*Journal of Chromatography*, 488 (1989) 341–348
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4591

# DIRECT SERUM INJECTION IN MICELLAR LIQUID CHROMATOGRAPHY

## RECOVERY OF SERUM PROTEINS AND ASSAY OF HYDROPHILIC DRUGS

JUN HAGINAKA*, JUNKO WAKAI and HIROYUKI YASUDA

*Faculty of Pharmaceutical Sciences, Mukogawa Women's University, 11-68, Koshien Kyuban-cho, Nishinomiya, Hyogo 663 (Japan)*

(First received August 16th, 1988; revised manuscript received November 22nd, 1988)

SUMMARY

The recovery of serum proteins from reversed-phase and internal-surface reversed-phase (ISRP) silica supports following direct serum injection was investigated using an eluent containing a micellar solution of sodium dodecyl sulphate (SDS). The results indicated that the recoveries of serum proteins were 98–103% for both supports. On the basis of the above findings, the separation and recovery of hydrophilic drugs (cephalosporins and salicylic acid) from human serum were investigated using acidic eluents including micellar solutions of SDS. They were completely separated from the components of serum, and the recoveries were 94–98% despite protein binding. Although the recommended eluent pH range is 6.0–7.5 for the ISRP support, eluents of pH 2–8 can be used with the micellar chromatographic system.

## INTRODUCTION

For the high-performance liquid chromatographic (HPLC) assays of drugs in biological samples, tedious pretreatment procedures such as extraction or deproteinization are needed, because the direct injection of proteinaceous samples such as serum on to conventional reversed-phase (RP) silica supports results in the accumulation of denatured proteins, which leads to obstruction of the interparticulate space and column clogging, thus decreasing the column efficiency [1, 2]. Hagestam and Pinkerton [3] prepared an internal-surface reversed-phase (ISRP) silica support which can be utilized for the determination of drugs in serum by direct injection. The novel property of the ISRP is due to the internal and external surfaces of the support: the former has a hydrophobic partitioning phase (glycyl-L-phenylalanyl-L-phenylalanine attached to the glycerylpropyl group) and

0378-4347/89/$03.50 

the latter a hydrophilic phase (glycerylpropyl group and glycine residue) that is non-adsorptive towards proteins. To avoid the precipitation of serum proteins, they recommended the use of an eluent with a pH from 6.0 to 7.5, an ionic strength from 0.1 to 0.2 and an organic modifier concentration from 0 to 25%. However, it is difficult to separate hydrophilic drugs (acidic drugs having a carboxyl group such as salicylic acid, or amphoteric drugs having carboxyl and amino groups in a molecule such as aminocephalosporins) from the components of serum under the recommended conditions described above, because they are hardly retained on the ISRP support.

On the other hand, Cline Love and co-workers [4, 5] reported an HPLC method involving the direct injection of untreated serum samples using a solvent containing a micellar solution of sodium dodecyl sulphate (SDS) or Brij-35 on a conventional RP column (e.g., $C_{18}$ or CN column). They stated that in this system the serum proteins were solubilized without the formation of a precipitate and the drugs bound to proteins were displaced by the surfactant monomers and/or micelles. In a previous study [6], we found that on being separated from the components of serum using an acidic eluent (pH 3.3–2.9) containing a micellar solution of SDS following direct serum injection, cephalosporins were not always observed as single peaks, depending on the composition of the eluent (especially the pH).

This paper describes the recovery of serum proteins on the ISRP and RP supports using an eluent containing a micellar solution of SDS, and the separation and recovery of hydrophilic drugs from the components of serum with direct serum injection.

## EXPERIMENTAL

### *Materials and reagents*

Cephalothin (CET), cefamandol (CMD), cefotaxime (CTX), cephalexin (CEX), cefradine (CED) and cefmenoxime (CMX) were kindly donated by Shionogi (Osaka, Japan), Hoechst Japan (Tokyo, Japan), Sankyo (Tokyo, Japan) and Takeda Chemical Industries (Osaka, Japan). The structures are shown in Fig. 1. Salicylic acid and control human serum (Control Serum I) was purchased from Wako (Osaka, Japan). BCA (bicinchoninic acid) protein assay reagent [7] was obtained from Pierce (Rockford, IL, U.S.A.). SDS and other reagents of analytical-reagent grade (except for 2-propanol) were obtained from Nacalai Tesque (Kyoto, Japan).

