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RETENTION PROPERTIES OF INTERNAL-SURFACE REVERSED-PHASE SILICA PACKING AND RECOVERY OF DRUGS FROM HUMAN PLASMA

TERUMICHI NAKAGAWA*, AKIMASA SHIBUKAWA, NORIHITO SHIMONO, TOMOKO KAWASHIMA and HISASHI TANAKA

Faculty of Pharmaceutical Sciences, Kyoto University, Yoshida Shimoadachi-cho, Sakyo-ku, Kyoto 606 (Japan)

and

JUN HAGINAKA

Faculty of Pharmaceutical Sciences, Mukogawa Women's University, 4-16 Edagawa-cho, Nishinomiya 663 (Japan)

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SUMMARY

The properties of internal-surface reversed-phase silica packings developed for use in high-performance liquid chromatography, were investigated with regard to the effects of mobile phase conditions (pH, ionic strength, nature and concentration of organic modifiers, ion-pairing agent and surfactant) on the retention of some selected compounds having different polarities. The results indicated that π-electron interactions play the main role in solute retention, although it is weaker than that on phenylsilica supports, and that weak cation-exchange properties exert a secondary effect on the retention of ionic solutes. The ion-pairing effect was found to be markedly weakened in mobile phases containing an anionic counter ion. In contrast, the addition of a surfactant (SDS) caused a marked increase in the retention of certain drugs at low pH and expanded the pH range of the mobile phase applicable to plasma samples. Based on these findings, the separation and recovery of several drugs (probenecid, lidocaine, cefpiramide and cefaclor) from human plasma were investigated, with emphasis on their protein bindings. The contour chromatogram of human plasma is also demonstrated in relation to the selection of the detection wavelength.

INTRODUCTION

In the usual high-performance liquid chromatographic (HPLC) determination of drugs in plasma or serum, pre-treatment of the sample, such as deproteinization and solvent extraction, is necessary in order to prevent the accumulation of blood proteins which cause clogging of the column and deterioration of the

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separation efficiency. However, such pre-treatment is time consuming and does not always give reproducible and quantitative results. In order to simplify or eliminate the sample pre-treatment, various types of disposable solid extractors have been prepared and are commercially available. Studies have been reported on the "in-line deproteinization" method [1], in which a pre-column packed with adsorbent gel is connected in-line with an analytical column, and procedures such as the separation of drugs from blood protein, detection of drugs and regeneration of the pre-column are carried out automatically by means of column switching devices.

Recently, two types of HPLC supports have been developed to allow the direct injection of plasma or serum samples without sample pre-treatment. One is the "protein-coated ODS" support prepared by Yoshida et al. [2] and the other is the "internal-surface reversed-phase (ISRP)" silica support developed by Hagestam and Pinkerton [3]. These supports have a hydrophilic external surface and a hydrophobic internal (pore) surface. When a plasma or serum sample is injected directly on to these supports, the blood proteins, as their molecular size is too large to enter the pores of the support, are excluded from the column without precipitation or accumulation, whereas drugs with a small molecular size can enter the pores and are retained on the hydrophobic partitioning phases.

The ISRP silica support used in this study has a hydrophobic tripeptide partitioning phase (glycyl-L-phenylalanyl-L-phenylalanine) on the internal surface and hydrophilic diol–glycine groups on the external surface. The synthesis, fundamental properties and some applications of this column have recently been published [4–6]. Another type of ISRP silica support has also been prepared by bonding *tert.*-butoxycarbonyl(Boc)-L-phenylalanine to the silica via an amine spacer, followed by cleavage of the amino acid residues from the external surface in the presence of chymotrypsin [7, 8]. However, this type of column is not commercially available.

When using an ISRP column, the ionic strength and pH of the mobile phase are limited in order to prevent precipitation of blood proteins. Acetonitrile, isopropanol and tetrahydrofuran are the preferred organic modifiers because they can afford a wide selectivity in controlling solute retention on the hydrophobic stationary ligand and are known to bring about less protein denaturation than methanol [6].

In this paper we deal systematically with the retention behaviour of several selected compounds with different polarities on the ISRP support, and discuss the effects of mobile phase conditions such as pH, ionic strength and concentration of organic modifiers, ion-pair reagents and surfactant on sample retention, comparing the results with those observed on a phenylsilica column. We also investigated the release of several drugs from human plasma proteins and discuss the effect of the drug–protein binding site and mobile phase conditions on the separation and recovery of drugs from plasma proteins. The elution profile of human plasma itself under some mobile phase conditions is also reported.

EXPERIMENTAL

Reagents and materials

Phenethylamine, *p*-toluic acid, methylparaben, L-phenylalanine and phenol of guaranteed-reagent grade and lidocaine for biochemical use were purchased from Nakarai Chemicals (Kyoto, Japan) and Wako (Osaka, Japan). Metoprolol tartrate, naproxen, probenecid and β-lactam antibiotics were commercial products available for clinical use. Tetrabutylammonium bromide, sodium heptanesulphonate and sodium dodecylsulphate were purchased from Nakarai Chemicals. Tetrahydrofuran and isopropanol of special-reagent grade, glass-distilled water and acetonitrile were used to prepare the mobile phases.

HPLC conditions

A liquid chromatograph equipped with a UV detector (LC-6A; Shimadzu, Kyoto, Japan) was used. The chromatograms of human plasma were recorded with an MCPD-3500PC photodiode array detector system (Otsuka Electronics, Japan). The ISRP column, commercial name "Pinkerton column" (GFF-S5-80, 5 μm), manufactured by Regis (Morton Grove, IL, U.S.A.), was kindly donated by Koken (Tokyo, Japan). The phenylsilica column (Cosmosil 5Ph, 15 cm$\times$4.6 mm I.D.) was purchased from Nakarai Chemicals. The mobile phase conditions are given in the figure captions. The operations were carried out at ambient temperature.

