ELSEVIER

Journal of Chromatography B, 705 (1998) 55–62

JOURNAL OF CHROMATOGRAPHY B

Method for the preparation of restricted access media by low-temperature plasma treatment

Yoshihisa Sudo[1,*], Ryuji Miyagawa, Yasuyo Takahata

Chemicals Inspection and Testing Institute, Division of Research and Development, 4-1-1 Higashimukojima, Sumida-ku, Tokyo 131, Japan

Received 8 July 1997; received in revised form 3 October 1997; accepted 6 October 1997

Abstract

A new method for the preparation of restricted access media (RAM) was developed. A low-temperature plasma treatment removed octadecyl groups on the external surface of octadecylsilylated silica gel before those on the internal surface to produce silanol groups. The silanol groups produced were glycerylpropylsilylated to give RAM, DIOL-ODSs, consisting of internal octadecyl groups and external glycerylpropyl groups. DIOL-ODSs were inert to serum proteins and retained low-molecular-mass analytes adequately. Direct injection analysis of anticonvulsants in serum was accurately performed by column-switching high-performance liquid chromatography using a pre-column packed with DIOL-ODS. © 1998 Elsevier Science B.V.

Keywords: Restricted access medium; Proteins

1. Introduction

Time-consuming pretreatments for removal of protein are necessary for direct injection analysis of drugs in serum by reversed-phase high-performance liquid chromatography (RP-HPLC). Serum proteins are denatured and accumulate on the packing materials when they contact the packing materials and mobile phases containing organic solvents [1], so that the column efficiency decreases and the back-pressure of the column increases. Methods for direct injection of serum samples on to RP-HPLC columns have been studied to overcome these problems [2–4]. Column-switching techniques [5–7], micellar liquid chromatography [8] and restricted access media (RAM) [9–11] have been developed for direct injection. These methods eliminated the troublesome pretreatments. However, the second and third methods have the following disadvantages: (1) the column efficiency is lower than that of a C_{18} column under conventional conditions [8,12], (2) the amount of organic solvents in the mobile phases is restricted to avoid denaturation of serum proteins [13], (3) a large amount of sample cannot be injected and (4) the large peak tailing of serum eluted at the void volume sometimes prevents detection of analytes and fast analysis. On the other hand, although column-switching techniques require added apparatus, they have the following advantages: (1) a large amount of sample can be injected, (2) a highly efficient analytical column can be used and (3) most components

*Corresponding author.

[1]Present address: Chemicals Inspection and Testing Institute, Chemical Biotesting Center, 19-14 Chuo-machi, Kurume, Fukuoka 830, Japan

PII S0378-4347(97)00496-9

of serum are discarded before the analytical column and do not prevent the detection of analytes. Reversed-phase [14], ion-exchange [15], polystyrene gel [16] and size-exclusion packing materials [17] have been used for the pre-columns in column-switching techniques. However, these packing materials require washing after every injection of serum and periodic replacement because the accumulation of serum proteins on them causes them to deteriorate. To solve this problem, restricted-access stationary phases such as protein-coated ODS [18] and internal-surface reversed phase (ISRP) [19] have been used.

The packing materials for the pre-column in column-switching techniques require inertness to proteins and sufficient retention of analytes. RAM, consisting of glycerylpropyl groups inert to proteins [20] on the external surface and octadecylsilyl groups on the internal surface, meets these requirements. RAM with an ISRP structure is usually prepared as follows: the entire surface of the silica gel is covalently modified with hydrophobic groups, and then the groups on the external surface only are removed, so that hydrophillic groups or silanol groups are exposed on only the external surface. Neutral hydrophillic groups such as glycerylpropyl groups are covalently bonded to the silanol groups. The selective removal of the modifying groups on the external surface is an important step in the preparation of this type of RAM. Thus far, hydrolysis with enzymes [19,21,22] and with hydrochloric acid [23] have been reported. The former method can be applied only to the packing materials with modifying groups that can be hydrolysed with enzymes, while the latter can be applied only to the packing materials with modifying groups such as octadecylsilyl groups that are too hydrophobic to be hydrolysed with HCl on the internal surface. In this study, we applied ashing with low-temperature plasma for the selective removal of modifying groups on the external surface.

