Separation and determination of aspoxicillin in human plasma by micellar electrokinetic chromatography with direct sample injection

HIROYUKI NISHI*, TSUKASA FUKUYAMA and MASAAKI MATSUO

Analytical Chemistry Research Laboratory, Tanabe Seiyaku, Co., Ltd., 16–89, Kashima 3-chome, Yodogawa-ku, Osaka 532 (Japan)

ABSTRACT

Both the separation and determination of aspoxicillin in human plasma by micellar electrokinetic chromatography (MEKC) were investigated. Selectivity in the separation of seven penicillin antibiotics was improved by using MEKC in comparison with capillary zone electrophoresis. Plasma proteins, which might interfere with drug analysis in conventional chromatography, were solubilized by the micelles employed in MEKC and eluted later than the drugs. This permitted the determination of the drugs in plasma by a direct sample injection method. One analysis of a plasma sample was performed within *ca.* 20 min without pretreatment. Good linearity and recovery were also obtained in the range of plasma levels usually encountered in clinical analysis with a correlation coefficient $r = 0.999$ and 94–104% recovery. The limit of detection for aspoxicillin was 1.3 μg ml^{-1} at a signal-to-noise ratio of 3.

INTRODUCTION

Micellar electrokinetic chromatography (MEKC) is a new type of separation method[1,2] based on micellar partitioning of the solute and electrophoretic migration of the micelle, and can be classified as an individual kind of chromatography on the basis of its separation principle, although it is performed with the same apparatus as capillary zone electrophoresis (CZE). Although this technique is called micellar electrokinetic capillary chromatography (MECC)[3], the term micellar EKC or MEKC will be used in order to stress that the partition mechanism in electrokinetic chromatography (EKC) has a wider scope than micellar solubilization as described elsewhere[4].

MEKC has many attractive advantages in addition to the capability of electrophoretic separation of electrically neutral substances. The selectivity and peak shapes are even better for the separation of ionic substances[5,6], although some of them can be separated by conventional CZE. Closely related isotopic compounds have been successfully separated by MEKC[7,8]. Chiral separation of some amino acid deriva-

tives[9,10] and drugs[11,12] has also been achieved by MEKC with a mixed micelle of sodium dodecyl sulphate (SDS) and a chiral additive or with chiral cholate micelles. The application of MEKC to the determination of drugs in preparations has been well studied by using an internal standard method[13-16] with the same reproducibility as in high-performance liquid chromatography (HPLC). Purity testing by the area percenta ge method has also been reported[16].

In a previous paper, we reported the migration behaviour of penicillins and cephalosporins in MEKC with sodium dodecyl sulphate (SDS) and sodium N-lauroyl-N-methyltaurate[6]. The selectivity was greatly improved for these ionic substances by MEKC in comparison with CZE.

In this paper, we describe the separation and determination of aspoxicillin in human plasma by MEKC with SDS. Aspoxicillin is a new broad-spectrum semi-synthetic penicillin, which has a amino acid residue N^4-methyl-D-asparagine in the molecule[17,18]. The determination of aspoxicillin in human serum, urine and bile by HPLC[19] and by bioassay[20] has been reported elsewhere. In MEKC, plasma proteins, which might interfere with drug analysis, are solubilized by the micelle, and hence elute later than the drugs. Consequently, a direct palsma sample injection can be performed without any pretreatment such as deproteinization or extraction. Nakagawa and co-workers[21,22] first reported the determination of drugs in plasma by MEKC using a direct injection method.
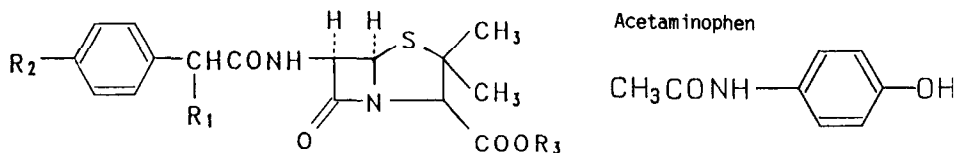
EXPERIMENTAL