Preparation of human plasma sample

Fresh plasma was obtained by centrifugation of human blood after anticoagulate treatment using citric acid and sodium citrate. Probenecid, lidocaine, cefpiramide and cefaclor were dissolved in plasma at a known concentration, and an appropriate volume of plasma solution was applied to the ISRP column after filtration through a 0.22-μm membrane filter.

RESULTS AND DISCUSSION

Elution profile of human plasma from ISRP column

Prior to optimization of the mobile phase conditions for the separation of drugs in plasma, the elution profile of plasma itself was investigated. Fig. 1 shows the contour chromatogram of human plasma eluted from the ISRP column with 0.1 *M* phosphate buffer solution (pH 7.0) as the mobile phase. A large peak due to plasma proteins and bilirubin (λ_{max} 450 nm) appeared at the void volume, and tailing up to 3 min was observed by detection at 240 nm. The recovery of plasma from the ISRP column has been reported to be 99$\pm$3% [6]. This means that most of the endogenous plasma components were excluded from the ISRP column, and that the tailing would interfere with the complete separation of a drug if the drug had a retention time shorter than 3 min under the selected mobile phase conditions and weak absorption above 240 nm. Therefore, it is necessary to enhance the retention of such drugs by using a longer column (e.g., 25 cm) or by selecting suitable mobile phase conditions such as pH, ionic strength and organic modifier. The use of other detection methods is obviously feasible. The

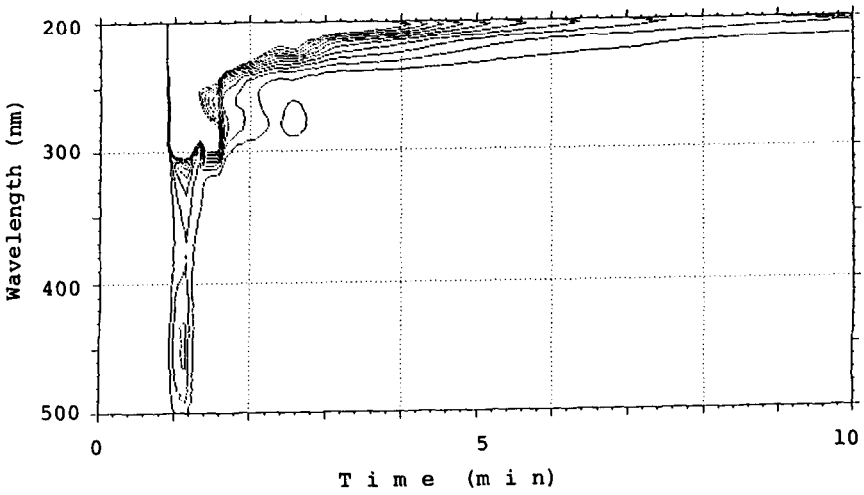

Fig. 1. Contour chromatogram of human plasma. A 10-μl volume of human plasma was injected. The plotting was made from 0.01 to 0.1 a.u.f.s. at intervals of 0.01 abs. Mobile phase, 0.1 *M* potassium phosphate buffer (pH 7.0); stationary phase, ISRP (GFF-S5-80); column 15 cm × 4.6 mm I.D.; flow-rate, 1.0 ml/min.

elution profile shown in Fig. 1 was almost unchanged even when 10% of acetonitrile, isopropanol or tetrahydrofuran was present in the mobile phase.

In order to characterize the nature of the ligand on the internal surface, the retention behaviours of some selected compounds with different ionic properties were investigated as functions of pH, ionic strength (μ), nature and concentration of the organic modifier and some additives in the mobile phases.

Effect of pH

Fig. 2 shows the changes in the capacity factor (k') of methylparaben, phenethylamine and *p*-toluic acid as a function of the pH of the mobile phase with a constant ionic strength ($\mu = 0.2$). The k' vs. pH profiles obtained with use of the ISRP column (Fig. 2A) are compared with those shown in Fig. 2B, where a phenylsilica column was used with a mobile phase consisting of 0.1 *M* phosphate buffer ($\mu = 0.2$) containing 20% of acetonitrile. In the absence of acetonitrile in this mobile phase, the strong adsorption of these compounds on the phenylsilica surface caused their k' values to become undesirably large. It is found from Fig. 2 that the decrease in the k' value of *p*-toluic acid (pK_a 4.37) on both columns results from ion exclusion with increasing pH of the mobile phase, and that in the ISRP column the ionic repulsion between the carboxylate groups of *p*-toluic acid and of the phenylalanine residue at the C-terminus on the internal surface can also be attributed to the decrease in k'. Such an ionic effect is also found with methylparaben; a slight decrease in the k' of methylparaben at pH > 7 found in both Fig. 2A and B could be ascribed to the partial dissociation of a phenolic hydroxy group (pK_a 8.47), while another decrease observed at pH 4 in Fig. 2A could result from the increasing dissociation of the carboxylic acid group on the internal surface. In contrast, the k' value of phenethylamine (pK_a 9.83) obtained with the ISRP column was markedly increased with increasing pH of the mobile

