ELSEVIER

Journal of Chromatography A, 887 (2000) 479–487

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Comparison of separation behavior of benzodiazepines in packed capillary electrochromatography and open-tubular capillary electrochromatography

Kiyokatsu Jinno[a,*], Hirokazu Sawada[a], Alicia P. Catabay[a], Hiroshi Watanabe[a], Nordin Bin Haji Sabli[a], Joseph J. Pesek[b], Maria T. Matyska[b]

[a]*School of Materials Science, Toyohashi University of Technology, Toyohashi 441-8580, Japan*
[b]*Department of Chemistry, San Jose State University, San Jose, CA 95192-0101, USA*

Abstract

Packed column capillary electrochromatography (CEC), open-tubular CEC and microcolumn liquid chromatography (LC) using a cholesteryl silica bonded phase have been studied to compare the retention behavior for benzodiazepines. It has been found that packed column CEC gives better resolution, faster analysis time than microcolumn LC for benzodiazepines maintaining similar selectivity except for some solutes which are charged species under the separation conditions. However, open-tubular CEC gave different selectivities to a larger extent for charged benzodiazepines from that which should be produced by the chromatographic properties of the cholesteryl silica phase. Charged species migration times are mainly influenced by electrophoretic mobility rather than the chromatographic interactions.

Keywords: Packed column capillary electrochromatography; Open-tubular capillary electrochromatography; Benzodiazepines

1. Introduction

Recently capillary electrochromatography (CEC), which combines advantages of capillary zone electrophoresis (CZE) and liquid chromatography (LC), has received considerable attention. Most CEC applications are performed with capillary columns packed with small LC packing particles, i.e., packed column CEC [1–4]. However, some problems must be solved in this type of CEC before its use becomes routine: it is difficult to pack small LC particles into the capillary column and to fabricate the frits, which are required to retain the packing particles within the column, in a reproducible manner. In addition to the difficulty of fabricating the frit, pressurization of both ends of the column is required to prevent bubble formation inside the capillary during the separation.

The use of packed columns in CEC is based on the premise that the electroosmotic flow (EOF) profile will be identical to that in an open tube, and independent of particle size provided that thermal effects and double layer overlap do not occur. Therefore the chromatographic performance of the packed column is dependent on the proper choice of electrolyte concentration [5]. In CEC, packed capillary columns are preferred over the open-tubular format because the former is expected to demonstrate

*Corresponding author. Tel.: +81-532-470-111; fax: +81-532-485-833.

E-mail address: jinno@chrom.tutms.tut.ac.jp (K. Jinno)

0021-9673/00/$ – see front matter
PII: S0021-9673(99)01198-X

greater selectivity due to stronger solute-bonded phase interactions. However uniform packing, bubble formation, thermal effects and choice and design of frit materials present some serious difficulties for the practical applications. Therefore, further progress in CEC column technology is required [2–4]. Pesek and Matyska have demonstrated an alternative to packed columns [6–8] through an open-tubular approach to CEC. They have proposed the use of etched capillaries that have been modified by the attachment of an organic moiety. This method is similar to the etching process done to modify long conventional gas chromatography (GC) capillaries to reduce the length of column needed for separation [9]. The larger surface area, up to 1000-fold, allows for an increase in the time for solute-bonded phase interactions and a plug flow velocity profile is similarly achieved when a potential is applied. As the bonded phase is coated and fixed onto the capillary walls by attachment to silanol groups, the problems encountered in packed columns are eliminated. This format has already been employed successfully in the analysis of such compounds as tetracyclines [6,7] and proteins [8].

For a comparative evaluation of packed column CEC and open-tubular CEC separation performance we have selected the cholesteryl-10-undecenoate bonded phase as a packing material for packed column CEC and the capillary surface modifier for open-tubular CEC. This phase has shown a remarkable difference in selectivity compared to the ODS phase in microcolumn LC studies, especially in the analysis of polycyclic aromatic hydrocarbons and benzodiazepines as we have reported [10–12]. Successful applications of such cholesteric bonded phases have also been described by Delaurent et al. [13] and Buszewski et al. [14] in LC.

Benzodiazepines were chosen as the probe because their retention behavior using the cholesteryl bonded phase in microcolumn LC has already been established [12] and translation to the CEC mode will be an interesting demonstration of the applicability of this fairly new separation methodology in pharmaceutical analysis. Benzodiazepines are important in pharmacological, clinical and forensic studies due to their widespread use as psychotropic agents either as anxiolytic, anticonvulsant, sedative or mild hypnotic drugs.

