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

II*. DETERMINATION OF HYDROPHILIC DRUGS

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SUMMARY

The determination of hydrophilic drugs in whole blood by direct injection high-performance liquid chromatography was investigated. A pre-column equipped with an inlet filter of pore size 40 μm and an outlet filter of pore size 2 μm was packed with Butyl Toyopearl 650-M. A whole blood sample was injected directly into the pre-column to trap proteins, hydrophobic compounds and blood cytomembranes, and hydrophilic compounds emerged into an analytical column (Nucleosil 5SA, particle size 5 μm) and were determined after column switching. Proteins in 40 μl of rabbit whole blood were adsorbed in the pre-column (0.63 ml of wet gel) in 0.4% perchloric acid solution. The recovery of procainamide and N-acetylprocainamide from whole blood was quantitative with good reproducibility (coefficient of variation less than 4%). It was shown that procainamide added to rabbit whole blood was subjected to N-acetylation by N-acetyltransferase in blood cells.

INTRODUCTION

In Part I [1] we reported a high-performance liquid chromatographic (HPLC) method with direct injection of whole blood samples. This method, however, cannot be applied to hydrophilic compounds. Recently, we reported the hydrophobic

*For Part I, see p. 147.

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interaction chromatography of proteins with Butyl Toyopearl 650-M (BT 650-M) using 0.4% perchloric acid solution (PCA) [2], and applied this method to the on-line deproteinization of serum samples for the determination of hydrophilic compounds [3,4]. The method has now been modified for application to whole blood samples and the details are presented in this paper.

As examples of hydrophilic substances, the antiarrythmic drug procainamide (PA) and its active metabolite N-acetylprocainamide (NAPA) were used. PA in whole blood not only exists in plasma but also is incorporated into blood corpuscles [5] and is metabolized to N-acetylprocainamide (NAPA) by N-acetyltransferase [6]. Some problems involved in this metabolic reaction both in vivo and in vitro are discussed in connection with the direct injection method for whole blood samples.

EXPERIMENTAL

Materials

Both PA hydrochloride and NAPA hydrochloride were obtained from Sigma (St. Louis, MO, U.S.A.). Nucleosil 5SA was purchased from Machery, Nagel (Düren, F.R.G.) and BT 650-M was from Toyo Soda (Tokyo, Japan). Other reagents, materials and apparatus were as reported in Part I [1]. Rabbit blood was used throughout.

Preparation of columns

The pre-column equipped with an inlet filter of pore size 40 μm and an outlet filter of pore size 2 μm, was packed with BT 650-M (particle size 44–88 μm) (50×4 mm I.D.) and the analytical column (50×4 mm I.D.) with Nucleosil 5SA (particle size 5 μm).

RESULTS AND DISCUSSION

BT 650-M pre-column and its application to the determination of hydrophilic compounds in whole blood

BT 650-M resin, which adsorbs proteins by hydrophobic interaction, was used as the packing in the pre-column. We have reported the adsorption of proteins on BT 650-M in solutions containing conventional denaturating reagents such as PCA at very low concentrations [2]. In this work, PCA solution (0.4%) was used as the mobile phase for pre-treatment of whole blood samples.

Fig. 1 shows schematically the behaviour of whole blood components in the pre-column. An end-fitting inlet filter of pore size 40 μm was used. By using this filter, entry of blood corpuscles or cytomembranes was not hindered. The pore size of the outlet filter was 2 μm, through which blood cytomembranes could not pass. Whole blood samples were injected directly into the pre-column equilibrated with 0.4% PCA solution, and the proteins eluted from the pre-column were determined by the Coomassie Brilliant blue (CBB) method [7]. As shown in Table I, the effluents obtained by injection of less than 35 μl of whole blood samples did not contain proteins, but volumes above 50 μl showed the existence of

