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Short communication

Development of a method to measure free and bound ropivacaine in human plasma using equilibrium dialysis and Hydrophilic interaction chromatography coupled to high resolution mass spectrometry

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ABSTRACT

The pharmacodynamics of absorption of local anaesthetics used during surgical procedures into tissues is governed by the amount of free drug in plasma. Toxicity may occur during continuous infusions if the levels of free drug become too high which may occur if the binding capacity of the α-1- acid glycoprotein present in plasma is exceeded. In order to monitor this a method was developed for the determination of the amount of free and bound ropivacaine in human plasma during knee and hip surgery. Rapid equilibrium dialysis units were used to separate free and bound drug then protein and buffer salts were removed by solvent precipitation. Analysis was carried out using a ZICHILIC HPLC column interfaced with an LTQ Orbitrap mass spectrometer. The following extracted ion ranges ($[M+H]^+$) were monitored: m/z 275.21-275.22 for ropivacaine and m/z 235.175-235.185 for lidocaine. The method was calibrated by spiking ropivacaine, and a fixed amount of lidocaine as internal standard, into plasma over the range 0.01–1.5 μg/ml. The equation of the line was $y=0.886x \pm 4.2\%$ ($n=2$), forcing the curve through zero since blank plasma was free of the analyte. The values obtained for the accuracy and precision of the analysis of plasma spiked at 0.03 μg/ml and 1.5 μg/ml were 93.2% ± 2.8% and 95.4% ± 1.5% respectively ($n=5$). Repeat analysis of a patient sample for free and bound drug gave the following values for levels of ropivacaine: bound 1.63 μg/ml ± 1.48%, unbound 0.0671 μg/ml ± 1.68% ($n=5$).

1. Introduction

Drug binding with plasma protein has a significant role in disposition of a drug within the body. Various pharmacokinetic (PK) parameters like rate of hepatic metabolism, renal excretion rate, transport rate and volume of distribution are all dependent on the free fraction of drug. The degree of drug protein binding is also used in determining PK parameters in animals as well as humans. In pharmacodynamics it is also important to know the free fraction of the drug because it can reach the target area more easily to produce an effect as compared to the bound drug. This principle can be applied to all other compartments which are in equilibrium with plasma requiring that there is no active transport, so accurate information about the free fraction of the drug is important in new drug development and also in clinical trials in order to determine the safety of drugs [1].

The drug after reaching the blood binds to proteins in the plasma either in lesser or greater extent. The main proteins responsible for drug binding are α1-acid-glycoprotein and albumin. The binding of protein with the drug might be changed if there was a sudden change in the concentration level of plasma proteins either because of surgical conditions, in geriatric or paediatric patients or because of certain disease states. It is known that the free portion of the drug only is responsible for the pharmacological activity, however the free portion of the drug can vary among individuals e.g. because of protein concentration variation, so it is very important to analyse the free fraction of the drug rather than total drug in determining a safe dosage [2].

Local anaesthetics are generally amino-amide, amino-ester and amino-ether compounds. These drugs are used for the treatment of pain during and after a surgical procedure. They can be used either intravenously, epidurally, subcutaneously or topically. The absorption and distribution of the drug in the body depends on various factors such as the route and method of administration, plasma protein binding, pH of the plasma, blood flow characteristics, physical properties and chemical structure of the anaesthetic administered [3]. For many decades local anaesthetics of the amide type have been used for anaesthesia during surgery. One of the first drugs in this class was bupivacaine which has been used for over 50 years. In 1996 Astra Pain Control introduced a new

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member of this group called ropivacaine into the clinic. Ropivacaine (Propyl-2′, 6′-pipecoloxylidide hydrochloride hydrate) is used as the enantiomerically pure (S)-isomer. Ropivacaine is generally used for peripheral nerve blocking and to induce analgesia [4]. It is similar to bupivacaine chemically [5] but the toxicity of ropivacaine to the cardiac system is less as compared to other anaesthetics because of its low affinity for sodium channels in the heart, also ropivacaine blocks sensory nerve fibres selectively so these advantages make ropivacaine a safe option to be used clinically [4,6]. The most important feature of these drugs is their high degree of plasma protein binding and ropivacaine is $>94\%$ bound to plasma protein [5,7]. The protein responsible for binding it is α1-acid-glycoprotein (AGP). AGP is an acute phase protein that increases after surgery and in some diseases [5]. The concentration of AGP in plasma shows variation among humans and ranges from 10 to 50 μM [6].

