Journal of Chromatography, 423 (1987) 147–153
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3925

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

I. DETERMINATION OF MODERATELY HYDROPHOBIC DRUGS INCORPORATED INTO BLOOD CORPUSCLES

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

SUMMARY

Amounts of a moderately hydrophobic drug incorporated into blood corpuscles were determined by column switching high-performance liquid chromatography with direct injection of whole blood samples. Some modifications were required in order to apply the conventional direct injection method to whole blood samples, as follows. Clogging of the pre-column by blood corpuscles or cytomembranes was avoided by using an end-fitting filter with pore size larger than 40 μm, and accordingly a packing with particle size larger than 40 μm was used. Haemoglobin was decomposed in contact with ODS silica gel and reduced the column efficiency. The protein-coated ODS pre-column (pore size less than 7 nm) was restored by washing with 0.1 *M* phosphate buffer (pH 3.0) containing 50% acetonitrile. Sodium dodecyl sulphate solution (0.5%, w/v) was preferable for rinsing and removes material remaining in the pre-column. A whole blood sample containing carbamazepine was directly injected into the pre-column, then blood cells were haemolysed promptly in contact with a mobile phase of low salt concentration. The total amount of carbamazepine could be determined with a coefficient of variation of 1.9% (within-run). Carbamazepine incorporated into rabbit blood cells (haematocrit value 33.6%) was determined to be 1.1–1.3 times higher than the concentration in plasma. Adsorption on the cytomembranes was not observed.

0378-4347/87/$03.50

INTRODUCTION

When evaluating the significance of drug levels in blood, it is important to know whether the analysis is carried out on whole blood or on plasma, because many kinds of drugs are unevenly distributed between blood plasma and the corpuscles. In clinical chemical laboratories, generally plasma or serum levels are monitored. Drug analyses in legal medicine are usually carried out on whole blood, the sample being more or less haemolysed. Apparently three fractions of a drug are present in blood, a free fraction in plasma, a plasma protein- or lipoprotein-bound fraction and a fraction in blood corpuscles. The drug easily shifts its equilibrium among these fractions. As the amounts of proteins, corpuscles and other endogenous biological components differ among individuals, sexes and species, a sequence of protein binding [1] and variation of the ratio between blood cell and plasma concentrations [2,3] was investigated. It is suggested that drug levels in plasma can be varied among individual samples even when the whole blood levels are the same.

Generally, the distribution of drugs in blood cells has been determined by radioisotope methods, which necessitate a special laboratory and cannot be applied directly to patients.

Recently, several automated high-performance liquid chromatographic (HPLC) methods using on-line deproteinization of serum or plasma samples have been reported [4–6]. We applied this direct injection method to the determination of carbamazepine (CBZ) in whole blood. CBZ, chosen as an example of a mildly hydrophobic substance, is expected to distribute in blood corpuscles. The details of the method are reported in this paper.

EXPERIMENTAL

Materials

Carbamazepine was obtained from Sigma (St. Louis, MO, U.S.A.) and bovine haemoglobin (assay 80%) from Wako (Osaka, Japan). Heparinized rabbit whole blood was collected from a rabbit vein prior to use, and plasma samples were obtained by centrifugation of heparinized blood (1000 *g* at 25 °C for 10 min).

CBZ (1 m*M* in 0.9% sodium chloride solution) was added to plasma or whole blood samples and determined whithin 5–10 min. Purified cytomembranes were prepared by haemolysis with water (5 volumes), then washing ten times and centrifugation.

All other reagents were of analytical-reagent grade and used as received.

Pre-column

The pre-column (50×4 mm I.D.) with a large pore end-fitting (pore size 40 μm) was packed with Fuji Davison ODS (particle size 30–50 μm, pore size 7 nm) (Fuji Gel, Tokyo, Japan), and used after coating with protein [6]. Haem adsorbed on the pre-column was eluted with a Model CCPM HPLC gradient system (Toyo Soda, Tokyo, Japan) with a UV-8000 spectrophotometric detector (Toyo Soda).

