ELSEVIER

Journal of Chromatography B, 668 (1995) 91–98

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Determination of free concentration of ropivacaine and bupivacaine in blood plasma by ultrafiltration and coupled-column liquid chromatography

Torbjörn Arvidsson*, Eivor Eklund

Pharmaceutical and Analytical R&D, Bioanalysis, Astra Pain Control AB, S-151 85 Södertälje, Sweden

First received 22 September 1994; revised manuscript received 13 January 1995; accepted 30 January 1995

Abstract

A coupled-column liquid chromatographic method for determining the free concentration of ropivacaine and bupivacaine in blood plasma was developed. Following adjustment of the temperature and pH, the plasma samples were ultrafiltrated. Ropivacaine or bupivacaine in the ultrafiltrate was determined by direct injection into a coupled-column liquid chromatographic system, consisting of one reversed-phase and one ion-exchange column.

The system was highly selective. Ropivacaine and bupivacaine were detected by UV at 210 nm. The limit of determination was 10 n*M* and the inter-assay precision at a concentration level of about 100 n*M* was 6% (R.S.D., $n = 30$) for ropivacaine and 7% (R.S.D., $n = 30$) for bupivacaine.

1. Introduction

Ropivacaine hydrochloride monohydrate (ropivacaine) is a long-acting local anaesthetic agent chemically homologous with bupivacaine and mepivacaine. Unlike bupivacaine, which is a racemate, ropivacaine is the single *S*(−)-enantiomer. Ropivacaine has a lower central nervous and cardiotoxic potential than bupivacaine in animals [1], and in human volunteers it has been shown to be less prone than bupivacaine to produce central nervous system symptoms and cardiovascular changes after intravenous infusion [2]. Ropivacaine in high concentration is used for surgical anaesthesia and in lower concentration for postoperative pain relief.

For local anaesthetics it is of special interest, from a pharmacological point of view, to determine the free concentration (i.e. the non-protein-bound concentration). Reasons for this have been discussed by Tucker [3–5].

Ropivacaine is administrated as the pure (*S*)-enantiomer; however, no metabolic racemisation of ropivacaine was found in vivo [6]. Therefore, for simplicity, in this paper a nonchiral methodology was applied for the determination of the free concentration of ropivacaine or bupivacaine in blood plasma.

Ropivacaine and bupivacaine are extensively protein-bound (in human >90%), which is why determination of the free concentration requires

* Corresponding author.

0378-4347/95/$09.50
SSDI 0378-4347(95)00059-3

highly sensitive methods. The free concentration is prepared by ultrafiltration, which is a simple technique often used in the preparation of the free concentration of other local anaesthetic agents, e.g. lidocaine [7,8], prilocaine [9,10], etidocaine [11] and bupivacaine [12,13].

In our laboratory the total concentration of ropivacaine or bupivacaine in blood plasma is usually determined by gas chromatography with nitrogen-sensitive detection after liquid–liquid extraction [14]. In this paper, the free concentration is determined by direct injection of the ultrafiltrate into a coupled-column liquid chromatographic system consisting of a reversed-phase and a cation-exchange column. This technique is used in order to minimize further sample treatment and to optimize performance with respect to precision and accuracy.

2. Experimental

2.1. Chemicals

Ropivacaine hydrochloride monohydrate and bupivacaine hydrochloride monohydrate were obtained from Astra Production Chemicals (Södertälje, Sweden) (ropivacaine base, M_r 274.4 and bupivacaine base, M_r 288.4). RBS 50 solution was from Lab Kemi (Stockholm, Sweden). CO_2 (50% in O_2) came from AGA GAS AB (Stockholm, Sweden). Acetonitrile of LiChrosolv grade was obtained from E. Merck (Darmstadt, Germany). All the other chemicals were of analytical grade, obtained from the usual commercial sources.

2.2. Equipment

The pH was measured using a XEROLYTE microelectrode (Mettler-Toledo, Greifensee, Switzerland) and temperature-controlled centrifugation was performed using a Hettich Rotanta/TRC (Hettich, Tuttlingen, Germany) with a 35° fixed-angle rotor. For collecting blood plasma, heparinized Venoject tubes (Terumo, Leuven, Belgium) were used. The plasma was stored in Nunc Cryo tubes (Intermed, Roskilde, Denmark). The ultrafiltrate was prepared using a micropartition system, Amicon MPS-1, with a YMT 30 membrane (W.R. Grace and Co., Danvers, MA, USA).

