ELSEVIER

Journal of Chromatography A, 736 (1996) 313–320

JOURNAL OF CHROMATOGRAPHY A

Separation of tetracyclines by high-performance capillary electrophoresis and capillary electrochromatography

Joseph J. Pesek*, Maria T. Matyska

Department of Chemistry, San Jose State University, One Washington Square, San Jose, CA 95192, USA

Received 29 August 1995; revised 15 November 1995; accepted 4 December 1995

Abstract

Separations of various tetracycline mixtures by high-performance capillary electrophoresis (HPCE) and a new form of electrochromatography (CEC) are compared. The new CEC method involves etching the inner wall of the capillary surface with an appropriate reagent (ammonium dihydrogen fluoride) in order to produce a significant increase in surface area. The etched surface is then modified by a silation/hydrosilation reaction sequence to first produce a hydride intermediate which is then further reacted to attach a C_{18} moiety. The bare and hydride capillaries are tested under HPCE conditions while the C_{18} capillary functions in the CEC mode. The effects of pH and the presence of an organic modifier (methanol) are also studied. Detection limits (<10 μg/ml) are comparable to previous HPLC and HPCE results. Resolutions for mixtures which simulate real analytical problems are equal to or better than those reported for separations on polymeric and diol columns by HPLC and in earlier studies by HPCE and MECC.

Keywords: Capillary columns; Pharmaceutical analysis; Tetracyclines; Antibiotics

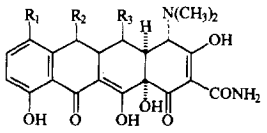
1. Introduction

The importance of tetracyclines as antibiotics for both humans and animals has been well documented [1,2]. Some structures of typical tetracyclines and their degradation products which illustrate both their amine and hydroxyl functionality are shown in Fig. 1. HPLC has proved to be an excellent separation technique for the analysis of many tetracycline mixtures which result from the original antibiotic and their chemical or physiological degradation products [3–8]. However, many of the applications which have been developed for the analysis of tetracyclines involve mobile phases which have aqueous components at either low pH (<2.5) or high pH (>8.0). Since these conditions are not conducive to use with many silica-based stationary phases, recent applications have focused on the use of polymer-based materials for the separation medium [9]. However, certain limitations exist for the polymer stationary phases, particularly the use of gradients, so that some tetracycline mixtures are not amenable to separation on these materials. Recently we reported that a new type of diol and mono-ol phases, which are silica-based and synthesized via a hydride intermediate, is successful for a broader range of tetracycline analyses at low pH than either the traditional ODS or polymeric materials used previously [10].

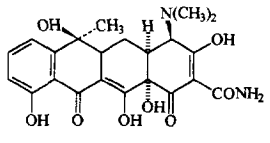
The synthetic route to the stationary phases cited above involves the controlled deposition of the hydrolysis product of triethoxysilane (TES) on a silica surface and subsequent addition of a terminal

*Corresponding author.

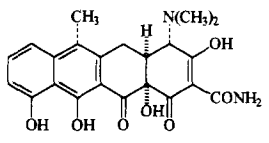
002-9673/96/$15.00
SSDI 0021-9673(95)01316-4



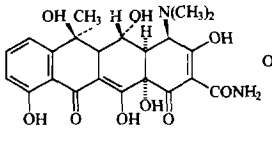
Compound	R_1	R_2	R_3
1. Minocycline	$N(CH_3)_2$	H, H	H, H
2. Tetracycline	H	HO, CH_3	H, H
3. Chlorotetracycline	Cl	HO, CH_3	H, H
4. Oxytetracycline	H	HO, CH_3	H, OH
5. Doxycycline	H	H, CH_3	H, OH
6. Methacycline	H	$=CH_2$	H, OH
7. Meclocycline	Cl	$=CH_2$	H, OH



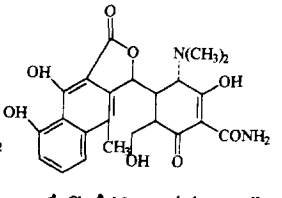
a. 4-Epitetracycline



b. Anhydrotetracycline



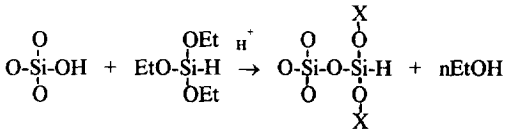
c. 4-Epioxytetracycline



d. α-Apo-oxytetracycline

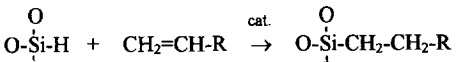
Fig. 1. Structures of the tetracyclines, degradation products and impurities.

olefin to the hydride-modified species [11–13]. The first step (silation) is as follows:



where X=Si or H and $n=1-3$.

