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Electrochromatography in chemically modified etched fused-silica capillaries

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Abstract

A new method of electrochromatography is described in which a 50-μm capillary is etched with ammonium hydrogen difluoride, followed by modification of the new surface via a silation reaction with triethoxysilane to produce a hydride intermediate, and then subsequently subjected to hydrosilation using 1-octadecene in the presence of a platinum complex catalyst. The C_{18} bonded phase is then compared with bare capillaries, etched bare capillaries and the hydride etched capillary to determine if any solute–bonded phase interactions are present. With bradykinin as a test solute, peak efficiencies are quite similar for all capillaries without C_{18} but become noticeably broader when the organic moiety is attached to the etched capillary wall. A test mixture of peptides and proteins displays shorter retention for some of the components when methanol is added to the mobile phase which is typical of reversed-phase behavior. The same result is also obtained when a mixture of tetracyclines is separated on the C_{18} capillary with and without methanol as part of the mobile phase. The reproducibility of retention times for two proteins is ±1.5%. A few results for several neutral compounds indicate small but measurable k' values.

Keywords: Capillary columns; Proteins; Peptides; Tetracyclines; Antibiotics; Bradykinin

1. Introduction

The first electrochromatography experiments were described more than 20 years ago by Pretorius et al. [1] and the technique was further developed seven years later by Jorgenson and Lukacs [2]. While capillary electrochromatography (CEC) is not a new technique chronologically, it is still relatively undeveloped because of many perceived as well as actual problems. In a theoretical treatment, it was predicated that CEC would only be practical if both narrow-bore capillaries (<100 μm) and submicron packing materials were used [3]. However, a subsequent study demonstrated that although the expected Joule heating effects were present, it was possible to use capillaries with a diameter as great as 320 μm because the amount of heat generated was reduced by a high content of organic in the mobile phase and the presence of the packing material [4]. Because in most applications of CEC pressure is not used to drive the solute through the column, it should be possible to utilize extremely small particle sizes to achieve maximum efficiency. It has been demonstrated that electroosmotic flow is independent of particle size to at least a diameter of 1.5 μm [3,5]. Other problems include bubble formation [4–7], packing difficulties [8–10], and the design and use of frits [2,4,5,11,12]. Another approach to CEC has

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been the fabrication of a complete column system on a microchip [13]. However, on such a small scale detection can be difficult and the geometry of the etched channel is important to reduce the effects of band broadening. Recently it has been demonstrated in a theoretical study that useful electrochromatographic separations might be possible in wall-coated open-tubular capillaries with diameters as great as 50 μm [14]. In spite of the many difficulties associated with current CEC methods and equipment, it is possible to successfully solve some separation problems such as the analysis of pharmaceutical compounds with current methodology [15].

In this paper the open tubular approach to CEC is utilized [16,17]. The fused-silica capillaries have been etched to increase the surface area via a process similar to a method described earlier by Onuska et al. [18] for the modification of larger diameter capillaries used in gas chromatography. In 400 μm I.D. capillaries the surface was etched in order to increase the area available for coating by conventional stationary phases used in typical GC analyses. Surface area increases up to 1000-fold were obtained which led to a great reduction in the length of capillary needed to achieve a particular separation. While little has been done with this concept in gas chromatography, the principle established could be transferred readily to CEC. Using smaller capillaries, i.e. diameters which are typical for HPCE such as 50 μm, the etching process will not only increase the surface area but produce radial extensions from the surface which should facilitate solute–bonded phase interactions. Application of a potential should drive the solutes via a plug flow profile similar to HPCE rather than a parabolic flow which is encountered in pressure-driven systems. In this format without packing, the difficulties associated with bubble formation should be greatly reduced as well with frits since there is no need for them in this open tubular configuration. In fact, the principle has already been demonstrated in 30-μm base-etched soda lime glass [19] and 8–11 μm acid/base-etched borosilicate glass [20,21]. Finally, the chemistry used to modify the etched capillary is based on the silation/hydrosilation reaction scheme (shown in simplified form below) which leads to a direct silicon–carbon bond on the surface and enhanced stability [22–27].