There are many techniques available to separate the free portion and bound portion of a drug e.g. equilibrium dialysis, ultrafiltration and microdialysis [2,8]. However, equilibrium dialysis is considered as the reference method for the separation of the free and bound portion of a drug [8]. A previous study showed that equilibrium dialysis was the most thermodynamically stable and robust method for the determination of protein binding and following the introduction of a 96 well plate format and it has emerged as an investigator's first choice over the last decade [9,10]. Microdialysis is another technique which has been widely used and has the advantage of connecting with on line monitoring systems [11] and also allows the determination of unbound drug in the plasma in vivo.

There have been a limited number studies monitoring free and bound levels of ropivacaine in clinical samples. Corso et al. monitored free and bound levels of ropivacaine in patients receiving ropivacaine infusions following bowel surgery [12]. The levels of free ropivacaine were ca 1.5% throughout the 96 h post-surgery infusion and the lack of accumulation of free levels could be related to a 63% increase in the levels of AGP measured at 48 h post-surgery. A study of the PK of ropivacaine in neonates following the use of ropivacaine to carry out a single caudal block found that the levels of free drug ranged between 1 and 12% of bound drug and there was a slight increase in levels of AGP post-surgery [13]. In a similar study examining drug disposition in neonates following both single shot and continuous infusion of ropivacaine, the levels of unbound drug were $<10\%$ of the bound drug and AGP levels were found to increase post-surgery [14].

The current study aimed to develop a method for measuring free and bound ropivacaine levels in plasma during ropivacaine infusion following a surgical procedure for hip or knee replacement. Following on from our previously developed the equilibrium dialysis method we took advantage of the rapid microscale equilibrium dialysis method described by Curran et al. [9] and combined this with analysis using hydrophilic interaction chromatography in combination with high resolution full scan mass spectrometry. The structures of ropivacaine and lidocaine, which was used as an internal standard, are shown in Fig. 1.

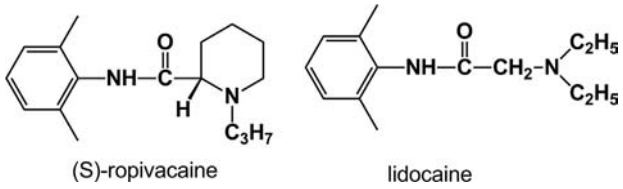

Fig. 1. The chemical structures of ropicavaine and lidocaine.

2. Experimental

2.1. Chemicals and materials

HPLC grade acetonitrile (ACN) was purchased from Fisher Scientific, UK. HPLC grade water was produced by a Direct-Q 3 Ultrapure Water System from Millipore, UK. AnalaR grade formic acid (98%) was obtained from BDH-Merck, UK. Sodium phosphate monobasic dihydrate, sodium phosphate dibasic and sodium chloride were purchased from Sigma-Aldrich, UK. The Thermo Scientific Pierce Rapid Equilibrium Dialysis (RED) units and base plate were purchased from Fisher Scientific, Leicestershire, UK. Ropivacaine hydrochloride monohydrate was obtained from the European Pharmacopoeia Laboratories, Strasbourg, France. Lidocaine hydrochloride monohydrate was obtained from Sigma Aldrich, UK.

2.2. Patient plasma samples

Blank plasma was provided by the Blood Transfusion Service (Gartnavel Hospital, Glasgow). Samples of plasma from patients were obtained from Dr. Mike Gill from patients undergoing knee joint surgery at the Golden Jubilee Hospital in Clydebank. Full risk assessments were produced for the procedures and samples were obtained with informed patient consent and ethics committee approval. In this procedure a total of 200 ml of 0.2% w/v ropivacaine hydrochloride was injected into the anterior and posterior compartments of the knee and subcutaneously. An intra-articular catheter was left in the knee. Then the local anaesthetic administered as three 40 ml top-up doses via the intra-articular catheter in the first 24 h with two additional 'as needed' top-up boluses at least 20 min. apart and 4 ml of blood were taken for a baseline level of local anaesthetic at the start of the procedure before the spinal anaesthetic. Then further samples of blood were taken at 5, 10, 15, 20, 25 and 30 min, 1 h and 24 h following local anaesthetic infiltration.