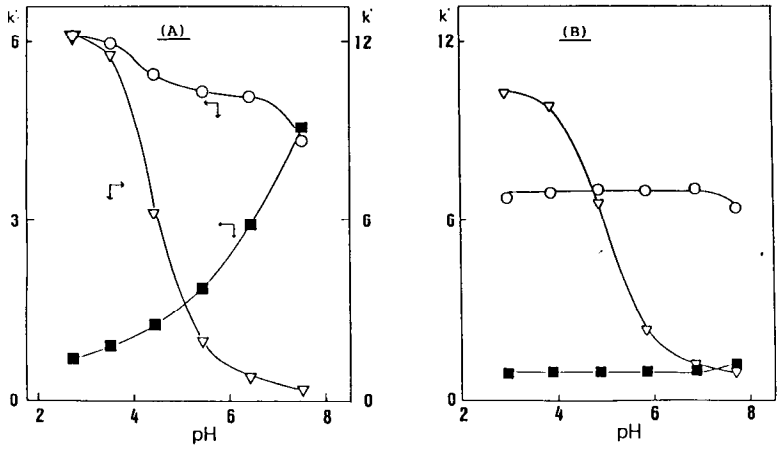

Fig. 2. Dependence of capacity factors of methylparaben (○), *p*-toluic acid (▽) and phenethylamine (■) on pH of mobile phase observed with (A) ISRP column and (B) phenylsilica column. Mobile phase, (A) potassium phosphate buffer (pH 2.71–7.48, ionic strength 0.20), (B) potassium phosphate buffer (ionic strength 0.20)–acetonitrile (8:2), pH 2.97–7.71; flow-rate, 1.0 ml/min; stationary phase, (A) ISRP (GFF-S5-80), (B) Cosmosil 5 Ph; column, 15 cm×4.6 mm I.D.; detection, UV (220 nm).

phase, whereas that with the phenylsilica column remained small and almost unchanged over the whole pH range examined, and a slight increase in k' ascribable to deprotonation of the ammonium group was observed at pH 8. This confirmed that the weak ion-exchange ability in addition to π-electron interactions contributed to the retention of phenethylamine on the ISRP column.

Effect of ionic strength

Changes in the salt concentration in the mobile phase are generally known to affect the retention of solutes on hydrophobic stationary ligands. The salt effects with the ISRP column were investigated with varying ionic strength of the mobile phase between 0.02 and 0.3 at a constant pH of 6.7. The results obtained with some aromatic compounds used as test samples are shown in Fig. 3, and are compared with those obtained on the phenylsilica column. The k' values measured on both columns for phenol and methylparaben, which do not have an electric charge at this pH, were almost unaffected by changes in the ionic strength. For the charged compounds, the k' values of *p*-toluic acid on both columns were increased to almost the same extent with increasing ionic strength, whereas those of phenethylamine exhibited a much greater decrease with the ISRP column (Fig. 3A) than with the phenylsilica column (Fig. 3B). These results suggest a complicated mechanism of the salt effect on the retention of ionic aromatic compounds on the ISRP column, as it involves at least two different modes of solute retention, as mentioned above.

Effect of organic modifier

Fig. 4 shows the relationship between acetonitrile concentration in the mobile phase (pH 7.2, μ=0.2) and log k' values of basic compounds (metoprolol, lido-

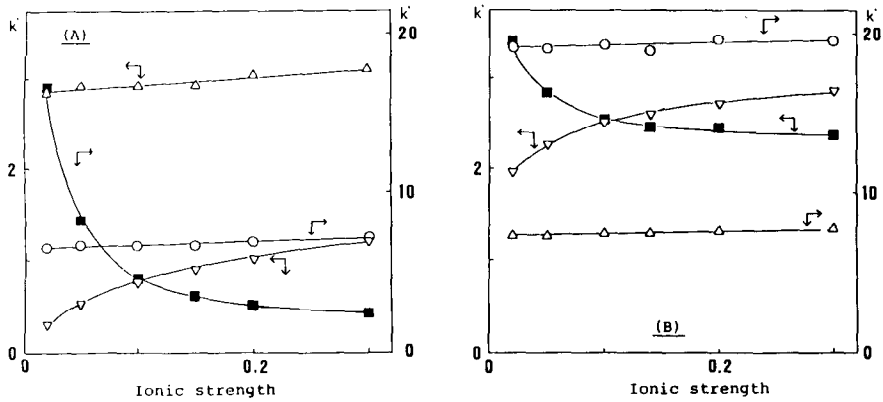

Fig. 3. Dependence of capacity factors on ionic strength of mobile phase observed with (A) ISRP column and (B) phenylsilica column. ■ = Phenethylamine; ▽ = *p*-toluic acid; ○ = methylparaben; △ = phenol. Mobile phase, (A) potassium phosphate buffer (pH 6.7, ionic strength 0.02–0.3), (B) potassium phosphate buffer (ionic strength 0.02–0.3)-acetonitrile (9:1), pH 6.7; flow-rate, 1.0 ml/min; stationary phase, (A) ISRP (GFF-S5-80), (B) Cosmosil 5 Ph; column, 15 cm × 4.6 mm I.D.; detection, UV (220 nm).

caine and phenethylamine), acidic compounds (naproxen, probenecid and cefpiramide) and a neutral compound (methylparaben) observed on the ISRP and phenylsilica columns. The linear decrease in log k' with increasing concentration of acetonitrile found in all instances means that hydrophobic interactions involving π-electrons predominate in the retention of aromatic solute on both columns. However, the former column retained all the solutes (except for phenethylamine) more weakly than the latter. The elution order from the phenylsilica column is obviously dependent on the aromaticity and electric charge of the solute but not on its ionic properties. In contrast, it is noticeable that on the ISRP column, the basic solutes showed much higher log k' values and a smaller dependence on the change in acetonitrile concentration than the acidic solutes. These results allow one to speculate that the ionic attraction of cationic solutes and the ionic repulsion of anionic solutes with the carboxylate ions on the ligand of the internal surface became stronger with decreasing polarity of the mobile phase. The reversal of the elution order of phenethylamine between the ISRP and phenylsilica columns, as mentioned above, suggests that the effect of such an ion-exchange property may exceed that of the hydrophobic interaction, and be responsible for the gentlest decrease in log k' among those in Fig. 4.