Apparatus

An untreated fused-silica capillary tube (Scientific Glass Engineering, Ringwood, Victoria, Australia) with dimensions of 650 mm × 50 μm I.D. (effective length 500 mm) was used as a separation tube. Detection of separated solutes was achieved by on-column UV absorption measurement at 210 nm using a Uvidec-100-VI (Jasco, Tokyo, Japan) with a time constant of 0.05 s. A Chromatopac C-R5A (Shimadzu, Kyoto, Japan) was used for data processing. A high voltage was applied with a Model HJLL-25PO d.c. power supply (Matsusada Precision Devices, Kasatsu, Japan), which delivered up to +25 kV. Other instruments were the same as described previously[5].

Reagents and samples

SDS was purchased from Nacalai Tesque (Kyoto, Japan). Aspoxicillin (ASPC) was obtained from Tanabe Seiyaku (Osaka, Japan). Benzylpenicillin, ampicillin, carbenicillin, sulbenicillin, piperacillin and amoxicillin were obtained either commerci ally or from the National Institute of Hygienic Sciences (Tokyo, Japan), and were used as test samples. The structure of each penicillin is shown in Fig. 1 together with that of acetaminophen from Wako (Osaka, Japan), which was used as an internal standard (I.S.). All other reagents and solvents, of analytical-reagent grade, were obtained from Katayama Kagaku (Osaka, Japan) and used without further purification.

SDS was dissolved in a buffer solution prepared by mixing a 0.02 M sodium dihydrogen phosphate solution with a 0.02 M sodium tetraborate solution to give the appropriate pH value, and the micellar solutions were passed through a 0.45-μm membrane filter (Gelman Science Japan, Tokyo, Japan) and degassed by sonication before use. The samples were dissolved in water at a concentration of *ca.* 1 mg ml^{-1} to

Fig. 1. Structures of penicillins and acetaminophen.

Penicillins	Symbol	R_1	R_2	R_3
Benzylpenicillin	1	H	H	K
Ampicillin	2	NH_2	H	Na
Carbenicillin	3	COONa	H	Na
Sulbenicillin	4	SO_3Na	H	Na
Piperacillin	5	*1)	H	Na
Aspoxicillin	6	*2)	OH	H
Amoxicillin	7	NH_2	OH	H

*1)

NH
CO
N O
N O
C₂H₅

*2)

NH
CO
CHNH₂
CH₂CONHCH₃

give adequate peak heights for separation studies. Human plasma was prepared from fresh human blood in the usual manner. Known amounts of aspoxicillin and I.S. were dissolved in human plasma or water for concentration studies (for concentrations, see later).

Procedure

A sample solution was introduced manually into the positive end of the capillary tube by siphoning (about a 10-cm height for 5–10 s) as described previously[5] and MEKC was performed at ambient temperature (*ca.* 20°C).

The capillary tube was flushed with the operating buffer solution once per five runs by using a syringe and washed with an alkaline solution daily, usually at the end of an experiment as follows: the capillary tube was filled manually with 0.1 *M* potassium hydroxide solution using a syringe and allowed to stand for 30 min, flushed with water and allowed to stand for a further 5 min, then finally filled with the working buffer solution.

RESULTS AND DISCUSSION

CZE of seven penicillin antibiotics

The separation of seven penicillins by CZE was investigated with a 0.02 M phosphate–borate buffer solution of pH 7 ∼ 9, in which all the solutes must be completely ionized. A typical electropherogram at pH 8.5 is shown in Fig. 2A. All the solutes migrated toward the negative electrode, that is, the electroosmotic velocity was always higher than the electrophoretic velocity of any solute employed under the above experimental conditions. Although these ionic antibiotics can be separated by conventional CZE, their migration times are similar, except for carbenicillin and sulbenicillin, and the separation was not successful. This is because that these solutes, except for carbenicillin and sulbenicillin, have similar structures (molecular weights) and electric charges (one carboxyl group), leading to the similar electrophoretic mobilities. Carbenicillin and sulbenicillin, which have additional anionic groups in molecules, are retarded more strongly than other solutes by the electrophoretic effect. The migration times of carbenicillin and sulbenicillin are also similar. These results show that the migration of the solutes in CZE depends greatly on the electric charge in the buffer solution used, in which the solutes are completely ionized. Acetaminophen migrated with the same velocity as methanol, which is a tracer of the electroosmotic velocity[2], because this is electrically neutral.