The liquid chromatographic system consisted of two Shimadzu LC-9A pumps (Shimadzu, Kyoto, Japan) connected to a CMA/200 auto-injector (CMA/Micro-dialysis AB, Stockholm, Sweden) containing two six-port switching valves with 250-μl and 1000-μl loops.

The columns were a Superspher RP-select B (125 mm × 4.0 mm I.D., 4 μm) (E. Merck) and a Nucleosil 5 SA (150 mm × 4.6 mm I.D., 5 μm) (Macherey-Nagel, Dueren, Germany). The column oven was a BAS LC-22C (Bioanalytical Systems, West Lafayette, IN, USA).

The detectors were a Spectra 100 UV–Vis (Spectra Physics, San José, CA, USA) for the assay and a LDC Spectro Monitor III (Riviera Beach, FL, USA) to check the switching time in the coupled-column system. PE Nelson Access*Chrom (Perkin-Elmer Nelson Systems, Cupertino, CA, USA) was used to interpret the data for the integration and calculation of the results.

2.3. Procedures

RBS solution 3% (v/v) was prepared in de-ionized water. Phosphate buffer pH 7.4 (ionic strength 0.2) was prepared by diluting 18 ml of 1 *M* sodium dihydrogen phosphate and 121 ml of 0.5 *M* disodium hydrogen phosphate to 1000 ml with de-ionized water.

Phosphate buffer pH 3 (ionic strength 0.02) was prepared by diluting 3.1 ml of 1 *M* phosphoric acid and 20 ml of 1 *M* sodium dihydrogen phosphate to 1000 ml with de-ionized water. Ammonium-phosphate buffer pH 2.6 (ionic strength 0.3) was prepared by diluting 149 ml of 2 *M* ammonium hydroxide and 348 ml of 1 *M* phosphoric acid to 1000 ml with de-ionized water.

Standard stock solutions (about 2 mg/25 ml) of ropivacaine or bupivacaine were prepared in de-ionized water and stored in a refrigerator. External standards were prepared by dilution of the stock solution of ropivacaine or bupivacaine

with phosphate buffer pH 7.4 (ionic strength 0.2) to a suitable concentration.

The eluents were prepared by mixing specified volumes of acetonitrile and buffer. Control plasma samples (4 μM) were prepared by adding ropivacaine or bupivacaine to pooled blank human plasma. The control plasma samples were stored frozen at −20°C.

2.4. Patient plasma sample

Human blood was collected in heparinized Venoject tubes and centrifuged within one hour. The plasma was transferred to polypropylene tubes (Nunc) and frozen within 30 min. The plasma samples were kept frozen (−20°C) until analysis.

2.5. Analytical method

The blood plasma sample was brought to 37°C and the pH was adjusted to 7.4 by adding CO_2 (gas). An aliquot (1 ml) of the sample was transferred to an ultrafiltration device, MPS-1, with a YMT 30 membrane and centrifuged at 37°C and 2000 rpm for 15 min (~500 g) to give 200–250 μl of the ultrafiltrate. The ultra-filtrate (150 μl) was injected, without further pre-treatment, into a coupled-column LC system (Fig. 1). After elution from the first column (Superspher RP-select B) the analyte was collected into a 1000-μl trapping loop. In a second step the collected analyte was transferred to and enriched at the top of the second column (Nucleosil 5SA) and then eluted with the second eluent.

The eluent for the first column consisted of acetonitrile–phosphate buffer pH 3 (ionic strength 0.02) (30:70, v/v), and that for the second column of acetonitrile–ammonium phosphate buffer pH 2.6 (ionic strength 0.3) (40:60, v/v). For both columns the flow-rate was 1.0 ml/min and the temperature 27°C. The eluate was detected by UV at 210 nm. The peak heights were measured and the concentrations of ropivacaine or bupivacaine in the ultrafiltrate were calculated by comparison with external standards.

