BIOMEDICAL CHROMATOGRAPHY, VOL. 10, 131–134 (1996)

Rapid and Specific Precolumn Extraction High-Performance Liquid Chromatographic Assay for Bupivacaine in Human Serum

Samy Emara, Alaa Khedr and Hassan Askal*

Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, Assiut University, Assiut, Egypt

A rapid and specific HPLC assay for quantitative determination of bupivacaine in human serum is described. The technique incorporates an on-line sample clean-up system followed by reversed-phase chromatography with UV detection. The proposed method uses a column-switching technique and protein-coated Lichrosorb RP-8 as a precolumn together with Lichrosorb RP-18 as an analytical column. The total run time for an injection of serum sample was 10 min. This procedure offers a sensitive assay without the need for time-consuming extractions. The average bupivacaine recoveries over a concentration range of 150–600 ng/mL ranged from 99.12 to 101.02%, and relative standard deviations ranged from 1.15 to 1.78%.

INTRODUCTION

Bupivacaine is a potent long acting local anaesthetic which is a valuable with or without epinephrine. A number of high-performance liquid chromatographic (HPLC) assays have been developed to monitor serum level of bupivacaine in patients (Lindberg *et al.*, 1986; Sullivan and Weiner, 1987; Clark *et al.*, 1991; Kastrissios *et al.*, 1992 and Chen, 1993). Determination of bupivacaine in serum has frequently been associated with extraction of the drug with a non-polar organic solvents (Louis *et al.*, 1991; Le Geuvello *et al.*, 1993; Murillo *et al.*, 1993 and Wang, 1994). Generally, sample clean-up by solvent extraction is tedious, slow, less precise and difficult to automate. Moreover, an internal standard must be used to obtain good quantitative measurements.

The importance of a high selectivity in the chromatographic system in combination with high resolution efficiency in the separating column is very noticeable when separating drugs from biological fluids. Also, precision and accuracy are the most important criteria for evaluating an analytical method. In addition, the time required to perform the analytical procedure cannot be neglected because time depends so strongly on analytical conditions, especially in chromatographic methodology. Therefore, our study was involved in a research effort aimed at developing and improving a direct injection chromatographic procedure and the application of such a technique for the separation and quantitation of bupivacaine in human serum. For this purpose, a column switching technique and protein-coated precolumns were used. The preparation of such precolumns is simple and economic and reduces the time and cost of renewal. It is essential that the precolumn has sufficient capacity and that it makes only a small contribution to band broadening.

EXPERIMENTAL

Apparatus. The HPLC system (Fig. 1) consisted of two pumps (Model CCPD for pump A, and Model HLC-803D for pump B, Toyo Soda, Japan). A model 7125 sample injection valve (VI) and a model 7010 flow direction-switching valve (VII) were applied (Rheodyne, Berkeley, CA, USA). For stepwise elution, solvent switching was performed by a solvent selector, model 8 V (Kyowa Seimitus, Tokyo, Japan), controlled by a sequence programmer, model SCY-POC Omron (Tokyo, Japan). This system was equipped with two columns: one was a short protein-coated Lichrosorb RP-8 or μBondapak CN silica (20×4.6 mm i.d.) for deproteinization and also for trapping of bupivacaine. The protein-coated columns were prepared manually in our laboratory (Sorbent, E. Merck). The other was an analytical column of Lichrosorb RP-18 silica (250×4.6 mm i.d.) (E. Merck, Darmstad, Germany). The flow cell detector used for UV spectrophotometry was a high speed liquid chromatograph UV detector, UV-8 model II spectrophotometer (Toyo Soda) operated at 245 nm. Peak heights were computed on a Shimadzu (Kyoto, Japan) C-R 3A chromatopac integrator. HPLC was operated at room temperature.

Reagents and materials. Bupivacaine HCl was kindly supplied by Astra Pharmaceuticals Pty. Ltd., Sydney, Australia. Methanol and acetonitrile were HPLC grade. All other chemicals and reagents used were of analytical grade.

Stock solution of bupivacaine HCl (400 μg/mL) was prepared by dissolving an accurately weighed amount of the drug in 100 mL distilled water. Aqueous calibration solutions of bupivacaine were prepared by dilution of stock solution to give a final concentration range of 2–20 μg/mL (at 2 μg/mL intervals). The stock and standard solutions were stored at 4°C until use. Spiked serum samples for calibration curve were prepared daily by diluting the appropriate volume of standard solutions of bupivacaine with free serum in a ratio of 1:9 to produce a concentration range of 200–2000 ng/mL (at 200 ng/mL interval). Spiked serum samples were stored at −20°C.

