Journal of Chromatography, 423 (1987) 163–168
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3927

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DRUG ANALYSIS BY DIRECT INJECTION OF WHOLE BLOOD SAMPLES

III*. DETERMINATION OF HYDROPHOBIC DRUGS ADSORBED ON BLOOD CELL MEMBRANES

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(First received May 25th; revised manuscript received August 7th, 1987)

SUMMARY

The determination of strongly hydrophobic drugs in whole blood by high-performance liquid chromatography was investigated and the amount adsorbed on cytomembranes was measured. A polyvinyl resin, TSK Gel HW-65, was used as the pre-column packing. Proteins, cytomembranes and endogeneous hydrophilic components flowed through the pre-column in aqueous medium, but strongly hydrophobic substances such as chlorpromazine were adsorbed and then eluted by backflushing into an ODS analytical column. The recovery of chlorpromazine from whole blood was 103.3% with a coefficient of variation of 3.4% ($n=10$). This method gave the total amounts in blood, representing not only the amount bound to proteins but also that bound to cytomembranes. The difference in the concentrations determined in whole blood and the supernatant of haemolysed whole blood gave the value adsorbed on cytomembranes.

INTRODUCTION

In Parts I and II we reported a high-performance liquid chromatographic (HPLC) method for the determinations of moderately hydrophobic [1] and hy-

*For Parts I and II, see pp. 147 and 155, respectively.

0378-4347/87/$03.50

drophilic [2] drugs by direct injection of whole blood samples. The total amount (free and bound to proteins) in blood plasma and in corpuscles could be determined, but adsorption on cytomembranes was not observed.

Chlorpromazine (CPZ), a phenothiazine, is a basic tranquillizer widely used in therapy. The ratio of the concentration of CPZ in plasma and cells (C/P ratio) was determined to be approximately unity by an in vitro isotope method [3]. Mohandas and Feo [4] found a ten times higher level of CPZ in red cells.

An on-line HPLC method for the direct injection of plasma samples containing CPZ was reported previously [5]. In this work, the method was applied to the determination of CPZ in whole blood and the amount adsorbed on cytomembranes was calculated.

EXPERIMENTAL

Materials

Chlorpromazine hydrochloride was obtained from Wako (Osaka, Japan). TSK Gel Toyopearl HW-65F (30–60 μm) and TSK Gel ODS 120T (5 μm) were kindly provided by Toyo Soda (Tokyo, Japan). All other reagents and materials were as reported in Part I [1].

Columns

A pre-column (50×4 mm I.D.) with an end-fitting of pore size 40 μm was packed with TSK Gel Toyopearl HW-65F and an analytical column (50×4 mm I.D.) was packed with TSK Gel ODS 120T (particle size 5 μm). The HPLC instrumentation was described in Part I [1].

Homogenization

CPZ added to whole blood at the 1 μM level was homogenized in an ice-bath. After 10–15 min, the supernatant obtained by centrifugation (2000 g for 10 min) was analysed by HPLC to determine the CPZ adsorbed on cytomembranes.

Dialysis cell

A 10-ml flow-through dialysis cell (Sanko Plastic, Tokyo, Japan) was used. CPZ (1 μM) added to a haemolysed whole blood sample was dialysed with water (flow-rate 0.8 ml/min) through the membrane (cut-off molecular mass 10 000) (Spectrum Medical Industries, Los Angeles, CA, U.S.A.).

RESULTS AND DISCUSSION

In Part I [1] we described a direct injection method for moderately hydrophobic substances in whole blood using a protein-coated ODS pre-column. This method was applied to the determination of CPZ in whole blood, but the recovery was poor owing to the too strong adsorptivitiy of CPZ. We found that TSK Gel Butyl Toyopearl was suitable for trapping CPZ in the pre-column, as reported in a previous paper [5]. Recently, we found that TSK Gel Toyopearl HW-65F shows superior characteristics especially with respect to reproducibility of the results.