Gleit et al. [24] applied low-temperature ashing of organic substances with a low-temperature plasma to the pretreatment of analytical samples. The characteristics of the ashing with low-temperature plasma are that it proceeds gradually only on the contact face of the organic substance and plasma gas, and the whole sample is not heated [25,26]. Sufficient space is necessary for the acceleration of electrons so that the energy of the electrons is high enough to generate a low-temperature plasma. Therefore, when octadecylsilylated silica gel (ODS) is treated by low-temperature plasma, plasma may preferentially contact the external surface of the packing material because the pores of packing materials are too narrow to generate plasma. Hollahan et al. [27] reported that $\equiv SiCH_2OH$ and $\equiv SiOH$ were formed by the plasma treatment of polydimethylsiloxane. This suggests that octadecyl groups on the external surface only are converted to alcoholic hydroxyl groups, which are inert to proteins, or to silanol groups. The product of plasma treatment may be one type of RAM in itself if octadecylsilyl groups are converted to alcoholic hydroxyl groups. On the other hand, RAM, consisting of octadecyl groups on the internal surface and glycerylpropyl groups on the external surface, is prepared by bonding glycerylpropyl groups to the silanol groups if the octadecyl groups are converted to silanol groups. Therefore, this method is expected to be applicable for preparing packing materials with various modifying groups. We report here the effect of the conditions of the plasma treatment on the characteristics of the prepared RAM and the application of the RAM to direct injection analysis of serum samples.

2. Experimental

2.1. Reagents and materials

All silicon chemicals were purchased from Shin-Etsu Chemical Co. (Tokyo, Japan). The silica gel used was Develosil 60-10 (particle size 10 μm, pore size 60 Å and surface area 495 $m^2\ g^{-1}$) from Nomura Chemicals Co. (Seto, Japan).

2.2. Preparation of packing materials

2.2.1. Preparation of ODS

A suspension of 100 g of silica gel in 400 ml of conc. HCl was heated at 100°C for 16 h. The suspension was then cooled to about 25°C and the silica gel was collected, washed with water until free of acid and dried under vacuum at 140°C for 8 h. To the stirred suspension of 50 g of the dried silica gel

in 150 ml of dry toluene under N_2 atmosphere were added 19 g (55 mmol) of dimethyloctadecylchlorosilane and 4.8 g (61 mmol) of dry pyridine. The suspension was refluxed for 16 h and then cooled. The silylated silica gel was washed sequentially with toluene and methanol and then dried under vacuum at 140°C for 4 h. To the stirred suspension of the silylated silica gel in 150 ml of dry toluene under N_2 atmosphere was added 9 g (56 mmol) of an end-capping agent, hexamethyldisilazane. The suspension was refluxed for 16 h and then cooled. The silylated silica gel obtained was washed sequentially with toluene and methanol and then dried under vacuum at 140°C for 4 h to give ODS. The carbon content (C%) was 17.3%.

2.2.2. Treatment of ODS by plasma

Fig. 1 shows the capacitively coupled plasma reactor used in this study. A 300-ml flask attached to a rotary evaporator was surrounded with a pair of copper bands 10 mm wide at intervals of 10 mm. The bands were connected to a radio-frequency (RF) generator of 13.56 MHz (RFG-200; Samco International Inc., Kyoto, Japan). In the reactor was placed 10 g of ODS, and then oxygen was supplied under flow control to an internal pressure of 75 Pa while the reactor was evacuated by a vacuum pump. While the reactor was rotated, plasma was generated with 80 W of RF power for a prescribed time, and then the generation of plasma was stopped for 3 min. The generation of plasma followed by a pause was repeated 18 times. The plasma treatment time recorded here is the total plasma generation time. The plasma-treated ODS was washed sequentially with water and methanol and then dried at 120°C under vacuum for 4 h.