Washout of polar constituents by the protein-coated column. Two elution experiments were run through the protein-coated RP-8 precolumn, one using phosphate buffer saline, pH 7.4 (mobile phase I) and the other using weak mobile phase of 20% methanol in 0.1 M phosphate buffer, pH 5.5 (mobile phase II).

Mobile phase I was used to deliver sample to the extraction protein-coated column, clean-up and enrichment of analyte, while mobile phase II was introduced as a second washing step to provide low interference background signals. The protein-coated

*Author to whom correspondence should be addressed.

CCC 0269-3879/96/030131-04

Received 1 December 1995
Accepted 17 January 1996

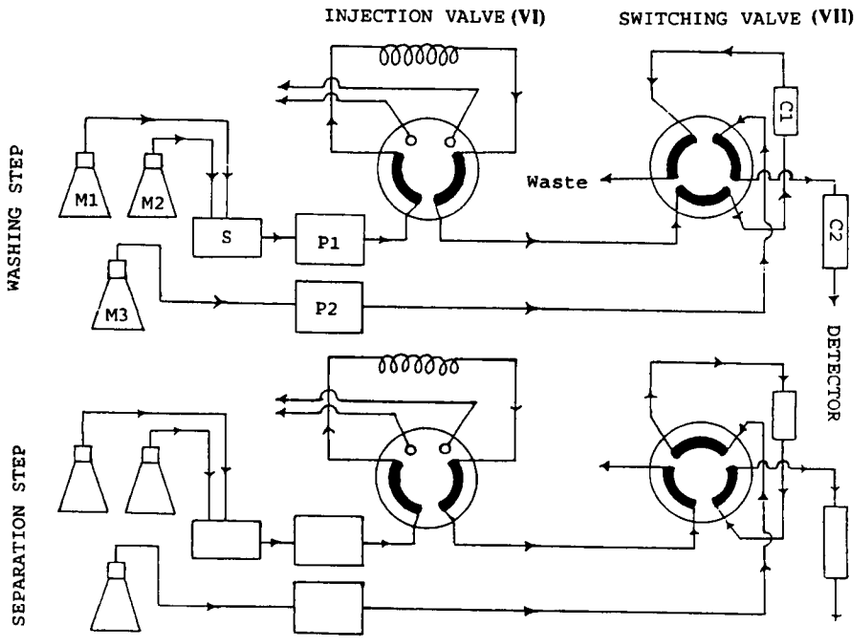

Figure 1. Schematic diagram of the column-switching system. M1, M2, M3, mobile phase I, II and III; C1, precolumn; C2, analytical column; P1, P2, pump A and B; S, solvent selector.

precolumn, into which the serum samples were injected, was equilibrated with the mobile phase I for 4 min. To drain out serum proteins and hydrophilic compounds, the precolumn was washed with the same solvent for 2 min at a flow-rate of 1 mL/min by pump A. For an additional 2 min, the precolumn was washed with the mobile phase II at a flow-rate of 1 mL/min by the same pump. The mobile phases were prepared daily and were filtered and degassed before use.

Operation of the column-switching chromatographic system. In injection mode, human serumn (100 μL) was injected without any sample preparation directly onto the protein-coated precolumn (extraction column), where bupivacaine was retained and unwanted components passed into waste in mobile phase I. Two minutes after sample injection, a second washing step was introduced using mobile phase II to provide extremely low interference background signals. The switching valve was then automatically rotated to the elution mode. The rotation allowed the analytical mobile phase of 30% methanol, 30% acetonitrile and 40% phosphate buffer, together with 0.3% ethylamine (pH 6.4) (mobile phase III) to flow through the protein-coated precolumn and elute the loaded content (bupivacaine and other polar constituents of human serum) onto the analytical column, at a flow-rate of 1 mL/min by pump B, for further separation and quantitation. The elution mode lasted for 2 min to ensure complete elution of the drug. Thereafter, the switching valve was rotated back to the injection mode where the protein-coated precolumn was reequilibrated with the mobile phase I for 4 min while bupivacaine underwent chromatographic separation and quantitation. The total run time for an injection of serum sample was 10 min.