The buffer pH and composition were very critical in relation to peak symmetry of the solutes. In the pH range examined, the peak symmetry of each solute, especially carbenicillin and sulbenicillin, was improved with increasing pH. Mikkers et al.[23] suggested that unsymmetrical peaks are usually generated when the electrophoretic mobilities of the solute and that of the buffer constituent are different.
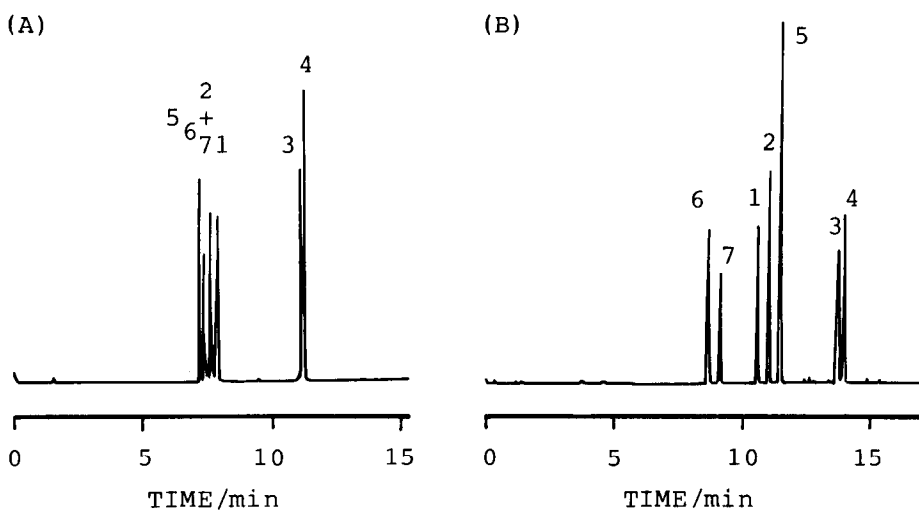

Fig. 2. Separation of seven penicillins by (A) CZE and (B) MEKC. (A) 0.02 M phosphate–borate buffer of pH 8.5; (B) 0.1 M SDS added to A. Applied voltage, 20 kV; temperature, ambient; detection, 210 nm; attenuation, 0.04 a.u.f.s. Sample numbers are given in Fig. 1.
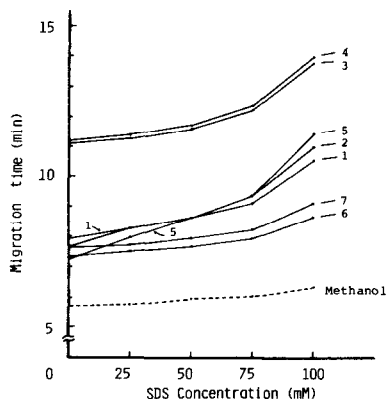
Fig. 3. Effect of SDS concentration on the migration times of penicillins. Buffer, 0.02 M phosphate–borate buffer (pH 8.5). Other conditions as in Fig. 2.

MEKC of seven penicillin antibiotics

   The effect of SDS concentration on the migration time of the solutes using a phosphate–borate buffer solution of pH 8.5, in which all the solutes are completely ionized, is shown in Fig. 3. The migration time increased gradually with increasing SDS concentration. The solubilization into the micelle or interaction with the micelle of the solutes probably increased with increase in SDS concentration even for these ionic solutes, as was observed previously[6]. The change in the migration time of piperacillin was remarkable, indicating that this was more readily incorporated into the SDS micelle owing to its high lipophilicity. A typical chromatogram at 0.1 M SDS is shown in Fig. 2B, showing an improvement in the selectivity in comparison with CZE (Fig. 2A).

