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Reversed-phase and chiral high-performance liquid chromatographic assay of bupivacaine and its enantiomers in clinical samples after continuous extraplural infusion

BRIAN J. CLARK* and ABDUL HAMDI
Pharmaceutical Chemistry, School of Pharmacy, University of Bradford, Bradford, BD7 1DP (U.K.)
and
RICHARD G. BERRISFORD, SABARATNAM SABANATHAN and ALAN J. MEARNS
Department of Thoracic Surgery, Bradford Royal Infirmary, Bradford, BD9 6RJ (U.K.)

ABSTRACT

A previously unreported coupled achiral–chiral high-performance liquid chromatographic method has been developed to assay the levels of bupivacaine and its enantiomers in plasma samples, after the local anaesthetic had been given as a continuous extrapleural intercostal nerve block for 4 days, to relieve postoperative pain following thoracotomy. The method has been used to determine maximum, individual enantiomer and steady state levels in conjunction with an assessment of whether accumulation of bupivacaine occurs. An off-line extraction sample preparation is involved before determination of the "total" levels and final sample clean-up on a cyanopropyl silica column prior to "heart cutting" of the bupivacaine peak to a Chiral-AGP column for assay of the enantiomeric ratio. For an initial 5 patient number the mean maximum level was 5.43 μg/ml against a reported toxic level of around 5 μg/ml, which was reached in 72 h and the *S*-enantiomer gave slightly increased concentrations over the *R*-enantiomer for which there is some evidence of higher toxicity.

INTRODUCTION

The local anaesthetic, bupivacaine has a relatively long duration (active up to 8 h) and can be utilised for nerve blockade in extended term surgery. One area where the anaesthetic has proved particularly useful is in thorocotomy. Postoperatively, however, thorocotomy can produce a high incidence of severe pain and pulmonary complications. Patient comfort after this major surgery is regularly carried out by employing systematic opiates. But this form of pain relief is known in some cases to be inadequate [1] and added to this central respiratory depression is problematic. Numerous alternatives have been suggested and these include cryonalagesia, intercostal and epidural nerve blocks.

Recently Sabanathan *et al.* [2] have demonstrated that continuous infusion of bupivacaine over a 24-h period by an indwelling epidural catheter placed extrapleurally at thoracotomy can be beneficial to control pain. Subsequently this study has

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been enlarged and the period of induction of bupivacaine by this mode has been extended to 5 days. As a result decreased pain and improved lung function have been illustrated when comparisons have been made against conventional analgesia [3]. Improved coughing ability and restoration of normal breathing pattern have lead to improved recovery times and early patient mobility.

However, over the 5 day period of infusion, the dosage of bupivacaine is necessarily high, but in observation tests no clinical toxic reactions, which are apparent as myocardial depression and convulsions, were found over a large patient population [4].

Nevertheless it was considered important to establish the maximum levels of bupivacaine in patient samples during and after infusion. In order to carry this out, subsequent to liquid extraction from a plasma biological fluid, a reversed-phase high-performance liquid chromatography (HPLC) method was developed. A number of previous methods have utilised alkyl-bonded phases for determination of bupivacaine levels in serum [5,6]. The assay is however complicated by the presence of a chiral centre in bupivacaine and the reported differences in toxicity of the *R* enantiomer over the *S* enantiomer [7]. In addition the *S* enantiomer is considered to have a longer duration of action which manifests from the differences in absorption into the tissues [8]. The developed HPLC method therefore includes a conventional assay for the total bupivacaine and an resolution on a chiral stationary phase for the enantiomeric ratio, with HPLC column coupling through switching valves.

EXPERIMENTAL

Patient samples

Plasma samples were obtained from 10 consenting adults who were undergoing elective thoracotomy and were subjected to continuous extraplural infusion of bupivacaine over 4 days. The patients had no infection, lung resection, grossly obese or loosing more than 500 ml of blood peroperatively. Infusion rates varied from 5–10 ml/h of a 5-mg/ml bupivacaine solution according to body weight. Samples were taken through an indwelling central venous catheter at 0, 10, 20, 30, 45, 60, 80, 120, 180, 210, 300, 360, 480 and 24, 48, 72, 96, 120 h. They were subject to centrifugation without additives to give the plasma portion which was stored at −40°C prior to analysis.