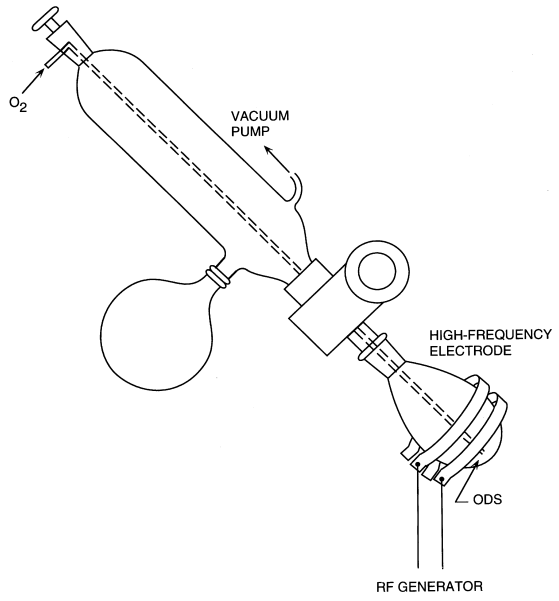

Fig. 1. Plasma reactor.

2.2.3. Glycerylpropylsilylation of plasma-treated ODS

In a 50-ml flask was placed 4 g of the plasma-treated ODS and, depending on the plasma treatment time, 67–400 μl of water was added. The flask was sealed, shaken and then allowed to stand for 24 h. Toluene (15 ml), 80 μl of 10% *p*-toluenesulfonic acid in acetonitrile and 2.2 g (9.3 mmol) of 3-glycidoxypropyltrimethoxysilane were added to the treated ODS. The suspension was stirred in an oil bath at 120°C for 16 h. The product was cooled and washed sequentially with toluene and acetone. Then, 15 ml of 0.02 mol l^{-1} H_2SO_4 was added and the suspension was refluxed to hydrolyse the epoxy groups. After cooling, the particles were washed sequentially with water and methanol. The particles were dried at 60°C under vacuum for 2 h to give DIOL-ODS consisting of external glyceryl groups and internal octadecyl groups.

2.2.4. Preparation of glycerylpropylsilylated silica gel

In a 50-ml flask was placed 4 g of silica gel treated with HCl and dried, and then 400 μl of water was added. The flask was sealed, shaken and then allowed to stand for 24 h. The silica gel was glycerylpropylsilylated by the method described above to give glycerylpropylsilylated silica gel (DIOL).

2.3. Chromatographic measurements

The packing materials prepared were packed into stainless steel tubes (30 mm×4.6 mm I.D.), whose ends were sealed by stainless steel frits, sequentially by tapping and pressurizing with a mixture of isopropanol and chloroform. The HPLC system

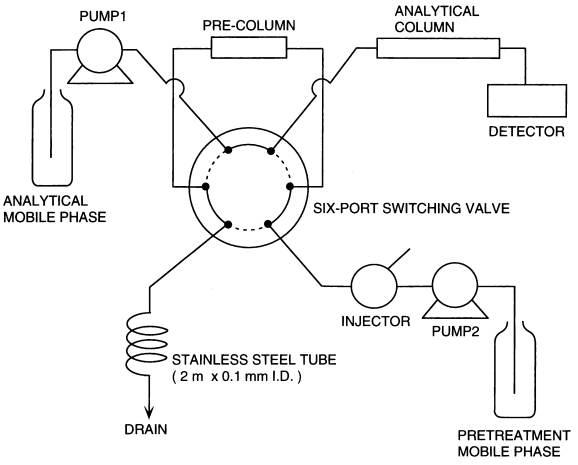

Fig. 2. Column-switching system.

consisted of a pump (LC-6A, Shimadzu, Kyoto, Japan), a UV detector (SPD-6A, Shimadzu), a data processor (C-R4A, Shimadzu) and an injector (model 7125, Rheodyne, CA, USA). Fig. 2 shows the column-switching system used in this experiment. A back-flash mode [28] was used to prevent the elution bands from broadening. A stainless steel tube (2 m×0.1 mm I.D.) was attached between the pre-column and the drain to prevent the generation of air in the pre-column by back pressure. The pre-column was equilibrated with the pretreatment mobile phase. Serum samples were injected and first chromatographed on the pre-column by the pretreatment mobile phase; serum proteins were then directly vented to waste. By rotating the six-port valve, the analytical mobile phase was passed through the pre-column to elute retained analytes onto the analytical column.