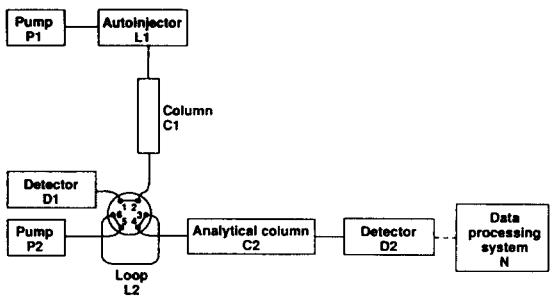

Fig. 1. Coupled-column liquid chromatographic system. System I, L1: autoinjector, six-port sample injector valve with a 200-μl loop. P1: eluent for rough separation; acetonitrile–phosphate buffer pH 3 (ionic strength 0.02) (30:70, v/v). Flow-rate 1.0 ml/min. C1: column for rough separation, Superspher RP-select B. D1: detector [for test of retention (C1)]. System II, L2: autoinjector, six-port switching valve with 1000-μl trapping loop. P2: eluent for fine separation on column 2; acetonitrile–ammonium phosphate buffer pH 2.6 (ionic strength 0.3) (40:60, v/v). Flow-rate 1.0 ml/min. C2: column for fine separation, Nucleosil 5SA. D2: detector UV 210 nm. N: PE Nelson Access*Chrom.

2.6. Calibration

The standards were prepared from the standard stock solution in phosphate buffer pH 7.4 (ionic strength 0.2) within the concentration range 10–1000 nM. The standards were injected directly into the chromatographic system.

Ropivacaine and bupivacaine in the plasma ultrafiltrate were determined by single-point calibration using the peak height of a standard at a concentration level of about 200–300 nM ($n = 6$). The standards at other concentration levels were used as controls.

2.7. Accuracy and precision

On each occasion of analysis, ultrafiltrated aqueous control standards were determined to check the adsorption to the ultrafiltration device. Plasma control samples, with a free concentration of about 100–400 nM ($n = 5$), were determined to measure intra- and inter-assay precision. Aqueous standards, 10–1000 nM, were measured to check the linearity.

2.8. Stability

The stability of the free concentration of ropivacaine and bupivacaine in plasma samples was tested at −20°C. The plasma samples were spiked with 2 μM of ropivacaine and bupivacaine, respectively, i.e. giving a free concentration of about 100 nM. The free concentration was determined on different occasions for a period of one year.

3. Results and discussion

3.1. Drug–protein binding

Ropivacaine and bupivacaine (Fig. 2) are extensively bound to plasma proteins, i.e. about 90–95% in human plasma. The major protein involved is α_1-acid glycoprotein (AAG). The level of AAG in humans shows high subject-to-subject variation (10–50 μM) and varies in different clinical situations [3–5]. The drug–protein binding is a reversible equilibrium and due to the physico-chemical nature the free concentration of the drug will depend on both the total concentration of the protein and the total concentration of the drug. Increased concentration of the binding protein results in decreased free concentration, and increased drug concentration results in decreased protein-binding due to saturation of the protein. In view of the high variation in the free fraction at different protein and drug concentrations, it is of interest from a pharmacological point of view to measure the free concentration, since the pharmacological effect is considered to be proportional to the free concentration of the drug. This is of especial interest in the evaluation of drug safety [3–5].

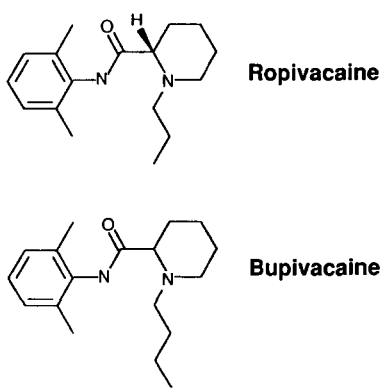

Fig. 2. Chemical structures of ropivacaine and bupivacaine.

3.2. Ultrafiltration

A simple technique of preparing the free concentration is ultrafiltration, which is the method chosen in this paper. The technique is often used to prepare free concentrations of other local anaesthetics [7–13]. To obtain accurate results, it is important to check factors affecting the drug–protein equilibria, e.g. the temperature, the pH and the adsorption of the drug to the ultrafiltration device.

The pH of the plasma will strongly affect the free concentration of ropivacaine and bupivacaine. When the pH was raised from pH 7.2 to pH 7.6, the free fraction decreased by a factor of two. Therefore, in the analytical method, the pH was adjusted to pH 7.4 by adding CO_2 (gas) to the plasma sample in order to obtain reliable results.

The temperature will also affect the drug–protein binding. The free fraction of ropivacaine increased from 6.7 to 8.7% when the temperature was raised from 32 to 40°C, and the free fraction of bupivacaine increased from 5.2 to 6.4% within the same temperature interval. The temperature was set at 37°C in the analytical method.