Water purified by a Barnstead Nanopure unit (Barnstead, Boston, MA, U.S.A.) and 2-propanol of HPLC quality were used for the preparation of the eluent.

### *Chromatography*

The liquid chromatograph consisted of a Bip-I pump (Japan Spectroscopic, Tokyo, Japan), a Model 7125 loop injector (Rheodyne, Cotati, CA, U.S.A.) equipped with a 100-$\mu$l loop and a Uvidec-100-V spectrophotometer equipped with an 8-$\mu$l flow-through cell. The columns used were as follows: column I, 15 cm$\times$4.6 mm I.D. packed with Develosil ODS (5 $\mu$m) (Nomura Chemicals, Seto,

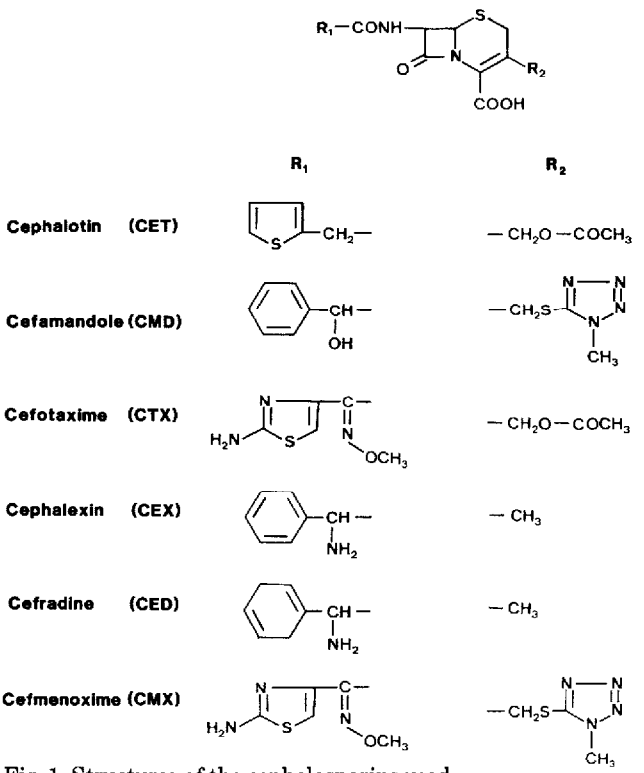


Fig. 1. Structures of the cephalosporins used.

Aichi, Japan); column II, 15 cm×4.6 mm I.D. ISRP [Pinkerton column (GFF-S5-80, 5 μm)] (Regis Chemical, Morton Grove, IL, U.S.A.); column III, 5 cm×4.6 mm I.D. packed with Nucleosil 100-5 $C_{18}$ (5 μm) (Macherey-Nagel, Düren, F.R.G.). Columns I and II were used together with guard columns packed with the same packing materials. The eluents used are specified in the figure captions. All the separations were carried out at ambient temperature.

*Recovery of serum proteins*

The recovery of human serum proteins from the ISRP and RP supports was examined as follows. A 20-μl portion of a control human serum sample was loaded on to column II or III using 40 m*M* SDS–50 mM $NaH_2PO_4$ plus 50 m*M* $Na_2HPO_4$ (pH 6.8) or 40 m*M* SDS–95 m*M* $NaH_2PO_4$ plus 5 m*M* $H_3PO_4$ (pH 3.4) as the eluent at a flow-rate of 0.8 ml/min; the eluate was collected at 8-min intervals for 24 min. A 0.2-ml portion of the eluate was mixed with 4 ml of BCA reagent, the mixture was incubated for 60 min at 37°C and the absorbance of the sample was measured at 562 nm using the eluent treated in the same manner as described above as a reference solution. The recovery was calculated from the absorbance ratio with and without the column.

*Preparation of serum samples*

Control serum samples in which a known amount of drug was dissolved were filtered with a 0.22-$\mu$m membrane filter. An appropriate volume of the filtrate was loaded on to the column.