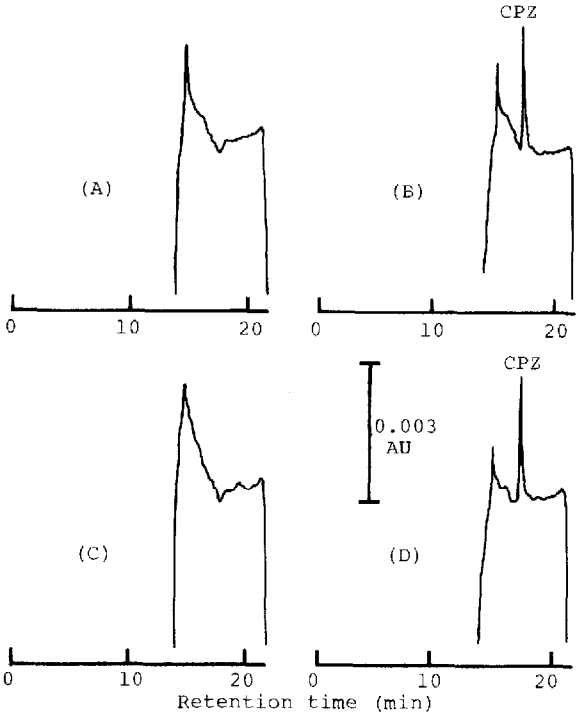

Fig. 1. Chromatograms of (A) blank plasma, (B) plasma spiked with 0.5 μM CPZ, (C) blank whole blood and (D) whole blood spiked with 0.5 μM CPZ. Injection volume: 200 μl.

The polyvinyl resin TSK HW-65F has hydroxy groups on its surface and has been used for gel filtration with an exclusion limit of $5 \cdot 10^6$ daltons for proteins. In this study we used HW-65F to trap CPZ from whole blood.

All apparatus and the column switching system were as reported in Part I [1], except the pre-column packing. Whole blood samples (50–500 μl) were injected directly into the pre-column equilibrated with water and haemolysed. Strongly hydrophobic substances were trapped in the pre-column and proteins, cytomembranes and other endogenous hydrophilic components in whole blood flowed through. After 3 min, the pre-column was washed with 0.1 M phosphate buffer (pH 7.0) containing 10% acetonitrile for 5 min in order to remove interfering substances. The CPZ adsorbed in the pre-column was then eluted by backflushing with 0.2 M citrate buffer (pH 4.0) containing 28% acetonitrile for 3 min. CPZ emerged completely on to the analytical column, and then the two columns were disconnected. The eluent was introduced into the analytical column and CPZ was detected by measuring the absorption at 254 nm. During the analysis, the pre-column was washed with 0.5% sodium dodecyl sulphate (SDS) solution for 5 min and then methanol for 5 min. The pre-column was used at ambient temperature and the analytical column was kept at a constant temperature of 45 °C. The flow-rate was 0.8 ml/min throughout.

A 200-μl sample of whole blood or plasma containing CPZ was injected directly. Fig. 1 shows the chromatograms of a rabbit plasma blank (A), CPZ added to

TABLE I

ADSORPTION OF CPZ ON BLOOD CYTOMEMBRANES

After haemolysis by three volumes of water, each sample was allowed to stand for 20 min, then centrifuged for 10 min. The difference between the whole blood and supernatant concentrations was ascribed to the amount adsorbed.

Concentration (μM)	Adsorbed quantity (%)
0.2	64.5
0.5	57.9
1	47.4
2	48.0
5	46.5

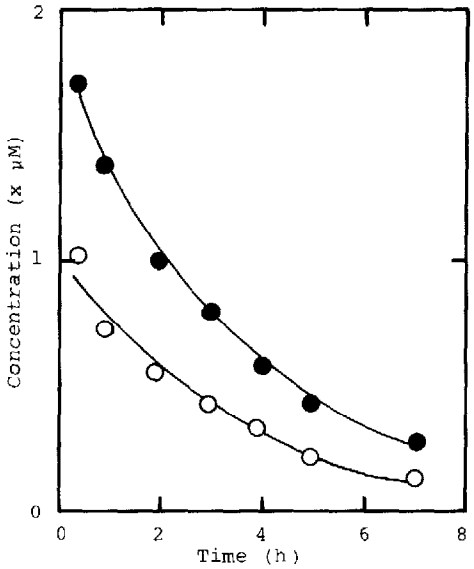

Fig. 2. Time course of CPZ in (○) plasma and (●) whole blood after intravenous injection of 40 mg of CPZ into a rabbit (3.5 kg).