   The pH dependence of the migration times of seven penicillins and acetaminophen (I.S.) was examined using 0.05 M SDS solutions in the pH range 7–9 (Fig. 4).
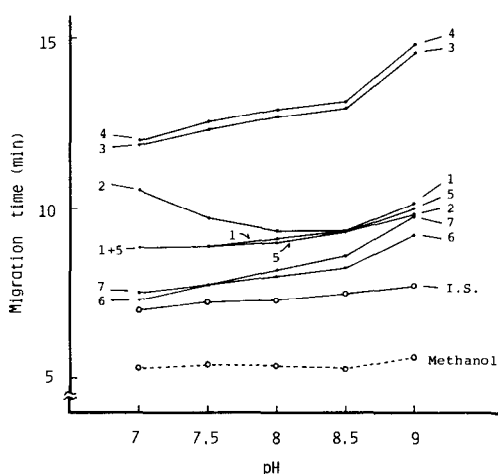

Fig. 4. pH dependence of the migration time of penicillins and acetaminophen (I.S.) in MEKC. Buffer, 0.02 M phosphate–borate buffer containing 0.05 M SDS. Other conditions as in Fig. 2.

The migration time of each solute except ampicillin increased with increasing pH, although those of methanol (electroosmotic velocity) and acetaminophen remained almost constant. It can be seen that the range of migration times increased. This can be ascribed to the increase in the electrophoretic velocity of the micelle with increasing pH[24]. For ampicillin, however, the migration time decreased from pH 7 to 8.5 and increased from pH 8.5 to 9. This may be due to the amino group in ampicillin.

MEKC of human plasma sample

The migration times of aspoxicillin, acetaminophen and methanol, which is considered as a tracer of the electroosmotic velocity, and the range of the migration times of blood proteins as a function of SDS concentration in a phosphate–borate buffer solution of pH 8.5 are shown in Fig. 5. In CZE, where SDS was absent, aspoxicillin coeluted with proteins. Acetaminophen migrated with the same velocity as that of methanol because it is electrically neutral under the above experimental conditions. After a plasma sample had been injected in CZE mode, aspoxicillin and acetaminophen, even in the standard solution, which was prepared by dissolving each standard compound in water, migrated with slower velocities in the following run. This was probably due to the adsorption of plasma proteins on the inside surface of the fused-silica capillary tube, leading to a decrease in the electroosmotic velocity. Therefore, it was necessary to wash the capillary tube with an alkaline solution to regenerate the capillary tube (surface) or to avoid such a phenomenon after the injection of plasma samples in the CZE mode.

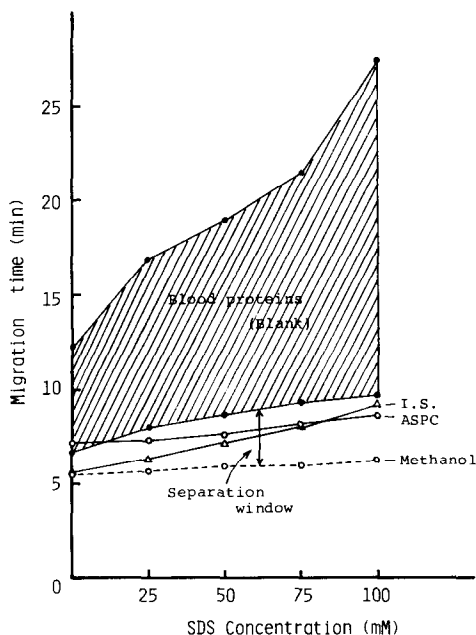The complete separation of aspoxicillin and acetaminophen from the protein

Fig. 5. Effect of SDS concentration in the migration times of aspoxicillin (ASPC), acetaminophen (I.S.) and plasma proteins. Other conditions as in Fig. 3.