Similar results were also obtained with the use of tetrahydrofuran and isopropanol as the organic modifier. Fig. 5 shows a comparison of solvent effects on the log k' values of acidic, basic and neutral aromatic compounds on the ISRP column. The preferential retention of basic solutes was also found with these solvent systems. In the practical application of the ISRP column to plasma samples, the selection of the organic modifier and its concentration in the mobile phase is limited in order to retain the column efficiency. The recommended concentration to prevent deterioration by protein coalescence is $<25\%$ for acetonitrile, $<20\%$

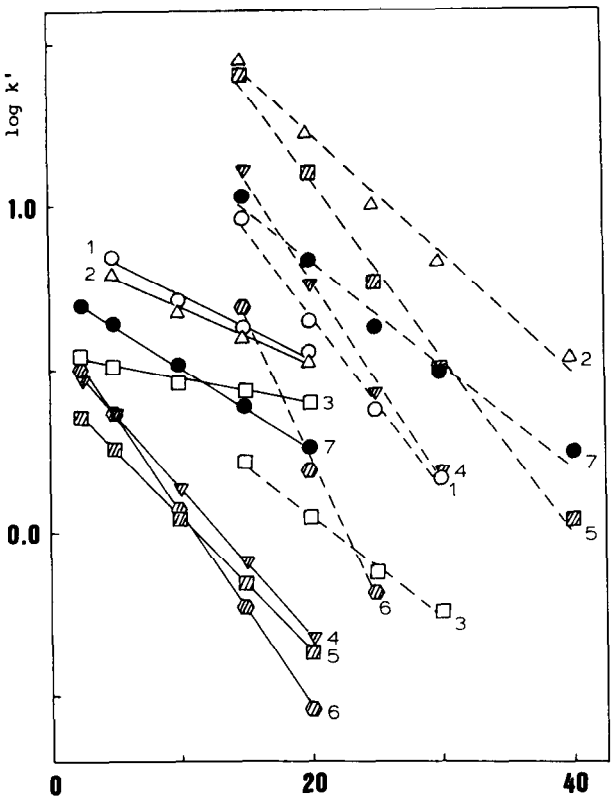

Fig. 4. Dependence of log k' on acetonitrile concentration in mobile phase observed with ISRP column (solid lines) and phenylsilica column (dashed lines). 1 = Metoprolol; 2 = lidocaine; 3 = phenethylamine; 4 = naproxen; 5 = probenecid; 6 = cefpiramide; 7 = methylparaben. Mobile phase, 0.01 *M* potassium phosphate buffer containing 0–20% acetonitrile, pH 7.2, ionic strength 0.20, adjusted by addition of sodium sulphate; flow-rate, 1.0 ml/min; stationary phase, (a) ISRP (GFF-S5-80), (b) Cosmosil 5Ph; column, 15 cm × 4.6 mm I.D.; detection, UV (220 nm).

for isopropanol, $<10\%$ for tetrahydrofuran and $<25\%$ for the total [6]. As for the separation of a relatively hydrophilic compound from plasma components, isopropanol or acetonitrile seems to provide better selectivity than tetrahydrofuran. However, the effect of the organic modifier on the recovery of drug from plasma sample should also be taken into account, as mentioned later.

Effect of ion pairs

The ion-pair effects on the retention of several compounds having different ionic properties were compared between the ISRP and phenylsilica columns. Fig. 6 shows the dependence of the k' values of some β-lactam antibiotics on the concentration of tetrabutylammonium bromide (TBAB) in the mobile phase. The k' vs. TBAB concentration profiles obtained with the phenylsilica column (Fig. 6B) are those often found in the usual reversed-phase ion-pair mode, whereas the k' values on the ISRP column (Fig. 6A) showed a smaller increase than on the phenylsilica column. For instance, the degree of enhancement of retention

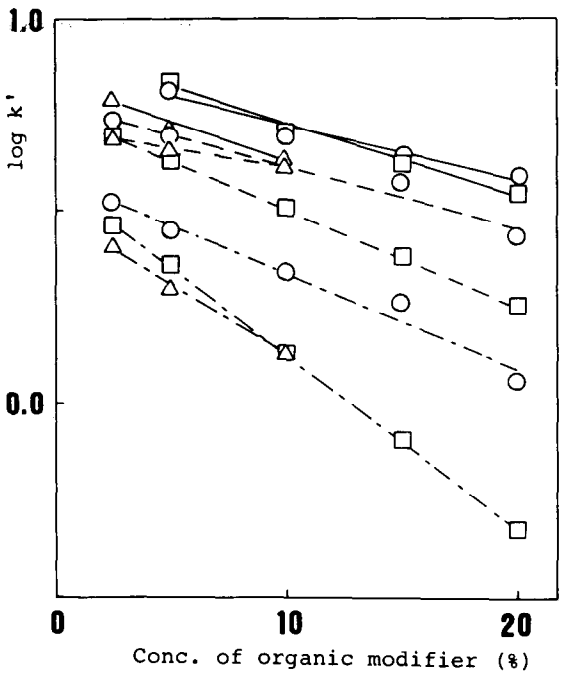

Fig. 5. Dependence of log k' of metoprolol (——), methylparaben (- - - -) and naproxen (— - —) on concentration of isopropanol (○), acetonitrile (□) and tetrahydrofuran (△) observed with ISRP column. Mobile phase, 0.01 M potassium phosphate buffer containing 0–20% acetonitrile, 0–20% isopropanol or 0–10% tetrahydrofuran, ionic strength 0.20, adjusted by addition of sodium sulphate; stationary phase, ISRP (GFF-S5-80); column, 15 cm×4.6 mm I.D.; detection, UV (220 nm).

