Determination of the Free Concentration of Ropivacaine in Plasma by Packed Capillary Liquid Chromatography: A Comparison of Ultrafiltration and Microdialysis as Sample Preparation Methods

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Abstract: In this study, ultrafiltration and microdialysis have been compared as sample preparation methods to separate the free fraction of ropivacaine from the protein bound in 150 μL plasma. A liquid chromatographic system with packed capillary columns (0.2 mm internal diameter) was used to enhance sensitivity when analyzing the small sample volumes obtained after the ultrafiltration and the microdialysis. The microdialysis was performed with probes of our own construction, and to save analysis time, the microdialysis sampling was coupled on-line to the liquid chromatographic system. The reduction of back pressure in the microdialysis outlet tube and in the injector was found to be essential. The free fraction obtained with each method was equivalent: both gave a free fraction of 6%. J Micro Sep 13: 197–201, 2001

Key words: *microdialysis; ultrafiltration; free fraction; ropivacaine; capillary liquid chromatography*

INTRODUCTION

It has been known for a long time that drugs bind to plasma proteins in the body to a greater or lesser extent. The two main proteins involved in drug–protein binding are albumin and α_1-acid glycoprotein (AGP) [1]. The protein binding may be altered if the concentration of plasma proteins suddenly changes, which may be the case in surgery, certain disease states, and for pediatric and geriatric patients [1]. It is generally believed that only the free fraction of a drug is the pharmacologically active fraction [2]. Whereas the free concentration can vary between individuals, e.g., due to variations in protein concentrations, the free fraction is more interesting to analyze than the total fraction when determining proper dosages.

Many techniques have been used to separate the free fraction of a drug from the protein-bound fraction. Sample preparation methods like conventional solid-phase extraction, liquid–liquid extraction, and protein precipitation are not a choice when extracting only the free fraction of the drug. Instead, ultrafiltration [3–5] and equilibrium dialysis [6–8] are the most commonly used techniques. Ultrafiltration is a rapid and simple technique, whereas equilibrium dialysis is more time-consuming. Microdialysis has been used in some applications [9–11] to determine free concentrations of drugs *in vitro*. The advantage of microdialysis over equilibrium dialysis and ultrafiltration is the possibility of coupling on-line to liquid chromatography (LC). On-line coupling to the analytical system may reduce the required time and offers the possibility of total automation of the microdialysis and the subsequent analysis. In addition, the problems usually associated with handling small volumes are avoided. Microdialysis can be used for both *in vitro* and *in vivo* analyses.

Only small volumes are collected when using microdialysis sampling and this may lead to detection problems. Hence, microcolumn LC is a proper choice of separation technique because the sample is thus less diluted in these columns, giving increased sensitivity if a concentration sensitive detector is used. When studying analytes in plasma there is always an advantage to using as small a volume of blood as possible, especially when samples are taken from small children and in animal studies. Ultrafiltration can be used to prepare small sample volumes, and a study of the combination of ultrafiltra-

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tion and packed capillary LC in which only 40 μL of plasma was used was reported by us earlier [12]. *In vitro* microdialyses are often performed in volumes of 1 mL or more. It should, however, be possible to perform the microdialysis with smaller sample volumes also.

Ropivacaine is a long acting local anaesthetic. The protein binding in plasma is higher than 90% for ropivacaine and the binding mainly occurs to AGP [13]. Previous studies on free fractions of ropivacaine have been made using ultrafiltration [5] and solid-phase microextraction [14]. In these studies, the plasma sample volume was, however, rather large (i.e., 1.0 mL). Stumpe et al. [15] recently published a small-scale equilibrium dialysis study of ropivacaine where only 100 μL plasma was used. This technique was, however, quite time-consuming. It took 3 h before the sample was equilibrated, which shows the major drawback of equilibrium dialysis.

In this study, packed capillary LC [internal diameter (i.d.) = 0.2 mm] was used to analyze the free concentration of ropivacaine in 150 μL of plasma. Ultrafiltration and microdialysis were compared as sample preparation methods, because they are rather fast methods for separating the free fraction of ropivacaine from the protein-bound fraction. Also, some manufacturing aspects of the microdialysis probe and its use in on-line coupling with the capillary LC system are discussed.