2.3. Preparation of different standards

Standard stock solutions of ropivacaine HCl and lidocaine HCl were prepared at 1 mg/ml in methanol.

2.4. Buffer preparation and equilibrium dialysis

First of all a buffer containing 100 mM sodium phosphate and 150 mM NaCl pH 7.40 was prepared by the following method [9]. A basic solution was made by dissolving 14.2 g/l Na_2HPO_4 and 8.77 g/l NaCl in deionized water. An acidic solution was made by dissolving 15.6 g/l $NaH_2PO_4 \cdot 2H_2O$ and 8.77 g/l NaCl in deionized water. The basic solution was then titrated with the acidic solution to pH 7.40. Immediately before dialysis plasma samples and buffer were temperature equilibrated at 37 °C. The free and bound portions of the drug were separated by equilibrium dialysis using RED (rapid equilibrium dialysis) device insets (Fisher Scientific, Loughborough, UK) along with the required base plate. Each insert is composed of two side-by-side chambers which is separated by an O-ring-sealed vertical cylinder of dialysis membrane (MWCO ~8000). The base plate is high grade, reusable, and made of durable and chemically inert high-grade PTFE eliminating non-specific binding. Each insert was filled with 300 μl of patient or control plasma and 500 μl of buffer respectively. Equilibration was then carried out for 6 h at 37 °C with shaking at 300 rpm in an Eppendorf Thermomixer.

2.5. Preparation of samples and the extraction method

After 6 h shaking the device was removed from the thermomixer. Then 50 μl was taken from the plasma and buffer compartments and transferred into an Eppendorf tube and 50 μl of IS (lidocaine 1 μg/ml in water) solution and 10 μl of 0.1% v/v aqueous formic acid were added to each tube. Then 50 μl of blank plasma was added to the buffer sample and 50 μl of buffer was added to the plasma sample [9] and the samples were vortexed. Then 340 μl ACN was added to both the plasma and buffer samples and the samples were vortexed for 2 min and centrifuged at 9000 rpm for 10 min. After centrifuging the supernatant layer was taken directly for LCMS analysis.

LC–MS analysis

Measurement of standards and samples was carried out on a Surveyor HPLC system combined with an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK). The column used was a ZIC-HILIC column (150 × 4.6 mm, 5 μm Hichrom, Reading, UK). Mobile phase A consisted of 0.1% v/v formic acid in water and Mobile phase B consisted of 0.1% v/v formic acid in ACN. The flow rate was 300 μl/min. The ESI interface was operated in a positive ion mode with a spray voltage of 4.5 kV. The temperature of the ion transfer capillary was 275 °C and the flow rates of the sheath and auxiliary gases were 50 and 17 arbitrary units respectively. The full scan range was 75–1200 m/z. The data was recorded using Xcalibur 2.1.0 software (Thermo Fisher Scientific). Mass calibration was performed for both ESI polarities before the analysis using the standard Thermo Calmix solution and the signals at 83.0604 m/z (2 × ACN+H) was selected as a lock masses for positive ion mode during each analytical run. The following extracted ion ranges ($[M+H]^+$) were monitored: m/z 275.21-275.22 for ropivacaine and m/z 235.175-235.185 for lidocaine.

2.6. Calibration curve

Diluted stock solutions of ropivacaine were prepared at a concentration of 10 μg/ml and 1 μg/ml in water and stored at 4 °C and a diluted stock solution of the lidocaine internal standard was prepared at 10 μg/ml. Then 1 ml aliquots of plasma were spiked with 0 μg, 0.01 μg, 0.03 μg, 0.05 μg, 0.1 μg, 0.2 μg, 0.5 μg, 1 μg and 1.5 μg of ropivacaine and 1 μg of lidocaine internal standard. Then these samples were processed in the same way as patient samples but without a dialysis step. A linear range was thus established over the range of 0.01–1.5 μg/ml.

2.7. Precision and accuracy of the method for determination of bound and unbound drug

The precision and accuracy of the method was determined by repeat preparation (× 5) of the 0.03 and 1.5 μg spiked samples of plasma. These samples were processed in the same way as the patient samples.

The recovery from the solvent treatment procedure was determined by extracting six replicates of plasma (for high level) and buffer (for low level) each without the internal standard. The internal standard was then added to both the extracts from plasma and buffer in the reconstitution step.