The ideal situation is for $n=3$ so that a monolayer of hydride is formed on the surface and there are a minimum number of residual silanols. The second step (hydrosilation) in this process can be described as follows:



Typically a metal complex such as hexachloroplatinic acid is used to catalyze the above reaction [14]. In the previous HPLC study [10] the diols were synthesized using allyl glycidyl ether or 7-octene-1,2-diol while the mono-ol was made from 1-octene-8-ol. The value of this synthetic method is that it removes many of the silanols from the surface and replaces them with hydrides and attachment of the organic moiety occurs through a direct Si–C linkage. Therefore, it was not surprising that these basic compounds gave good peak shapes and the phases were able to withstand prolonged exposure to aqueous eluents in the pH range of 2.0–2.5.

High-performance capillary electrophoresis (HPCE) is an emerging technique that has been shown to possess distinct advantages over HPLC, particularly its extremely high efficiency which can often approach a million theoretical plates [15]. While not all of the advantages have been fully exploited, there already are numerous examples of the resolving power of HPCE for the separation of complex mixtures of both small molecules and macromolecules. Tetracyclines, with their basic functional groups that are capable of being positively charged at low pH, are a typical class of molecules which is amenable to analysis by HPCE [16–18]. Capillary electrochromatography (CEC) is a hybrid technique between HPLC and HPCE. It involves partition of a solute between a stationary phase and a mobile phase as in HPLC but the means of transporting the analytes through the column is based on electrophoretic mobility and/or electroosmosis as in HPCE [19]. To date, most CEC experiments involve the use of capillary tubes (100–400 μm I.D.) packed with stationary phases which are used in HPLC, i.e. alkyl moieties bonded to 5–10 μm porous silica particles [20–26]. While in principle these methods have proved to be successful for a variety of separations, there are many problems associated with CEC in the packed capillary format. Among these are the need to install a frit near the end of the capillary but before the detector, Joule heating, bubble formation and packing problems especially with the narrowest bore capillaries. Recently we have introduced a new format for CEC which involves etching the inner walls of a 50-μm capillary in order to increase the surface area and produce radial extensions of up to 5 μm and then subsequently bonding an appropriate organic moiety to this etched surface [27]. The bonding scheme used was

the silation/ hydrosilation described above. It has been shown theoretically that electrochromatography in an open tube should lead to efficiencies which are higher than those which can be achieved via a packed capillary format [28]. This paper evaluates the potential of both HPCE and CEC for the separation of various tetracycline mixtures and compares these techniques to typical results which are obtained for similar analyses by HPLC.

2. Experimental

2.1. Apparatus

All HPCE and electrochromatography experiments were performed on a Perkin-Elmer/Applied Biosystems (Foster City, CA, USA) Model 270A-HT capillary electrophoresis system. The oven used for etching and modification of capillaries in the electrochromatography experiments was a Hewlett-Packard Model 5890 gas chromatograph which was altered slightly to accommodate several tubes through both the inlet and outlet.

2.2. Etching and modification procedures

The etching process has been described in detail elsewhere [27]. The basic process involves etching the inner wall with a saturated solution of ammonium hydrogen difluoride. The extent of surface etching depends on both the time and temperature. The modification process involves two steps: silation of the etched surface with TES followed by hydrosilation with 1-octadecene in the presence of Spier's catalyst (hexachloroplatinic acid) [27].