2. Experimental

The CEC system is laboratory-assembled consisting of a Model HCZE-30 PNO.25 high-voltage power supply (Matsusada Precision Devices, Kusatsu, Japan), a Model CE-970 UV detector (Jasco, Tokyo, Japan) set at 240 nm. The glass buffer reservoirs and high-voltage power supply were enclosed in a protective casing made of acrylate resin. For microcolumn LC experiments the same set-up was used with the addition of pumping system (Microfeeder MF-2, Azuma Electric, Tokyo, Japan).

Benzodiazepine samples were kindly provided by Dr. M. Hayashida of Nippon Medical School, Tokyo, Japan. The structures of nine benzodiazepines are shown in Fig. 1. Buffers were made from tris(hydroxymethyl)aminomethane (Tris), standard hydrochloric acid solutions and acetonitrile obtained from Kishida (Osaka, Japan). Other solvents were of chromatographic grade. Deionized water was obtained from a Milli-Q water system (Millipore, USA). For packed column CEC the fused-silica capillaries for the columns were obtained from GL Sciences (Tokyo, Japan). The cholesteryl bonded silica was packed into a 200 mm effective length$\times$ 0.1 mm I.D. fused-silica capillary. The synthetic procedures for preparing the cholesteryl bonded silica are found in our previous publications [9–11]. The cholesteryl silica bonded phase has the following basic properties as the LC stationary phase: Vydac Silica TP (The Separations Group, Hesperia, CA, USA) is the support material having a particle diameter of 6.5 μm, a pore size of 300 Å, carbon content of 6.5% and a surface coverage of 1.5 μmol/m^2. Since the EOF is independent of particle size, it is possible to get higher efficiency by using a smaller particle size such as 3 μm. However, in this study we selected 6.5 μm silica due to its availability in the laboratory. For comparison an ODS capillary column was also prepared, in which Shiseido (Tokyo, Japan) Superiorex ODS-5 was the stationary phase. The frits for packed columns were made by heating a potassium silicate–silica mixture using a hot wire.

After the frits were made, the column was conditioned with a flow of acetonitrile for overnight and then the mobile phase before the measurements.

For open-tubular CEC the column was made by

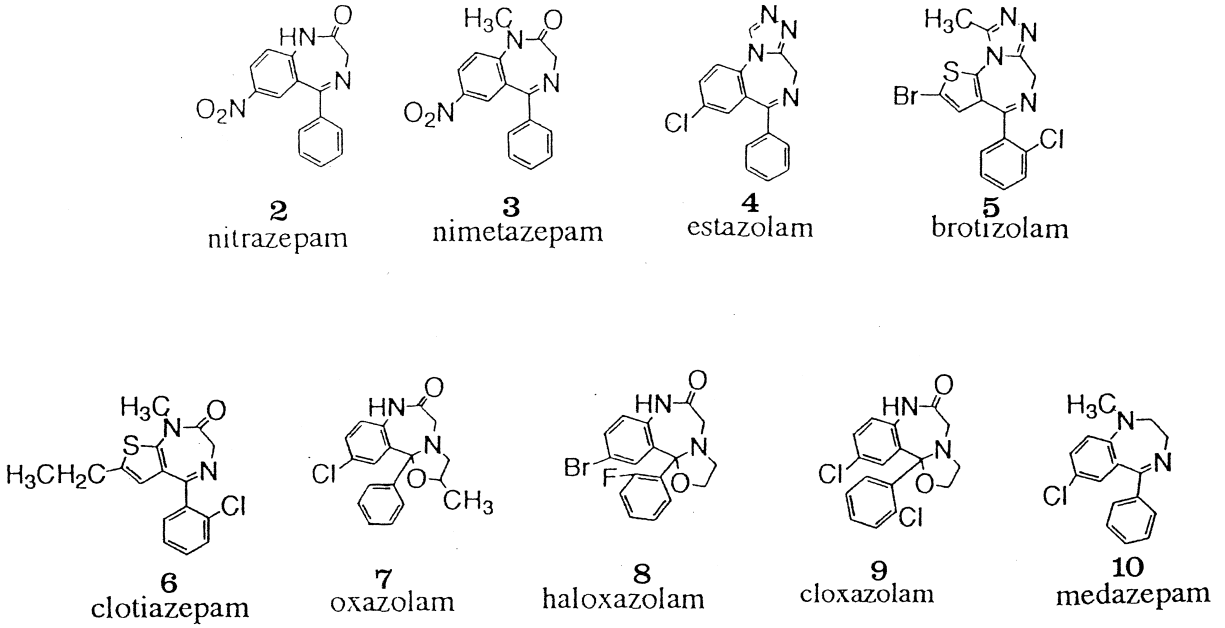

Fig. 1. Chemical structures of benzodiazepines used in this work.

the following procedure. The preparation of the etched capillaries is a two-step process, as described by Pesek and co-workers [6–8,15].