due to the presence of 10 mM TBAB on the ISRP and phenylsilica columns were 1.72 and 3.73 for dichloxacillin, 1.49 and 3.04 for cefpiramide and 1.41 and 3.23 for benzylpenicillin, respectively. On the other hand, the addition of an anionic ion-pairing agent (heptanesulphonate) to the mobile phase gave rise to different effects on the retention of cationic compounds between the ISRP column (Fig. 7A) and the phenylsilica column (Fig. 7B). On the phenylsilica column, the retentions of metoprolol, lidocaine and phenethylamine were markedly increased, as expected, with increasing concentration of heptanesulphonate, whereas those of methylparaben and phenylalanine (pI=5.5) were unaffected because of almost no apparent charge at this pH (6.0). In contrast, at the same pH of the mobile phase, the retention of these basic compounds and neutral compounds on the ISRP column underwent no apparent ion-pair effect. It follows from these results that the cationic counter ion reduced the ionic repulsion between the anionic solute and the carboxylate group on the hydrophobic ligand of the ISRP silica, which contributed to the prolonged retention of acidic compounds. In contrast, the anionic ion-pairing agent interfered with the ionic interaction between the cationic solute and the stationary ligand, resulting in a decrease in ion-exchange capability. Such considerations will be applicable to compounds that undergo weak reversed-phase retention.

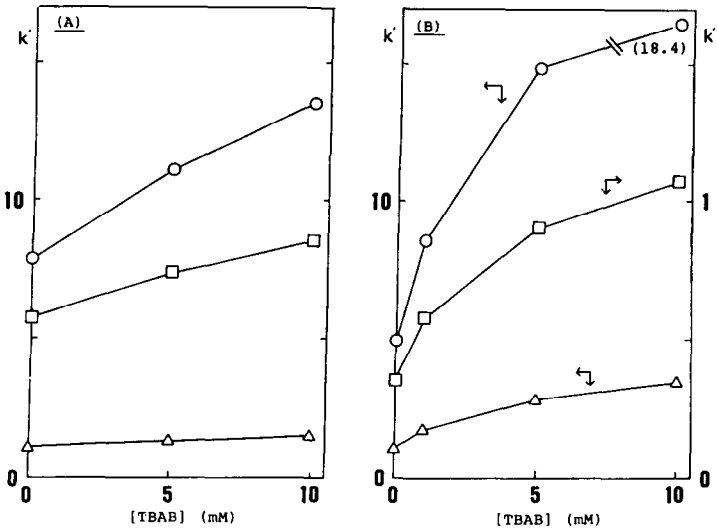

Fig. 6. Dependence of capacity factors on concentration of tetrabutylammonium bromide in mobile phase observed with (A) ISRP column and (B) phenylsilica column. Mobile phase, (A) 0.1 *M* potassium phosphate buffer containing 0–10 m*M* TBAB, pH 7.0, (B) 0.1 *M* potassium phosphate buffer–acetonitrile (7:3) containing 0–10 m*M* TBAB, pH 7.0; flow-rate, 1.0 ml/min; stationary phase, (A) ISRP (GFF-S5-80), (B) Cosmosil 5Ph; column, 15 cm × 4.6 mm I.D.; detection, UV (220 nm). △ = Benzylpenicillin; ○ = dicloxacillin; □ = cefpiramide.

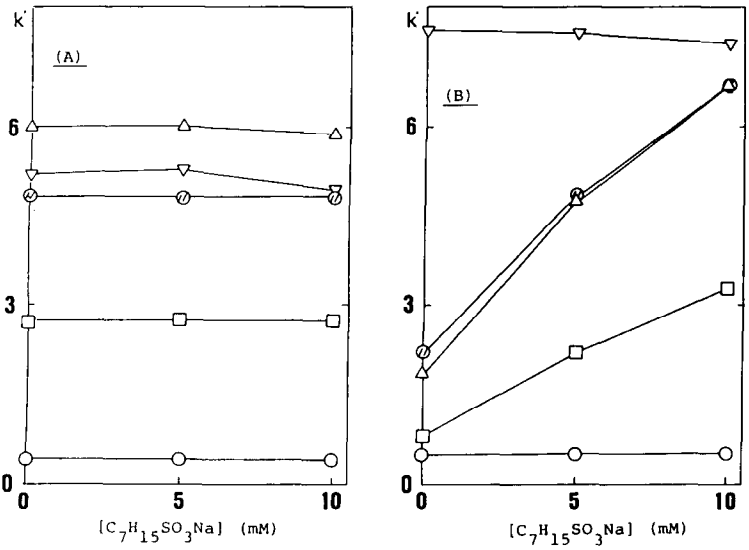

Fig. 7. Dependence of capacity factors on concentration of sodium heptanesulphonate in mobile phase observed with (A) ISRP column and (B) phenylsilica column. △, Metoprolol; ▽, methylparaben; ⓓ, lidocaine; □, phenethylamine; ○, phenylalanine. Mobile phase, (A) 0.1 *M* potassium phosphate buffer–isopropanol (97.5:2.5) containing 0–10 m*M* sodium heptanesulphonate, pH 6.0, (B) 0.1 *M* potassium phosphate buffer–isopropanol (8:2) containing 0–10 m*M* sodium heptanesulphonate, pH 6.0; flow-rate, 1.0 ml/min; stationary phase, (A) ISRP (GFF-S5-80), (B) Cosmosil 5Ph; column, 15 cm × 4.6 mm I.D.; detection, UV (220 nm).

Effect of surfactant

It is known that a surfactant-containing mobile phase, which solubilizes serum proteins, can afford the determination of drugs by direct introduction of serum samples on to a hydrophobic stationary phase [9]. Although the separation on the ISRP column is achieved on the basis of a different principle, we examined the effect of anionic surfactants on the retention of drugs in order to expand the selectivity of the mobile phase conditions. Fig. 8 shows the plots of the capacity factor of cefaclor (CCL) (for structure, see Fig. 8) vs. pH of the mobile phase containing 20 m*M* sodium dodecylsulphate (SDS), the results obtained without SDS and with sodium heptanesulphonate of the same concentration also being shown for reference. It is interesting that the addition of SDS gave rise to a marked increase in k' in a low pH region, and resulted in a much longer retention than that given by a mobile phase containing heptanesulphonate, which probably did not form micelles in the mobile phase. Such a retention enhancement became weaker with increasing pH of the mobile phase.