2.3. HPCE and CEC procedures

Three types of capillaries were tested: a bare, unetched and unmodified one for HPCE; hydride etched for CEC; and C_{18} etched for CEC. The endosmotic flow was measured using dimethyl sulphoxide (DMSO) as the neutral marker. Injection of the samples was done hydrodynamically for 0.8 s at 12.6 cmHg vacuum ($1.68 \cdot 10^4$ Pa) (bare and hydride capillaries) or electrokinetically for 5–10 s at 10 kV (bare, hydride and C_{18} capillaries). The electrolyte solutions were degassed by ultrasonication followed by purging with He. The C_{18} capillary was conditioned with methanol prior to use by purging it with at least 10 column volumes forced through with a syringe. Bare capillaries were purged with 0.01 *M* NaOH for 2 min after each injection. Hydride capillaries were flushed with the run buffer for 2 min after each injection. Tetracyclines were detected at 254 nm.

2.4. Materials

The capillary tubing used was 375 μm O.D.×50 μm I.D. (Polymicro Technologies, Phoenix, AZ, USA). The analytes, tetracyclines (Sigma, St. Louis, MO, USA) and their degradation products (Janssen Chimica, Beerse, Belgium) are as follows (see Fig. 1): 1=minocycline HCl; 2=tetracycline HCl; 3=chlorotetracycline HCl; 4=oxytetracycline HCl; 5=doxycycline HCl; 6=methacycline HCl; 7=melocycline sulphosalicylate salt; a=4-epitetracycline HCl; b=anhydrotetracycline HCl; c=4-epioxytetracycline; d=α-apo-oxytetracycline. The analytes were generally prepared at a concentration of 20 μg/ml for those components which were commercially available. Buffer constituents and methanol were obtained in the highest purity available. Water was purified on a Milli-Q system and then passed though a 0.45-μm filter. The etching agent, ammonium hydrogen difluoride, was purchased form Aldrich (Milwaukee, WI, USA). TES (Hüls America, Bristol, PA, USA) and 1-octadecene (Aldrich) for modification of the etched capillaries were used as received from the manufacturer. The buffers used in the HPCE and CEC experiments were 30 m*M* phosphate–19 m*M* Tris (pH 2.14) and 30 m*M* citric acid–24.5 m*M* β-alanine (pH 3.0).

3. Results and discussion

HPLC has proved to be a valuable technique for separating various tetracycline mixtures. In particular the most promising studies to date involve polymeric columns [9] and diol phases synthesized via a hydride intermediate [10]. However, even these phases have some limitations with respect to certain practical separations for a particular pair of com-

pounds, usually a tetracycline and one of its degradation products or impurities. Therefore, instead of trying to improve separations on these columns through mobile phase optimization which might be difficult considering the effort already expended on this approach or synthesizing new bonded materials, it was decided to explore the use of two electrically driven separation methods: HPCE and CEC. Tetracyclines should be particularly amenable to these techniques due to their ability to acquire one or more positive charges at low pH depending on their structure and the exact hydrogen ion concentration in the buffer. The general ability of HPCE to separate a reasonably complex mixture of seven tetracyclines is shown in Fig. 2. Six peaks appear in the electropherogram indicating that all but two of the components (doxycycline and methacycline) have been separated under these experimental conditions. Therefore, this preliminary test displays promise for the analysis of various tetracyclines and their degradation products using an electrically driven technique such as HPCE. As shown previously [16], the pH of the buffer controls the migration of tetracyclines and