Instances where high adsorption has affected accurate determination of the free concentration have been reported [15]. No specific adsorption of ropivacaine and bupivacaine to the filtration membrane was observed. However, adsorption to the ultrafiltration device did occur. The Amicon MPS-1 ultrafiltration device adsorbed less than other ultrafiltration devices tested. In addition, initial aqueous washing of the ultrafiltration device significantly decreased the adsorption of the drugs. The effect of the adsorption of the drug on the free concentration can be estimated by simple calculations. The total concentration of a drug, C_X, in a plasma sample

placed in an ultrafiltration device can be expressed as:

$$C_X = [X] + [XP] + [XA]_s \quad (1)$$

where [X] is the free concentration, [XP] the protein-bound concentration and $[XA]_s$ the adsorbed concentration of the drug. Eq. (1) can be rearranged to give:

$$\frac{C_X}{[X]} = 1 + \frac{[XP]}{[X]} + \frac{[XA]_s}{[X]} \quad (2)$$

where [XP]/[X] and $[XA]_s$/[X] are the binding ratios of protein binding and adsorption, respectively. The binding ratio of adsorption can be determined in buffer solutions (pH 7.4) of the drug. If the adsorption is 10%, the ratio $[XA]_s$/[X] is 0.1. If the binding is assumed to be independent of the drug concentration (linear binding isotherm), the effect of adsorption on the free concentration in plasma can be estimated. The adsorption in buffer solution was found to be constant within the concentration range used in the method. If the adsorption is less than 10% in buffer solution (pH 7.4), and if the protein-binding exceeds 90%, the maximum error due to adsorption, calculated from Eq. (2) is less than 1%. However, if the adsorption is high ($>20\%$) and the protein-binding $<90\%$, errors exceeding 10% may occur. The adsorption to the ultrafiltration device must therefore be checked. Aqueous control samples of ropivacaine and bupivacaine are ultrafiltrated and determined on each occasion of analysis. In general the adsorption of ropivacaine or bupivacaine is less than 10% in buffered solutions. Therefore, the adsorption in plasma can be regarded as negligible. External standardisation using standards dissolved in aqueous buffer, pH 7.4, can be used with accuracy.

3.3. Liquid chromatographic system

To minimize pre-treatment of the sample and to achieve optimum precision, direct injection of the ultrafiltrate is preferred. Initial tests using direct injection into a single column resulted in large front peaks and low selectivity. UV-detection at 210 nm was used to obtain high detection sensitivity. A coupled-column system was developed to increase the selectivity, and such systems are often used in bioanalytical applications [16,17]. Chiral determination of bupivacaine enantiomers has also been performed using coupled-column systems [18–20].

In the present study a coupled-column system of a reversed-phase and an ion-exchange column was used. On the first column a rough separation between the analyte and the ultrafiltrate is obtained. The zone where the analyte is eluted is transferred to the second column and the analyte has to be enriched at the top of the column to avoid band-broadening. In the last step the analyte is eluted from the second column with a suitable eluent. The system set-up is given in Fig. 1.

To obtain high selectivity, a reversed-phase C_8 column was used as the first column and a cation-exchange column as the second. By choosing a low ionic strength of the eluent on the first column, it was possible to transfer and enrich the analyte on the second column. At a low ionic strength the retention was very high ($k' > 50$ for both ropivacaine and bupivacaine) on the cation-exchange column (Fig. 3), i.e. the analytes are easily enriched. On the transfer between the columns, the analyte was trapped in

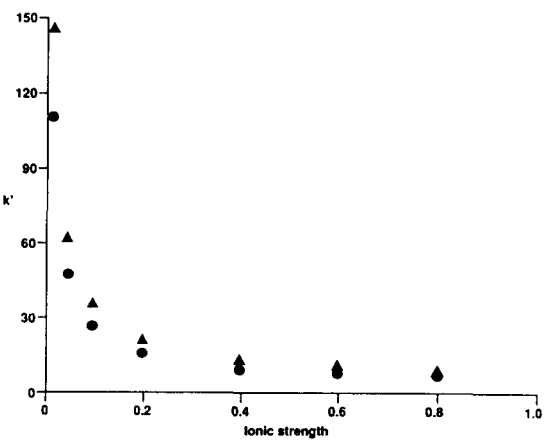

Fig. 3. Effect of the ionic strength on the retention (k') of ropivacaine (●) and bupivacaine (▲) on a strong cation-exchange column. Eluent: acetonitrile–phosphate buffer pH 3 (30:70, v/v). Column: Nucleosil 5SA (150 × 4.6 mm I.D., 5 μm).

a 1000-μl loop. The trapping loop was used to avoid changes in back pressure when coupling the two columns in series. Such pressure changes result in strong disturbances in the UV-detection.