Fig. 9 demonstrates the separation of CCL from plasma proteins with the use of phosphate buffer solution containing 20 m*M* SDS as the mobile phase. The complete separation was achieved in a short period of time (retention time of CCL, 13 min) with a recovery of CCL of $101 \pm 2.4\%$ ($n=5$), although the chromatogram of plasma proteins (Fig. 9A) under the same conditions was different from those shown in Figs. 10–12. It is noticeable that the pH of the mobile phase (4.38) is lower than that previously recommended (pH 6.0–7.5) [4]. Clogging of the column and deterioration of the separation efficiency were not observed, even after repeated injection of plasma samples (total volume about 1 ml), when the mobile phase contained SDS. Nevertheless, it is obviously recommended that the column is cleaned with isopropanol or tetrahydrofuran after use in order to maintain its high efficiency.

Recovery of drug

The proteins in blood have some different sites for binding drugs in different modes, and a drug in blood is bound to plasma (or serum) protein to a greater or lesser extent. There is an equilibrium between bound and unbound species, and the unbound drug is believed to show pharmacological activity. Prior to the determination of drug concentrations in plasma, it is necessary to investigate the recovery of the drug from the sample. The pre-treatment procedure for the deproteinization and solvent extraction often involved in the conventional methods allows, in most instances, the total amount of the drug to be recovered, because the drug is released by protein denaturation. In most instances, drugs can easily be released by dilution of plasma samples with a buffer solution, because of the break of equilibrium of the protein binding. When using an ISRP column, which allows direct injection of plasma samples without pre-treatment, the cleavage of protein binding seems to occur in mobile phases with an aqueous organic solvent. Although the details of the mechanism are not clear, it is certain that the drug can be released without precipitation of plasma proteins, because the binding is possibly cleaved by the partial denaturation of the protein at the particular site.

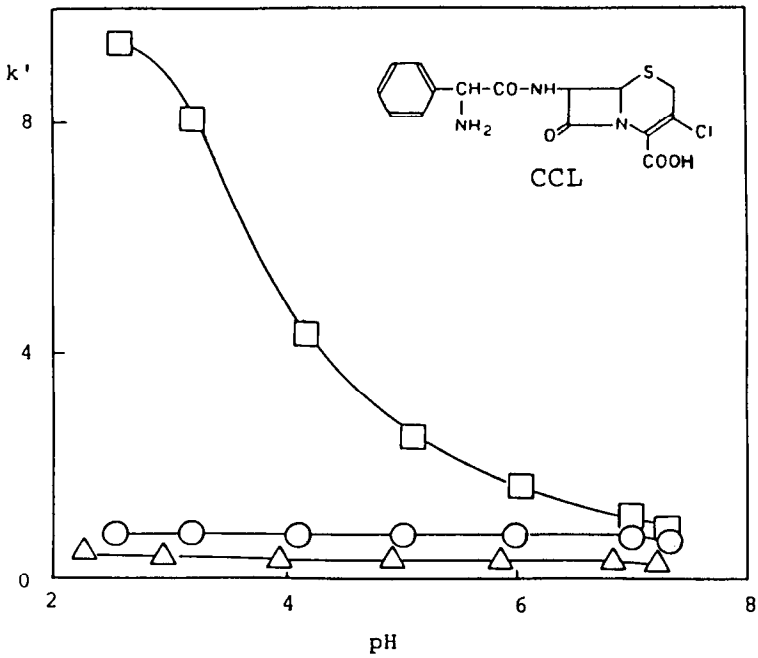

Fig. 8. Effect of sodium dodecylsulphate (SDS) on the capacity factor (k') of cefaclor (CCL) at various pH of the mobile phase. Mobile phase, (a) 0.05 *M* sodium phosphate buffer (○), (b) (a) +20 m*M* SDS (□) and (c) (a) +20 m*M* sodium heptanesulphonate (△); flow-rate, 1.0 ml/min; stationary phase, ISRP (GFF-S5-80); column, 15 cm×4.6 mm I.D.; detection, UV (220 nm).

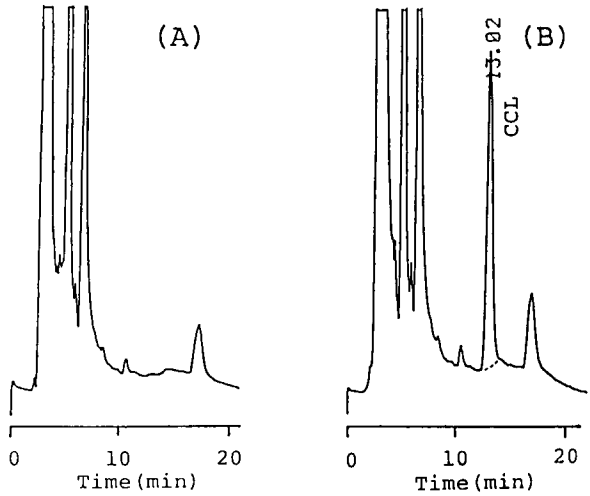

Fig. 9. Separation of cefaclor (CCL) from human plasma. Mobile phase, 0.1 *M* sodium phosphate buffer (pH 4.38) containing 20 m*M* SDS; flow-rate, 0.8 ml/min; stationary phase, ISRP (GFF-S5-80); column, 25 cm×4.6 mm I.D.; detection, UV (254 nm); injection, 10 μl of (A) human plasma and (B) 20 μg/ml of CCL human plasma solution.