$$-\mathrm{Si}-\mathrm{OH} + (\mathrm{OEt})_3\mathrm{Si}-\mathrm{H} \rightarrow -\mathrm{Si}-\mathrm{O}-\mathrm{Si}-\mathrm{H} + 3\mathrm{EtOH}$$

$$-\mathrm{Si}-\mathrm{H} + \mathrm{R}-\mathrm{CH}=\mathrm{CH}_2 \xrightarrow{\text{cat}} -\mathrm{Si}-\mathrm{CH}_2-\mathrm{CH}_2-\mathrm{R}$$

The feasibility of using this approach for the modification of capillary walls in HPCE has already been demonstrated [28]. In this method a significant number of silanols is converted to hydrides which lowers the electroosmotic flow. The result should be an increase in the time for solute–bonded phase interactions at constant applied potential and an enhancement of separation provided by differences in electrophoretic mobility for charged species. With some electroosmotic flow, separation of neutral species should also be possible. In this initial paper the processes of etching small diameter capillaries is established and the principle of CEC in this particular format is proved as a viable alternative to packed column methods described in the literature.

2. Experimental

2.1. Materials

The capillary tubing used was 375 μm O.D.×50 μm I.D. (Polymicro Technologies, Phoenix, AZ, USA). The proteins (turkey lysozyme and ribonuclease A), polypeptides (bradykinin, angiotensin I and III) and tetracyclines used as test solutes were obtained from Sigma (St. Louis, MO, USA) and used without further purification. Buffer constituents and methanol were obtained in the highest purity available. Water was purified on a Milli-Q system and then passed through a 0.45-μm filter. The etching reagent, ammonium hydrogen difluoride was purchased from Aldrich (Milwaukee, WI, USA). Triethoxysilane (TES, Hüls America, Bristol, PA, USA) and 1-octadecene (Aldrich) for modification of the capillaries were used as received from the manufacturer.

The following buffers were used in the various electrochromatographic and electrophoretic experiments: pH 2.14, 30 m*M* phosphate–19 m*M* Tris; pH

3.0, 30 m*M* citric acid–24.5 m*M* β-alanine; and pH 3.7, 30 m*M* lactic acid–36 m*M* β-alanine.

2.2. Apparatus

All electrochromatography experiments were performed on a Perkin-Elmer/Applied Biosystems (Foster City, CA, USA) Model 270A-HT capillary electrophoresis system. SEM pictures of bare and etched capillaries were obtained on a Hitachi Model s5205 scanning electron microscope. The oven used for the etching and modification procedure was a Hewlett-Packard Model 5890 gas chromatograph which was altered slightly to accommodate several capillaries through both the inlet and outlet.

2.3. Etching and modification procedures

The bare capillary (2 m) was filled with concentrated HCl, sealed, and then heated overnight at 80°C. After opening, the capillary was successively washed with distilled water, acetone and finally diethyl ether. The tube was then dried with nitrogen for 1 h. Next the capillary was filled with a 5% (w/v) (saturated) solution of ammonium hydrogen difluoride in methanol and allowed to stand for 1 h. The methanol solvent was removed by a uniform nitrogen flow through the capillary for 0.5 h. The capillaries were then sealed at both ends and heated at either 300°C or 400°C (under nitrogen) for 3 h. After the heating process, the capillaries were washed with methanol for 1 h and then dried with nitrogen for 0.5 h.

Before modification, the capillaries were preconditioned at room temperature by first treating them with a pH 10 (about 6 m*M*) ammonia solution for 20 h at a flow-rate of 0.1–0.2 ml/h. After rinsing for 1 h with deionized water, the capillary was flushed with 0.1 *M* HCl solution for 4 h to remove any ammonia adsorbed on the surface. After a 2-h rinsing with deionized water, the tube was dried with nitrogen at 100°C for at least 20 h. Modification of the etched capillary is a two-step process. First an Si–H monolayer was formed by washing the capillary with dioxane and then treating it with a 1.0 *M* TES solution in dioxane at 90°C for 90 min. The hydride-modified capillary was then washed for 2 h with THF–water and then for 2 h with THF at room temperature. Finally, the capillary was dried for 0.5 h with nitrogen. The second step is the attachment of the organic moiety (1-octadecene) via the hydrosilation reaction. The hydride-modified capillary was first washed with dry toluene. Then 2 ml of pure 1-octadecene was mixed with 70 μl of 10 m*M* Spiers catalyst (hexachloroplatinic acid in 2-propanol), heated to 60–70°C for 1 h before being flushed through the capillary at 100°C for 45 h. At the completion of this cycle, the capillary was washed successively with toluene and THF for 1 h. Finally, the C_{18}-modified capillary was dried with nitrogen at 100°C overnight.