First step (etching process): The capillary walls are etched by ammonium hydrogendifluoride (Aldrich, Milwaukee, WI, USA) to increase the surface area. A 2-m section of a bare capillary (Polymicro Technologies, Phoenix, AZ, USA), 75 μm I.D. was filled with concentrated HCl, sealed and heated overnight at 80°C. The tube was flushed successively with deionized water, acetone and diethyl ether upon opening. The tube was then dried for 1 h under nitrogen flow at ambient temperature. The capillary was filled with a 5% solution of ammonium hydrogendifluoride in methanol and allowed to stand for 1 h. Methanol was then removed by nitrogen flow for 0.5 h. After the capillary was sealed at both ends, it was heated in an oven at temperatures between 300 and 400°C for a period of 3 to 4 h.

Second step (chemical modification process): Subsequent modification by the attachment of the organic moiety, cholesterol-10-undecenoate (Sigma, St. Louis, MO, USA), via a silanization/hydrosilation process (as shown in Fig. 2). The capillary after etching is treated with 6 m*M* ammonia solution, pH 10 for 20 h at a flow-rate of 0.1–0.2 ml/h. The capillary was rinsed with deionized water and then washed with 0.1 *M* HCl, and a second rinsing with

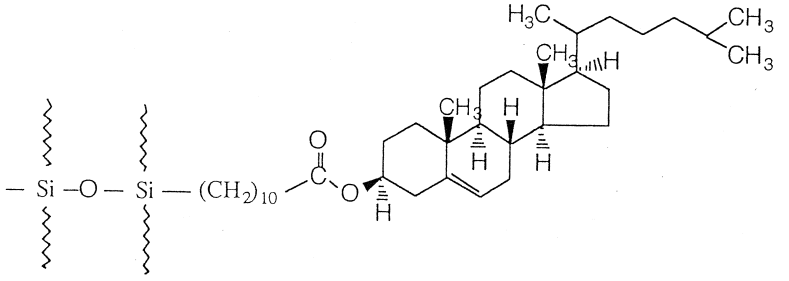

Fig. 2. Chemical structure of cholesteryl silica bonded phase.

water. The tube was dried with nitrogen, filled with dioxane, and then flushed with 1.0 *M* TES (triethoxysilane, United Chemical Technologies, Bristol, PA, USA) solution for 90 min at 90°C. After the TES treatment, the capillary was washed with water for 2 h and dried under a stream of nitrogen for 0.5 h. The resulting hydride capillary was then flushed with dry toluene, followed by a constant flow of the olefin, cholesteryl-10-undecenoate, dissolved in toluene containing Speier's catalyst [10 m*M* hexachloroplatinic acid (Aldrich) in 2-propanol] for a period of 45 h at 100°C. Prior to this the olefin/catalyst solution was heated at 60–70°C for 1 h. Then the capillary was washed with toluene and tetrahydrofuran for 1 h. After washing the capillary was dried under nitrogen flow at 100°C overnight.

Prior to use the capillary is conditioned by flushing with one volume of methanol, one volume of water and finally with one volume of the buffer. Capillaries to be re-used are flushed with water and stored with methanol overnight and re-conditioned again before use.

Benzodiazepine samples were available in methanol stock solutions. Methanol was evaporated under a stream of nitrogen and the residue is re-dissolved in the buffer.

All sample injections were performed electrokinetically for 5 s at 12 kV at the positive end. All chemicals not specified were obtained commercially in the highest purity available.

3. Results and discussion

In order to compare CEC to LC, the retention behavior of benzodiazepines in packed column CEC and microcolumn LC was examined. The chromatograms obtained using the same column with a 200 mm effective length×0.1 mm I.D. packed with the cholesteryl silica bonded phase as the stationary phase are shown in Fig. 3. In this case microcolumn LC conditions were adjusted to give similar retention times for benzodiazepines as those obtained with packed column CEC. It appears that slightly better resolution is obtained by CEC but cloxazolam (peak 9) behaves unusually in the CEC separation compared to the microcolumn LC separation.