Sample preparation

An aliquot of plasma (1 ml) was shaken with 2 *M* sodium hydroxide (100 μl) and hexane (7 ml) in a separating funnel for 10 min. From the upper hexane layer 4 ml was transfered to a centrifuge tube (15 ml) and the solvent evaporated under nitrogen. The residue was redissolved in 200 μl of mobile phase (mobile phase 1) and 50 μl was injected onto the cyanopropyl HPLC column.

HPLC equipment and methods

The HPLC instrumentation for the coupled column experiments consisted of two dual reciprocating pumps, a variable-wavelength detector, two high-pressure switching valves and a low-pressure valve (Fig. 1).

For the reversed-phase separation (total bupivacaine) and LDC/Milton Roy

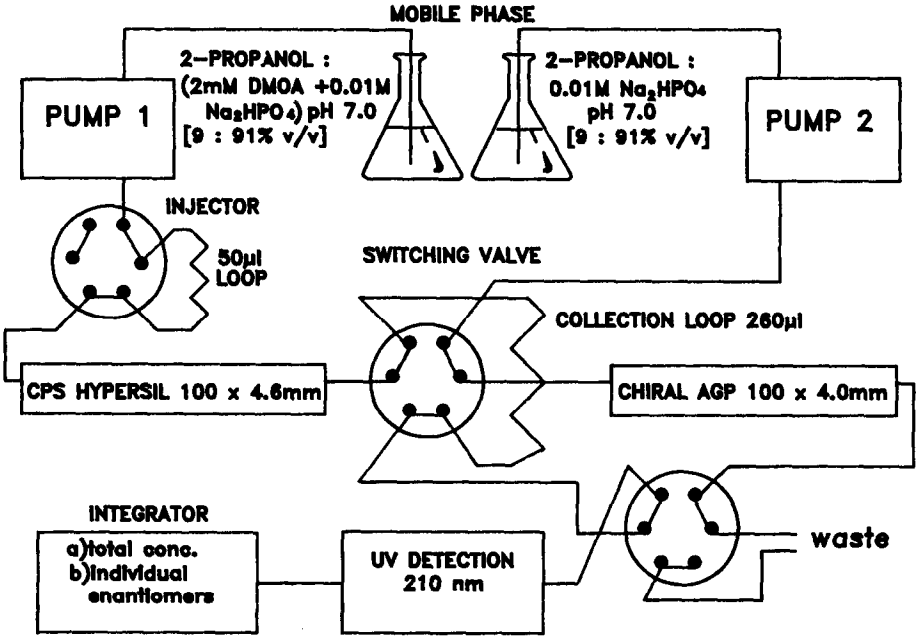

Fig. 1. Configuration of the coupled system, where after initial off-line sample preparation the CPS-column is used for final clean-up and for the determination of the "total" bupivacaine. The chromatogram is then heart-cut to a 260-µl loop for injection onto the Chiral-AGP column in order to obtain the enantiomeric ratios for bupivacaine. Apart from the slight differences in mobile phase compositions between the two columns, there are differences in flow-rate (CPS, 2 ml/min; AGP, 0.9 ml/min).

constametrics 3000 pump was used (LDC/Milton Roy, FL, U.S.A.) with a Rheodyne injection valve fitted with a 50-µl loop (Model 7125, Rheodyne, CA, U.S.A.). The detector for the total levels and individual enantiomeric ratios was an LDC/Milton Roy Spectromonitor 3000 variable-wavelength detector fitted with a 14-µl flow cell. The switching valve between columns and detector was a low-pressure Omnifit Model 1105 valve (Omnifit, Cambridge, U.K.).

The reversed-phase HPLC column was a 100 × 4.6 mm I.D. stainless-steel column packed with 5 µm Cyanopropyl (CPS) Hypersil (Chromex, Cheshire, U.K.). The mobile phase was 2-propanol–0.01 *M* disodium hydrogen orthophosphate + 2 m*M* dimethyl-*n*-octylamine (9:91, v/v) (pH 7.0) (mobile phase 1). The flow-rate was 2 ml/min and detection wavelength 210 nm,.