## RESULTS AND DISCUSSION

*Recovery of human serum proteins from ISRP and RP supports*

The ISRP support prepared by Hagestam and Pinkerton [3] has the disadvantage that the eluent pH range recommended is limited to 6.0–7.5. It is reported that the ISRP support might be used in acidic regions (pH 2.0–3.0) [8]. However, the recovery of serum proteins was only about 0 and 20%, respectively, using the eluents (0.1 *M* phosphate buffer) at pH 3.5 and 2.5. On the other hand, direct serum injection was effected on the conventional RP support using a solvent containing a micellar solution of SDS or Brij-35 [4, 5]. However, the recovery of serum proteins from the support was not examined precisely. As our preliminary study revealed that the use of Brij-35 gave a low column efficiency compared with that of SDS, in the subsequent study SDS was used as an additive to the solvent for direct serum injection. The recovery of serum proteins from the ISRP and RP ($C_{18}$) supports was examined using acidic and neutral eluents containing micellar solutions of SDS. The results, summarized in Table I, revealed that the serum proteins were completely recovered from both the supports with both neutral and acidic eluents. Similar results were obtained in the presence of an organic modifier. Although the recommended eluent pH range is 6.0–7.5 for the ISRP support, eluents of pH 2–8 can be used in the present system.

*Recovery of drugs from the ISRP support*

We also investigated the application of the micellar chromatographic system to the assay of hydrophilic drugs by direct serum injection.

Fig. 2 shows the plots of the capacity factors of CET, CMD and CTX against pH of the eluent with and without addition of 20 m*M* SDS. When the eluent pH

TABLE I

RECOVERY OF SERUM PROTEINS FROM ISRP AND RP SUPPORTS

The values given are means of five analyses ± S.D.

| Support | Recovery (%) | |
|---|---|---|
| | Eluent A[a] | Eluent B[b] |
| ISRP[c] | 102 ± 3.4 | 98.4 ± 5.3 |
| RP[d] | 103 ± 6.1 | 97.6 ± 3.8 |

[a]40 m*M* SDS–50 m*M* $NaH_2PO_4$ plus 50 m*M* $Na_2HPO_4$ (pH 6.8).
[b]40 m*M* SDS–95 m*M* $NaH_2PO_4$ plus 5 m*M* $H_3PO_4$ (pH 3.4).
[c]Column II.
[d]Column III.

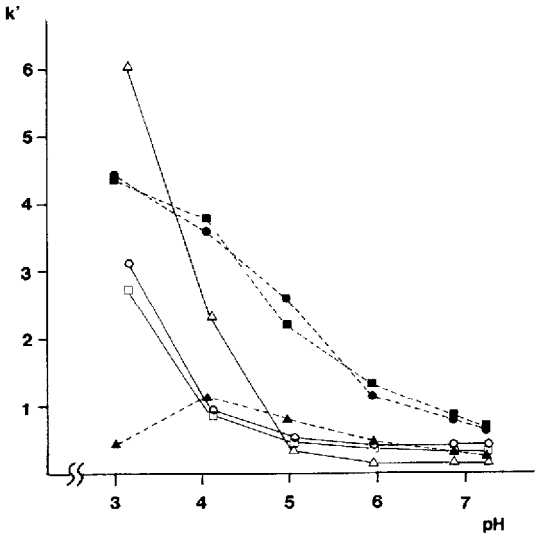


Fig. 2. Dependence of capacity factors on the eluent pH observed with (solid lines) and without (broken lines) addition of SDS. ○ and ●, CMD; △ and ▲, CTX; □ and ■, CET. Column: column II. Eluent: 50 m*M* phosphate buffer + 20 m*M* SDS (solid lines) and 50 m*M* phosphate buffer (broken lines). Flow-rate: 0.8 ml/min.