This behavior can be easily identified in Fig. 4 where the retention factors (k_{CEC}) of nine solutes with CEC were plotted against the retention factors (k_{LC}) with microcolumn LC. Two solutes, cloxazolam and medazepam (peak 10), deviate from the linear relationship between k_{LC} and k_{CEC}.

At pH 7.3 cloxazolam (pK_a=7.1) is mostly ionized and this charged species can be greatly influenced by electrophoretic mobility even in the packed column CEC format as well as the chromatographic interaction between the solute and the cholesteryl bonded phase. Medazepam (pK_a=6.2) is partly charged under this separation condition and there is some influence from the electrophoretic mobility on its retention factor. In order to confirm

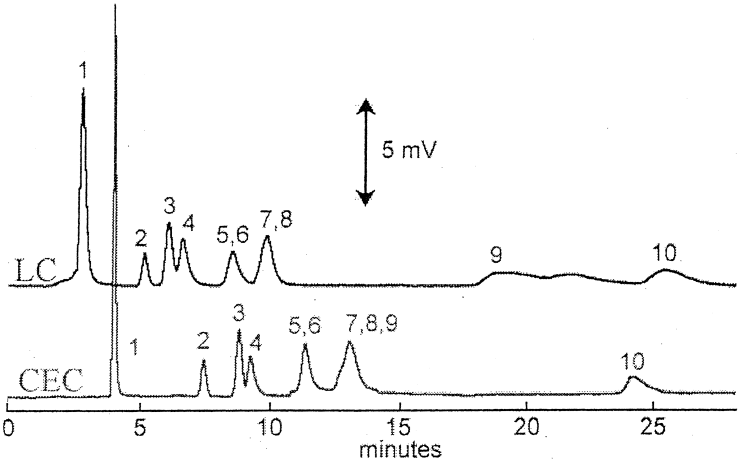

Fig. 3. Separation of benzodiazepines by microcolumn LC and packed column CEC with the cholesteryl bonded silica stationary phase. LC conditions; mobile phase acetonitrile–5 m*M* Tris–HCl buffer (35:65) with flow-rate 2 μl/min, pH 7.3. CEC conditions; mobile phase acetonitrile–5 m*M* Tris–HCl buffer (35:65) pH 7.3, applied voltage 300 V/cm.

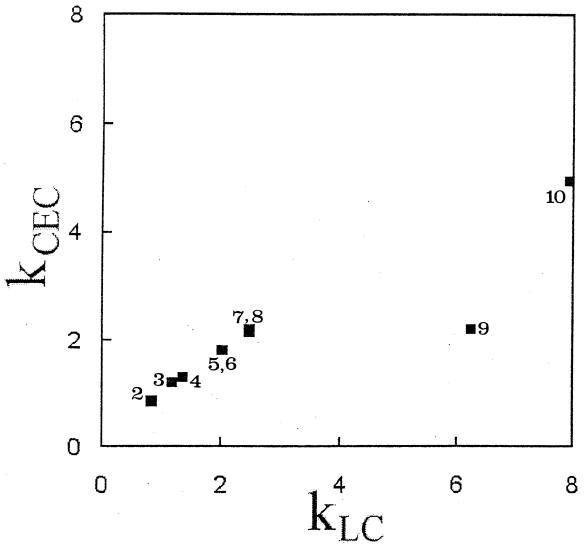

Fig. 4. Relationship between retention factors with packed column CEC (k_{CEC}) and microcolumn LC (k_{LC}). Conditions as in Fig. 3.

this hypothesis a mixture of five solutes including thiourea (a void volume marker), clotiazepam, nitrazepam, medazepam and cloxazolam was injected into a bare fused-silica capillary without any packing material. A high voltage was applied to both ends of the capillary (CZE separation mode) and the migration times of the solutes were measured. The migration times of the solutes were as follows: cloxazolam 3.24 min, medazepam 3.36 min, other solutes including thiourea 3.47 min. The results clearly indicate that two solutes cloxazolam and medazepam were influenced by electrophoretic mobility under this CZE separation conditions. Therefore one can conclude that charged species under these separation condition would be influenced by electrophoretic mobility even in packed column CEC and this fact produces some selectivity differences in packed column CEC in comparison to microcolumn LC separations.