EXPERIMENTAL

Chemicals. Ropivacaine hydrochloride monohydrate (Figure 1) was obtained from AstraZeneca (Södertälje, Sweden). Methanol of LiChrosolv grade was purchased from Merck (Darmstedt, Germany). All other chemicals were of analytical grade and were obtained from the usual commercial sources. Water was purified with a Milli-Q purification system (Millipore, Bedford, MA).

Chromatography. The mobile phase [methanol-ammonium formate pH 3.0 (ionic strength 0.01), 50:50, v/v] was delivered by a LC pump (PU-980, Jasco, Tokyo, Japan) operated in constant pressure mode at a flow rate of 1 μL/min. A manual six-port valve (C6W, Valco instruments, Houston, TX) was used for injection. A large loop volume (2.5 μL) could be used, because the sample was dissolved in a solvent of lower elution strength than the mobile phase, thus giving a preconcentration on top of the column. The column (200 × 0.2 mm i.d.) was packed with 5 μm C_{18} particles (ODS A, YMC Europe GmbH, Scermbeck/Weselerwald, Germany) in fused silica capillaries (Polymicro Technologies, Phoenix, AZ) using a supercritical carbon dioxide packing method [16]. A Jasco UV detector (UV-975) with a capillary flow cell (UZ-JA97-CAP, LC Packings, Amsterdam, The Netherlands) was used at a wavelength of 210 nm. In the initial microdialysis recovery studies, a UV detector (μPEAK Monitor, Amersham Pharmacia Biotech, Uppsala, Sweden) with optical fibers for on-column detection was used. The chromatography software Borwin™ (JMBS Developments, Le Fontanil, France) was used to integrate the peaks.

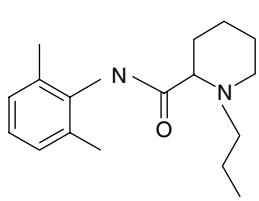

Figure 1. *Chemical structure of ropivacaine.*

Sample preparation. Stock solutions of 100 and 1000 μM ropivacaine were prepared in water and stored in a refrigerator. Calibration standards were prepared from the 100 μM stock solution by diluting with a phosphate buffer pH 7.4 (ionic strength 0.2) to 100–400 nM.

Human plasma was purchased from the blood bank at the University Hospital, Uppsala, Sweden. The plasma was adjusted to pH 7.4 by adding a few microliters of acetic acid (5%, v/v) to about 1 mL of plasma. The plasma was spiked to a concentration of 4.0 μM by adding 1000 μM stock solution. Prior to ultrafiltration and microdialysis, the plasma was incubated for 15 min at 37°C in a block thermostat (DB-2D, Techne, Cambridge, UK) to assure drug–protein binding.

Ultrafiltration. The ultrafiltration device was an Ultrafree-MC with 30,000 as the molecular weight cutoff (Millipore). A centrifuge (2K15, Sigma, Osterode am Harz, Germany) equipped with a 33° fixed angle rotor and a heating element was used to perform the ultrafiltration. 150 μL of sample was ultrafiltrated at 37°C for 10 min at 2000g to give 40 μL filtrate. The filtrate was injected directly into the LC system.

Microdialysis. Microdialysis was performed using homemade probes. The construction is shown in Figure 2. The probes were made of a polyamide membrane with a cutoff of 20,000 Da [i.d. = 0.5 mm, outer diameter (o.d.) = 0.6 mm, CMA, Stockholm, Sweden]. A polypropylene (PP) tubing (i.d. = 65 μm, o.d. = 250–260 μm; Polymers Inc., Middlebury, VT) served as the inlet tube and was inserted into the membrane. Poly(ether ether ketone) (PEEK) tubing of 0.064 mm i.d. and 0.51 mm o.d. (Chromtech AB, Hägersten, Sweden) or fluorinated ethylenepropy-