2.4. Electrochromatography procedures

Four types of capillaries were tested: bare and unetched; bare and etched; hydride etched; and C_{18} etched. The electroosmotic flow was measured using dimethyl sulphoxide (DMSO) and/or mesityl oxide as the neutral marker. Injection of the samples was done hydrodynamically for 0.8 s at 167 mbar ($1.69 \cdot 10^4$ Pa) except for the C_{18} capillary which was done electrokinetically for 10–20 s at 10 kV. The mobile phases 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. Proteins were detected by UV absorbance at 211 nm and all other solutes at 254 nm.

3. Results and discussion

The effects of the etching process on the inner surface of fused-silica capillaries are clearly seen in the SEM photographs shown in Fig. 1. The smooth surface of the starting material is shown in Fig. 1A. A large increase in the surface area is obtained when the etching process is allowed to proceed for 3 h at 300°C as seen in the SEM shown in Fig. 1B. Under these conditions it appears that a considerable amount of the original surface was dissolved by the etching agent and then precipitated onto the wall in rather large particles. If the process is allowed to proceed for the same period of time but at a higher

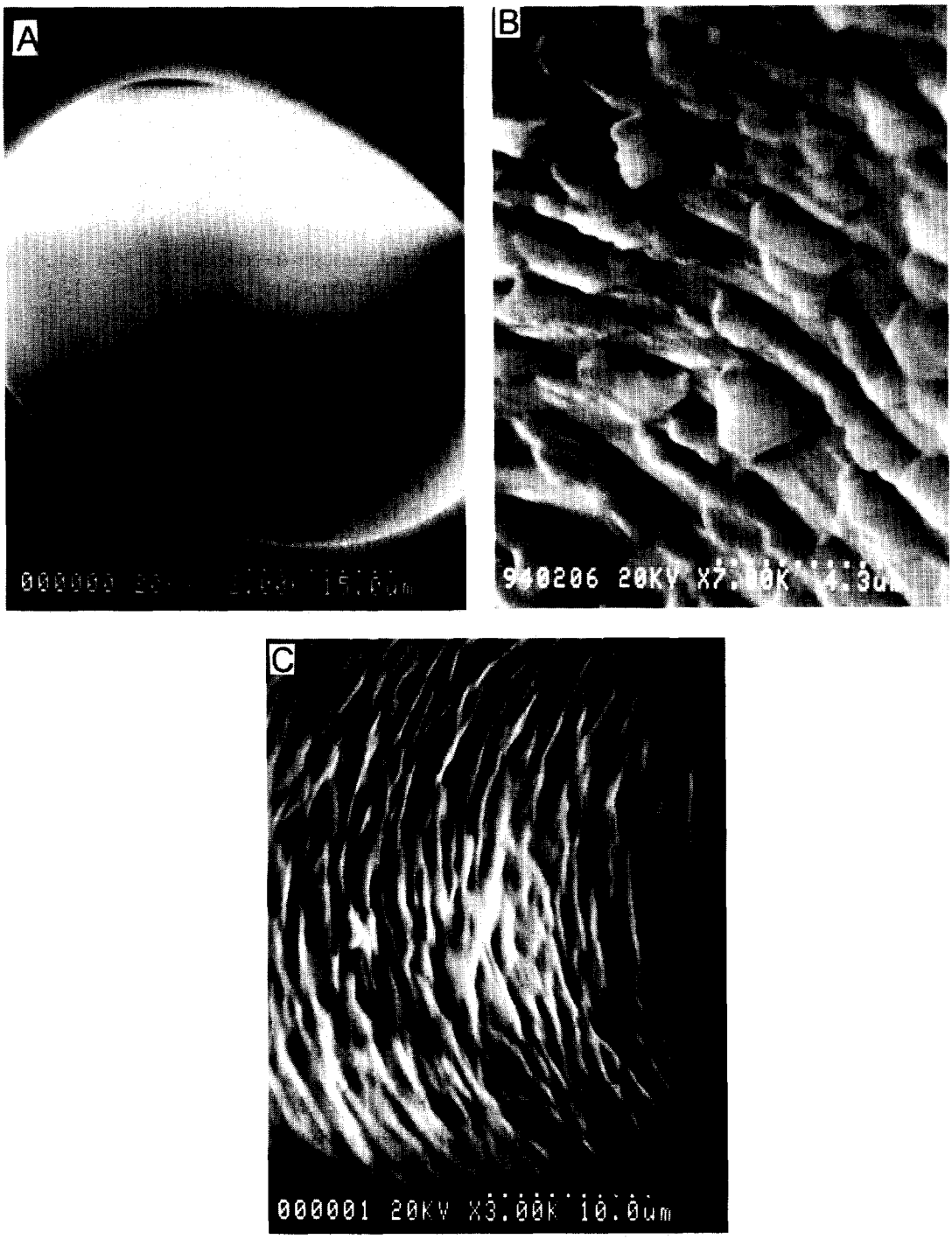

Fig. 1. Scanning electron micrographs of (A) bare capillary; (B) capillary etched with ammonium hydrogen difluoride for 3 h at 300°C; and (C) capillary etched with ammonium hydrogen difluoride for 3 h at 400°C.

temperature (400°C), then the new surface formed is somewhat more uniform and better defined (Fig. 1C). Inspection of a variety of SEMs taken at different times for several temperatures from 200 to 400°C leads to the assumption that in the early stages of the etching process the surface is composed of large spikes protruding from the wall which then proceed to more defined particles as shown in Fig. 1B and finally become a more uniform pattern as illustrated by Fig. 1C. However, the surface morphology which results from a given set of etching conditions is quite reproducible if the time, temperature and reagent concentration are well controlled. Differences between the bare surface and the various etched surfaces can also be seen in an optical microscope at a magnification of 1000. These differences are not as detailed as the SEM photos but the patterns observed provide some information about the degree of etching. At this point it cannot be determined which of the etched surfaces will be best suited to the proposed electrochromatography method. The mechanical stability of the surface structures described above were tested by vibration of the capillaries for 30 m. The surfaces which are obtained in the early stages of the etching process (large spikes protruding from the wall) are severely damaged by the mechanical stress of vibration. However, the surfaces which are produced later in the etching process do not appear to sustain any damage after prolonged mechanical stress from vibration.

