/100 ml at 1 h; although it had increased significantly to 33 mg/100 ml at 24 h of infusion, it was still below the normal level.

Despite reduced dosing in neonates, toxic effects of epidurally administered local anaesthetics are still seen, which might be due to an underestimation of the true free plasma concentrations by currently used methods. The use of microscale equilibrium–dialysis provides a useful alternative to ultrafiltration where volumes are small. For example, Peutrell et al. [24] failed to separate the free fraction of bupivacaine in samples taken from babies using a ultrafiltration micro-partition system (Amicon) and measure its concentration by HPLC. The volume of plasma obtained was insufficient for this separation method or the concentration of the free drug was below the sensitivity of the assay (ca. 0.1 μg/ml for 100 μl) for the volumes of plasma available.

In summary, the microscale equilibrium–dialysis method requires only 100 μl of plasma for the separation of free drug. Use of a very sensitive LC–MS method for the determination of free drug concentrations further enhances the performance of the whole procedure. The adsorption of ropivacaine or bupivacaine on to the dialysis membrane was found to be acceptable and introducing a mathematical factor compensated for composition differences from the buffer to the plasma compartment.

Table 5
AGP concentrations in control and patient plasma samples[a]

| Sample | Fluorescence | AGP concentration (mg/100 ml) |
|---|---|---|
| Control 1 | 16.2 | 64 |
| Control 2 | 17.2 | 76 |
| Control 3 | 16.6 | 69 |
| Patient 1 (30 min) | 7.8 | –[b] |
| Patient 1 (45 min) | 8.5 | – |
| Patient 1 (60 min) | 9.5 | – |
| Patient 2 (45 min) | 12.0 | 13 |
| Patient 3 (60 min) | 7.4 | – |
| Patient 4 (15 min) | 8.8 | – |
| Patient 4 (30 min) | 6.9 | – |
| Patient 5 (60 min) | 10.1 | – |

[a] Equation of calibration line $y$ (fluorescence) $= 0.0828x + 10.9$, $R^2 = 0.996$.
[b] –: Below limit of detection.

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