3. Results and discussion

3.1. Preparation of packings

ODS adhered extensively to the inner surface of the plasma reactor during plasma treatment but did not during pauses in the plasma generation. This extensive adhesion is effective in plasma treatment because it expands the contact area between ODS and plasma, but it is difficult for the plasma to enter inside the ODS layer. Therefore, to treat ODS homogeneously, two processes were applied alternatively: plasma generation followed by a pause during which ODS was mixed by rotating the reactor. The discharge colour was blue during the plasma treatment of ODS, which indicates the generation of carbon dioxide and the occurrence of low-temperature ashing.

Fig. 3 shows the relationship between the plasma treatment time and the carbon content (*C*%) of plasma-treated ODS, and between the time and the *k′* value of theophylline on DIOL-ODS. Removal of the octadecyl groups proceeded during plasma treatment. Even the ODS that was plasma-treated for 9 min was wettable by water. The results suggest that the octadecyl groups on the external surface were removed more easily than those on the internal surface, although they were also removed as treatment time increased. When ODS that was plasma-treated for 30 min was prepared six times, the average *C*% of the plasma-treated ODSs was 13.7% and the relative standard deviation (R.S.D.) was 1.32%. These results show the good reproducibility of this technique.

The *C*% was increased from 13.7% to 16.3%

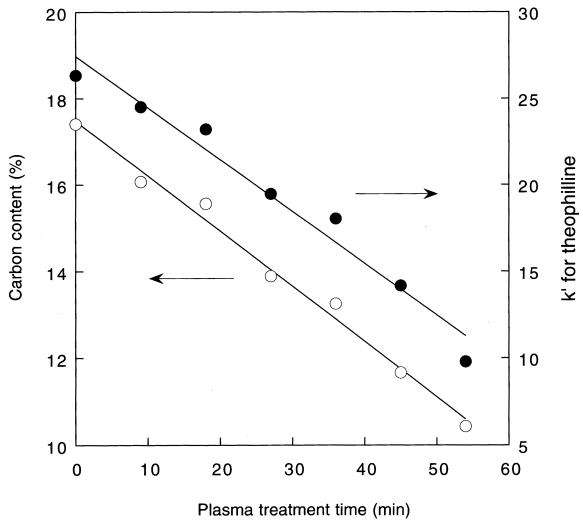

Fig. 3. Relationship between plasma treatment time and carbon content (*C*%) of plasma-treated ODS, and between the time and *k′* value of theophylline on DIOL-ODS. (○) *C*% of plasma-treated ODS. (●) *k′* value of theophylline. HPLC conditions: mobile phase, acetonitrile–water (2:98, v/v); flow-rate, 1 ml min^{-1}; detection, UV 270 nm.

through glycerylpropylsilylation, which implies that the plasma treatment produced mainly silanol groups. If the plasma treatment had produced mainly $\equiv Si(CH_2)_nOH$, glycerylpropylsilylation would not have increased the *C*% because the glyceryl-propylsilyl groups bonded to hydroxyl groups of $\equiv Si(CH_2)_nOH$ would have been hydrolysed by treatment with 0.02 mol l^{-1} H_2SO_4.