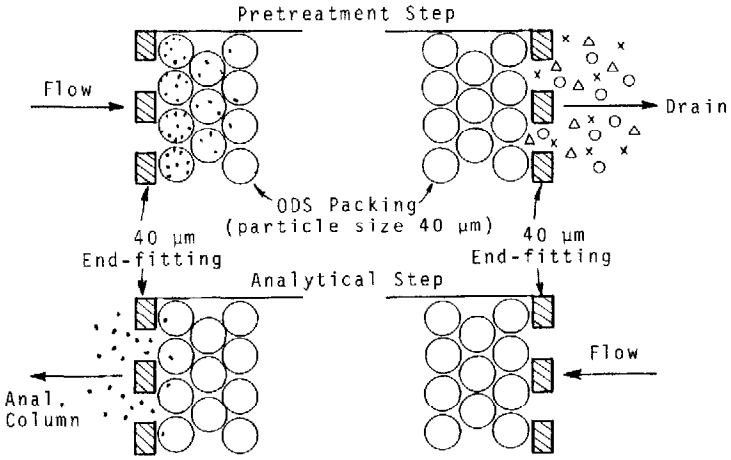

Fig. 1. Schematic representation of pre-column for the determination of hydrophobic components in whole blood. (•) Hydrophobic compounds; (△) blood proteins; (○) cytomembranes; (×) hydrophilic compounds.

Column switching HPLC

A Model HLC-803D liquid chromatograph (Toyo Soda) equipped with a Model GE-4 solvent selector (Toyo Soda) was used to deliver the mobile phase. Two columns, one a pre-column and the other an analytical column (80×4 mm I.D.) (TSK Gel ODS 120T, 5 μm) were used. The pre-column was used at ambient temperature and the analytical column was kept at a constant temperature of 45°C. A Model UV-8000 UV spectrophotometer was used for detecting CBZ. The flow-rate was 0.8 ml/min throughout the experiments. A six-way valve and other apparatus were used as reported in a previous paper [6].

RESULTS AND DISCUSSION

Preparation of pre-column and its applicability to whole blood analysis

Fig. 1 is a schematic representation of the inside of the pre-column on direct injection of a whole blood sample. The end-fitting of the pre-column (pore size 40 μm) was designed to avoid clogging by blood cell membranes (erythrocyte < 10 μm, leucocyte 10–20 μm, thrombocyte 2–3 μm). The particle size of the pre-column packing should be larger than the pore size of the end-fitting. Three kinds of protein-coated ODS packings (particle size 40 μm) were tentatively used for this purpose. (1) The spherical ODS packing (pore size 12 nm) adsorbed haem and turned brown. The reduction in the column efficiency could not be restored even by washing with absolute methanol or surfactant solutions [e.g., 1% Brij-35, 0.5% sodium dodecyl sulphate (SDS)]. (2) The crushed-type ODS packing (pore size 7 nm) did not adsorb haem, but a gap at the top of the pre-column appeared after successive column switchings. (3) The spherical ODS packing (pore size 7 nm) showed favourable characteristics.

Fig. 2 shows chromatograms of haemoglobin obtained by using the last packing with gradient elution from water to (A) 0.1 *M* phosphate buffer (pH 7.0), (B)