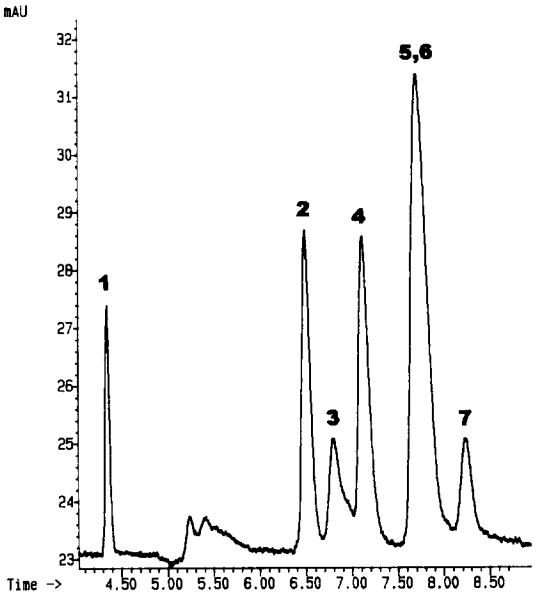


Fig. 2. Separation of a mixture of seven tetracyclines on a bare fused-silica capillary (for solute notation see Fig. 1). Conditions: buffer, pH 3.0 (30 m*M* citric acid and 24.5 m*M* β-alanine)–40% methanol, l=50 cm, V=30 kV, electrokinetic injection for 5 s at 10 kV. Time in min.

the change from one buffer system to another (Tris–phosphate to citric acid–β-alanine) does not seem to alter the dominant effect of the hydrogen ion concentration. Separation of samples at lower concentration indicate that the detection limit for most of the analytes is 5–10 μg/ml.

In terms of practical separations, the impurities and degradation products which might be found in commercially available tetracycline include 4-epitetracycline, 4-epianhydrotetracycline, chlorotetracycline and anhydrotetracycline [9]. Separation on a polymeric column was possible for such mixtures under isocratic conditions but elution of the last component (anhydrotetracycline) required more than 40 min. Using both mono-ol and diol columns synthesized via the silation/hydrosilation method [10], all five components of this mixture were separated under gradient conditions in less than 10 min. Fig. 3A shows the separation of this sample on a bare capillary in a pH 3.0 buffer. Three individual components are separated (component e, 4-epianhydrotetracycline, is assigned by comparison to HPLC data [10] using an identical sample) under these conditions. When a mixture of 40% methanol and the buffer is used as the electrolyte, the electropherogram obtained for the separation is presented in Fig. 3B. The same pattern is observed as in Fig. 3A but the peaks have become noticeably sharper. The result obtained on the hydride capillary (Fig. 3C) is similar to the bare capillary except that the peaks have become even sharper than those obtained on the bare capillary. Since basic solutes would interact strongly with residual silanols on the surface, the formation of the hydride layer eliminates a large fraction of these Si–OH groups. Finally, the C_{18} column (Fig. 3D) successfully resolves the two components which co-eluted on the bare and hydride capillaries (chlorotetracycline and anhydrotetracycline). However, component e became a shoulder on the first peak. While this minor component can still be detected and quantitated, it is often not observed in real mixtures. Further optimization of buffer and solvent conditions might also improve the separation so that all five components can be resolved as in the case of gradient elution on mono-ol and diol phases. However, the separation times here are comparable to the best (~10 min) reported by HPLC [10].