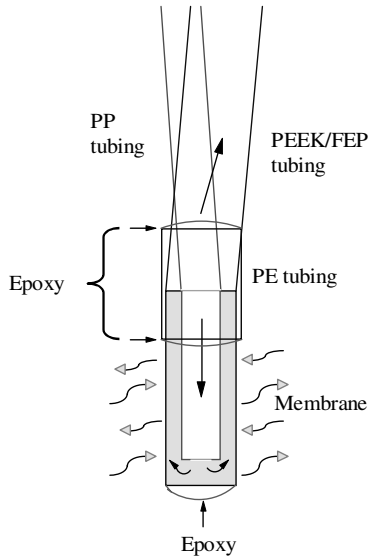

Figure 2. *Schematic of the microdialysis probe construction.*

lene (FEP) tubing of 0.12 mm i.d. and 0.65 mm o.d. (CMA, Stockholm, Sweden) served as the outlet tube. The outlet tube and the membrane (including the inlet tube) were connected by a short piece of 0.8 mm i.d. PP tubing. The different parts, as well as the end of the membrane, were fixated with epoxy glue (Araldit rapid, CIPA, Switzerland). The probe was placed in a vial containing 150 μL sample held at 37°C by a block thermostat (DB-2D, Techne). The probe was perfused with phosphate buffer pH 7.4 (ionic strength 0.2) using a syringe pump (CMA 102, CMA, Stockholm, Sweden). The flow rate was 0.5 μL/min and the sampling was performed for 20 min unless otherwise indicated. The outlet of the probe was coupled directly to the LC injection valve. After at least 4 min of injection, the injector was put into the load position to perform sampling during analysis.

RESULTS AND DISCUSSION

Ultrafiltration. Ultrafiltration is a rapid and simple technique for preparing the free nonbonded fraction of a drug in plasma. A disadvantage with ultrafiltration is, however, that the drug and/or its metabolites may adsorb to the ultrafiltration device. This potential problem is enhanced when the technique is miniaturized and the surface area gets proportionally larger. In a previous study, the adsorption of the drug tolterodine and its hydroxymethyl metabolite at a concentration of 20 nM to ultrafiltration devices of different material compositions was examined [12]. It was found that the recovery of the different ultrafiltration devices differed a lot, i.e., from 13 to 82% for tolterodine. Recovery of the polar metabolite was higher than for tolterodine. Possible adsorption to the ultrafiltration device was thus found to be an important factor to examine. The adsorption of ropivacaine to the ultrafiltration device was examined by filtrating 150 μL of 200 nM ropivacaine in phosphate buffer pH 7.4 (ionic strength 0.2). The recovery was around 85%. To approach the ultrafiltration of a real sample, ultrafiltrated plasma was spiked and ultrafiltrated again. The recovery was slightly improved with this procedure, giving a recovery of 88%.

Microdialysis. Microdialysis is a technique that is suitable for on-line coupling to an analytical system. A prerequisite is, however, to reduce the back pressure in the system. Initial problems were occasionally observed with the use of PEEK as the outlet tube. It was seen that the perfusate not only wetted the inside of the membrane but sometimes also penetrated the pores of the membrane. This "perspiration" indicated that the pressure in the outlet was too high, probably from restriction due to a nonuniform i.d. of the tube. After this insight, the probes were constructed with a FEP tubing for the outlet flow. The main advantage of this FEP tubing was that the i.d. is guaranteed to be 0.12 mm all the way. However, the i.d. of the FEP tubing was twice the i.d. of the PEEK tubing and the length of the FEP tubing was 15 cm, which resulted in a dead volume of 1.7 μL. The back pressure in the injection device also had to be minimized. Observations of low and irreproducible recoveries were occasionally made, and not until the injector was properly cleaned and filters in the injector were removed did the results become acceptable.

Decreasing the perfusion flow rate and increasing the length of the microdialysis membrane increased, as expected, the relative recovery (RR). The relative recovery of ropivacaine (1000 nM) in phosphate buffer pH 7.4, ionic strength 0.2, as a function of perfusion flow rate was examined (Figure 3) at a membrane length of 9 mm. The sampling time was 10–100 min, depending on flow rate, to achieve a proper injection volume. A flow rate of 0.2 μL/min gave acceptable recoveries (90%). Such low flow rates, however, result in very long sampling times (60 min). The membrane length was thus increased to 14–15 mm to further increase the recovery. Much further elongation was not possible if 150 μL volumes were to be used. A flow rate of 0.5 μL/min with a sampling time of 20 min gave a recovery of 90% for the two probes examined. The recovery in plasma was also investigated to see if it differed from the recovery in phosphate buffer. The plasma was ultrafiltrated to remove the proteins and spiked