The pump for the chiral stationary phase (CSP) method was an LKB Model 2150 (LKB Produkter, Bromma, Sweden) fitted with a Rheodyne Model 7000 switching valve and the integrator for both systems was a Hewlett Packard Model HP 3396A (Hewlett Packard, Waldbronn, Germany).

The CSP was a 100 × 4.0 mm I.D. stainless-steel column packed with 5 µm Chiral-AGP (α_1-acid glycoprotein) (ChromTech AB, Norsborg, Sweden). A mobile phase of 2-propanol–0.01 *M* disodium hydrogen orthophosphate (9:91, v/v), (pH 7.0) was used, with a flow-rate of 0.9 ml/min and a detection wavelength of 210 nm.

The mobile phase components: disodium hydrogen orthophosphate was Analar grade and obtained from BDH (BDH, Dorset, U.K.), the dimethyl-*n*-octylamine from Lancaster Synthesis (Lancaster Synthesis, Lancashire, U.K.) and 2-propanol and hexane were obtained as HPLC grade (Rathburn Chemicals, Perthshire, U.K.).

Bupivacaine hydrochloride injection (0.5%) was as a 10-ml ampoule (5 mg/ml) (Astra Pharmaceuticals, Herts, U.K.).

RESULTS AND DISCUSSION

The benefits of column switching in multicolumn HPLC for bioanalysis has been apparent for a number of years [9]. Biological sample preparation, analyte concentration through peak compression and selective component column transfer have all been actively practised. Among the most recent developments in this area are the applications in multidimensional chromatography for chiral drug and metabolite separations in bioanalysis, through the coupling of achiral–chiral columns [10]. Primarily the approach has been to directly inject biological samples for separation and sample clean-up through an initial reversed-phase column [11], then switching the chromatographic peak(s) of interest to the second chiral stationary phase for enantiomeric resolution. This initial step has been successfully illustrated on a number of efficient serum and urine fractionations by Wainer where a Pinkerton internal surface reversed-phase column has been utilised [12].

Although the use of such a column is advantageous, particularly where automation is to be involved, in the development of the bupivacaine HPLC method the cost was considered prohibative when this column's main purpose was in scaveging. Therefore a conventional reversed-phase method was investigated and a number of alkyl-bonded phases were examined. Unfortunately from this work it was not found possible to optimise the packing–mobile phase combination to allow efficient cleanup of the precolumn after every injection and occasional endogenous peak breakthrough occured on random multiple biological sample injections.

As it was intended that the second column would involve a Chiral-AGP packing, which has been shown by Hermansson [13] to be effective in resolving a racemic mixture of bupivacaine enantiomers, care was required in avoiding poisoning of the phase with endogenous material.

As a result an off-line liquid–liquid extraction sample preparation was developed with hexane, which allowed about a 3 times preconcentration, with final clean-up and separation on the achiral column with a 94% (w/w) recovery. For this a column packing of cyanopropyl-bonded silica was found to give both the best separation from the residual proteinatious background and good chromatographic peak shape for bupivacaine as the single "total" level peak. In achieving the optimum separation there was close agreement between the best mobile phase for the achiral and chiral stationary phases. The difference related to the use of ion-pairing mode in the CPS-column, with dimethyl-*n*-octylamine (2 m*M*) and the flow-rate of the mobile phase (CPS, 2.0 ml/min and AGP, 0.9 ml/min). But all other proportions including pH were the same.

The method thus consisted of sample preparation off-line and determination of the "total" bupivacaine levels for the patient samples on the CPS-packing through duplicate injections of 50 μl of prepared sample in mobile phase 1 (Fig. 2). The first