RESULTS AND DISCUSSION

Adsorbability of serum proteins on the protein-coated RP-8 and CN Columns

The use of BondElut C18 - Silica, and cyano extraction columns were previously described by Gupta and Dauphin (1994) and Groen *et al.* (1994), respectively, for precolumn extraction of bupivacaine from serum. The preliminary results indicated that the coated (immobilized) proteins were mainly adsorbed on the outside surface of porous silica and the inside surface was not coated with proteins owing to the molecular sieve effect of small pores. In addition the capacity factors for small molecules or the pore volumes of the silica were not affected so much by protein coating because, in the case of silica, of small pores, almost all the surface area being on the inside surface. The deproteinization in the protein-coated column could be considered as size-exclusion due to the small pores of packing materials. Serum proteins could not be adsorbed further on the external surface of the protein-coated column, and they could not enter into the interior of the small pores, thus they flowed out of the extraction column. However, the small pores still retained adsorptivity for small molecules. Hydrophobic small molecules could be adsorbed on the internal surface of small pores, and eluted by an appropriate eluent containing organic solvents. Therefore, the pore diameter of packing materials is of primary importance in protein-coated columns. Packing materials of wide pore diameter should tend to adsorb more proteins and to lose their surface area and pore volume. In other words, protein-coated columns prepared from small pores had similar pore structure to that of native packing substances. Protein-coated columns (typical pore size 10–20 μm) had reversed-phase characteristics for small hydrophobic molecules and lost adsorbability for serum proteins.

Breakthrough study

A breakthrough study was performed on the protein-coated precolumn for verification that bupivacaine was quantitatively retained on the precolumns from the volume of mobile phase I and II. It was found that the peak-height obtained upon elution of a fixed quantity of the tested compound was independent of the volume of mobile phase I passed through the precolumn up to at least 30 mL. Thus, it might be concluded that, in mobile phase I, bupivacaine

was strongly retained in protein-coated precolumns.

The loading time was found to have a significant influence upon recovery when the second washing step was introduced. Elution of bupivacaine from protein-coated CN columns is too fast and, consequently, a large proportion of the analyte will break through the outlet of the precolumn upon using 20% methanol. On the other hand, such problems did not appear with protein-coated RP-8 column, since the breakthrough volume of bupivacaine was determined as not less than 8 min. Therefore, the practical application of CN-bonded silica was limited under these conditions.

Protein binding

The effect of protein binding was studied by comparing the recovery of bupivacaine after injection of spiked phosphate and spiked serum. The recovery was identical, indicating that bupivacaine has a higher affinity for the packing material of protein-coated columns than for serum proteins. Thus it is noteworthy that the total amount of bupivacaine in serum was recovered, whether it was free or bound to serum proteins.

Protein-coated column life time

The protein-coated column lifetime in terms of being able to clean-up trapped samples was investigated as a function of the volumes of spiked serum injected onto the column. It was found that the protein-coated column could be used successfully for loading at least 300 times with 100 μL injected volume of serum samples. Increase of the injected volumes above this level led to gradual pressure build up at the head of the protein-coated column and ultimately to clogging. Accordingly, protein-coated columns should be routinely changed when excessive back pressure is seen.

Calibration curve

A standard curve from HPLC of directly injected spiked serum was constructed by plotting the measured peak-height against concentration of the drug. A linear relation between peak-height and concentration existed over the range of concentration examined (200–2000 ng/mL). The equation for the best-fit straight line was determined by the linear regression analysis $Y=0.4266+0.0604X$ ($r=0.9998$).

Recovery

Recovery was calculated over a concentration range of 150–600 ng/mL. Spiked serum and aqueous standard solutions of the same known bupivacaine concentration were prepared and injected onto the column. Comparison of peak-heights from spiked serum with those from the standard solutions was applied for calculating the analytical recovery. It was found that the recoveries were almost quantitative upon using protein-coated RP-8 silica column (Table 1). Quantitative reproducibility by the present method may be due to the fact that it was carried out without solvent extraction or without precipitation of serum proteins, in contrast to the classical deproteinization procedures.

Accuracy and variability

Ten spiked serum samples of an identical bupivacaine concentration of 300 ng/mL were analysed on five consecutive days. The coefficient of variation within the series of ten samples (within-day) was 1.42%. The day-to-day coefficient of variation was 2.13%.

Table 1. Recovery for bupivacaine from HPLC of direct injection spiked human serum at different concentrations

Spiked concentration (ng/mL)	Concentration found (ng/mL)	Recovery (%)* (±SD)	CV (%)
150	148.68	99.12 (1.78)	1.79
300	302.55	100.85 (1.39)	1.37
450	454.59	101.02 (1.67)	1.65
600	597.24	99.54 (1.15)	1.15

*Average of five determinations.

Interferences

Several samples of drug-free serum were checked for potential endogenous interferences. In addition, several drugs such as epinephrine, lignocaine, codeine, morphine and pethidine, which could be co-administered with bupivacaine, were examined for interference with the assay procedure by injecting their aqueous solutions onto the column and comparing the retention times with that of bupivacaine. The results obtained indicated that there was no interference from the endogenous components which co-eluted with bupivacaine or from the drugs examined.

