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On-line dialysis and weak cation-exchange enrichment of dialysate

Automated high-performance liquid chromatography of pholcodine in human plasma and whole blood

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ABSTRACT

An automated method for the determination of pholcodine in plasma and whole blood is described. The technique combines dialysis and trace enrichment prior to high-performance liquid chromatography. Dialysis, trace enrichment on a weak cation-exchange column, separation on a cyano column and fluorescence detection was shown to be an extremely selective and sensitive method. The method has been used successfully in the analysis of real samples after administration of pholcodine. The automated method can be used, after minor modification, to determine other basic drugs in whole blood and plasma.

INTRODUCTION

The opiate derivative pholcodine (3-O-morpholinoethylmorphine) (Fig. 1) is widely used as an antitussive in paediatric medicine and it has been reported to cross-react when biological samples are screened for opiate abuse by immunological methods [1]. The use of non-specific immunological methods has introduced the need for sensitive, specific and simple confirmation methods. We have recently reported two different chromatographic methods for the determination of pholcodine and metabolites in urine using capillary gas chromatography (GC) and column-switching high-performance liquid chromatography (HPLC) [2,3]. Only one chromatographic method has been reported for the specific determination of pholcodine in plasma [4]. The method was based on HPLC and included time-consuming liquid–liquid extraction.

There have been no reports on the specific determination of pholcodine in whole blood. By using dialysis as a purification step prior to HPLC, whole blood and plasma analysis is considerably simplified and fully automated methods can be developed. Only low-molecular-mass compounds

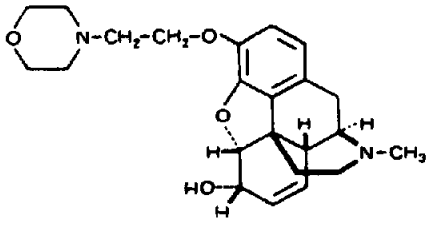

Fig. 1. Structure of the antitussive opiate pholcodine.

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diffuse through the dialysis membrane and the analytes collected in the dialysate are concentrated on a trace enrichment column. The trace enrichment column is connected to the HPLC unit with a column-switching system. This is the principle of the fully automated ASTED (automated sequential trace enrichment of dialysates) system. The purpose of this study was to develop an automated method for the determination of pholcodine in human plasma and whole blood based on the ASTED system for sample preparation. The method was to be used in analysis of real samples after administration of pholcodine.

EXPERIMENTAL

Chemicals

Pholcodine was obtained from Weiders Farmasøytiske (Oslo, Norway). HPLC-grade acetonitrile was supplied from May and Baker (Dagenham, UK). Triton X-100, orthophosphoric acid (H_3PO_4) and sodium acetate, all of analytical-reagent grade, were purchased from Merck (Darmstadt, Germany). Sodium azide of AnalaR grade was obtained from BDH (Poole, UK). HPLC-grade water was obtained from a Milli-Q water purification system (Millipore, MA, USA).

Preparation of standards

Stock standard solutions (0.2 μmol/ml) of pholcodine were prepared in distilled water. Working standard solutions of pholcodine (0.10–2.0 nmol/ml) in 0.02 *M* acetate buffer were prepared from the stock standard solution. Plasma was prepared by centrifuging citrated whole blood for 15 min at 1920 *g*. Spiked plasma and whole blood samples (0.10–2.0 nmol/ml) were prepared from the standard solution and kept at −24°C until analysis.

ASTED method

The sample preparation system shown in Fig. 2 was a Gilson ASTED unit (Gilson Medical Electronics, Villiers-le Bel, France) consisting of a Model 231 auto-sampling injector, two Model 401 diluters equipped with 1-ml syringes and a flat-bed dialyser with a donor channel volume of 375 μl and a recipient channel volume of 650 μl, fitted with a cuprophane membrane of molecular mass cut-off 15 kilodalton. A Model 7010 automated six-port valve (Rheodyne, Berkeley, CA, USA) connected a trace enrichment column either with the recipient channel of the dialyser or with the mobile phase used in the analytical column of the HPLC system. A Shimadzu (Kyoto,