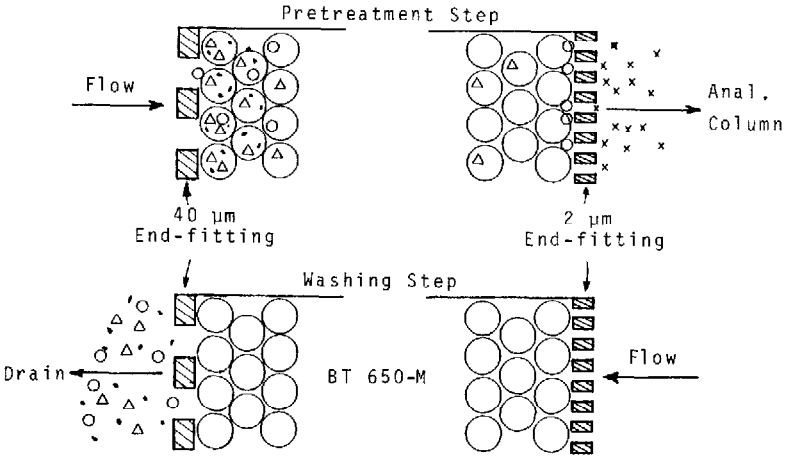

Fig. 1. Schematic representation of pre-column. (•) Hydrophobic compounds; (△) blood proteins; (○) cytomembranes; (×) hydrophilic compounds.

proteins. The trapping capacity of BT 650-M (0.63 ml of wet gel) for proteins corresponded to the content in about 40 µl whole blood. It was found that by injection of less than 40 µl of whole blood samples, proteins, blood cytomembranes and hydrophobic compounds were trapped in the pre-column, and hydrophilic compounds flowed into the analytical column.

Fig. 2 shows the elution profiles of whole blood components adsorbed on the pre-column. After trapping of proteins, cytomembranes and endogenous hydrophobic components, the flow direction was changed to backflushing, then stepwise washing of the pre-column was carried out with water, sodium dodecyl sulphate (SDS) (0.5% in 0.1 *M* sodium hydroxide solution), water and finally absolute methanol. The cytomembranes in the pre-column were eluted by forming micelles with 0.5% SDS solution and haemoglobin was eluted completely by

TABLE I

ADSORPTION CAPACITY OF BT 650-M* FOR PROTEINS IN RABBIT WHOLE BLOOD

Injection volume (µl)	Amount of proteins in effluent** (µg)
10	0–trace
20	0–trace
35	0–trace
50	60– 80
65	80–170
80	300–330

*0.63 ml/wet gel.

**Determined by CBB method (adsorption at 595 nm), expressed in terms of bovine serum albumin amount.

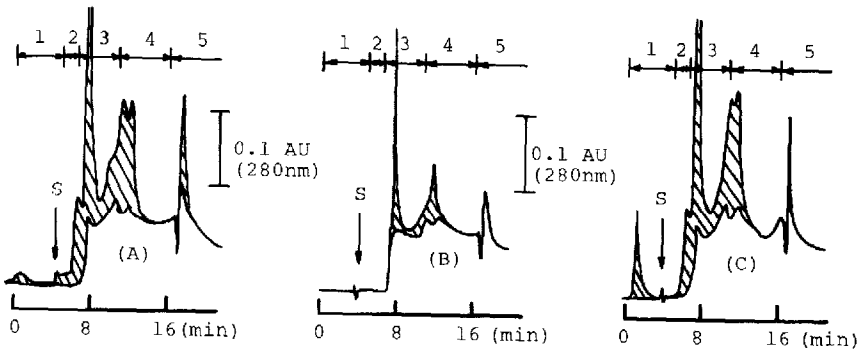

Fig. 2. Elution profiles from pre-column. (A) 12% haemoglobin, 10 μl; (B) purified blood cytomembranes corresponding to whole blood, 10 μl; (C) whole blood, 10 μl. Stepwise elution: (1) 0.4% PCA for 5 min; (2) water for 2 min; (3) 0.5% SDS for 5 min; (4) water for 5 min; (5) methanol for 5 min. S indicates the change in flow direction.

the methanol. The pre-column could be regenerated completely by this stepwise washing.