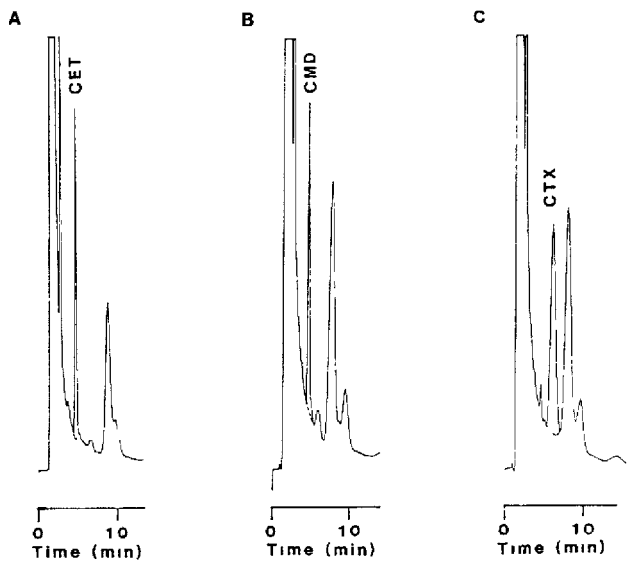


Fig. 3. Separation of (A) CET, (B) CMD and (C) CTX from the components of serum on the ISRP support. Column: column II. Eluent: (A) and (B) 40 m*M* SDS–43 m*M* $NaH_2PO_4$ plus 7 m*M* $H_3PO_4$ (pH 3.0); (C) 40 m*M* SDS–45 m*M* $NaH_2PO_4$ plus 5 m*M* $H_3PO_4$ (pH 3.2). Flow-rate: 0.8 ml/min. Detection: 254 nm. Sensitivity: (A) 0.064 a.u.f.s.; (B) and (C) 0.032 a.u.f.s. Concentration: 25 μg/ml. Injection volume: 10 μl. Broken lines indicate serum blank.

TABLE II

RECOVERY OF CEPHALOSPORINS FROM HUMAN SERUM

The values given are means of five analyses ± S.D.

| Support | Cephalosporin | Recovery (%) |
|---|---|---|
| ISRP[a] | CET | 95.8 ± 4.4 |
| | CMD | 98.0 ± 2.4 |
| | CTX | 96.7 ± 0.9 |
| RP[b] | CEX | 97.0 ± 2.4 |
| | CED | 97.8 ± 3.2 |
| | CMX | 97.3 ± 5.4 |
| | CTX | 97.9 ± 5.7 |

[a]Column II.
[b]Column I.

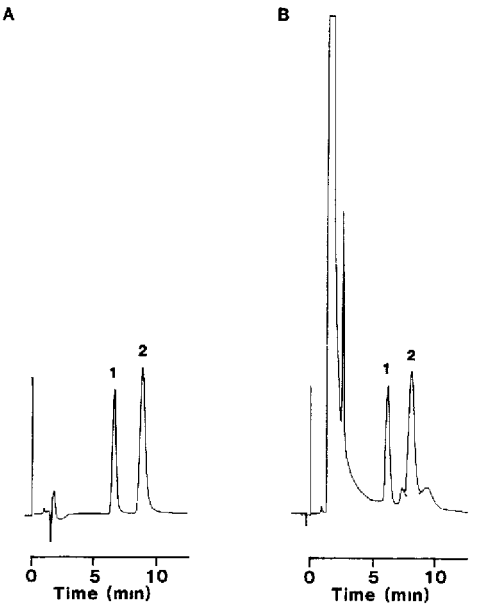


Fig. 4. Separation of salicylic acid and acetylsalicylic acid from the components of serum on the ISRP support. Column: column II. (A) Standard solution; (B) serum sample. Eluent: 50 m*M* SDS–43 m*M* $NaH_2PO_4$ plus 7 m*M* $H_3PO_4$ (pH 3.1). Peaks: 1 = acetylsalicylic acid; 2 = salicylic acid. Flow-rate: 0.8 ml/min Detection: 254 nm. Sensitivity: 0.08 a.u.f.s. Concentration: 50 μg/ml. Injection volume: 10 μl. Broken line indicates serum blank.

was decreased without addition of SDS, CET and CMD were more retained on the column, whereas the capacity factor of CTX had a maximum at pH 4 without addition of SDS. As CTX has an amino group which is protonated over the pH range studied, the retention of CTX could be affected by the dissociation of a carboxyl group of an L-phenylalanine residue on the ISRP support, as previously reported [9].

When an eluent of pH ca. 3 containing 20 m*M* SDS was used, these cephalosporins were well retained on the column. On the basis of these findings, the separations of CET, CMD and CTX from the components of serum were investigated. Fig. 3 shows the chromatograms obtained with direct serum injection. The recoveries of the cephalosporins were 96–98%, as shown in Table II.