3.2. Recovery of BSA from DIOL-ODS

The recovery of bovine serum albumin (BSA) from the columns has been measured to estimate the elution ratio of serum proteins in the direct injection analysis of serum samples by HPLC [23,29,30]. In addition, Haginaka et al. [29] reported that the recovery of BSA corresponded to that of serum proteins under the same conditions. Therefore, the inertness of the stationary phases to proteins was also evaluated through BSA recovery in this study. A dilute solution of BSA was used to detect slight differences among the packing materials. Because the recovery of BSA from the columns increased with the number of injections, a newly prepared column was used in each run. BSA was injected three times into each column and the recovery from each injection was measured. Table 1 shows the results. The recoveries of BSA from DIOL-ODS that was plasma-treated for shorter times were a little lower for the first injection, but thereafter, the recovery from all DIOL-ODS was almost equal to that from DIOL. These results also indicate that the octadecyl groups on the external surface were removed more easily than those on the internal surface and suggest that direct injection of serum causes neither accumulation of serum proteins on DIOL-ODS nor decreases in column efficiency and capacity.

3.3. Stability of DIOL-ODS

Fig. 4 shows the variation in the retention of theophylline on DIOL-ODS that was plasma-treated for 30 min when serum was directly injected onto it. Because ODS adsorbs proteins, the retention of low molecular weight analytes decreases with the number of direct injections of serum on ODS [23]. The k' value and the peak shape of theophylline on DIOL-ODS as well as the column pressure did not vary after 250 injections of 100 μl serum, which confirms that serum proteins did not accumulate on DIOL-ODS, although the recovery of the first injection of 1% BAS solution onto DIOL-ODS was a little lower than 100%, as mentioned above.

3.4. Direct injection analysis of anticonvulsants in serum by column-switching techniques

Various pretreatment mobile phases were examined for direct injection analysis of serum samples through column-switching HPLC using a pre-column packed with DIOL-ODS. After serum was injected on the pre-column, 10 ml of a pretreatment mobile phase was delivered. Then, serum components remaining on the pre-column were eluted with the analytical mobile phase, acetonitrile–100 mmol l^{-1}

Table 1
Recovery of BSA from DIOL-ODSs

Packing No.	Plasma treatment time (min)	Recovery of BSA (%)			
		1st injection	2nd injection	3rd injection	R.S.D. (%)
1	0	0	0	0	
2	9	83.3	94.3	95.2	5.68
3	18	88.4	95.9	98.3	4.48
4	27	90.4	99.5	97.5	4.08
5	36	90.6	100.2	100.8	4.81
6	45	95.0	99.5	101.8	2.86
7	54	96.1	99.6	100.5	1.92
DIOL	–	98.5	99.4	99.5	0.454

HPLC conditions: sample, 1% BSA in 100 mmol l^{-1} phosphate buffer (pH 6.8); sample volume, 20 μl; mobile phase, 100 mmol l^{-1} phosphate buffer (pH 6.8); flow-rate, 1 ml min^{-1}; detection, UV 290 nm.

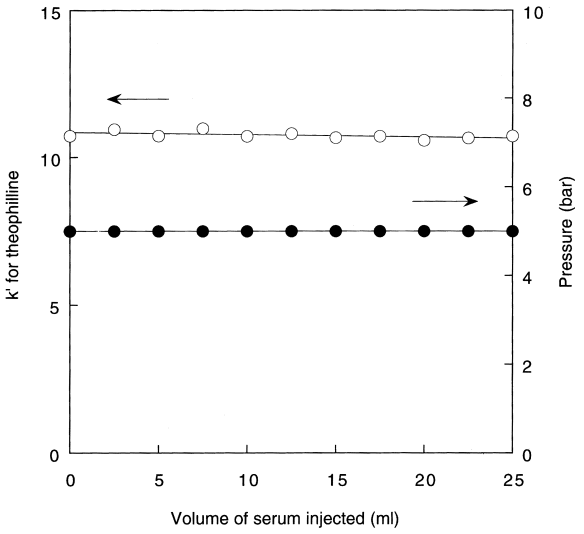

Fig. 4. Effect of volume of serum injection on k' value of theophylline. Test conditions: sample, serum; injection volume, 100 μl; stationary phase, DIOL-ODS plasma-treated for 30 min; mobile phase, 100 mmol l^{-1} phosphate buffer (pH 6.8); flow-rate, 1 ml min^{-1}. HPLC conditions for theophylline: mobile phase, acetonitrile–20 mmol l^{-1} phosphate buffer (pH 6.8) (3:97, v/v); flow-rate, 1 ml min^{-1}; detection, UV 270 nm. (○) k' value of theophylline. (●) pressure of the column.