Determination of PA and NAPA in whole blood

Owing to the hydrophilicity of PA, only 15–17% was bound to plasma proteins [8], and it was not adsorbed on ODS packings without ion-pair reagents. In acidic media, PA (pK_a 9.23) was protonated and adsorbed on a cation exchanger (Nucleosil 5SA). A whole blood or plasma sample (10 μl) was injected directly into the pre-column at room temperature, the pre-column having previously been equilibrated with 0.4% PCA solution. Haemolysis occurred just after injection of the whole blood sample. Proteins and hydrophobic components were adsorbed on BT 650-M and cytomembranes were trapped at the outlet end-fitting, but hydrophilic compounds passed through the pre-column into the analytical column. After 5 min, the two columns were disconnected and the analytical eluent, 0.1 *M* phosphate solution (pH 3.0) containing 30% acetonitrile, was introduced into the analytical column, the temperature being controlled at 37°C. A spectrophotometer was used to detect both PA and NAPA at 280 nm. During the analysis, the pre-column was washed with rinse solution, first water for 2 min, second 0.5% SDS in 0.1 *M* sodium hydroxide solution for 5 min, third water for 5 min and finally absolute methanol for 5 min. After each run, both the pre-column and the analytical column were again equilibrated with 0.4% PCA solution. The time required for the analysis was 25 min.

Fig. 3 shows chromatograms of PA and NAPA added to heparinized rabbit plasma and whole blood samples. In the elution profiles, no differences between plasma and whole blood were observed except for the fraction at the void volume. Quantitative recovery was obtained with good reproducibility, as shown in Table II. The calibration graphs for PA and NAPA were good straight lines with relative slopes of 1.4 and 2.5 (peak height/μM), respectively, at therapeutic concentrations (8.5–34 μM in plasma [8,9]). All spiked whole blood samples were prepared

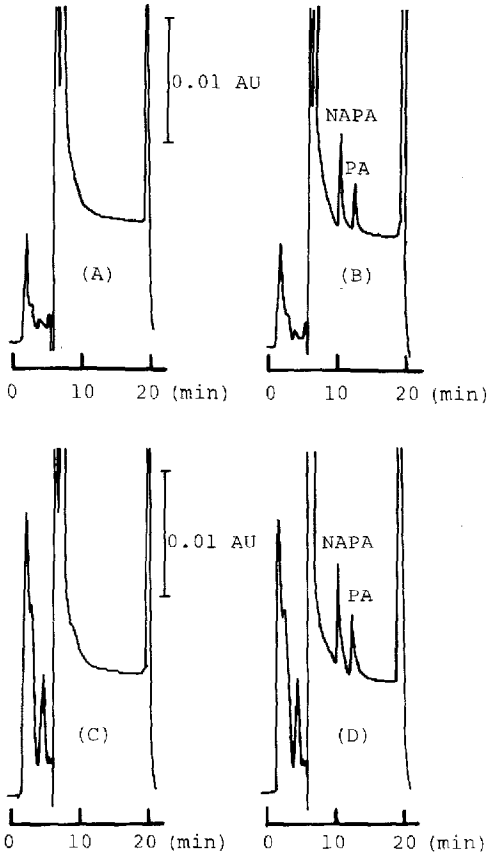

Fig. 3. Typical chromatograms of (A) control plasma, (B) PA and NAPA added at 20 μM to plasma, (C) control whole blood and (D) PA and NAPA added at 20 μM to whole blood.

10 min before injection into the HPLC system in order to avoid the effect of metabolic reactions.

The recoveries of PA and NAPA in samples with and without cytomembranes were almost the same as that for whole blood samples. Therefore, it seems that

TABLE II

RECOVERY OF PA AND NAPA ADDED TO PLASMA AND WHOLE BLOOD

Sample: 20 μM PA and NAPA, 10-μl injection.