plasma (B), a whole blood blank (C) and CPZ added to whole blood (D). No differences between the plasma and whole blood samples were observed in the elution profile. The recoveries were 101.1% [coefficient of variation (C.V.) $=3.4\%$, $n=10$] for plasma and 103.3% (C.V. $=3.4\%$, $n=10$) for whole blood. A calibration graph with a good straight line over the therapeutic concentration range (0.09–1.10 μM in plasma [7]) was obtained, and the enrichment of CPZ by using up to 500 μl of whole blood was confirmed to be quantitative. This method could be used for the analysis of at least 100 whole blood samples (200 μl) without any problems. When the pressure of the pre-column was increased by repeated direct injections of whole blood samples, the inlet end-fitting (pore size 40 μm) was disconnected and washed with 0.1 M sodium hydroxide solution containing 50% methanol under sonication. The regeneration was satisfactory.

The difference between the concentrations of CPZ in whole blood and in the

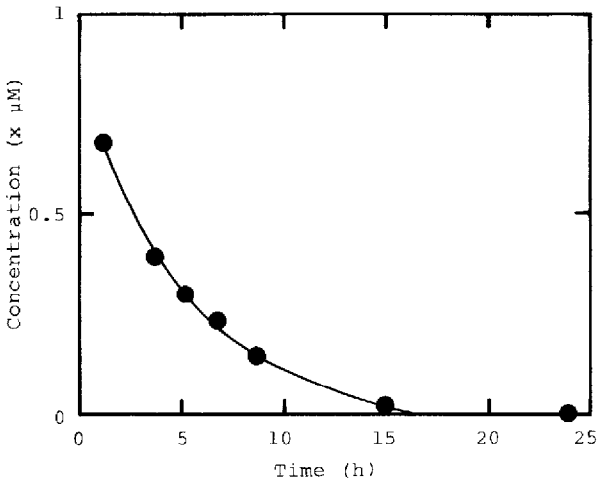

Fig. 3. Time course of CPZ in haemolysed whole blood by flow-through dialysis.

supernatant obtained by centrifugation (1000 *g* for 30 min) of whole blood haemolysed by adding three volumes of water represented the amount of CPZ adsorbed on cytomembranes. The adsorbed quantity decreased with increasing concentrations of CPZ, as shown in Table I. The time course of the CPZ concentration during homogenization reached a plateau at 0.67 μM in the supernatant after 20 min. The actual amount adsorbed on cytomembranes was calculated to be about 39% at 1 μM CPZ in whole blood [the volume of cytomembranes obtained by homogenizing whole blood was estimated to be about 10% (v/v) in haemolysed blood]. Fig. 2 shows the time course of CPZ in rabbit plasma and whole blood samples after intravenous injection of 40 mg of CPZ to a 3.5-kg rabbit. It was found that the CPZ concentration in whole blood was higher than that in plasma, and the C/P ratio obtained in vivo was 3.2–3.9 (the haematocrit value used was 33.4%).

Fig. 3 shows the time course of the CPZ concentration in a haemolysed whole blood sample by flow-through dialysis. Water was passed continuously at 0.8 ml/min. After 20 h CPZ was completely eliminated from the whole sample. The results suggest that the free fraction of CPZ was supplied from protein or cytomembrane fractions by the shift of mutual equilibria. It seems that the effective concentration of the drug is controlled by the free component in plasma, but the total amount in the various fractions in whole blood can be transferred into the free fraction by the shift of the equilibria.

CONCLUSION

An HPLC method was developed for the determination of strongly adsorptive drugs in whole blood. CPZ adsorbed on cytomembranes was trapped in a precolumn packed with TSK Gel HW-65F and was recovered quantitatively. It was found that even the entity strongly adsorbed on cytomembranes could be determined quantitatively by using a column switching technique.

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