phosphate buffer (pH 6.8) (2:8, v/v), and detected by a UV detector at 220 nm. Table 2 shows the peak area of serum blanks eluted with the analytical mobile phase. The pretreatment mobile phase consisting of sodium perchlorate and phosphate buffer gave the smallest peak area for the serum blanks. Because sodium perchlorate is an anti-tailing reagent for the HPLC of basic analytes [31,32], components with amine groups in serum may have eluted easily at the pretreatment step because of its addition to the pretreatment mobile phase. The examination of pretreatment time reveals that 10 ml or less of the pretreatment mobile phase was necessary to vent the component of serum to waste. Fig. 5 shows the chromatogram of serum on the pre-column packed with DIOL-ODS. The chromatogram reveals that the serum proteins were eluted at the void volume. The retention times of ethosuximide and primidone, which, among the anticonvulsants, are retained most weakly on the reversed-phase packing materials, were 11.2 min and 60.4 min, respectively. These results indicate that 7 ml of the pretreatment mobile phase is enough to elute almost all components of serum from the pre-column without losing these drugs. Fig. 6 shows the chromatogram of serum spiked with anticonvulsants by direct injection using the column-switching method. Table 3 shows the average recoveries and the relative standard deviations of the results by this method for the anticonvulsants. Each anticonvulsant was almost completely recovered and the results were reproducible.

4. Conclusions

The low-temperature plasma exclusively removed the octadecyl groups on the external surface of ODS to produce silanol groups. The resultant silanol groups were silylated to give restricted access media. These results suggest that this method for the preparation of restricted access media can be used for the preparation of various combinations of

Table 2
Effect of pretreatment mobile phases on peak area of serum blanks eluted with the analytical mobile phase after pretreatment

Pretreatment mobile phases	Volume of pretreatment mobile phases (ml)	Peak area of serum blank (μV s)
20 mmol l^{-1} phosphate buffer (pH 6.8)	10	101 910
100 mmol l^{-1} phosphate buffer (pH 6.8)	10	92 763
100 mmol l^{-1} phosphate buffer (pH 6.8)	20	92 463
10 mmol l^{-1} phosphate buffer (pH 6.8) +100 mmol l^{-1} sodium perchlorate	10	87 474

Pretreatment conditions: sample, serum; sample volume, 20 μl; flow-rate, 1 ml min^{-1}. Analytical conditions: mobile phase, acetonitrile–100 mmol l^{-1} phosphate buffer (pH 6.8) (2:8, v/v); flow-rate, 1 ml min^{-1}; detection, UV 220 nm.

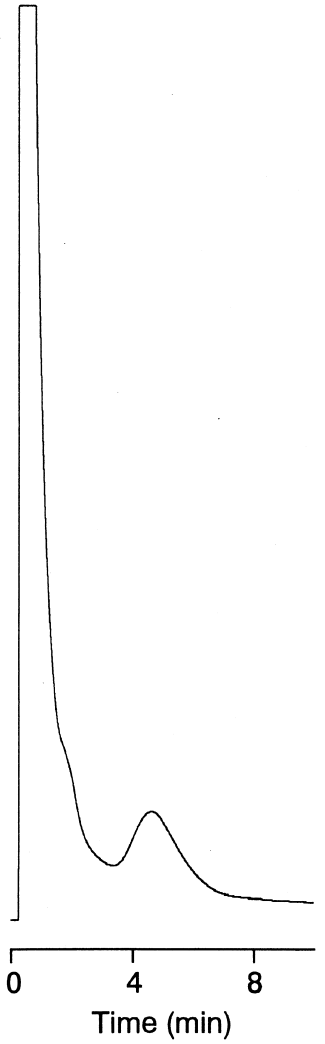

Fig. 5. Chromatogram of serum on DIOL-ODS. HPLC conditions: stationary phase, DIOL-ODS plasma-treated for 30 min; mobile phase, 100 mmol l^{-1} sodium perchlorate in 10 mmol l^{-1} phosphate buffer (pH 6.8); flow-rate, 1 ml min^{-1}; detection, UV 220 nm (0.16 a.u.f.s.); injection volume, 20 μl.