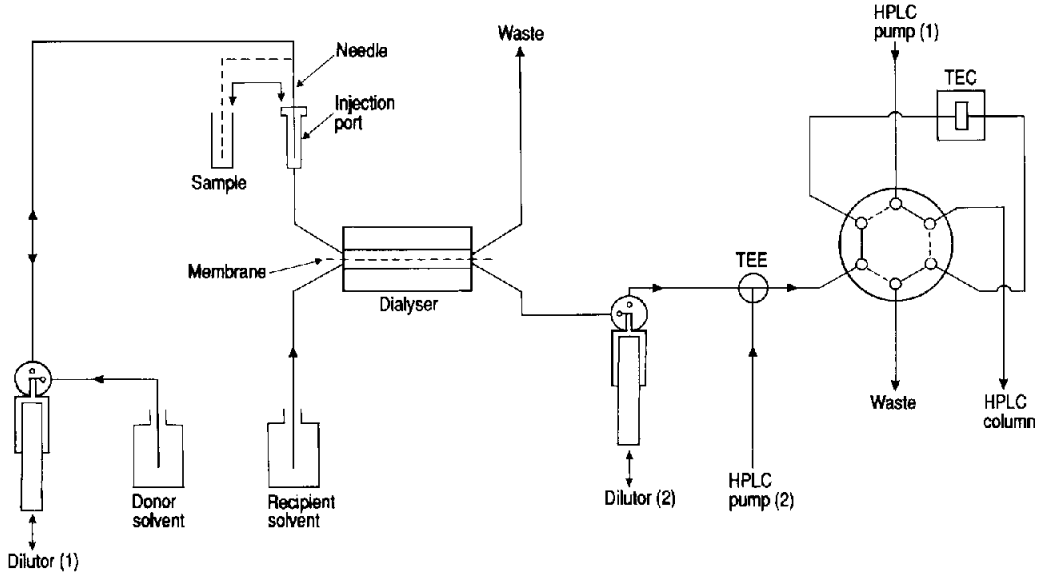

Fig. 2. Schematic diagram of the automated ASTED system.

Japan) Model LC-9A external solvent delivery pump was used to wash the trace enrichment column. This pump was programmable and able to communicate with the ASTED system. A tee-piece was coupled between the diluter (2) and the six-port valve, allowing the washing solvent to go to waste after the washing procedure. The two trace enrichment columns (TEC), each 10 mm × 2 mm I.D. from Chrompack (Middelburg, Netherlands), were connected in series and packed with Bond Elut 40-μm weak cation-exchange (CBA) material from Analytichem International (Harbor City, CA, USA).

High-performance liquid chromatography

The ASTED was coupled to the HPLC system via the six-port valve as shown in Fig. 2. The HPLC pump was a Model LC 6A (Shimadzu). The fluorescence detector (Model RF 535, Shimadzu) was operated with high sensitivity at an excitation wavelength of 280 nm and an emission wavelength of 345 nm monitoring pholcodine. The optimum excitation and emission wavelengths for monitoring pholcodine were determined with a Perkin-Elmer (Norwalk, CT, USA) LS 50 luminescence spectrometer. Signals from the detector were recorded on a Chromatopac C-R4A integrator (Shimadzu). The analytical cyano column (100 mm × 4.6 mm I.D.), Brownlee Columns, Spheri 5, was packed with 5-μm particles. The mobile phase was acetonitrile–0.005 *M* H_3PO_4 (20:80, v/v) (pH 2.5) delivered at a flow-rate of 1.0 ml/min. The New Guard cyano precolumn (15 mm × 3.2 mm I.D.), Brownlee Columns, Spheri 7, was packed with 7-μm particles.

ASTED procedure

Sample loading. One diluter was used to inject plasma or whole blood into the donor channel of the dialyser cell, with the six-port valve in the load position. The sample was held static in the donor channel.