Analytical application. The procedure is fast and sufficiently sensitive for the pharmacokinetics of bupivacaine to be followed for at least 8 h after drug administration. Figure 2

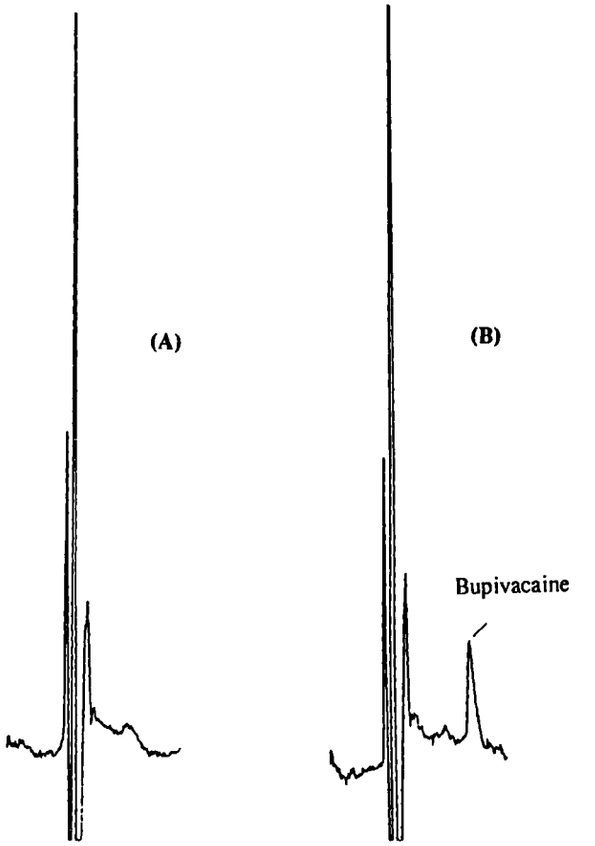

Figure 2. Chromatographic profiles of (A) blank serum before bupivacaine administration and (B) serum 60 min after administration of 20 mL (0.5%) bupivacaine (concentration of bupivacaine found was 0.61 μg/mL).

shows the chromatograms obtained from (A) blank serum and (B) from serum 60 min after administration of bupivacaine. Neither desbutylbupivacaine nor 4-hydroxybupivacaine, major metabolites of the parent drug in serum, were detected 60 min after administration.

The limit of detection was determined as the concentration the signal of which exceeded about triple the base line noise. The smallest detectable concentration of bupivacaine was 70 ng/mL. Yu *et al.* (1994) described a method for determination of bupivacaine by direct injection of plasma in a column with a switching HPLC system using a precolumn with a semipermeable surface (SPS). In this method, the SPS precolumn lifetime exceeded 200 direct injections of plasma sample. The method has detection limit of 8.2 ng and requires a total assay time of 15 min per plasma sample with 1.6–5.2% coefficient of variance of inter-day precisions.

In conclusion, the present method is a simple and accurate way to determine bupivacaine in serum under the usual therapeutic drug concentration levels without the usual sample preparation step. The recovery and reproducibility of the present method were superior to those of extraction procedures in view of its accuracy and simplicity. Since the recovery of the drug was close to 100%, the internal standard could be eliminated and this feature was one of the most important advantages over the classical HPLC methods.

REFERENCES

Chen, X. Q., Chen, K., Hu, W. S. and Yun, W. (1993). *Sepu* **11**, 371 (in Chinese), *Anal. Abstr.* **56**, 6G141.

Clark, P. G., Hamdi, A., Berrisford, R. G., Sabanathan, S. and Mearns, A. J. (1991). *J. Chromatogr.* **553**, 383.

Groen, K, Zeijlmans, P. W., Burm, A. G. and Van Kleef, J. W. (1994). *J. Chromatogr. Biomed. Appl.* **655**, 163.

Gupta, R. N. and Dauphin, A. (1994). *J. Chromatogr. Biomed. Appl.* **658**, 113.

Kastrissios, H., Hung, M.-F. and Triggs, E. J. (1992). *J. Chromatogr.* **577**, 103.

Le Geuvello, P., Le Corre, P., Chevanne, F. and Le Verge, R. (1993). *J. Chromatogr.* **622**, 284.

Lindberg, R. L. P., Kanto, J. H. and Pihlagamati, K. K. (1986). *J. Chromatogr.* **383**, 357.

Louis, P. S., Rybczynski, J., Das, I., Bissonnette, B. and Hartley, E. J. (1991). *Clin. Biomed.* **553**, 383.

Murillo, I., Costa, J. and Salva, P. (1993). *J. Liq. Chromatogr.* **16**, 350.

Sullivan, N. W. and Weiner, L. (1987). *J. Chromatogr.* **417**, 236.

Wang, X. (1994). *Fenxi Huaxue* **22**, 644 (in Chinese). *Chem. Abstr.* **121**, 98964G.

Yu, Z., Abdel Rehim, M. and Westerlund, D. (1994). *J. Chromatogr. Biomed. Appl.* **654**, 221.