An ionisation suppression test was carried out by mixing 0.4 ml of blank plasma+0.4 ml of buffer with 3.2 ml of acetonitrile and the sample was centrifuged and the supernatant was removed. The supernatant (500 μl) was spiked with 50 μl of ropivacaine (0.5 μg/ml); similarly 500 μl of acetonitrile was also spiked with 50 μl of ropivacaine (0.5 μg/ml). The samples were then analysed.

2.8. Precision of the method for the determination of free drug

The precision for the determination of free drug was also checked by the spiking 5 ml of blank plasma with 50 μl of a solution of ropivacaine (100 μg/ml) in water. Then 5 aliquots from this spiked plasma were treated using the RED method and the levels of the free drug in dialysate were determined.

2.9. Determination of the precision of analysis of unbound drug in samples

Repeat ($n=5$) determination of bound and unbound drug in a selected patient sample was carried out.

2.10. Determination of binding capacity of patient samples

An aliquot of patient plasma (1 ml) was spiked with ropivacaine (100 μg/ml) in water to produce a concentration of ropivacaine at 1 μg/ml above the original concentration in the patient sample.

3. Results

3.1. Calibration

The HILIC method proved convenient for the analysis of ropivacaine since when using a protein crash with organic solvent there was no need to remove the organic solvent since trapping in HILIC mode is most efficient with a high concentration of organic solvent in the sample solution [15]. Since in previous studies [15] a ZICHILIC column had proved robust when used in a bionalytical method it was chosen for the current study. Fig. 2 shows the chromatograms obtained for lidocaine and ropivacaine extracted from plasma and dialysate and then analysed the on a ZICHILIC column interfaced to an LTQ Orbitrap mass spectrometer. The method was calibrated over the range 0.01–1.5 μg/ml by using spiked blank plasma. Since the blank was found to be clear of ropivacaine the calibration curve was forced through zero otherwise the intercept had a disproportionate impact on the lower concentration samples. The equation of the line was $y=0.886x \pm 4.2\%$ ($n=2$) and R^2 was 0.997 ± 0.15% ($n=2$). The slope of the curve is close to the ratios of the molecular weight of the lidocaine divided by that of ropivicaine indicating a similar mass spectrometric response for the two compounds. Thus it was decided that monitoring any variations in the slope of the calibration curve could be carried out by including a single point calibration with each batch of samples.

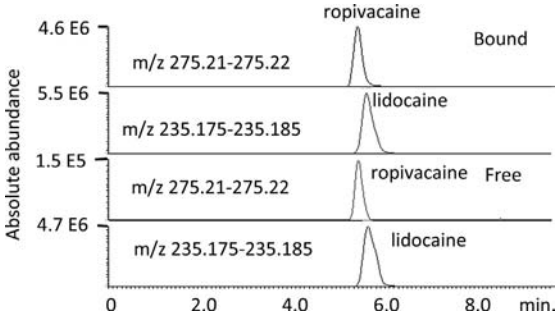

Fig. 2. Extracted ion chromatograms for bound and free ropivacaine and lidocaine internal standard (1 μg/ml).

3.2. Accuracy and precision

The values obtained for the accuracy and precision of the analysis of plasma spiked at 0.03 μg/ml and 1.5 μg/ml were 93.2% ± 2.8% and 95.4% ± 1.5% respectively ($n=5$). Repeat analysis of two patient samples for free and bound drug gave the following values for levels of ropivacaine: Patient 1: bound 1.63 μg/ml ± 1.48%, unbound 0.0671 μg/ml ± 1.68% ($n=5$); patient 2 bound 0.609 μg/ml ± 2.6%, unbound 0.0143 μg/ml ± 6.8% ($n=4$). Thus the microscale equilibrium dialysis process worked with a good degree of precision and was suitable for the analysis of free and bound ropivacaine in plasma samples from patients. The full data for the levels of free and bound drug in patients will be reported in a clinical journal. Having found a good degree of linearity for the full calibration curves the level of drug in each batch of patient samples was determined by using a single point calibration prepared by spiking ropivacaine and lidocaine at 1 μg/ml into blank plasma to run with each batch of patient samples. The mean ratio obtained across 25 such spikings on 25 different days over a period of seven months was 0.939 ± 9.9% ($n=25$) indicating that the variation in slope based on a one point spiking was within acceptable limits.