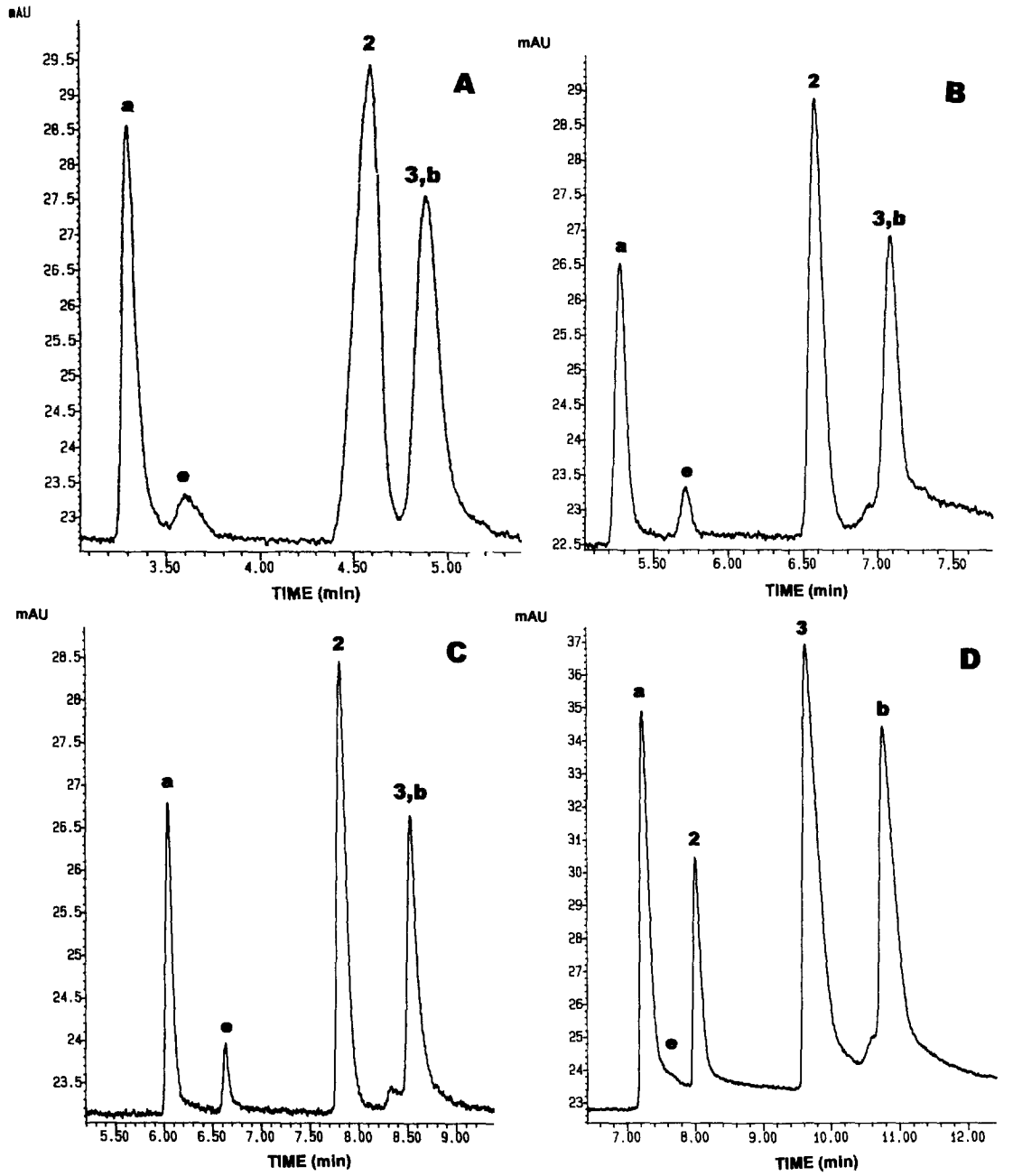


Fig. 3. Separation of some likely components found in commercial tetracycline (for solute notation see Fig. 1). (A) Bare capillary with electrolyte pH 3.0 buffer (30 m*M* citric acid and 24.5 m*M* β-alanine), *l*=50 cm, *V*=30 kV, electrokinetic injection for 5 s at 10 kV; (B) bare capillary same as for A but with buffer–methanol, 60:40; (C) hydride capillary, conditions same as for B except *l*=25 cm; and (D) C_{18} capillary, conditions same as for C.

In the analysis of commercial oxytetracycline, the impurities and degradation products include 4-epioxytetracycline, α-apo-oxytetracycline and tetracycline. Under isocratic conditions on the polymeric columns [9], the mixture is separated except for oxytetracycline and 4-epioxytetracycline which could not

be resolved according to the protocol reported. The total analysis time was in excess of 30 min. However, partial resolution of these two components was achieved on the mono-ol column by the proper choice of gradient with an analysis time of slightly longer than 10 min [10]. Testing this same mixture under HPCE conditions with a bare capillary using a pH 3.0 buffer gives the electropherogram shown in Fig. 4A. All four components, including the oxytetracycline and 4-epioxytetracycline pair which