In order to learn something about the effects of the etching and modification processes, one solute, bradykinin, was used as a test solute to probe the various capillary surface configurations. Fig. 2A is the electropherogram of bradykinin on a bare capillary. The peak shape is symmetric and relatively narrow. A similar result is obtained for the etched capillary surface (Fig. 2B). Even though the effective length of the etched capillary is twice as long as the bare capillary, the migration time is more than twice as long. This may be due to the fact that the etching process reduces the number of silanols, particularly free silanols, on the surface and hence lowers the electroosmotic flow. The hydride-modified etched capillary (Fig. 2C) gives a peak for bradykinin which is also narrow and symmetrical similar to that obtained on the unetched and etched unmodified capillaries. The migration cannot be easily predicted in this case since the surface is hydroxylated with ammonium hydroxide before the TES silation reaction. Therefore, some new silanols are created by the ammonium hydroxide treatment but then an unknown number is removed by the TES reaction. In any case, it appears that the hydride capillary does not exhibit significant interaction with the bradykinin solute. Finally, Fig. 2D shows the electrochromatogram of the solute obtained on the C_{18}-modified etched capillary. Under conditions which are identical to those of the hydride capillary, both an increase in the migration time as well as the peak width are readily apparent. Since the result of the hydrosilation reaction is to replace hydrides with octadecyl moieties, there should be no increase or decrease in the number of silanols which are on the surface. Therefore the change in the time for the solute to pass through the capillary must be due to increased interaction with the new modified surface. The increase in peak width also supports this conclusion since there should be some decrease in efficiency due to mass transfer effects.

Table 1 summarizes the above data as well as some additional experiments done at pH 3.0. The efficiency data show that the bare, etched and hydride capillaries are of the same order of magnitude. However, the C_{18} efficiency is significantly lower (factor of 10) due to appreciable interaction (k') between the solute and the bonded organic moiety. Peak symmetries for each of these four capillaries are quite similar. A decrease of pH to 3.0 on the C_{18} capillary results in an increase in the migration time. This result indicates that electroosmotic flow does have at lease some effect on solute migration because a lower pH would result in fewer ionized silanols and hence a longer migration time. This occurs despite the fact that the net charge on the polypeptide increases which would have the opposite effect on the migration time. The longer migration also results in lower efficiency due in some part to poorer peak symmetry. However, the importance of solute–bonded phase interactions is illustrated by the effects of adding 10% methanol to the electrolyte at pH 3.0. In the absence of any k' interactions, the above results in the absence of methanol would suggest an increase in migration time due to a further decrease in the number of ionized silanols. However, a decrease in migration