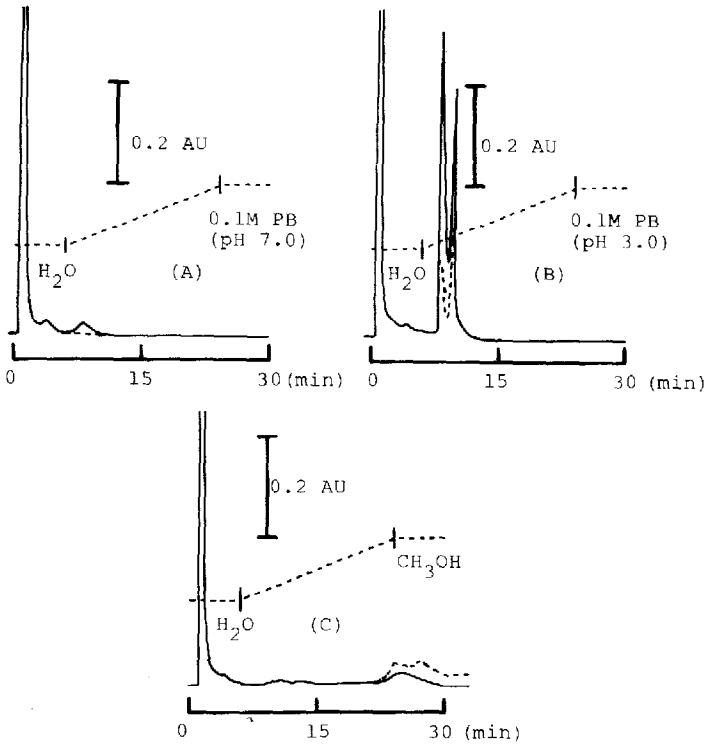

Fig. 2. Elution profiles of haemoglobin (12%, 50 μl) from the pre-column. The solid and dotted lines represent detection at 280 and 410 nm, respectively. PB = phosphate buffer.

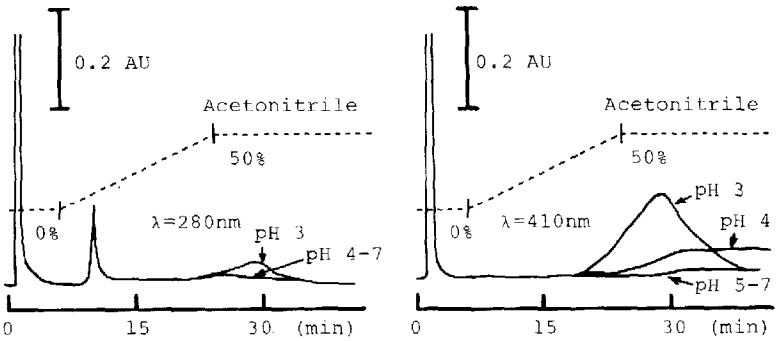

Fig. 3. Effect of pH and acetonitrile concentration on the retention of haemoglobin (12%, 50 μl). Elution: from 0 to 50% in 0.1 *M* phosphate buffer at various pH.

0.1 *M* phosphate solution (pH 3.0) or (C) absolute methanol. The concentration of haemoglobin in whole blood was calculated to be about 11.5% (w/v) by spectroscopy at 410 nm. It was found that a large portion of haemoglobin, which showed similar behaviour to plasma proteins, was eluted at the void volume from the pre-column by distilled water, and some haemoglobin was adsorbed on the pre-column packings, in spite of previous protein coating, and eluted with 0.1 *M*

phosphate solution (pH 3.0). However, these adsorbed components were not eluted by absolute methanol or 0.1 *M* phosphate buffer (pH 7.0).

Fig. 3 shows the effect of pH and acetonitrile concentration on the elution of haemoglobin or haem from the pre-column. Experiments were carried out with various acetonitrile concentrations from 0 to 50% in 0.1 *M* phosphate solutions of various pH (3–7), and the globin moiety was detected at 280 nm and haem at 410 nm. By using 0.1 *M* phosphate solution (pH 3.0) containing acetonitrile, it was found that haemoglobin components (haem and globin moieties) were eluted separately, showing that haemoglobin was decomposed in contact with ODS. Globin adsorbed on the pre-column was eluted by 0.1 *M* phosphate solution (pH 3–7) at 5% acetonitrile concentration, but haem was eluted only by 0.1 *M* phosphate solution (pH 3.0) containing 50% acetonitrile. On the other hand, most of blood cytomembranes obtained from rabbit whole blood (see Experimental) were eluted at the void volume from the pre-column, but on successive injections of whole blood samples (50 μl, about tweny times) the column pressure was gradually increased even when the end-fittings of 40 μm pore size were used. This problem was solved by rinsing with surfactant solution. Of several surfactant solutions tested, SDS solution (0.5%, w/v) was the most effective in solubilizing not only blood corpuscles but also blood cytomembranes clogging the pre-column.