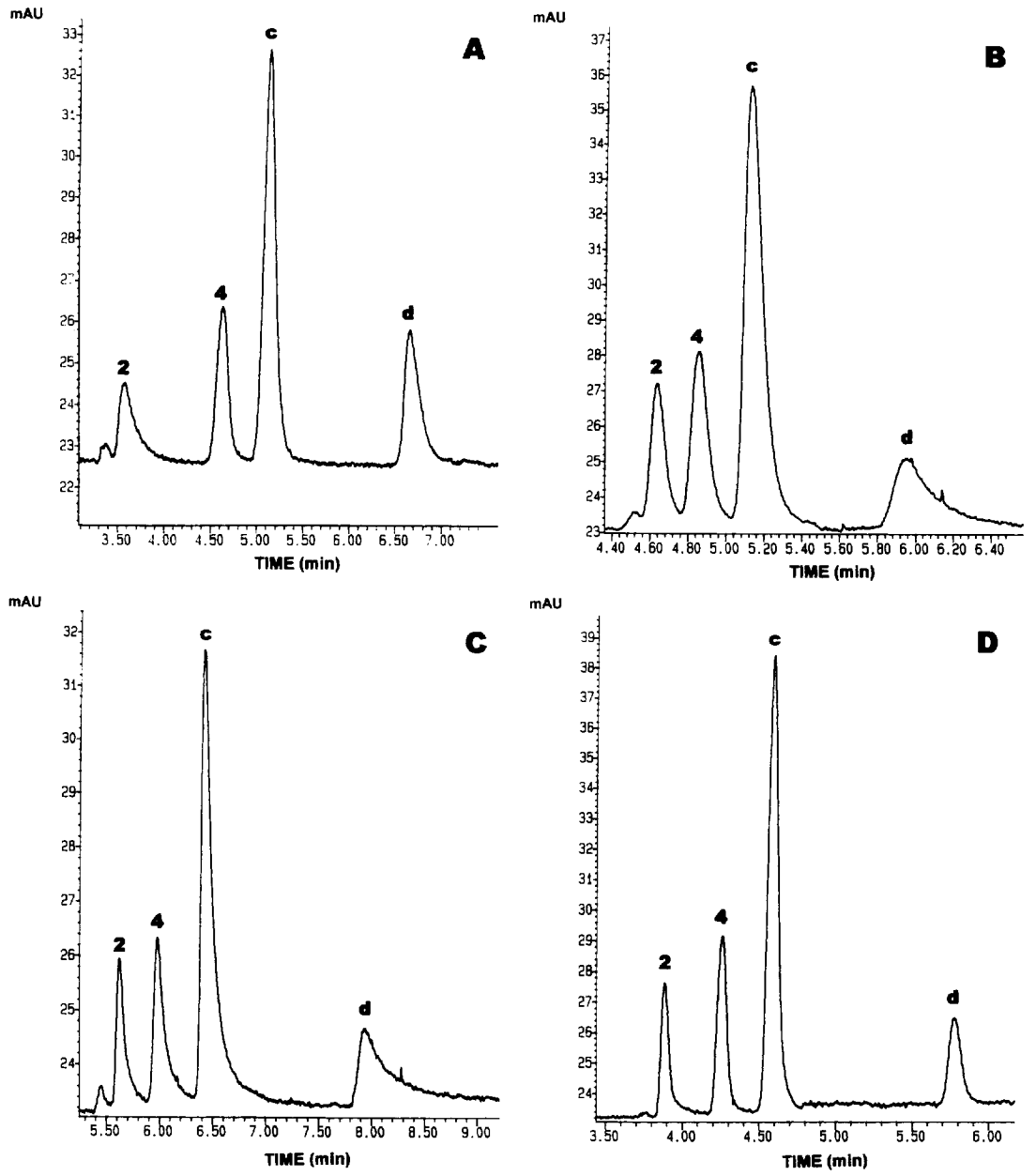


Fig. 4. Separation of some likely components found in commercial oxytetracycline (for solute notation see Fig. 1). (A) Bare capillary, conditions same as for Fig. 3A; (B) same as for Fig. 3A except electrolyte is pH 2.14 buffer (30 m*M* phosphate and 19 m*M* Tris) and 40% methanol; (C) hydride capillary, same as for B except l=25 cm; and (D) C_{18} capillary, conditions same as for C.

were unresolved on the polymeric columns and partially resolved on the mono-ol column, are well separated by this method. Peak symmetry is reasonable under these conditions and improves slightly except for the last component when changing the electrolyte to a mixture of the pH 2.14 buffer and 40% methanol (Fig. 4B). The hydride capillary under identical conditions (Fig. 4C) as the bare capillary shows a similar separation of the four components. Finally, a noticeable improvement in peak shape and a slightly shorter analysis time (less than 6 min) is obtained for the C_{18} capillary (Fig. 4D).