Dialysis, trace enrichment and washing procedure. The sample was held static in the donor channel while the other diluter pulsed 2.6 ml of 0.002 *M* sodium acetate buffer solution (pH 6.0) through the recipient channel of the dialyser and into the trace enrichment column, with the injection valve still in the load position. The recipient solution was transported through the dialyser in four pulses of 650 μl, corresponding to an average flow-rate of 0.32 ml/min. The total dialysis time was 8 min 15 s. After trace enrichment the column was washed with 1.5 ml of water–acetonitrile (50:50, v/v) at 0.5 ml/min with the HPLC pump (see Fig. 2, pump 2).

Injection and purging. On switching the six-port valve to the inject position, the analytes on the trace enrichment column were fore-flushed into the analytical column by elution for 4 min with the HPLC mobile phase. The recipient side of the dialyser was washed with 4 ml of 0.002 *M* sodium acetate buffer solution (pH 6.0) (flow-rate 6 ml/min). The donor side of the dialyser was simultaneously washed with 4 ml of 0.02 *M* sodium acetate solution containing 100 mg/l Triton X-100 and 0.05 g/l sodium azide (flow-rate 6 ml/min).

Regeneration. After 4 min, the six-port valve was switched back to the load position to bring the trace enrichment column back to the recipient channel of the dialyser. After regeneration of the trace enrichment column with 2 ml of 0.002 *M* sodium acetate buffer (pH 6.0) (flow-rate 0.32 ml/min), the next sample was injected into the dialyser. Automated injections were performed every 20 min.

Validation of the procedure

The calibration graphs, accuracy and precision of the method were evaluated by analysis of manually spiked plasma and whole blood samples. The calibration graphs were based on external calibration standards, measuring peak height *versus* concentration in plasma or whole blood.

Analysis of real samples

Three healthy volunteers received single oral doses of 100 and 400 mg of pholcodine and blood samples were collected 4, 8, 12, 24, 48 and 72 h after administration. Plasma samples were prepared as described above. The plasma and whole blood samples were kept at −24°C until analysis.

RESULTS AND DISCUSSION

Trace enrichment

During method development, several packing materials were investigated for trace enrichment. The reversed-phase materials polystyrene–divinylbenzene and silica-based C_{18}, C_8 and C_2 gave either unsatisfactory clean-up or poor recovery. However, as pholcodine is a basic compound it can be retained on a cation-exchange column used in combination with a recipient solution having a low ionic strength at a pH where the isolates and the cation exchanger are charged. A silica-based weak cation exchanger (CBA) was chosen as the packing material in the trace enrichment column because desorption of basic drugs from a weak cation exchanger is easily achieved by using an HPLC mobile phase which is sufficiently acidic (pH <3) to protonate the cation exchanger. In this study 0.002 *M* acetate buffer (pH 6.0) was selected as the recipient solvent and flushing eluent.

Different lengths of the trace enrichment column (10, 20 and 30 mm × 2 mm I.D.) packed with CBA were tried. In the ASTED system a syringe pump is used to bring the dialysate into the trace enrichment column. This syringe pump can only operate with trace enrichment columns having a low back-pressure (below 1.2 p.s.i.). The 30-mm trace enrichment column showed too high a back-pressure to be operated in the ASTED system. The 10-mm column had insufficient capacity, which resulted in breakthrough of pholcodine. The 20-mm trace enrichment column gave complete retention of pholcodine and no problems with back-pressure.

An extra washing step of the trace enrichment column with 1.5 ml of acetonitrile–water (50:50, v/v) was necessary before column switching and elution of the enriched drug by the HPLC mobile phase. Without this washing step both the trace enrichment column and the analytical column were seriously contaminated and clogging of the HPLC system occurred after only a few injections. The cut-off value of the dialysis membrane (15 kilodalton) was too large to avoid clogging of both the trace enrichment and the analytical column without a proper washing procedure. Acetonitrile–water removes impurities that are retained by the CBA material by secondary interactions and replaces acetate buffer in the trace enrichment column before column switching. Column switching with acetate buffer in the trace enrichment column will give rise to a solvent effect which disturbs the HPLC determination of pholcodine.