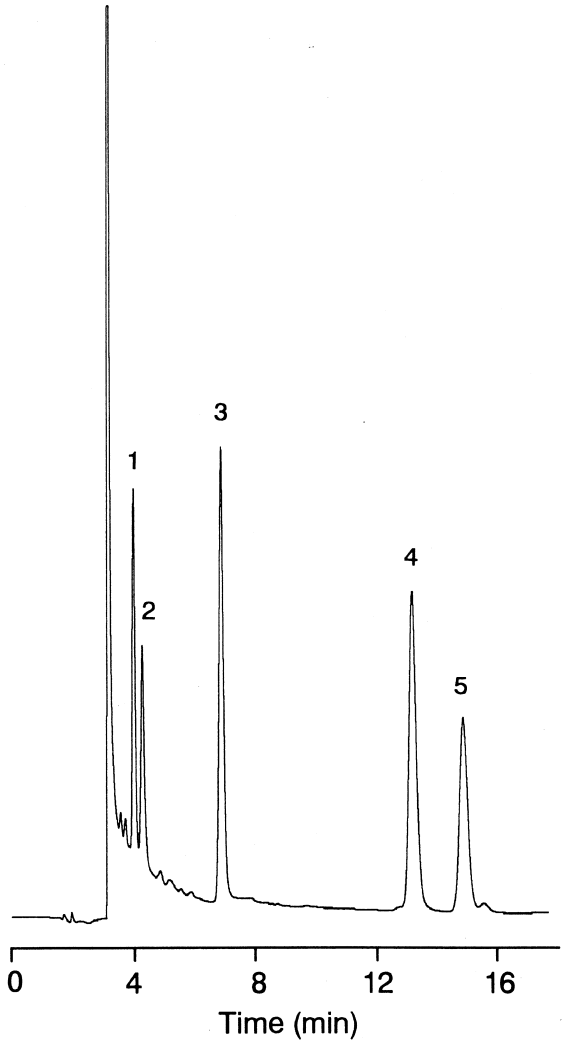

Fig. 6. Chromatogram of serum spiked with anticonvulsants by direct injection HPLC with column-switching technique using a pre-column packed with DIOL-ODS. Pretreatment conditions: pre-column, DIOL-ODS (30 mm×4.6 mm I.D.); mobile phase, 100 mmol l^{-1} sodium perchlorate in 10 mmol l^{-1} phosphate buffer (pH 6.8); flow-rate, 1 ml min^{-1}; pretreatment time, 7 min. HPLC conditions: analytical column, Lichrospher RP-18(e) (250 mm×4 mm I.D.); mobile phase, acetonitirile–100 mmol l^{-1} phosphate buffer (pH 6.8) (3:7, v/v); flow-rate, 1 ml min^{-1}; detection, UV 220 nm (0.16 a.u.f.s.). Peak: 1=primidone (10 mg l^{-1}); 2=ethosuximide (100 mg l^{-1}); 3=phenobarbital (20 mg l^{-1}); 4=phenytoin (10 mg l^{-1}); 5=carbamazepine (5 mg l^{-1}).

modifying groups on the external and internal surfaces of packing materials.

Acknowledgements

The authors are grateful to Professor Toshiyuki Hobo of Tokyo Metropolitan University for his

Table 3
Recovery and reproducibility for analysis of anticonvulsants

Drug	Recovery (%)[a]	R.S.D. (%)[b]
Primidone	92.4	0.40
Ethosuximide	95.9	0.83
Phenobarbital	102.5	0.25
Phenytoin	102.8	1.26
Carbamazepine	100.3	0.18

[a]Average of five analyses.
[b]Relative standard deviation of five analyses.

helpful discussions and the revision of the manuscript.