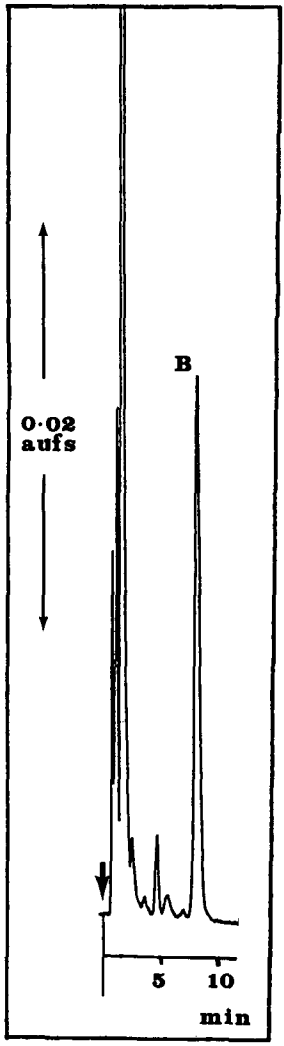

Fig. 2. Determination of the "total" bupivacaine levels on the CPS-Hypersil column, in a 72-h patient sample, after continuous infusion of bupivacaine, (bupivacaine 5.72 μg/ml, 50 μl injected) (for chromatographic conditions, see text).

switching valve allowed the chromatographic profile to be passed directly to the UV detector. Duplicate injections were then carried out (50 μl) where the bupivacaine peak was "heart-cut" from the first separation through manual valve positioning and passed through the switching valve into the 260-μl injection loop. With the manual controlled timing of the Rheodyne switching valve about 50% of the bupivacaine peak was transferred through injection onto the Chiral-AGP column. Baseline resolution of the enantiomorphs was achieved throughout the study with no interference apparent from background peaks as illustrated in Fig. 3 for a 72-h plasma sample.

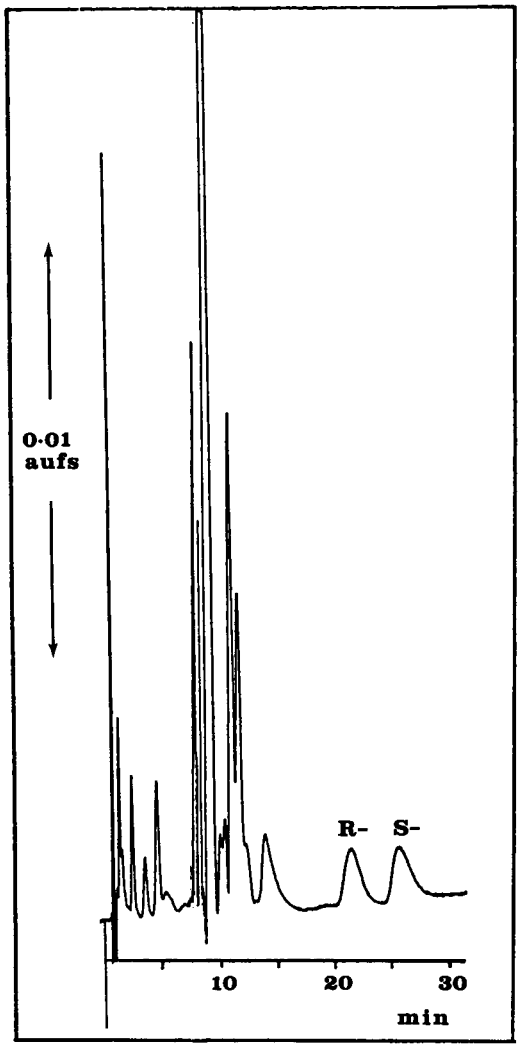

Fig. 3. Determination of the enantiomeric ratios from the chromatogram in Fig. 2 by heart cutting and transfering about 50% (v/v) of the bupivacaine peak to the 260-μl loop for injection and resolution of the *R* and *S* enantiomers on the Chiral-AGP column. The ratios are *R*–*S*, 45.7:54.3% (w/w) (for chromatographic conditions: see text).

Baseline disturbance is however observed in the chromatographic profile at 7–13 min which is considered to be caused by a combination of the 260-μl volume of mobile phase 1 containing dimethyl-*n*-octylamine present on the column and pressure differences associated with injection of this volume. Fortunately these disturbance peaks do not interfere with the resolution of the *R* and *S* enantiomers.