The chromatograms of drug-free whole blood without and with washing of the trace enrichment column with acetonitrile–water shown in Fig. 3 demonstrate that the washing step also removed impurities which may co-elute with pholcodine. No breakthrough of pholcodine was observed when up to 10 ml of acetonitrile water were pumped through the trace enrichment column. The trace enrichment column was repacked after 150 analyses with the CBA material being in-line with the acidic mobile phase for 4 min per

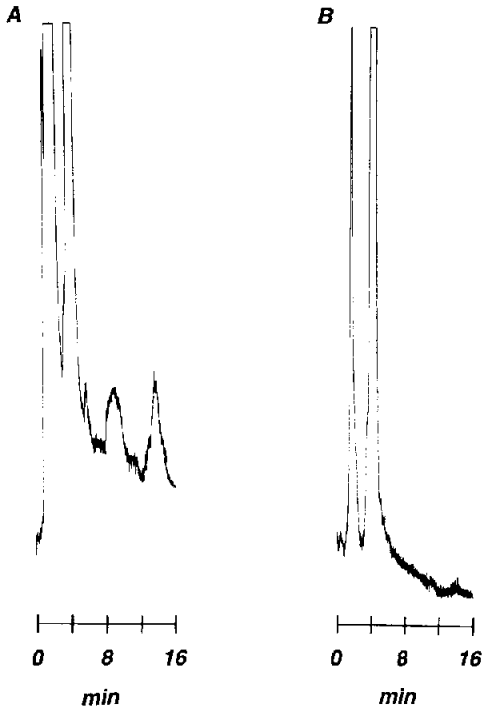

Fig. 3. Chromatograms of a drug-free whole blood sample (A) without washing of the trace enrichment column and (B) with washing of the trace enrichment column before column switching.

analysis. Degradation of the CBA material was observed after 200 analyses.

The use of a weak cation exchanger and washing with acetonitrile–water resulted in a very selective and robust clean-up procedure with no endogenous substances being detected from whole blood or plasma after elution of the solvent front.

Dialysis

Pholcodine was found to adsorb strongly to the cellulose acetate membrane. This was shown by soaking a cuprophane membrane (52 cm^2) in 9 ml of aqueous solution containing 1 nmol/ml pholcodine. After 10 min only one third of the pholcodine remained in solution, the remainder being adsorbed on the membrane. The strong adsorption of pholcodine caused problems during the dialysis process and serious carry-over effects between analysis were observed. A water sample analysed after a standard solution of 1 nmol/ml pholcodine was contaminated with 30% of the pholcodine concentration. Between these injections the donor channel of the dialyser was washed with water and the recipient channel with 0.002 *M* acetate buffer (pH 6.0) according to the experimental procedure. Dialysis of pholcodine in acetate buffer solutions (pH 6.0) of increasing molarity diminished the adsorption on the membrane and increased the peak heights of pholcodine, as shown in Fig. 4. The peak heights were almost doubled in acetate buffer solutions stronger than 0.025 *M* as compared with water. These results show that acetate buffer greatly reduces the binding of pholcodine to the membrane. Acetate buffer was therefore tried as solvent for washing of the donor side of the dialyser between analyses. Washing of the donor side with 0.02 *M* acetate buffer (pH 6.0) between injections completely washed out adsorbed pholcodine from the membrane. In biological samples, however, the situation was more complex. Addition of acetate buffer to plasma and whole blood samples reduced the peak heights of pholcodine due to dilution. Biological samples were therefore dialysed without addition of acetate buffer and the donor channel of the dialyser was washed with 0.02 *M* acetate buffer (pH 6.0) containing Triton X-100 between each injection while the acceptor side of the dialyser and the trace enrichment column was washed and regenerated with 0.002 *M* acetate buffer (pH 6.0). This procedure ensured no carry-over effect between analyses. The detergent Triton X-100 was added to reduce surface tension and ensure reproducible results.