The eluent on the ion-exchange column was chosen to give suitable retention and selectivity. Chromatograms of a plasma sample of ropivacaine eluted from the first column as well as in the coupled-column system are shown in Fig. 4. In the first column low selectivity was obtained, whereas the coupled-column system gave a completely resolved peak of ropivacaine.

To ensure the performance of the system, the retention characteristics on the first column were checked on a daily basis in order to eliminate errors in the transfer of ropivacaine or bupivacaine to the second column. Both retention and peak width must be monitored. The retention of bupivacaine was higher compared to ropivacaine, i.e. the switching time was different for the two compounds. The switching time of the trapping loop was set from the peak volume data. When the peak width volume exceeds 750 μl, the first column is replaced.

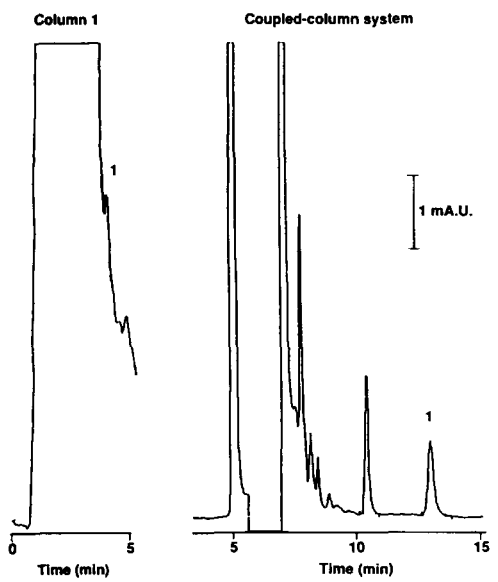

Fig. 4. Performance on direct injection of plasma ultrafiltrate containing ropivacaine. Left: rough separation on first column. Right: coupled-column separation. For conditions, see Fig. 1; 1 = ropivacaine, 140 n*M*.

3.4. Ruggedness

On the first column, Superspher RP-select B, the eluent consists of acetonitrile–phosphate buffer pH 3 (ionic strength 0.02) (30:70, v/v). Variations in the pH (2–4) and the ionic strength (0.01–0.1) of the eluent resulted in only small changes in retention. The acetonitrile content affects retention, as in ordinary reversed-phase systems, and is the major variable factor in the regulation of retention.

On the second column, Nucleosil 5SA, the eluent consists of acetonitrile–ammonium phosphate buffer pH 2.6 (ionic strength 0.3) (40:60, v/v). As shown above (Fig. 3), the ionic strength drastically affects retention at low ionic strength. However, close to the ionic strength chosen (0.3) a minor effect on retention was observed with small changes in ionic strength. This is also true for minor changes in pH or the acetonitrile concentration.

The cation-exchange columns show batch-to-batch variations; the retention varies possibly due to different amounts of charges on the column surface. In addition, cation-exchange columns from different manufacturers show variations in retention characteristics. To obtain similar retention from column to column, the optimal parameter to change is the content of organic modifier in the eluent. However, the acetonitrile concentration can not be raised above 50% due to solubility problems. If the retention is still too high, it is possible to add isopropanol to the eluent to lower it. Other columns tested, e.g. Partisil P10SCX, Spherisorb S5SCX and Exsil 100-SCX, can be used after eluent optimisation.

3.5. Selectivity, sensitivity and linearity

Typical patient plasma sample chromatograms for ropivacaine and bupivacaine as well as blank chromatograms from human plasma are shown in Fig. 5. The blank chromatograms show no peaks interfering with ropivacaine or bupivacaine.