In comparing packed column CEC to microcolumn LC, higher efficiency and faster analysis times as a result of using electrically driven flow are obtained, while the selectivity based on the stationary phase is maintained in CEC separations. As noted above, some solutes which are charged under particular experimental conditions show different migration behavior than that found in LC separations. Therefore the migration behavior of nine benzodiazepines on a packed ODS column and the cholesteryl silica column are compared in order to determine if the selectivity based on the stationary phase properties in CEC is maintained. The results are shown in Fig. 5 where the separation conditions for both columns were adjusted to give us the similar migration times for the solutes. The migration order

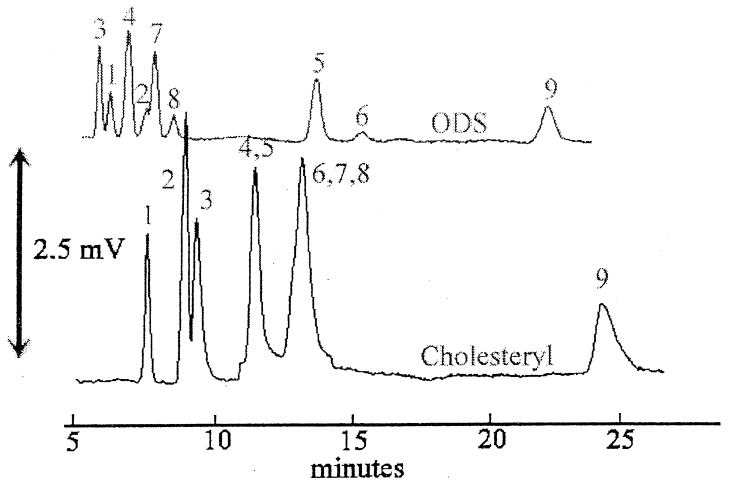

Fig. 5. Separation of benzodiazepines by packed column CEC with octadecyl bonded silica (ODS) phase and cholesteryl silica bonded phase. ODS column; acetonitrile–5 m*M* Tris–HCl buffer (60:40). Cholesteryl column; acetonitrile–5 m*M* Tris–HCl buffer (35:65). Other conditions for CEC as in Fig. 4.

of nine benzodiazepines in packed column CEC with the cholesteryl phase was totally different from that obtained with the packed ODS column CEC. The migration order of the former is mainly based on the molecular structure of the solutes [15], while on the latter the migration order is based mainly on the hydrophobicity of the solutes. As seen in Fig. 5 one can conclude that packed column CEC can maintain the selectivity which is caused by the properties of the stationary phase except for the solutes which are charged under the separation conditions (in this case cloxazolam and medazepam).

The organic solvent concentration in the buffer mobile phase in packed column CEC influences the retention behavior similar to reversed-phase LC separations. Two different acetonitrile concentrations in the mobile phase have been tested and the chromatograms obtained are shown in Fig. 6, where A utilizes a 25% acetonitrile concentration in Tris–HCl buffer solution at pH 7.3 and B has a 35% acetonitrile concentration in the same buffer system. As can be seen, the higher concentration of acetonitrile produces faster migration and better peak shapes as in reversed-phase LC separations. This result means the main interaction influencing the migration order is based on the molecular level chromato-