3.3. Recoveries, ion suppression and robustness

In the recovery test the recovery of 0.5 μg/ml spiking of ropivacaine into plasma was 91.7% ± 8.9% ($n=6$) and for 0.05 μg/ml spiking the recovery was 90.1 ± 3.7% ($n=6$). In the ion suppression test the ratio of the signal for a 0.05 μg/ml spike of ropivacaine into supernatant from a plasma crash compared with acetonitrile was 1.007 ± 5.6% ($n=2$). The robustness of the methodology was tested by spiking ropivacaine into a patient sample already containing ropivacaine in order to further assess ion suppression effects and recovery. In the sample selected the initial levels of drug were 0.389 μg/ml bound and 0.00433 μg/ml (1.12%) unbound. The level of bound drug after spiking at 1 μg/ml was 1.38 μg/ml ± 0.68% ($n=3$) and the level of unbound drug was 0.0263 μg/ml (1.92%) ± 1.6% ($n=3$) thus in this case the plasma proteins offered strong buffering against an increase in the level of free drug.

4. Conclusion

A robust and precise method was developed for the determination of free and bound ropivacaine in plasma. The RED devices proved convenient to use in comparison with a method we had developed previously for the determination of levels of unbound drug [8]. The use of HILIC chromatography allowed direct injection of the sample in high organic solvent without any loss in peak shape as we observed previously [15]. The method has been applied to the analysis of free and bound drug in 20 patients undergoing knee surgery and 20 patients undergoing hip surgery. The results indicate that the levels of free drug remain below levels which are regarded as safe. The clinical data will be reported elsewhere.

The levels of AGP have also been determined in each sample and will be correlated with the observed levels of free drug in the clinical paper.

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References

[1] Lloyd, T. (2012). Techniques for Determining Protein Binding in Drug Discovery and Development. ADME-Enabling Technologies in Drug Design and Development, p. 177.
[2] P. Koivisto, S.K. Bergstrom, K.E. Markides, Journal of Microcolumn Separations 13 (2001) 197.
[3] M. Baniceru, C.V. Manda, S.M. Popescu, J. Pharm. Biomed. Anal. 54 (2011) 1.
[4] K. Sawaki, M. Okubo, T. Shimomiya, E. Tukagoshi, T. Sakai, T. Yamazaki, M. Kenmochi, M. Miyao, Y. Kaneko, T. Ichinohe, M. Kawaguchi, Biomed. Res.-Tokyo 30 (2009) 319.
[5] L. Aarons, B. Sadler, M. Pitsiu, J. Sjovall, J. Henriksson, V. Molnar, Br. J. Anaesth. 107 (2011) 409.
[6] L.L. Bleckner, S. Bina, K.H. Kwon, G. McKnight, A. Dragovich, C.C. Buckenmaier III, Anesth. Analg. 110 (2010) 630.
[7] B. Atcheson, P.J. Taylor, P.I. Pillans, S.E. Tett, Anal. Chim. Acta 492 (2003) 157.
[8] M. Stumpe, N.S. Morton, D.G. Watson, J. Chromatogr. B 748 (2000) 321.
[9] R.E. Curran, C.R.J. Claxton, L. Hutchison, P.J. Harradine, I.J. Martin, P. Littlewood, Drug Metab. Dispos. 39 (2011) 551.
[10] M.J. Zamek-Gliszczynski, K.J. Ruterbories, R.T. Ajamie, E.R. Wickremsinhe, L. Pothuri, M.V.S. Rao, V.N. Basavanakatti, J. Pinjari, V.K. Ramanathan, A.K. Chaudhary, J. Pharm. Sci. 100 (2011) 2498.
[11] S.K. Bergstrom, K.E. Markides, J. Chromatogr. B—Anal. Technol. Biomed. Life Sci. 775 (2002) 79.
[12] O.H. Corso, R.G. Morris, P.J. Hewett, A. Karatassas, Ther. Drug. Monit. 29 (2007) 57.
[13] H.J. Rapp, V. Molnar, S. Austin, S. Krohn, V. Gadeke, J. Motsch, K. Boos, D.G. Williams, U. Gustafsson, G. Huledal, L.E. Larsson, Pediatr. Anesth. 14 (2004) 724.
[14] A. Calder, G.T. Bell, M. Andersson, A.H. Thomson, D.G. Watson, N.S. Morton, Pediatr. Anesth. 22 (2012) 430.
[15] S. Bawazeer, D.G. Watson, C. Knottenbelt, Talanta 88 (2012) 408–411.