The limit of determination for ropivacaine and bupivacaine in human plasma ultrafiltrate is 10

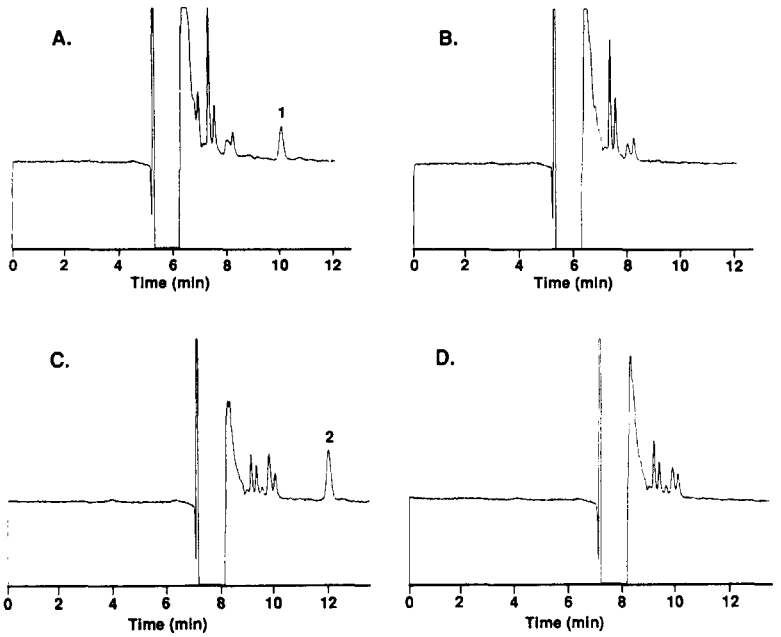

Fig. 5. Chromatograms of plasma samples obtained after epidural administration of ropivacaine and bupivacaine. For analytical conditions, see Fig. 1. (A) Patient plasma, 1 = ropivacaine, 35 n*M*. (B) Blank plasma sample, ropivacaine analysis. (C) Patient plasma, 2 = bupivacaine, 51 n*M*. (D) Blank plasma sample, bupivacaine analysis.

n*M*, which is low or similar, as compared to other published liquid chromatographic methods [21–28] for bupivacaine. The precision (R.S.D.) in aqueous standards at this concentration is $< 10\%$ ($n = 30$). Excellent linearity ($r^2 = 0.999$) is obtained in the concentration range 10–3000 n*M*.

3.6. Accuracy, precision and stability

The intra-assay precisions (R.S.D.) in plasma samples are normally between 2% and 8%. The intra-assay precisions obtained on about one hundred different occasions of analysis are given in Fig. 6. The inter-assay precision (R.S.D.) at a concentration level of about 100 n*M* is 6% ($n = 30$) for ropivacaine and 7% ($n = 30$) for bupivacaine.

No decrease was observed in the free concentration of ropivacaine or bupivacaine in plasma samples stored at −20°C for at least one year.

3.7. Routine use of the method

The method has been used routinely for two years and more than two thousand samples have been determined in pharmacokinetic and pharmacodynamic studies. The repeatability of the

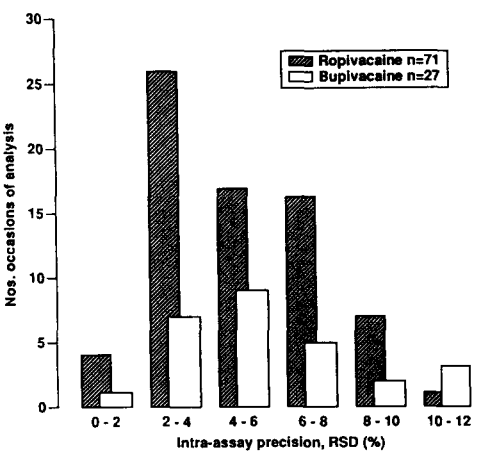

Fig. 6. Intra-assay precision (R.S.D.) with long-term use of the method.

method has been high, as shown by the intra-assay precision of the method given in Fig. 6.

The liquid chromatographic system is robust and can be used for more than six months without changing the columns.

4. Conclusion

The free concentration of ropivacaine and bupivacaine in blood plasma is determined by a simple and robust methodology involving ultrafiltration and coupled-column liquid chromatography. The free concentration is prepared simply by ultrafiltration and the ultrafiltrate is injected directly into the liquid chromatographic system. During ultrafiltration it is shown that drug–protein binding related factors such as the temperature, the pH and the adsorption must be controlled.