Sample	Coefficient of variation (%)		Recovery (%)	
	PA	NAPA	PA	NAPA
Plasma	1.1 ($n=10$)	1.9	101.1	100.0
Whole blood	2.7 ($n=20$)	3.8	102.7	102.6

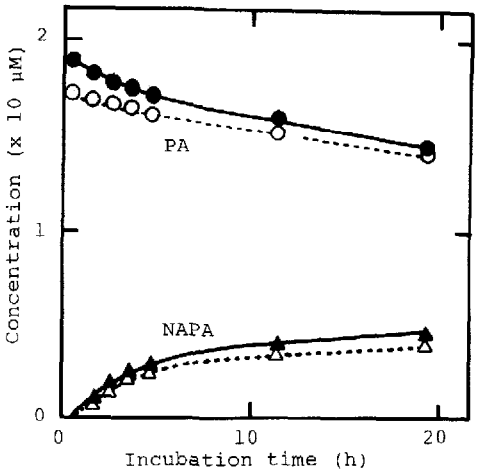

Fig. 4. Variation of concentrations of PA and NAPA added to whole blood with incubation time at 37°C. Closed symbols show the whole blood concentrations and open symbols the plasma concentrations.

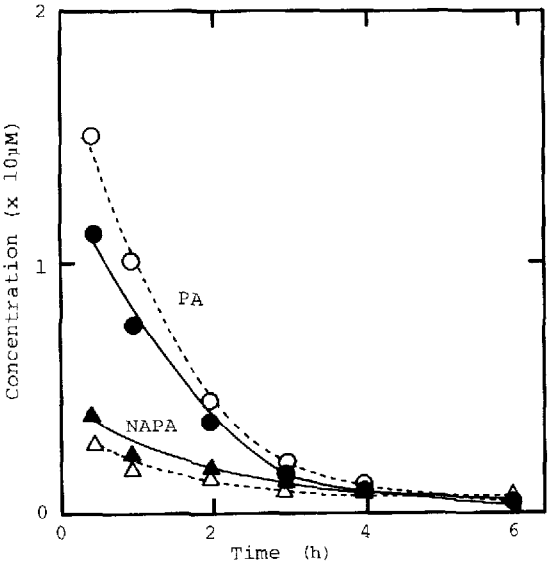

Fig. 5. Time course of PA and NAPA after intravenous injection of 28 mg of PA into a rabbit (3.2 kg). Symbols as in Fig. 4.

neither PA nor NAPA was adsorbed on cytomembranes. This method could be used for the analysis of more than 100 whole blood samples without any chromatographic problems.

Metabolic reaction in vitro and in vivo

PA added to whole blood is metabolized to NAPA by N-acetyltransferase [6]. Fig. 4 shows the time course of PA and NAPA concentrations with incubation at 37°C. The original sample contained only 20 μM PA added to rabbit whole blood. As shown in Fig. 4, the concentrations of PA in both plasma and whole blood

decreased, whereas the NAPA concentration increased. The ratio of the concentrations of PA in blood corpuscles and plasma (C/P ratio) varied from 1.3 after 1 h to 1.1 after 10 h, whereas for NAPA the final value was 1.6 (the haematocrit value of the rabbit whole blood used was 33.6%). Beyond 10 h the levels of PA and NAPA in plasma and whole blood could hardly be determined owing to haemolysis of erythrocytes during incubation.

Fig. 5 shows the time course of PA and NAPA in plasma and whole blood samples after intravenous injection of 28 mg of procainamide hydrochloride to a 3.2-kg rabbit. NAPA was produced within 30 min by the action of polymorphic N-acetyltransferase [10] and almost all of the PA and NAPA was eliminated from blood within 6 h. The C/P ratios of PA and NAPA determined in vivo were 0.3–0.6 and 1.6, respectively. The difference in the C/P ratios determined in vitro and in vivo might be due to differences in the enzyme activity.

CONCLUSION

The determination of a hydrophilic drug (PA) in whole blood by direct injection HPLC was investigated using a pre-column with an adsorbent gel for proteins. The pre-column had inlet and outlet end-fittings of pore size 40 and 2 μm, respectively. In this pre-column not only proteins but also blood cytomembranes could be trapped. The hydrophilic compounds flowed through the pre-column into the analytical column. PA and NAPA added to both plasma and whole blood were determined by this method. The recovery was quantitative with good reproducibility. The metabolic reaction of PA in whole blood proceeded by N-acetyltransferase. In therapeutic monitoring of PA and NAPA, it is necessary to use whole blood samples within 30 min after collection in order to avoid the effect of enzyme reactions. Similarly, spiked samples should be analysed as quickly as possible after preparation.

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