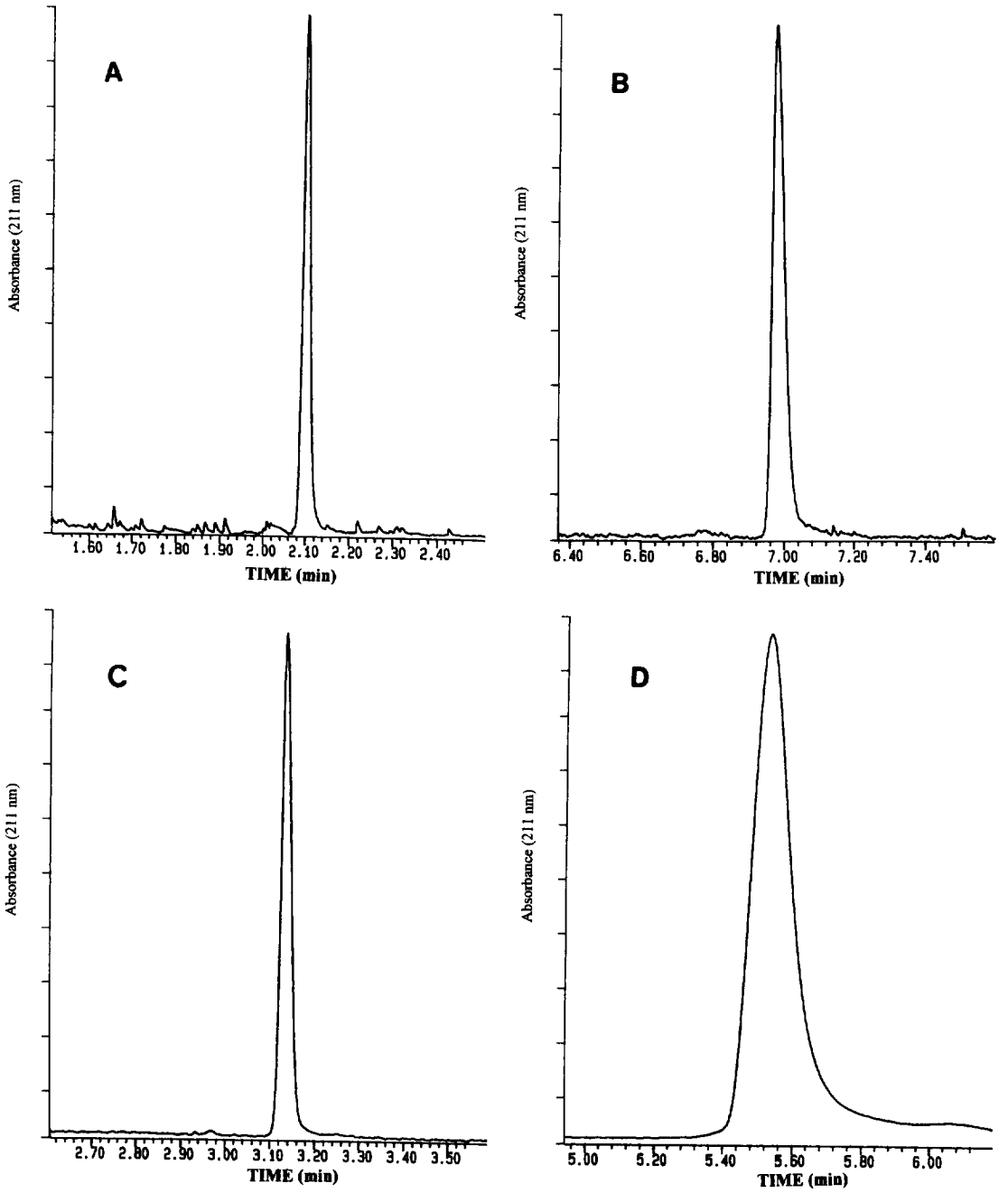

Fig. 2. Elution of bradykinin at 25 kV in lactic acid–β-alanine buffer at pH 3.7 in (A) bare capillary (L_{tot}=45 cm, L_{eff}=25 cm, I=18 μA) electrophoretically; (B) etched capillary (L_{tot}=70 cm, L_{eff}=50 cm, I=7 μA) electrophoretically; (C) hydride-modified etched capillary (L_{tot}=45 cm, L_{eff}=25 cm, I=10 μA) electrophoretically; and (D) C_{18}-modified etched capillary (L_{tot}=45 cm, L_{eff}=25 cm, I=12 μA) electrochromatographically.