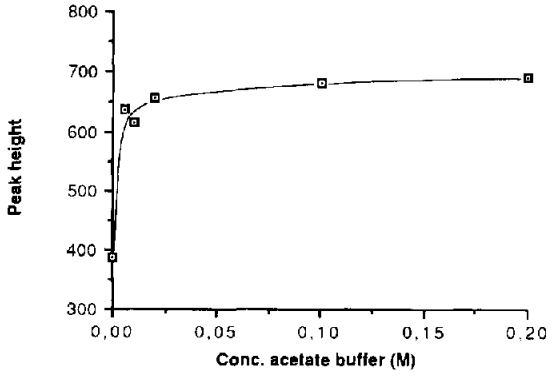

Fig. 4. Peak heights of pholcodine *versus* added molarity of acetate buffer prior to dialysis.

In a fixed dialysis cell the dialysis efficiency is influenced by the dialysis time and the concentration gradient across the membrane. By maintaining a steep concentration gradient across the membrane such as in static-pulsed dialysis or static-continuous dialysis, high dialysis efficiencies are obtained in a short time [5,6]. In this study the sample was held static in the donor channel of the dialyser while 2.6 ml of 0.002 *M* acetate buffer (pH 6.0) were delivered in pulses of 650 μl through the recipient channel. No breakthrough of pholcodine was observed when up to 10 ml of 0.002 *M* acetate buffer were pumped through. The recovery of pholcodine from plasma and whole blood was about 60% when the sample was dialysed for 8 min 15 s.

We have previously developed automated methods for antibiotics such as quinolones and tetracyclines [7,8] and very polar drugs such as the X-ray contrast agent iopentol [5]. Several opiates and other alkaline drugs were also run through the system described here. With these substances no adsorption to the dialysis membrane was found. We are not able to explain why pholcodine adsorbs so strongly to cellulose acetate.

HPLC separation

A satisfactory separation of pholcodine from endogenous substances in plasma and whole blood was obtained with a CN column and a mobile phase consisting of 20% acetonitrile in 0.005 *M* phosphoric acid. Elution of pholcodine from the CBA material was achieved by adjusting the pH of the mobile phase to 2.5. The chromatograms in Fig. 5 of drug-free plasma, plasma spiked with 1 nmol/ml pholcodine, drug-free whole blood and whole blood spiked with 1 nmol/ml pholcodine show that only pholcodine is detected after elution of the solvent front.

Validation of the method

The calibration graphs for plasma and whole blood were based on peak-height measurements of external standards. The calibration graphs were linear in the pholcodine concentration range 0.10–2.0 nmol/ml ($y = 574x + 10$ and $y = 598x + 7$; y = peak height and x = concentration of pholcodine) with correlation coefficients $r = 0.997$ and 0.998 for plasma and whole blood, respectively. Table I shows the intra- and the inter-assay variations of the ASTED method. The relative standard deviations are satisfactory considering the low concentrations and the adsorption of pholcodine on the membrane.

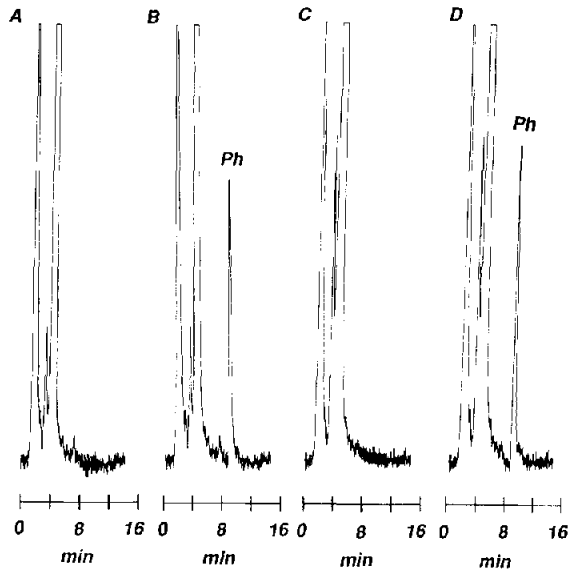

Fig. 5. Chromatograms of (A) drug-free plasma, (B) plasma spiked with 1 nmol/ml pholcodine, (C) drug-free whole blood and (D) whole blood spiked with 1 nmol/ml pholcodine after dialysis and trace enrichment of the dialysate on the trace enrichment column. For chromatographic conditions, see text. Ph = pholcodine.