Determination of CBZ in whole blood

CBZ in whole blood was determined using the pre-column. A whole blood or plasma sample (50 μl) was injected directly into the pre-column equilibrated with water and haemolyzed. A large portion of proteins, blood cytomembranes and hydrophilic components flowed out into the drain within 3 min, and some proteins remaining in the pre-column were eluted thoroughly with 0.1 *M* phosphate solution (pH 3.0) containing 10% acetonitrile within 5 min. The pre-column was then connected with the analytical column equilibrated with 0.1 *M* phosphate solution (pH 3.0) containing 25% acetonitrile. Using this eluent, CBZ trapped in the pre-column emerged into the analytical column within 3 min. After the two columns had been disconnected, the eluent was introduced only into the analytical column and the effluent was monitored by measuring the absorption at 285 nm. Column switching was devised so as to allow the rinse solution to flow in the pre-column during the analysis. First 0.5% SDS solution for 5 min and second absolute methanol for 5 min were allowed to flow through the pre-column. After the determination of CBZ, absolute methanol was used for 5 min to clean the analytical column. For the next run, the pre-column and analytical column were equilibrated with water.

Fig. 4 shows chromatograms of CBZ added to heparinized rabbit plasma and whole blood. No differences between plasma and whole blood were observed in the elution profile. Table I shows the recovery and reproducibility for plasma and whole blood. It is noteworthy that 70–80% of CBZ was reported to be bound to plasma proteins [7]. Nevertheless, quantitative recovery was obtained with good reproducibility [coefficient of variation = 1.0% (plasma), 1.9% (whole blood); $n = 10$]. The calibration graph for whole blood was a good straight line over therapeutic concentration range in plasma (21–42 μ*M* [8]). The ratio of concentra-

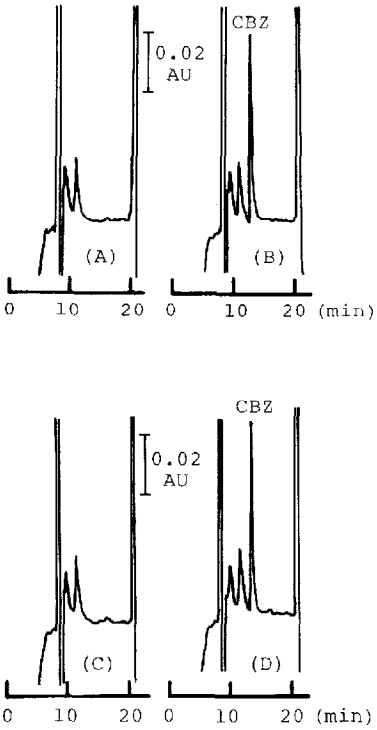

Fig. 4. Chomatograms of (A) blank plasma, (B) plasma spiked with 20 μM CBZ, (C) blank whole blood and (D) whole blood spiked with 20 μM CBZ.

tions in blood cells and plasma (C/P ratio) was calculated to be 1.1–1.3 by an in vitro experiment at therapeutic concentrations in plasma (the haematocrit value of rabbit whole blood used was 33.6%). Accordingly, 36–40% of the total amount was distributed in corpuscles. It is noteworthy that these data may vary when the measurement is carried out in vivo using human blood.

When a whole blood sample haemolysed by addition of three volumes of water was analysed after removal of cytomembranes by centrifugation, the recovery of CBZ was almost quantitative (95–100%). Thus, the absence of adsorption on blood cytomembranes was confirmed. This column switching method could be applied to at least 100 whole blood samples without any difficulty.

TABLE I

ANALYTICAL DATA FOR CARBAMAZEPINE

Spiked carbamazepine: 20 μM.

Sample	Coefficient of variation ($n=10$) (%)	Recovery (%)
Plasma	1.0	104.9
Whole blood	1.9	100.3

CONCLUSION

Direct injection HPLC was applied to the determination of drug concentration in whole blood samples. The pre-column was designed so as to pass the haemolysed cytomembranes with an end-fitting of pore size of 40 μm. A spherical ODS packing with a particle size larger than 40 μm and a pore size of about 7 nm was used to eliminate the interference by cytomembranes and haemoglobin. CBZ used as a model sample was recovered quantitatively from whole blood, and the ratio of CBZ concentrations in corpuscles and plasma was determined to be 1.1–1.3 in vitro at therapeutic concentrations. This method could be used for the determination of the total level of moderately hydrophobic drugs in whole blood without any pre-treatment.

ACKNOWLEDGEMENT

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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