Table 1
Efficiency and peak symmetry

Capillary	Buffer	$N^a_{1/2}$	A/B^b
Bare	pH 3.7	400 000	1.00
Etched; 300°C, 3 h	pH 3.7	337 000	0.73
Si–H modified	pH 3.7	542 000	0.75
C_{18} modified	pH 3.7	68 000	1.12
C_{18} modified	pH 3.0	33 600	2.47
C_{18} modified	pH 3.0 with 10% MeOH	79 000	2.22

[a] Efficiency calculated at half peak height (plates per meter).
[b] Peak symmetry calculated at 10% peak height.

time is observed due to the increased solvent power of the mobile phase. Some improvement in peak symmetry is obtained in the presence of methanol.

Confirmation of the effects of methanol in the mobile phase on the electroosmotic flow is seen in Fig. 3. Here an increase in the percentage of methanol in the mobile phase results in a decrease in the linear velocity of the neutral marker DMSO at both pH 2.14 and 3.0. These results are similar in trend to those reported in packed capillaries with acetonitrile as the modifier [10]. The conclusion in the previous study was that the decrease in electroosmotic flow was consistent with a decrease in the dielectric constant and the magnitude of the zeta potential as well as a change in viscosity when an organic modifier is present.

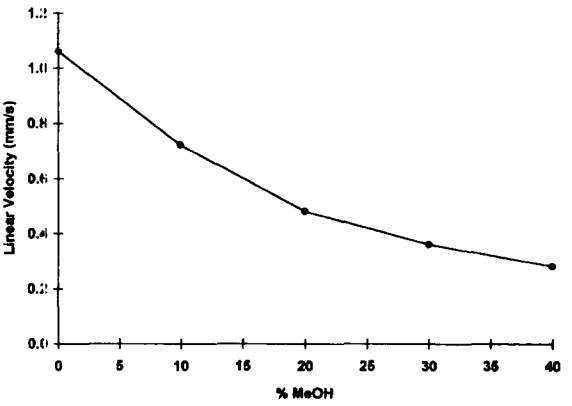

Fig. 3. Linear velocity of neutral marker (DMSO) in the C_{18}-modified etched capillary as a function of % methanol in the mobile phase at pH 2.14. ●=Buffer pH 2.14.

In order to further characterize the behavior of this new electrochromatography format, a test mixture of five polypeptides and proteins were run on the four types of capillaries. Table 2 lists the components of the test mixture as well as the migration times obtained. In the bare capillary there is a relatively small difference between the migration times of the five components (~20%). The etched capillary also has a relatively small range of migration times but each component has a lower linear velocity as noted above in the description of the behavior of bradykinin. Some improvement in separation as well as an increase in migration time over the bare capillary is seen for the hydride-modified column. The increase in elution time is probably the result of a lower electroosmotic flow which leads to separation based more on differences in electrophoretic mobility. There may be some effect of solute interaction with the more hydrophobic hydride surface but its contribution is probably relatively small since the efficiencies for the solutes are similar to those obtained on the bare capillary. When the same solutes were evaluated using the C_{18}-modified etched capillary, some solutes had long retention times and/or poor peak shapes. This was most likely due to the

Table 2
Migration times of various peptides and proteins

Compound	Capillary and buffer				
	Bare; pH 3.7	Etched (300°C, 3 h); pH 3.7	Si–H modified; pH 3.7	C_{18} modified; pH 3.0	C_{18} modified; pH 3.0 with 10% MeOH
a=Lysozyme (turkey)	1.83	Wide peak	2.23	4.06	4.67
b=Angiotensin III	2.05	6.82	3.13	4.68	5.37
c=Bradykinin	2.09	6.98	3.00	6.01	5.68
d=Ribonuclease A	2.02	6.89	2.89	6.95	6.47
e=Angiotensin I	2.24	7.55	3.66	8.01	7.00