On the CPS column linearity of chromatographic response was determined over two concentration ranges for aqueous bupivacaine standards, as at the outset the

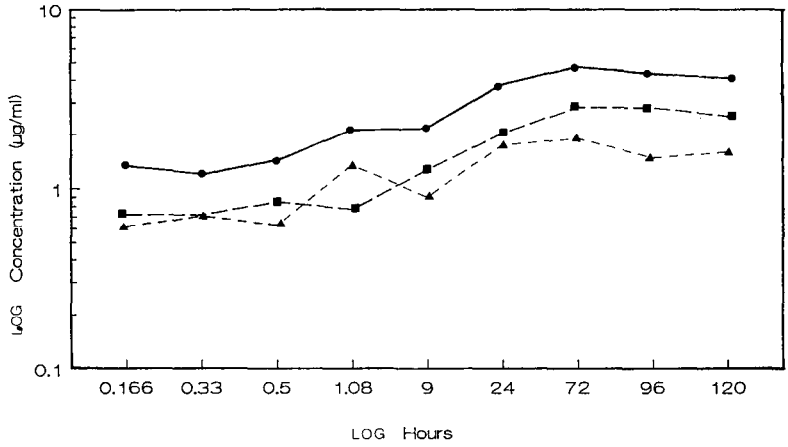

Fig. 4. The plasma concentration against time curve for a 10-patient mean over a 120-h period with a 96-h continuous infusion of bupivacaine. The "total" level (●) was determined on the CPS-Hypersil column and is indicated by where the maximum value (48 h) is at 5.43 µg/ml of bupivacaine. The mean value of the *R* and *S* enantiomers which were resolved on the AGP-column after heart-cutting from the reversed-phase CPS-column, illustrates the slight increase in the *S* enantiomer (■) over the *R* enantiomer (▲) from the original racemic pharmaceutical preparation (for chromatographic conditions, see text).

maximum levels of bupivacaine that the patient samples would reach was not apparent. However, as indicated no clinically toxic effects were shown in the patients and it was therefore expected that the bupivacaine levels would lie around the suggested toxic point of 5 µg/ml [7]. For the concentration range of 1–20 µg/ml of aqueous standards the regression equation was $y = 8.14x + 1.06$ ($R = 0.999$, $n = 12$). Repeatability (R.S.D.) for a standard at 10 µg/ml was 0.58% ($n = 8$). With overlapping higher concentration range 5.02–50.2 µg/ml; again linearity was given and the equation was $y = 2.72x + 1.27$ ($R = 0.999$, $n = 7$). In addition to aqueous standards, spiked standards were run from 5 to 40 µg/ml (spiked in pooled serum). The liquid–liquid extraction was completed as above and the regression equation was $y = 56.34x + 14.29$ ($R = 0.996$, $n = 8$). For the AGP-column linearity was achieved for the *R* isomer with $y = 0.45x + 0.22$ ($R = 0.998$, $n = 5$) and for the *S* isomer, $y = 0.35x + 0.17$ ($R = 0.976$, $n = 5$) for the range 2.5–25 µg/ml. In these assays, for the CPS-column the limit of detection (measured as twice baseline noise) was 13 ng on column for a 50-µl injection.

On the patient samples the plasma concentrations against time curve for a mean of 10 patient samples is shown on Fig. 4. The time to peak plasma concentration was 72 h and the level was 5.43 µg/ml with steady state being reached. There were no suggestions of accumulation of bupivacaine over the 96-h of infusion. After this period there is a slight fall in the mean plasma levels which indicates acceptable clearance from the body. The enantiomeric ratios suggest no significant difference between the enantiomers over the term of study, although the *S* enantiomer is present at a slightly higher level than the *R* enantiomer after 50 h, which is important in view of the reported increase in toxicity from the *R* enantiomer [7]. From these results it is possible to calculate the requisite pharmacokinetic profiles of apparent volume of

distribution, terminal half life and area under the plasma concentration time curve. This will be presented along with full patient data (10-patient sample) in a future report.

CONCLUSIONS

The continuous infusion of bupivacaine has been shown to have considerable benefits for therapy in chronic pain, without the effects of noticable toxic side effects. The analytical results obtained here, compliment these observations and indicate that accumulation of bupivacaine in the body is not problematic. The method developed is easily carried out, although more automation would have been useful in reducing the analyst involvement in sample preparation and switching valve operation. These points however may be considered in future studies of neonate samples and in adult samples through drug combination studies.

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