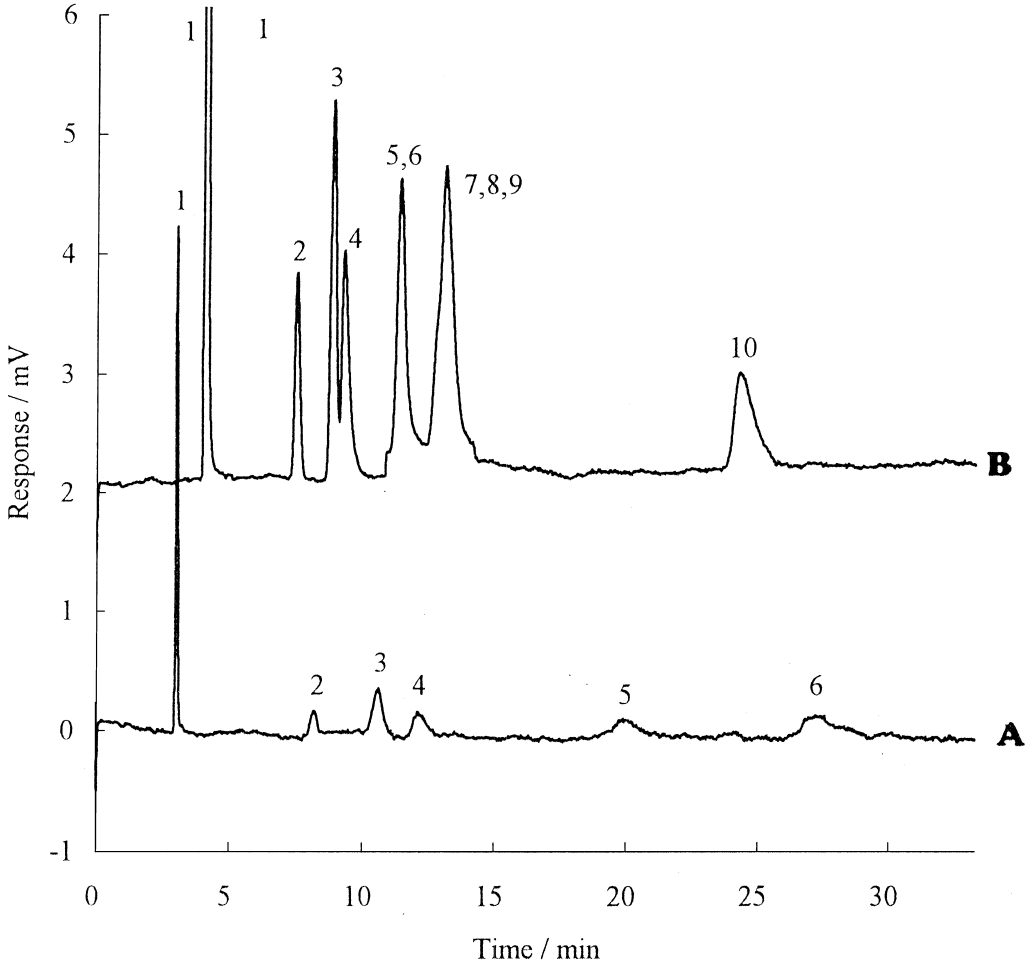

Fig. 6. Separation of benzodiazepines by packed column CEC with cholesteryl bonded stationary phase. (A) Acetonitrile–5 m*M* Tris–HCl buffer (25:75); (B) acetonitrile–5 m*M* Tris–HCl buffer (35:65) Other conditions as in Fig. 4.

graphic interaction between the solutes and the cholesteryl bonded phase, where the structural recognition by this material is the dominant factor [15].

Another important consideration in the mobile phase system is the pH of the buffer solution. The pH of the buffer was adjusted to either 7.3 or 7.7 and separations have been performed under these two conditions. The results are shown in Fig. 7 for the chromatograms of a mixture containing nine benzodiazepines. As can be seen, the higher pH gave better separation. One pair of solutes, brotiazolam (peak 5) and clotiazepam (peak 6) as well as three other solutes, oxazolam (peak 7), halozazolam (peak 8) and cloxazolam (peak 9), can be partially resolved at pH 7.7. However higher pH requires longer migration time and therefore, optimization to get better separation results is still required when practical applications are considered.

To confirm the performance of the open-tubular format CEC with the cholesteryl bonded moiety, a mixture of benzodiazepines (cloxazolam, clotiazepam,medazepam and nitrazepam), was tested using 10% acetonitrile in 10 m*M* Tris–HCl buffer, pH 7.3. The chromatogram obtained is shown in Fig. 8A.

The migration behavior is consistent with the data obtained by microcolumn LC and packed column CEC for three of the solutes, nitrazepam,