TABLE I

INTRA- AND INTER-ASSAY VARIATIONS AFTER ASTED SAMPLE PREPARATION, EXPRESSED AS MEAN OF PARALLEL SAMPLES ± SAMPLE STANDARD DEVIATION (S.D.) AND RELATIVE STANDARD DEVIATION (R.S.D.)

Matrix	Calculated concentration (nmol/ml)	Measured concentration (mean ± S.D.) (nmol/ml)	R.S.D. (%)
Intra-assay (n = 5)			
Plasma	0.20	0.177 ± 0.017	9.6
	0.40	0.337 ± 0.034	10.1
	1.00	0.994 ± 0.0008	8.8
Whole blood	0.20	0.196 ± 0.008	3.8
	0.40	0.396 ± 0.007	3.8
	1.00	0.984 ± 0.010	1.7
Inter-assay (n = 5)			
Plasma	0.20	0.194 ± 0.006	3.1
	0.40	0.363 ± 0.025	7.0
	1.00	0.994 ± 0.013	1.3
Whole blood	0.20	0.204 ± 0.023	11.4
	0.40	0.390 ± 0.036	9.4
	1.00	0.994 ± 0.017	1.7

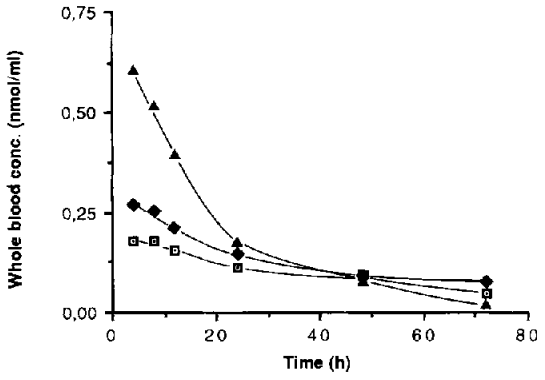

Fig. 6. Whole blood concentrations of pholcodine for three subjects after administration of 100 mg of pholcodine: □ = subject 1; ◆ = subject 2; ▲ = subject 3.

Limit of detection

The limit of detection is determined by the sample size injected into the dialyser. In this method a dialyser with a 375-μl donor channel volume was selected. The limit of detection at a signal-to-noise ratio of 3 was 0.1 nmol/ml pholcodine in both plasma and whole blood, which was considered satisfactory for the determination of pholcodine in real samples. The limit of detection could be improved by pulsing the sample through the donor channel. This method significantly reduced the membrane lifetime and was therefore rejected.

Analysis of real samples

Three healthy volunteers, one woman and two men, received 100 and 400 mg of pholcodine in single oral doses of Tuxi linctus with an interval of two months between the two treatments. Blood samples were collected for 72 h after each dosage administration. The recommended daily dose of pholcodine as an antitussive is 100 mg. The single oral dose of 400 mg of pholcodine was administered in order to be able to detect any differences between plasma and whole blood concentrations and to be able to detect possible metabolites of pholcodine. Fig. 6 shows plots of whole blood concentration *versus* time for all three subjects after administration of 100 mg of pholcodine. The concentration of pholcodine after 72 h was below the limit of detection. The