peaks was attained through the addition of SDS, that is, by MEKC. The migration time of plasma proteins increased with increase in concentration of SDS, as shown in Fig. 5. Plasma proteins were probably solubilized by the SDS micelles because the SDS concentration in the buffer solution is above the critical micellar concentration (CMC)[25]. Hence the proteins migrated later than the drugs. That is, the plasma proteins are solubilized by SDS and they acquire a strong negative charge and are consequently retarded strongly by the electrophoretic force operating in the opposite direction. In this way, the adsorption of plasma proteins on the capillary tube, which was encountered in CZE, was prevented in MEKC[21,22]. This permitted the determination of the drugs in plasma by a direct sample injection method, similar to micellar chromatography in HPLC developed by Armstrong[26] and Dorsey[27]. Typical MEKC results for a standad solution containing 50 μg ml^{-1} of aspoxicillin and 25 μg ml^{-1} of acetaminophen, blank plasma and plasma spiked with aspoxicillin and acetaminophen at the same concentration as in the standard solution are shown in Fig. 6, where an operating buffer solution of pH 8.5 containing 0.05 M SDS was used.

In MEKC, where SDS was added to the buffer solution, the migration times of aspoxicillin and acetaminophen of the plasma samples were consistent with those for the standard samples (aqueous solution) and these values were almost constant from run to run and from day to day. Good reproducibility of the migration time of each solute was obtained without frequent capillary washing (see below). The effect of SDS concentration on the migration time of acetaminophen, which is electrically neutral, was greater than that of aspoxicillin, which is ionized (Fig. 5).

In this method, in order to obtain a good separation, the drugs and I.S. substances should migrate more rapidly than the first-eluted plasma protein, or within the separation window (see Fig. 5) without coelution with blank plasma peaks shown in Fig. 6B. The SDS concentration, which is above the CMC, has to be adjusted to avoid such coelution. A buffer solution containing 0.05 M SDS was employed for concentration studies from the above investigations and with a relatively short analysis time.

Quantitation

Quantitation of aspoxicillin in human plasma was performed by the internal standard method using a buffer solution of pH 8.5 containing 0.05 M SDS. The reproducibility of the migration time and peak-area ratios of standards and spiked plasma samples was determined by repeated injections ($n = $ 5–9) (Tables I and II). The calculated relative standard deviations (R.S.D.) of 0.25–0.97% for migration times and 2.7–4.9% for peak-area ratios are comparable to those obtained in previous studies[13–16]. The calibration graph for aspoxicillin in water over the concentration range 25–300 μg ml^{-1}, which covers the plasma levels encountered in clinical analysis[19], shows excellent linearity with a correlation coefficient $r = 0.999$ and passes through the origin. The detection limit of aspoxicillin, calculated from the peak height for a standard solution containing 25 μg ml^{-1} of aspoxicillin, was *ca.* 1.3 μg ml^{-1} at a signal-to-noise ratio of 3.