Other challenging analyses involve commercially available doxycycline and methacycline. Both are always present with one being the major component and the other being an impurity. Another potential impurity in both of these products is oxytetracycline. The polymeric columns partially separate these two compounds (doxycycline and methacycline) in an analysis time of almost 40 min [9]. Mono-ol and diol columns give about the same degree of separation but with an analysis of time of about 8 min [10]. Fig. 5A shows the separation of these three compounds on a bare capillary in presence of a pH 3.0 buffer. Again partial separation is possible but with an analysis time of around 5 min. Slightly better separation is achieved with the hydride capillary (Fig. 5B) under identical conditions in about 6 min. The doxycycline/methacycline pair was not separated on the C_{18} column with the pH 3.0 buffer or at several other pH values both with and without methanol.

In conclusion, separation of various tetracycline mixtures can be readily achieved by HPCE as well as CEC. The etched columns with a bonded C_{18} moiety result in better separations for some but not all tetracycline samples. The separations reported here are comparable or better than those achieved in HPLC using polymeric columns and always are accomplished in considerably less time. In comparison to analysis by HPLC with mono-ol or diol columns, HPCE and CEC are at least equivalent, and in some instances better, for the mixtures tested and the analysis time is similar. The results reported here are also equivalent to or better than those obtained in earlier CE experiments [16–18]. The detection limits (5–10 μg/ml) are comparable to those reported in

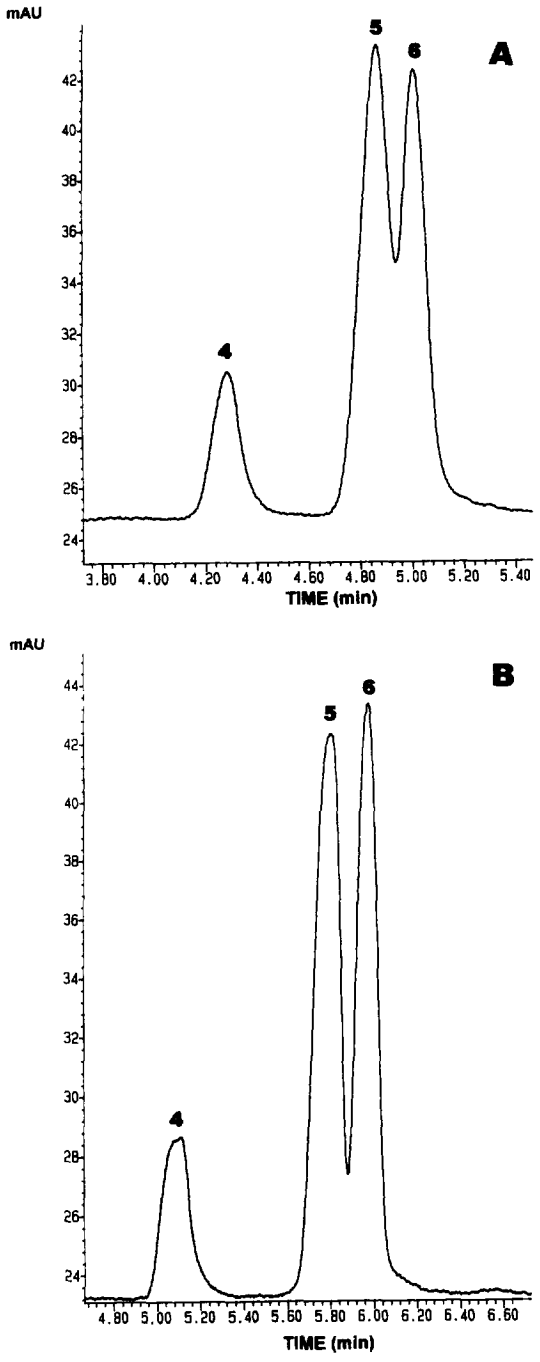


Fig. 5. Separation of some likely components found in commercial doxycycline and methacycline (for solute notation see Fig. 1). (A) Bare capillary, conditions same as for Fig. 3A; and (B) hydride capillary, conditions same as for Fig. 3A except l=25 cm.

both HPLC and HPCE. Therefore, the simpler instrumental system required for HPCE or CEC as well as considerably reduced solvent consumption makes these two methods attractive alternatives to HPLC for future practical analyses of various tetracycline mixtures.