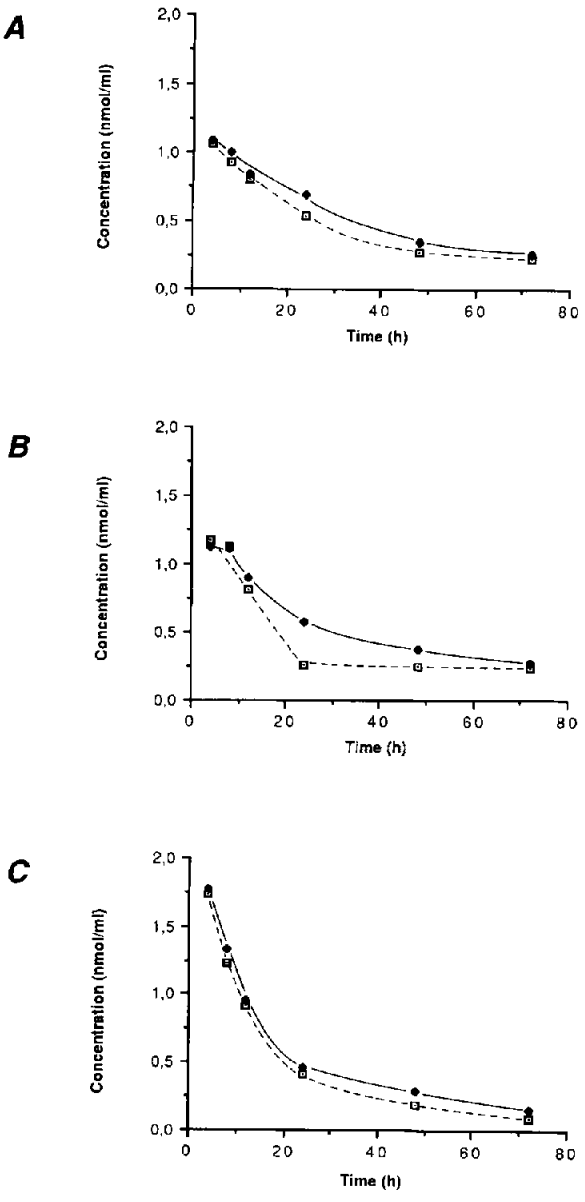

Fig. 7. Whole blood (◆) and plasma (□) concentrations of pholcodine for (A) subject 1, (B) subject 2 and (C) subject 3 after administration of 400 mg of pholcodine.

plasma concentration *versus* the whole blood concentration for all three subjects after a single 400-mg oral dose are shown in Fig. 7. The plasma concentrations measured are of the same order as those reported by Chen *et al.* [4], but analysis of whole blood was not included in their study. The

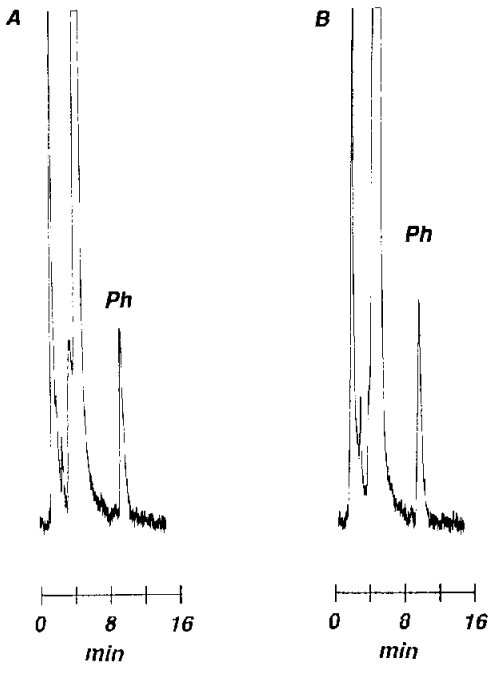

Fig. 8. Chromatograms of a corresponding (A) plasma and (B) whole blood sample collected 24 h after administration of 400 mg of pholcodine (Ph).

observed plasma concentrations were lower than the concentrations in haemolysed whole blood for all three subjects.

Chromatograms of corresponding plasma and whole blood samples obtained 24 h after administration of 400 mg of pholcodine are shown in Fig. 8.

We have previously reported morphine to be a metabolite of pholcodine [2]. In this system morphine had a retention time of about 4 min and any morphine present in the samples would co-elute with the solvent front. No other metabolites of pholcodine were detected.

CONCLUSIONS

A fully automated HPLC method for the determination of pholcodine in human plasma and whole blood has been developed. The method includes on-line dialysis and column switching and was shown to be highly reliable. The method has been successfully used in analyses of real samples after administration of pholcodine. The method can also be used, with minor modifications, for the determination of other basic drugs in whole blood and plasma. The only requirement is a mobile phase with a pH < 3 to desorb the cations from the CBA material. In this system HPLC separations can also be optimized by using ion-pair chromatography on reversed-phase columns with sulphonic acids as counter ions at pH 2.5.

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