The recovery was also examined over the concentration range 50–250 μg ml^{-1} (Table III). The average recovery with repeated injections ($n = $ 5–6) was 94–104%. Hence the total amount of bound together with unbound aspoxicillin could be determined by this method.
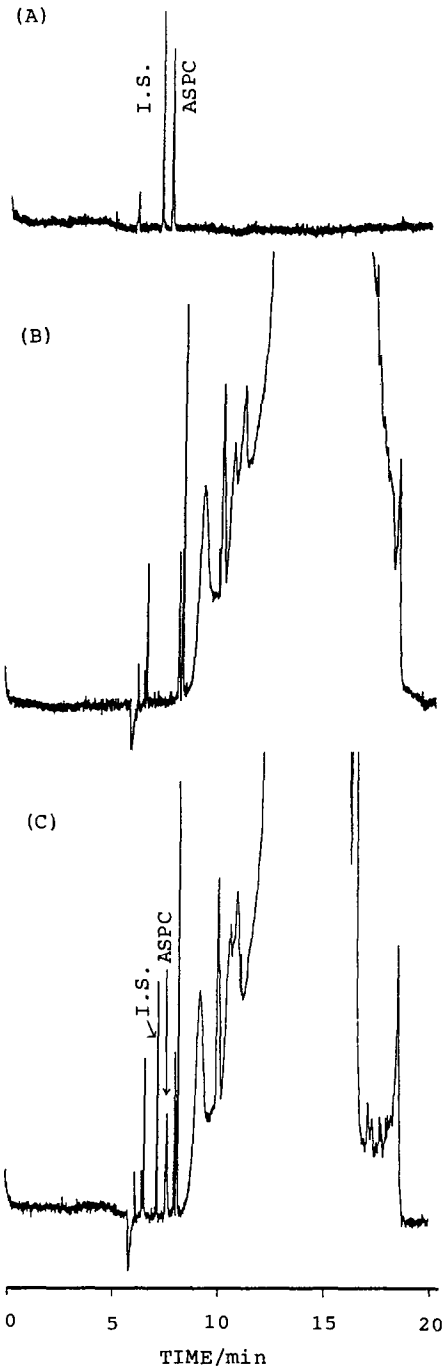
Fig. 6. MEKC separation of aspoxicillin (ASPC) and acetaminophen (I.S.). (A) Standard solution; (B) blank plasma; (C) plasma spiked with ASPC and I.S. Buffer, 0.02 M phosphate–borate buffer (pH 8.5) containing 0.05 M SDS. Other conditions as in Fig. 2.

TABLE I

REPRODUCIBILITY OF MIGRATION TIMES

Concentration ($\mu g\ ml^{-1}$)	Solvent	n	Acetaminophen		Aspoxicillin	
			t_R (min)	R.S.D. (%)	t_R (min)	R.S.D. (%)
50	Water	9	7.17	0.85	7.62	0.73
50	Plasma	9	7.31	0.97	7.75	0.88
100	Plasma	5	7.26	0.83	7.71	0.75
150	Plasma	5	7.35	0.89	7.78	0.96
200	Plasma	5	7.30	0.27	7.74	0.25
250	Plasma	5	7.41	0.35	7.83	0.48

TABLE II

REPRODUCIBILITY OF PEAK-AREA RATIOS

Concentration ($\mu g\ ml^{-1}$)	Solvent	n	R.S.D. (%)
50	Water	9	4.07
50	Plasma	9	3.90
100	Plasma	5	4.92
150	Plasma	5	4.48
200	Plasma	5	3.66
250	Plasma	5	2.73

TABLE III

RECOVERY TEST

Aspoxicillin added to plasma ($\mu g\ ml^{-1}$)	n	Recovery (%)
50	6	94.3
100	5	103.9
150	6	100.1
200	5	103.4
250	5	97.7

TABLE IV

THEORETICAL PLATE NUMBERS

Concentration ($\mu g\ ml^{-1}$)	Water		Plasma	
	I.S.[a]	ASPC[b]	I.S.[a]	ASPC[b]
50	276 000	98 000	256 000	39 100
100	267 000	104 000	267 000	42 500
150	245 000	89 900	272 000	46 300
200	279 000	98 800	277 000	46 200
250	275 000	96 700	277 000	51 800

[a] Acetaminophen.
[b] Aspoxicillin.

The theoretical plate numbers (N) calculated for aspoxicillin and acetaminophen in water and plasma samples over the concentration 50–250 μg ml^{-1} are summarized in Table IV. The N value for aspoxicillin at a given concentration in a plasma sample was half that obtained in water (standard solution) (see Fig. 6A and C). However, the migration time and peak-area ratios were not affected (Tables I and II). In contrast, the N value of acetaminophen did not change between water and plasma samples. These results indicate that a higher protein binding of aspoxicillin than acetaminophen caused a slower release of aspoxicillin from proteins, which resulted in the broadening of the aspoxicillin peak.

In conclusion, MEKC improved the separation of ionic penicillins in comparison with CZE, in addition to the high separation efficiency. Under the experimental conditions of an effective capillary tube length of 500 mm and a voltage of 20 kV, one analysis of a plasma sample was performed within *ca.* 20 min without pretreatment such as deproteinization and extraction. Recently, a few commercial instruments have become available, but it will be necessary to develop an ultra-microinjector which allows quantitative and reproducible injection or highly sensitive detectors for the assay of drugs in body fluids.