Although salicylic acid and acetylsalicylic acid were eluted at the void volume over the recommended pH range (6.0–7.5), their separation from the components of serum was achieved by using an eluent of pH 3.1 containing 50 m*M* SDS, as shown in Fig. 4. The recovery of salicylic acid was 93.6% (S.D. = 2.5%, $n = 5$).

*Recovery of drugs from the RP support*

Fig. 5 shows the separation of CEX, CED, CMX and CTX from the components of serum. With direct serum injection, CEX and CED were observed as single peaks, as shown in Fig. 5A. However, the direct injection of CMX and CTX in a serum sample gave two peaks when an acidic eluent (pH 3.3–3.1) was used, as reported previously [6]. After acidification of the serum sample by the addition of one fifth volume of 1 *M* hydrochloric acid to one volume of the serum sample, they were observed as single peaks, as shown in Fig. 5B. The recovery of these cephalosporins, as listed in Table II, was 97–98%.

*Repetitive serum injection*

With both the ISRP and RP supports the analytical columns maintained their efficiency if the guard column was replaced after about 200 repetitive injections of a 10-$\mu$l serum sample.

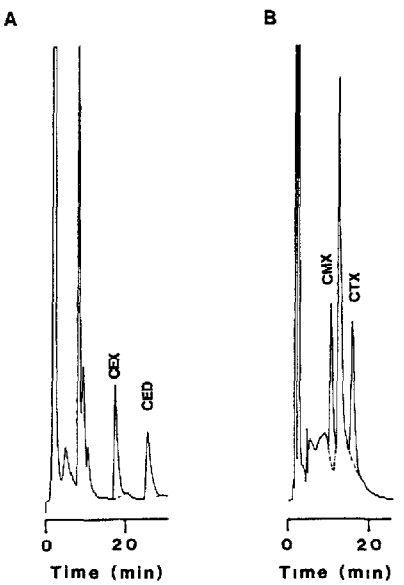


Fig. 5 Separation of (A) CEX and CED and (B) CMX and CTX from the components of serum on the RP support. Column: column I. Eluent: (A) 20 m*M* SDS–45 m*M* $NaH_2PO_4$ plus 5 m*M* $Na_2HPO_4$ (pH 6.1); (B) 150 m*M* SDS–45 m*M* $NaH_2PO_4$ plus 5 m*M* $H_3PO_4$ (pH 3.1). Flow-rate: 0.8 ml/min. Column temperature: 40°C. Detection: 254 nm. Sensitivity: 0.016 a.u f.s. Concentration: 20 $\mu$g/ml. Injection volume: 10 $\mu$l. (A) Direct injection; (B) injection after addition of one fifth volume of 1 *M* hydrochloric acid to one volume of the serum sample. Broken lines indicate serum blank.

ACKNOWLEDGEMENTS

The authors thank Koken (Tokyo, Japan) for the loan of the Pinkerton column. They thank also Miss M. Mizuno for technical assistance.

REFERENCES

1 T. Arvidsson, K.-G. Wahlund and N. Daoud, J. Chromatogr., 317 (1984) 213.
2 U. Juergens, J. Chromatogr., 310 (1984) 97.
3 I.H. Hagestam and T.C. Pinkerton, Anal. Chem., 57 (1985) 1757.
4 F.J. DeLuccia, M. Arunyanart and L.J. Cline Love, Anal. Chem., 57 (1985) 1564.
5 L.J. Cline Love, S. Zibas, J. Moroski and M. Arunyanart. J. Pharm. Biomed. Anal., 3 (1985) 511
6 J. Haginaka, J. Wakai, H. Yasuda and T. Nakagawa, Anal. Chem., 59 (1987) 2732.
7 P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K Mallia, F.H. Gartner, M.D. Provenzano, E.K Fujimoto, N.M. Goeke, B.J. Olson and D.C. Klenk, Anal. Biochem., 150 (1985) 76.
8 T.C. Pinkerton, T.D. Miller, S.E. Cook, J.A. Perry, J.D. Rateike and T.J. Szczerba, BioChromatography, 1 (1986) 96.
9 T. Nakagawa, A. Shibukawa, N. Shimono, T. Kawashima, T. Tanaka and J. Haginaka, J. Chromatogr., 420 (1987) 297