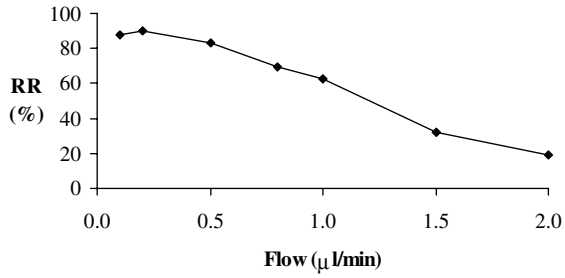

Figure 3. *Effect of flow rate on the relative recovery of 1000 nM ropivacaine in phosphate buffer pH 7.4 (ionic strength 0.2) at a membrane length of 9 mm. The sampling time varied from 10 to 100 min depending on flow rate.*

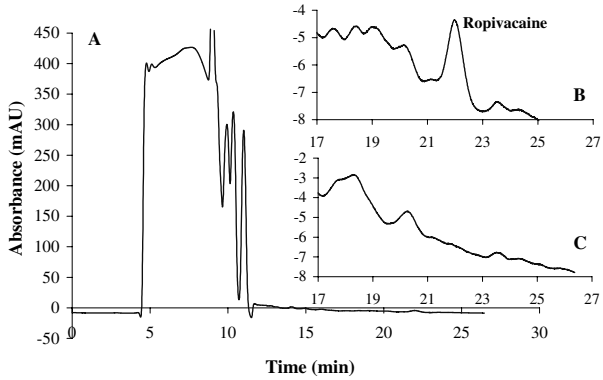

Figure 4. *Chromatogram of plasma sample spiked to a total concentration of 4.0 μM (A and B) compared to a blank plasma (C) after ultrafiltration. (A) Total chromatogram of spiked plasma. (B) Enlargement of the ropivacaine peak in spiked plasma, 224 nM (t_R = 22 min). (C) Enlargement of the blank ultrafiltrated plasma sample in the time interval where ropivacaine would elute.*

with 1000 nM ropivacaine. The recovery in filtrated plasma was found to be the same as in phosphate buffer, i.e., about 90%. Before plasma analysis, the microdialysis probe was checked to give proper recovery in a standard solution of 200 nM ropivacaine in phosphate buffer. The same probe was used throughout the study with recoveries of not less than 95%.

Determination of free fraction. Plasma samples were spiked with ropivacaine to a total concentration of 4.0 μM and 150 μL was ultrafiltrated or microdialyzed using the optimized conditions. The free concentration of ropivacaine was quantified against standard solutions prepared in phosphate buffer. The calibration curve was linear in the interval studied (0–400 nM), with correlation coefficients of not less than 0.998. Figure 4 shows a chromatogram of spiked and ultrafiltrated plasma samples. An ultrafiltrated plasma sample without ropivacaine gave no peak at the same retention time as ropivacaine. The experiments were repeated over three different days and the results of the free fraction determination together with precision data are illustrated in Table I. No significant difference with respect to the free fraction between the two sample preparation methods was observed: both gave a free fraction of 6%. However, the precision of the ultrafiltration was better. In this microdialysis experiment, a new sample was taken for every injection. However, in a later study, three subsequent injections were made from the same sample (n = 3) to see if the free concentration would decrease due to slow protein binding or change of free fraction when the total concentration decreases. No such trend was observed, at least not at the studied concentration (4000 nM total concentration) and at the obtained precision (mean RSD = 5%).

Concentrations down to 10 nM in phosphate buffer (signal to noise ratio = 3) could be detected with the described method. Due to endogenous compounds that elute close to ropivacaine, the method has to be modified for plasma samples at these low levels. In a pharmacokinetic study [17] and in a prediction of the adverse effects [18], the free concentration of ropivacaine was in the range of 10–400 nM. Further studies will include a mass spectrometer as a detector to improve sensitivity and selectivity in the detection. A column-switching system will then be used to get rid of salts that may disturb the detection, but also to prolong the lifetime of the separation column.

This study has shown that ultrafiltration and microdialysis give equivalent results in free fraction determination of relatively small plasma volumes.

Table I. *Results and precision data for analysis of ropivacaine in plasma.*

Method	Mean free concentration (nM)	Mean free fraction (%)	Interassay precision (n = 3) (RSD, %)	Mean intraassay precision (RSD, %)
Ultrafiltration	240	6.0	3.4	4.2
Microdialysis	244	6.1	8.4	7.4

Microdialysis can favorably be coupled on-line to the analytical system and the same device can be used for several samples, which is not possible with ultrafiltration. Ultrafiltration is, however, a simpler technique to use and commercial devices are available at a relatively low cost.

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