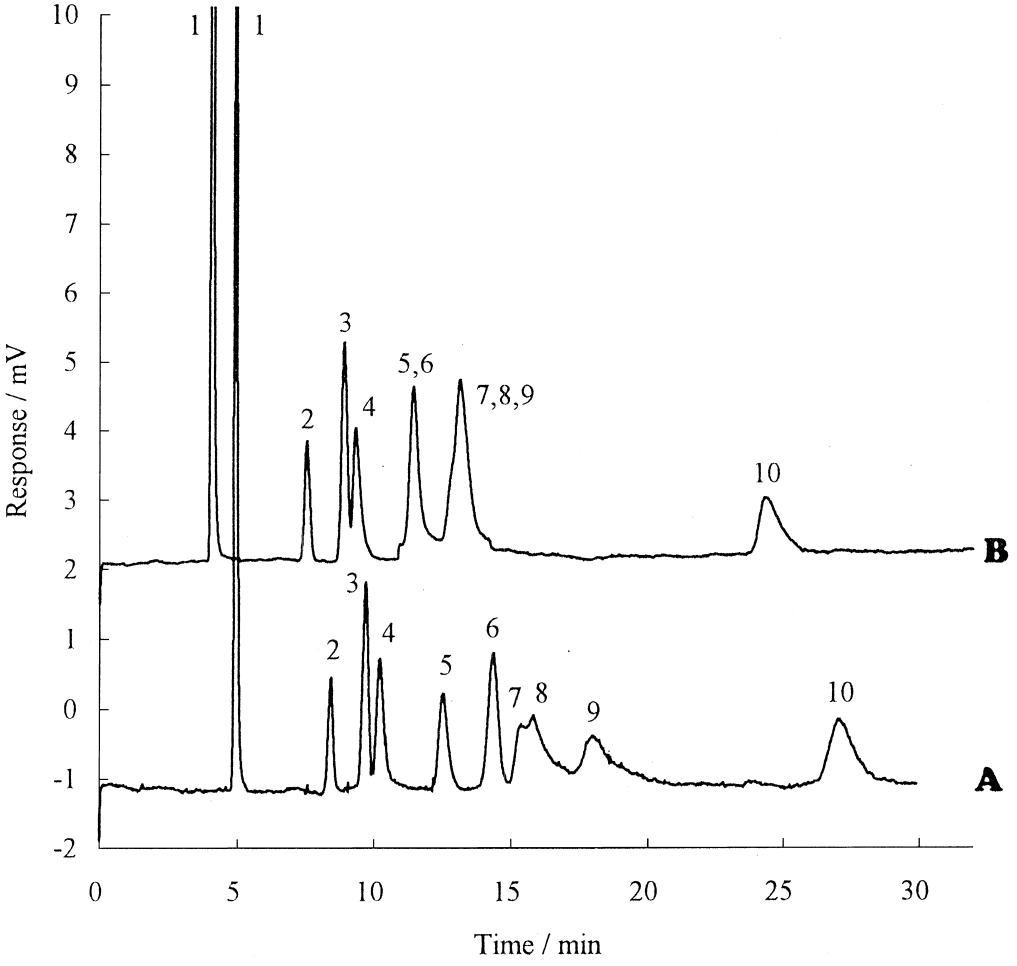

Fig. 7. Separation of benzodiazepines by packed column CEC with cholesteryl bonded stationary phase. (A) Acetonitrile–5 m*M* Tris–HCl buffer (35:75), pH 7.7; (B) acetonitrile–5 m*M* Tris–HCl buffer (35:65), pH 7.3. Other conditions as in Fig. 4.

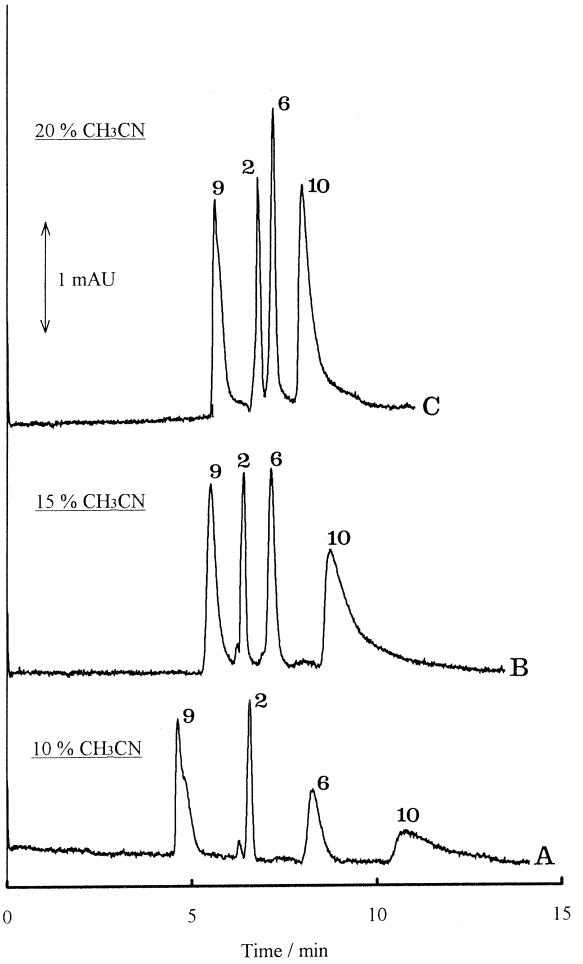

Fig. 8. Effect of acetonitrile concentration in the mobile phase for the separation of four benzodiazepines with open-tubular CEC using the cholesteryl modified etched capillary column. CEC conditions; acetonitrile–10 m*M* Tris–HCl buffer mobile phase, pH 7.3.