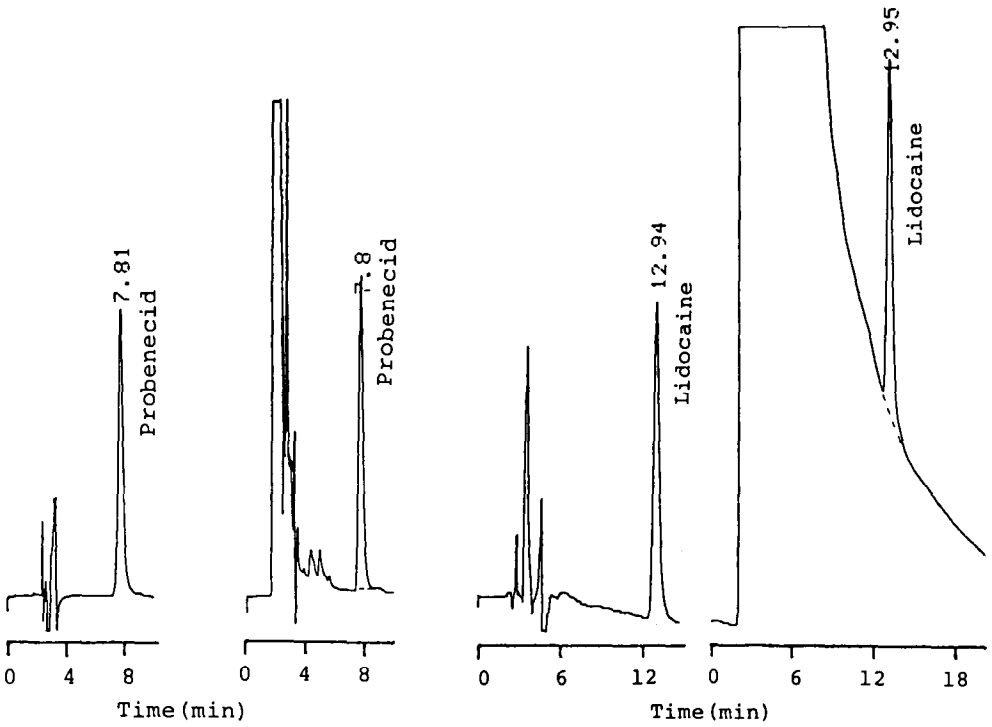

Fig. 10. Separation of probenecid from human plasma. Mobile phase, 0.1 *M* potassium phosphate buffer-tetrahydrofuran (95:5), pH 7.0; flow-rate, 1.0 ml/min; stationary phase, ISRP (GFF-S5-80); column, 15 cm×4.6 mm I.D.; detection, UV (254 nm); injection volume, 10 μl.

Fig. 11. Separation of lidocaine from human plasma. Mobile phase, 0.1 *M* potassium phosphate buffer-tetrahydrofuran (9:1), pH 7.2; flow-rate, 0.8 ml/min; stationary phase, ISRP (GFF-S5-80); column, 15 cm×4.6 mm I.D.; detection, UV (220 nm); injection volume, 10 μl.

Naturally, the entire denaturation may make the protein so hydrophobic as to result in precipitation.

The effect of the mobile phase conditions on the recovery of drugs in the ISRP column has not previously been reported, although it was found that 98% of the total amount of phenytoin added to human serum was recovered when the ISRP column was used with a mobile phase consisting of 20% acetonitrile in 0.1 *M* phosphate buffer (pH 6) [3]. Phenytoin is known to be bound mainly to the "warfarin site" of human albumin [10] in excess of 90% [11]. We therefore examined the recovery of probenecid and lidocaine which are bound to the "diazepam site" of human albumin [10] and to the α_1-acid glycoprotein [12], respectively. It was reported that 83–94% of probenecid [13] and 65–77% of lidocaine [12] are bound in human serum. The recovery was calculated from the peak-area ratio of a given concentration of the drug dissolved in plasma and methanol. The chromatograms of plasma spiked with these drugs at clinical levels (50 μg/ml for probenecid, 5.94 μg/ml for lidocaine) are shown in Figs. 10 and 11, together with