Acknowledgments

The authors wish to thank the National Institutes of Health (Grant R15 GM 49452-01) and the National Science Foundation (CHE 9119933) for partial support of the research reported here.

References

[1] D. Gotlieb and P.D. Shaw, Antibiotics, Vol. 1, Springer Verlag, New York, 1967.
[2] J.R.D. McCormick, in Z. Vanek and Z. Hostalek (Editors), The Biogenesis of Antibiotic Substances, Academic Press, New York, 1965, p. 83.
[3] W. Naidong, J. Thuranira, K. Vermeulen, E. Roets and J. Hoogmartens, J. Liq. Chromatogr., 15 (1992) 2529.
[4] W. Naidong, K. Verresen, E. Roets and J. Hoogmartens, J. Chromatogr., 586 (1991) 61.
[5] N.H. Khan, P. Wera, E. Roets and J. Hoogmartens, J. Liq. Chromatogr., 13 (1990) 1351.
[6] W. Naidong, E. Roets and J. Hoogmartens, J. Pharm. Biomed. Anal., 7 (1989) 1691.
[7] N.H. Khan, E. Roets, J. Hoogmartens and H. Vanderhaeghe, J. Chromatogr., 405 (1987) 229.
[8] K. Wolfs, E. Roets, J. Hoogmartens and H. Vanderhaeghe, J. Chromatogr., 385 (1986) 444.
[9] P.D. Bryan and J.T. Stewart, J. Pharm. Biomed. Anal., 12 (1994) 675.
[10] M.T. Matyska, J.J. Pesek and A.M. Siouffi, Chem. Anal., 40 (1995) 517.
[11] J.E. Sandoval and J.J. Pesek, Anal. Chem., 61 (1989) 2067.
[12] J.E. Sandoval and J.J. Pesek, Anal. Chem., 63 (1991) 2634.
[13] C.-H. Chu, E. Jonsson, M. Auvinen, J.J. Pesek and J.E. Sandoval, Anal. Chem., 65 (1993) 808.
[14] J. Marciniec, J. Gulinski, W. Urbaniek and Z.W. Kornetka, in J. Marciniec (Editor), Comprehensive Handbook on Hydrosilation, BPCC Wheatons, Exeter, 1992, p. 8.
[15] P.J. Camellini, Capillary Electrophoresis, CRC Press, Boca Raton, 1993.
[16] C.-X. Zhang, Z.-P. Sun, D.-K. Ling and Y.-J Zhang, J. Chromatogr., 627 (1992) 281.
[17] S. Croubels, W. Baeyens, C. Dewaele and C. Van Peteghem, J. Chromatogr. A, 673 (1994) 267.
[18] M.F.M. Tavares and V.L. McGuffin, J. Chromatogr. A, 686 (1994) 129.
[19] J.H. Knox, J. Chromatogr. A, 680 (1994) 3.
[20] Y. Yan, D. Schaufelberger and F. Erni, J. Chromatogr. A, 670 (1994) 15.
[21] J.H. Knox and I.H. Grant, Chromatographia, 32 (1991) 317.
[22] H. Rebscher and U. Pyell, Chromatographia, 38 (1994) 737.
[23] F. Yang, J. Chromatogr., 326 (1982) 265.
[24] T. Ishii and T. Takeuchi, J. Chromatogr., 255 (1983) 349.
[25] H. Alborn and G. Stenhagen, J. Chromatogr., 323 (1985) 47.
[26] H. Yamamoto, J. Baumann and F. Erni, J. Chromatogr., 593 (1992) 313.
[27] J.J. Pesek and M.T. Matyska, J. Chromatogr. A, in press.
[28] M. Martin and G. Guiochon, Anal. Chem., 56 (1984) 614.