clotiazepam and medazepam. However cloxazolam shows a totally different migration behavior. It eluted in microcolumn LC and packed column CEC between clotiazepam and medazepam but in this open-tubular format its migration was faster than nitrazepam. This results indicates that charged species show different migration behavior in CEC. In open-tubular CEC the influence is very large compared to packed column CEC. Two reasons can be used to explain this phenomenon: one is the possibility of the interaction between the solutes and the cholesteryl bonded moiety. Packed column CEC should have a greater possibility than open-tubular CEC for such molecular interactions since the surface coverage density of the former is larger than that of the latter. Second the electrophoretic contribution to the migration behavior in the latter should be larger than that in the former, since open-tubular columns sometimes function as the separation medium for CZE. This fact indicates that the main interaction in controlling the migration of three solutes is the chromatographic interaction between the solutes and the cholesteryl bonded phase on the inner surface of the fused-silica capillary. If this is true, increasing the concentrations of acetonitrile in the mobile phase is expected to result in better peak symmetry for the modified capillary since the addition of an organic modifier tends to decrease the chromatographic interaction between the solutes and the stationary phase. This assumption is confirmed by the chromatograms in Fig. 8. Likewise, the migration times should also decrease. If these interactions are not present, the addition of organic modifier should have no effect on the peak widths. An increase of the organic modifier concentration in the mobile phase did improve peak symmetry especially medazepam, as seen in Fig. 8. A decrease in the migration time, however, was noted only for clotiazepam and medazepam. These results also indicate that cloxazolam has little or no interaction with the stationary phase at this buffer pH (7.3). The migration time of cloxazolam actually increased with an increase in acetonitrile concentration. These results show the influence of the stationary phase on the separation, i.e., cloxazolam has a net charge at pH 7.3 and therefore does not exhibit solute–surface interactions to any large extent, but is mostly influenced by its electrophoretic mobility.

4. Conclusion

The comparative studies on packed column CEC, open-tubular CEC and microcolumn LC using the same bonded moiety, cholesteryl undecenoate, indicated that packed column CEC gives better resolution and faster analysis time than microcolumn LC separations of benzodiazepines while maintaining similar selectivity except for some solutes which are

charged species under the separation conditions. However, the open-tubular format of CEC gave a different selectivity for benzodiazepines from that which should be produced by the chromatographic properties of the cholesteryl silica phase. In most cases charged species migrate mainly on electrophoretic mobility rather than the chromatographic selectivity. Based on the above results packed column CEC is the most promising method for determining benzodiazepines in clinical and forensic drug analysis based on faster analysis time, higher resolution, low solvent consumption and high throughput of the analytical data.

References

[1] C. Fujimoto, Y. Fujise, E. Matsuzawa, Anal. Chem. 68 (1996) 2753.
[2] J.-L. Liao, N. Chen, C. Ericson, S. Hjerten, Anal. Chem. 68 (1996) 3468.
[3] A. Palm, M.V. Novotny, Anal. Chem. 69 (1997) 4499.
[4] E.C. Peters, K. Lewandowski, M. Petro, F. Svec, J.M.J. Frechet, Anal. Commun. 35 (1998) 83.
[5] Q.-H. Wan, J. Chromatogr. A 782 (1997) 181.
[6] J.J. Pesek, M.T. Matyska, J. Chromatogr. A 736 (1996) 255.
[7] J.J. Pesek, M.T. Matyska, J. Chromatogr. A 736 (1996) 313.
[8] J.J. Pesek, M.T. Matyska, J. Chromatogr. A 763 (1997) 307.
[9] F. Onuska, M.E. Comba, T.B. Bistriki, R.J. Wilkinson, J. Chromatogr. 142 (1977) 117.
[10] A.P. Catabay, C. Okumura, Y. Saito, K. Jinno, J. Microcol. Sep. 9 (1997) 81.
[11] A.P. Catabay, C. Okumura, K. Jinno, J.J. Pesek, E. Williamsen, J.C. Fetzer, W.R. Biggs, Chromatographia 47 (1998) 13.
[12] A.P. Catabay, M. Taniguchi, K. Jinno, J.J. Pesek, E. Williamsen, J. Chromatogr. Sci. 36 (1988) 111.
[13] C. Delaurent, V. Tomao, A.M. Siouffi, Chromatographia 45 (1997) 355.
[14] B. Buszewski, M. Jezierska, M. Welniak, D. Berek, J. High Resolut. Chromatogr. 21 (1998) 267.
[15] A.P. Catabay, H. Sawada, K. Jinno, J.J. Pesek, M.T. Matyska, J. Cap. Electrophoresis 5 (1998) 89.