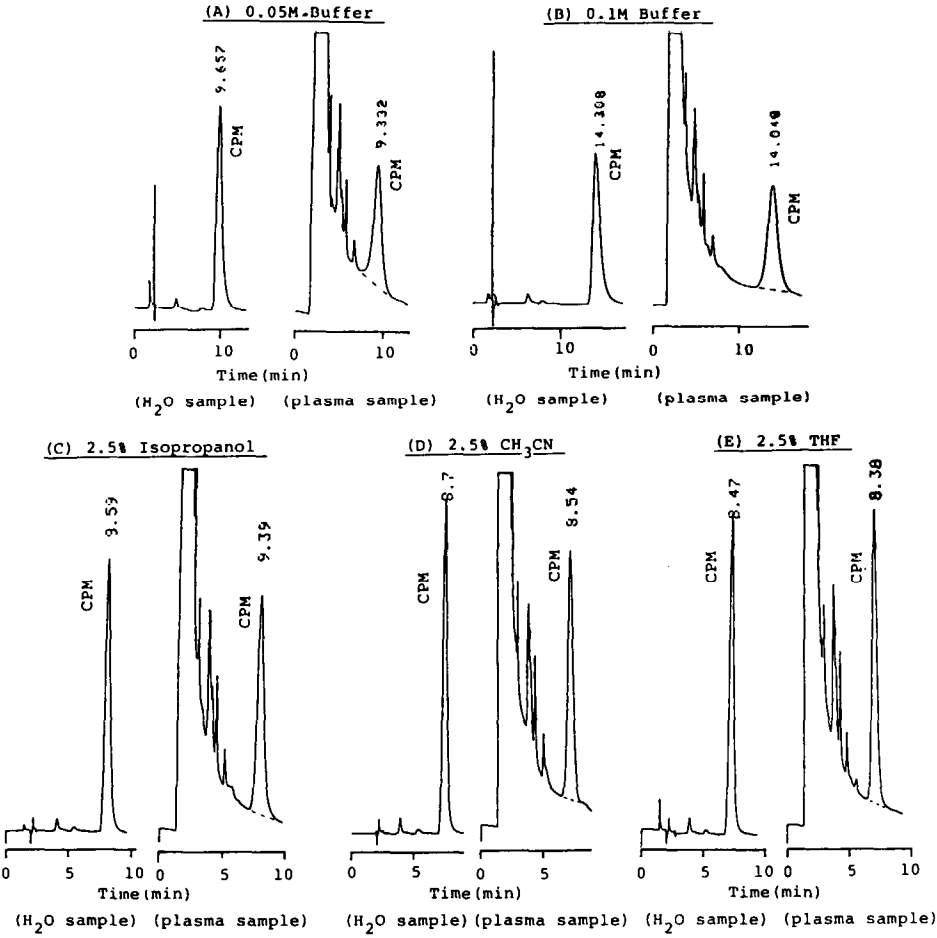

Fig. 12. Separation of cefpiramide (CPM) from human plasma. Mobile phase: (A) 0.05 *M* potassium phosphate buffer, pH 7.0; (B) 0.1 *M* potassium phosphate buffer, pH 7.0; (C) 0.1 *M* potassium phosphate buffer–isopropanol (97.5:2.5), pH 7.0; (D) 0.1 *M* potassium phosphate buffer–acetonitrile (97.5:2.5), pH 7.0; (E) 0.1 *M* potassium phosphate buffer–tetrahydrofuran (97.5:2.5), pH 7.0. Flow-rate, 1.0 ml/min; stationary phase, ISRP (GFF-S5-80); column, 25 cm × 4.6 mm I.D.; detection, UV (270 nm); injection volume, 10 μl.

those for methanolic solutions of the same concentration. The large difference in the intensities of the background peaks in these chromatograms is due to the difference in the detection wavelengths. Naturally, the shorter wavelength (220 nm for lidocaine) can detect more peaks with stronger intensity than the longer wavelength (254 nm for probenecid).

The quantitative recoveries of both drugs averaged over repeated determinations ($n = 5$) are given in Table I together with the coefficient of variation (C.V.) for the peak area. Almost complete recoveries with low C.V. indicate that the total amounts of these drugs can be determined under the mobile phase conditions employed regardless of the difference in their bindings to plasma protein.

Fig. 12 shows the separation of cefpiramide from the plasma components on

TABLE I

REPRODUCIBILITY (C.V. FOR PEAK AREA) AND RECOVERY OF PROBENECID AND LIDOCAINE IN HUMAN PLASMA

HPLC conditions as in Figs. 10 and 11.

Drug	C.V. ($n=5$) (%)		Recovery from human plasma sample (%)
	Plasma sample	Methanol sample	
Probenecid	1.47	1.10	98.0
Lidocaine	2.53	1.42	100.0

the ISRP column under different mobile phase conditions (A–E), where a 10-μl portion of a 30 μg/ml plasma solution was injected in each run. It has been reported that over 96% of cefpiramide is bound in human serum [14]. The effects of these mobile phase conditions on the column efficiency (HETP) and on the recovery of cefpiramide from plasma samples are shown in Table II. An increase in the phosphate buffer concentration from 50 mM (A in Table II) to 100 mM (B) resulted in an increase in both the retention time and the recovery of cefpiramide together with a slight enhancement of column efficiency, although complete recovery was not obtained. The addition of a small amount of organic modifier further improved the peak shape and recovery. With mobile phases consisting of 0.1 M phosphate buffer containing 2.5% of isopropanol (Fig. 12C), acetonitrile (Fig. 12D) or tetrahydrofuran (Fig. 12E), cefpiramide was sharply separated from plasma proteins in a short period of time and was recovered completely with good reproducibility. These results suggest that the organic solvent accelerates the rate of release of the drug from plasma proteins. Such an effect is clearly shown in Figs. 10 and 11, where probenecid and lidocaine were released very quickly in the mobile phase containing higher concentrations (5 or 10%) of

TABLE II

EFFECT OF MOBILE PHASE CONDITIONS (A)-(E) ON COLUMN EFFICIENCY (HETP), REPRODUCIBILITY (C.V. FOR PEAK AREA) AND RECOVERY OF CEFPIRAMIDE FROM HUMAN PLASMA

Mobile phase conditions as in Fig. 12.

Mobile phase conditions	HETP* (μm)		C.V. ($n=5$) (%)		Recovery from human plasma (%)
	Plasma solution	Aqueous solution	Plasma solution	Aqueous solution	
(A) 0.05 M buffer	470	200	1.64	1.29	71.4
(B) 0.1 M buffer	350	170	7.22	0.78	84.7
(C) 2.5% isopropanol	190	110	0.53	0.41	99.5
(D) 2.5% acetonitrile	190	100	0.90	0.24	96.5
(E) 2.5% tetrahydrofuran	140	118	1.28	0.44	100.5

*Height equivalent to a theoretical plate for the peak of cefpiramide.

tetrahydrofuran, so that each of these drugs in plasma samples had almost the same retention time and HETP value as those in methanol solutions. Hence it is considered that the use of an organic modifier is advantageous for the rapid separation of hydrophobic drugs with high recoveries and the use of an aqueous mobile phase is effective for the separation of hydrophilic drugs (including drug metabolites) but disadvantageous with respect to the recovery. It seems difficult to separate extremely hydrophilic compounds from plasma proteins by using an ISRP column. During this study, neither a significant increase in column pressure nor a decrease in column efficiency was observed after hundreds of plasma sample injections. It has been reported [6] that the use of titanium frits and a PTFE filter instead of stainless-steel frits and the use of a short guard column packed with ISRP silica allow approximately 1200 serum sample injections without a decrease in column efficiency.

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