The methodology described is well-suited for determining the free concentration of ropivacaine and bupivacaine since the method shows high selectivity, sensitivity and precision. The chromatographic system also shows a high degree of ruggedness and stability and is suitable for routine analysis.

Acknowledgements

We thank Dr K. Gröningsson for valuable comments on the manuscript.

References

[1] H.S. Feldman, in S.A. Rice and K.J. Fish (Editors), *Anaesthetic Toxicity*, Raven Press, New York, 1994, pp. 107–133.
[2] D.B. Scott, A. Lee, D. Fagan, G.M.R. Bowler, P. Bloomfield and R. Lundh, *Anesth. Analg.*, 69 (1989) 563.
[3] G.T. Tucker, *Acta Anaesthesiol. Belg.*, 39 (1988) 147.
[4] G.T. Tucker, *Reg. Anesth.*, 17 (3 Suppl.) (1992) 1.
[5] G.T. Tucker, *Reg. Anesth.*, 19 (1994) 155.
[6] T. Arvidsson, H. Forsmo-Bruce and M.M. Halldin, *Chirality*, in press.
[7] C.L. Beach, T.M. Ludden, W.A. Clementi and S.R.B. Allerheiligen, *Ther. Drug Monit.*, 8 (1986) 326.
[8] D.E. Coyle and D.D. Denson, *Biopharm. Drug Dispos.*, 5 (1984) 399.
[9] B. Bachmann-Mennenga, J. Biscoping, R. Schürg, E. Sinning and G. Hempelmann, *Arzneim-Forsch./Drug Res.*, 41 (1991) 520.
[10] B. Bachmann, J. Biscoping, E. Sinning and G. Hempelmann, *Acta Anaesthesiol. Scand.*, 34 (1990) 311.
[11] D.J. Morgan, B.B. Koay and J.D. Paull, *Eur. J. Clin. Pharmacol.*, 22 (1982) 451.
[12] J.X. Mazoit, D.D. Denson and K. Samii, *Anesthesiology*, 68 (1988) 387.
[13] B. Bachmann-Mennenga, J. Biscoping, T. Violka, R. Schürg and G. Hempelmann, *Reg. Anaesth.*, 14 (1991) 32.
[14] M. Björk, K.-J. Pettersson and G. Österlöf, *J. Chromatogr.*, 533 (1990) 229.
[15] Yu.A. Zhirkov and V.K. Piotrovskii, *J. Pharm. Pharmacol.*, 36 (1984) 844.
[16] L.-E. Edholm and L. Ögren, in I.W. Wainer (Editor), *Liquid Chromatography in Pharmaceutical Development: An Introduction.*, Alster, Springfield, OR, 1985, pp. 345–373.
[17] L.-E. Edholm, A. Blomgren, C. Roos and L. Ögren, *Lab. Inf. Manag.*, 17 (1992) 233.
[18] B.J. Clark, A. Hamdi, R.G. Berrisford, S. Sabanathan and A.J. Mearns, *J. Chromatogr.*, 553 (1991) 383.
[19] J.J. Butter, J.C. Kraak and H. Poppe, *J. Pharm. Biomed. Anal.*, 11 (1993) 225.
[20] A. Walhagen and L.E. Edholm, *J. Chromatogr.*, 473 (1989) 371.
[21] U.-W. Wiegand, R.C. Chou, E. Lanz and E. Jähnchen, *J. Chromatogr.*, 311 (1984) 218.
[22] R.L.P. Lindberg and K.K. Pihlajamäki, *J. Chromatogr.*, 309 (1984) 369.
[23] H.-R. Ha, B. Funk, H.R. Gerber and F. Follath, *Anesth. Analg.*, 63 (1984) 448.
[24] H.A. Adams, J. Biscoping, K. Ludolf, A. Börgmann, B. Bachmann-Mennenga and G. Hempelmann, *Reg. Anaesth.*, 12 (1989) 53.
[25] H.C. Michaelis, W.P. Geng, G.F. Kahl and H. Foth, *J. Chromatogr.*, 527 (1990) 201.
[26] A. Ruzafa, M.C. Pastor, J.L. Aguilar and R. Galimany, *J. Liq. Chromatogr.*, 14 (1991) 2937.
[27] H. Kastrissios, M.-F. Hung and E.J. Triggs, *J. Chromatogr.*, 577 (1992) 103.
[28] I. Murillo, J. Costa and P. Salvá, *J. Liq. Chromatogr.*, 16 (1993) 3509.