REFERENCES

1 S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya and T. Ando, *Anal. Chem.*, 56 (1984) 111.
2 S. Terabe, K. Otsuka and T. Ando, *Anal. Chem.*, 57 (1985) 834.
3 D. E. Burton, M. J. Sepaniak and M. P. Maskarinec, *Chromatographia*, 21 (1986) 583.
4 S. Terabe, *Trends Anal. Chem.*, 8 (1989) 129.
5 H. Nishi, N. Tsumagari, T. Kakimoto and S. Terabe, *J. Chromatogr.*, 465 (1989) 331.
6 H. Nishi, N. Tsumagari, T. Kakimoto and S. Terabe, *J. Chromatogr.*, 477 (1989) 259.
7 M. M. Bushey and J. W. Jorgenson, *Anal. Chem.*, 61 (1989) 491.
8 M. M. Bushey and J. W. Jorgenson, *J. Microcolumn Sep.*, 1 (1989) 125.
9 A. S. Cohen, A. Paulus and B. L. Karger, *Chromatographia*, 24 (1987) 15.
10 S. Terabe, M. Shibata and Y. Miyashita, *J. Chromatogr.*, 480 (1989) 403.
11 H. Nishi, T. Fukuyama, M. Matsuo and S. Terabe, *J. Microcolumn Sep.*, 1 (1989) 234.
12 H. Nishi, T. Fukuyama, M. Matsuo and S. Terabe, *J. Chromatogr.*, 515 (1990) 233.
13 K. Otsuka, S. Terabe and T. Ando, *J. Chromatogr.*, 396 (1987) 350.
14 S. Fujiwara and S. Honda, *Anal. Chem.*, 59 (1987) 2773.
15 H. Nishi, T. Fukuyama, M. Matsuo and S. Terabe, *J. Pharm. Sci.*, 79 (1990) in press.
16 H. Nishi, T. Fukuyama, M. Matsuo and S. Terabe, *J. Chromatogr.*, 513 (1990) 279.
17 M. Wagatsuma, M. Seto, T. Miyagishima, M. Kawazu, T. Yamaguchi and S. Ohshima, *J. Antibiot.*, 36 (1983) 147.
18 S. Okuno, I. Maezawa, Y. Sakuma, T. Matsushita and T. Yamaguchi, *J. Antibiot.*, 41 (1988) 239.
19 T. Yanagida, Y. Kojima, K. Yamasaki, I. Daira and T. Ishikawa, *Chemotherapy*, 32 (1984) 112.
20 K. Tani, I. Maezawa, Y. Sakuma, N. Ishii, H. Yoshida, T. Yamaguchi, M. Ichikawa, M. Itoh, T. Ehara, M. Inoue and T. Ishikawa, *Chemotherapy*, 32 (1984) 99.
21 T. Nakagawa, Y. Oda, A. Shibukawa and H. Tanaka, *Chem. Pharm. Bull.*, 36 (1988) 1622.
22 T. Nakagawa, Y. Oda, A. Shibukawa, H. Fukuda and H. Tanaka, *Chem. Pharm. Bull.*, 37 (1989) 707.

23 F. E. P. Mikkers, F. M. Everaerts and Th. P. E. M. Verheggen, *J. Chromatogr.*, 169 (1979) 11.
24 S. Terabe, H. Utsumi, K. Otsuka, T. Ando, T. Inomata, S. Kuze and Y. Hanaoka, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 9 (1986) 666.
25 W. L. Hinze, *Ordered Media in Chemical Separations* (*ACS Symposium Series*, No. 342), American Chemical Society, Washington, DC, 1987, p. 4.
26 D. W. Armstrong, *Anal. Chem.*, 59 (1987) 84A.
27 J. G. Dorsey, *Advances in Chromatography*, Vol. 27, Marcel Dekker, New York and Basle, 1988, pp. 167–214.