References

[1] T. Arvidsson, K.-G. Wahlund, N. Daoud, J. Chromatogr. 317 (1984) 213.
[2] D. Westerlund, Chromatographia 24 (1987) 155.
[3] Z.K. Shihabi, J. Liq. Chromatogr. 11 (1988) 1579.
[4] D.J. Anderson, Anal. Chem. 65 (1993) 434R.
[5] B.L. Karger, R.W. Giese, L.R. Snyder, Trends Anal. Chem. 2 (1983) 106.
[6] H. Imai, T. Masujima, I. Morita-Wada, G. Tamai, Anal. Sci. 5 (1989) 389.
[7] P. Campíns-Falcó, R. Herráez-Hernández, A. Sevillano-Cabeza, J. Chromatogr. 619 (1993) 177.
[8] M.J. Koenigbauer, J. Chromatogr. 531 (1990) 79.
[9] J. Haginaka, Trends Anal. Chem. 10 (1991) 17.
[10] K.K. Unger, Chromatographia 31 (1991) 507.
[11] T.C. Pinkerton, J. Chromatogr. 544 (1991) 13.
[12] D. Bentrop, F.V. Warren Jr., S. Schmitz, B.A. Bidlingmeyer, J. Chromatogr. 535 (1990) 293.
[13] T.C. Pinkerton, T.D. Miller, S.E. Cook, J.P. Perry, J.D. Rateike, T.J. Szczerba, Biochromatography 1 (1986) 96.
[14] G.J. Jong, J. Chromatogr. 183 (1980) 203.
[15] B.P. Crawmer, J.A. Cook, R.R. Brown, J. Chromatogr. 530 (1990) 407.
[16] R.A. Hux, H.Y. Mohammed, F.F. Cantwell, Anal. Chem. 54 (1982) 113.
[17] H. Takahagi, K. Inoue, M. Horiguchi, J. Chromatogr. 301 (1986) 369.
[18] H. Yoshida, I. Morita, T. Masujima, H. Imai, Chem. Pharm. Bull. 30 (1982) 3827.
[19] I.H. Hagestam, T.C. Pinkerton, Anal. Chem. 57 (1985) 1757.
[20] F.E. Regnier, R. Noel, J. Chromatogr. Sci. 14 (1976) 316.
[21] J. Haginaka, N. Yasuda, J. Wakai, H. Matsunaga, H. Yasuda, Y. Kimura, Anal. Chem. 61 (1989) 2445.
[22] S. Vielhauer, A. Rudolphi, K.-S. Boos, D. Seidel, J. Chromatogr. B 666 (1995) 315.
[23] K. Kimata, R. Tsuboi, K. Hosoya, N. Tanaka, T. Araki, J. Chromatogr. 515 (1990) 73.
[24] C.E. Gleit, W.D. Holland, Anal. Chem. 34 (1962) 1454.
[25] K. Hiraoka, H. Jin, S. Oda, H. Eguchi, H. Kamada, Bunseki Kagaku 19 (1970) 349.
[26] K. Hoseki, M. Matsumoto, Bunseki Kagaku 21 (1972) 206.
[27] J.R. Hollahan, G.L. Carlson, J. Appl. Polym. Sci. 14 (1970) 2499.
[28] J. Lankelma, H. Poppe, J. Chromatogr. 149 (1978) 587.
[29] J. Haginaka, J. Wakai, Anal.Chem. 62 (1990) 997.
[30] R.A. Grohs, F.V. Warren Jr., B.A. Bidlingmeyer, Anal. Chem. 63 (1991) 384.
[31] J.H. Kennedy, B.A. Olsen, J. Chromatogr. 389 (1987) 369.
[32] J.A. De Schutter, P. De Moerloose, J. Pharm. Biomed. Anal. 6 (1988) 879.