relatively strong interactions between the solutes and the bonded octadecyl moiety which may have caused unfolding of the proteins and polypeptides. In order to alleviate this problem in the biomolecules, the pH was lowered from 3.7 to 3.0 which increased the charge on each of these species. Under these conditions, each solute was eluted in a reasonable time and the peaks had good symmetry (Fig. 4A). A relatively large difference in the elution times was also obtained (about a factor of 2). Both the increase in peak width and the larger range of elution times indicate that significant interaction between the solutes and the bonded moiety has occurred (electrochromatography as opposed to pure electrophoresis). Further support to this conclusion is obtained when methanol is added to the mobile phase causing a decrease in elution times for some solutes and a slight improvement in efficiency indicating a decrease in k' (Fig. 4B). It should be noted that the first two components in the mixture have a slight increase in elution time when methanol is added to the electrolyte indicating that electrophoretic mobility as well as bonded phase interactions are contributing to the separating ability of the C_{18}-modified capillary. The small broader peaks near base of each solute peak could be due to partial unfolding of some of the analytes in the presence of methanol.

Another example of the potential of electrochromatography in the etched capillary format is shown in Fig. 5. Fig. 5A is a separation of a mixture of tetracyclines at pH 2.14 using the C_{18}-modified capillary. Under these conditions five of the six tetracyclines are resolved because the first peak in the electrochromatogram is a decomposition product of minocycline, the next peak observed. Minocycline has one more nitrogen than the rest of the compounds and possesses a higher charge. Therefore, electrophoretic mobility is an important factor in determining the order in which the compounds are eluted. Second, it also is the sharpest peak of the group which indicates that is has the smallest degree of interaction with the bonded C_{18} moiety. The other peaks are significantly broader which reflect their lower charge, greater association with the hydrophobic bonded phase and slower mass transfer between the stationary and mobile phases. The importance of the k' interactions are illustrated in Fig. 5B which represents the separation of the same

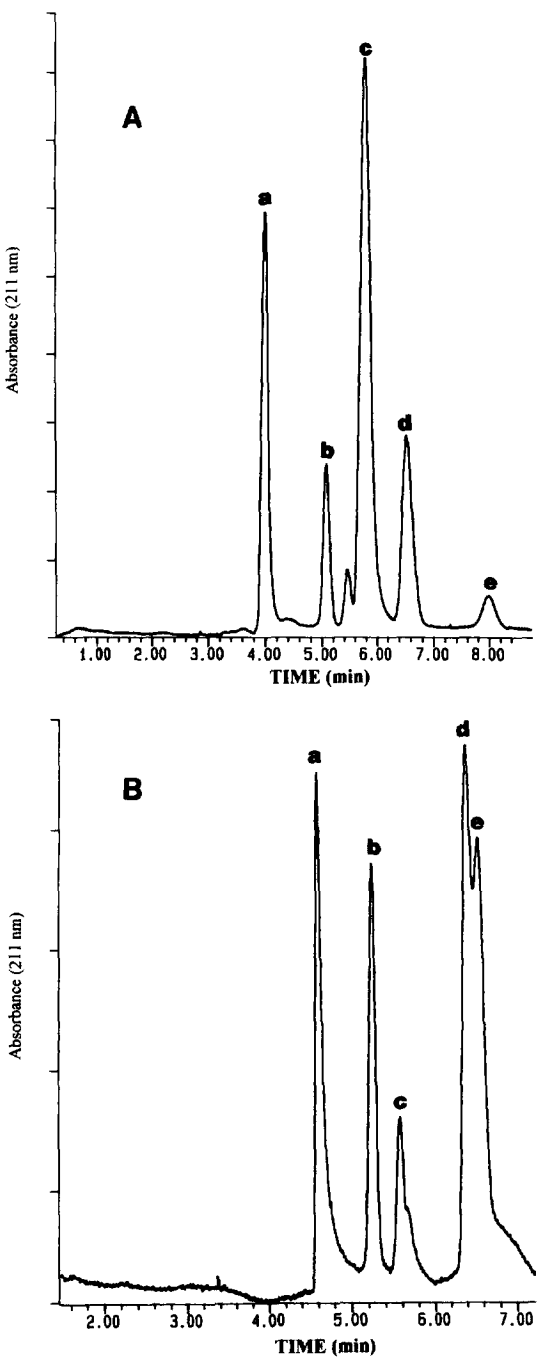

Fig. 4. Separation of a mixture of peptides and proteins on a C_{18}-modified etched capillary at pH 3.0 with (A) 0% methanol ($I = 18$ μA) and (B) 10% methanol ($I = 14$ μA) in the electrolyte. $L = 45$ cm, $l = 25$ cm, $V = 25$ kV. Solutes: a = lysozyme (turkey); b = angiotensin III; c = bradykinin; d = ribonuclease A; and e = angiotensin I.

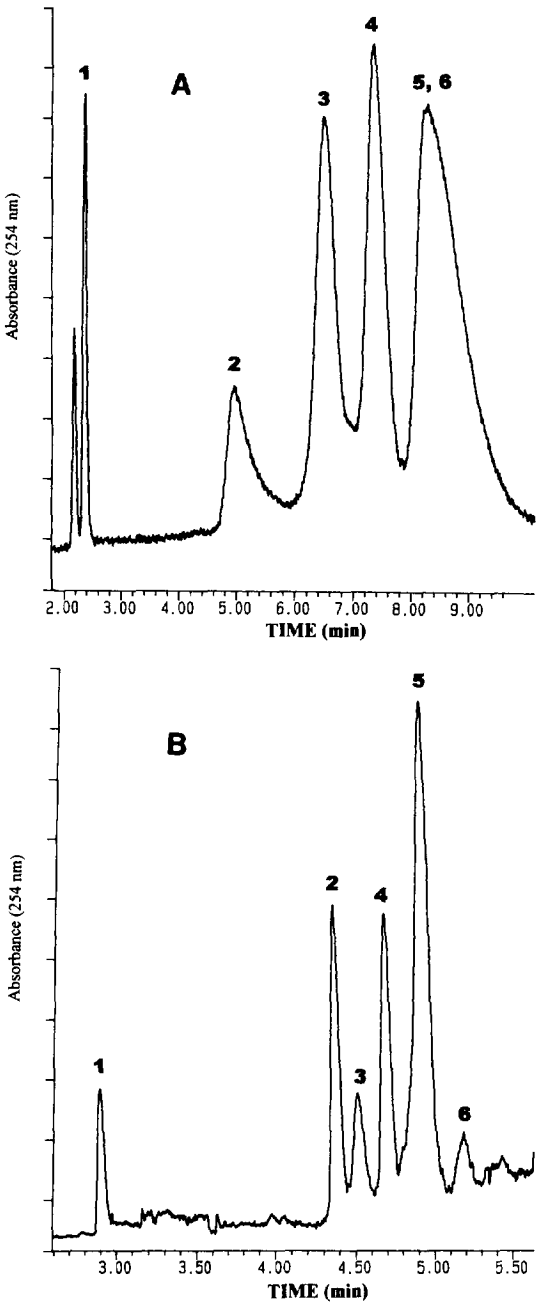

Fig. 5. Separation of a mixture of tetracyclines on a C_{18}-modified etched capillary at pH 2.14 with (A) 0% methanol ($I = 35$ μA) and (B) 40% methanol ($I = 4$ μA) in the electrolyte. $L = 45$ cm, $l = 25$ cm $V = 30$ kV. Solutes: 1 = minocycline; 2 = tetracycline; 3 = oxytetracycline; 4 = doxycycline; 5 = methacycline; and 6 = meclocycline.

mixture using an identical electrolyte but with the addition of 40% methanol to the mobile phase. Two noticeable effects can be readily seen. First, retention for all the components is reduced and second all the peak efficiencies have improved considerably, except for minocycline, which results in better resolution for the mixture. For minocycline, the charge has been lowered to a greater extent than the other components which results in slightly longer retention and larger peak width. In fact the peak widths of all the components are more similar. In this mixture the decomposition product of minocycline is not present and the six components are now resolved. If k' interactions were not present, the presence of the organic component in the mobile phase should not have a significant effect on the peak width. With k' effects present, the presence of the methanol in the mobile phase decreases the interaction between the solute and the stationary phase resulting in shorter elution times and narrower peak widths. To further amplify the importance of the bonded C_{18} moiety, when the same experiments with and without methanol are run on this mixture using a bare capillary, retention for all the components increases when the mobile phase contains the organic modifier. This is the opposite effect observed when C_{18} is present but is the same trend observed in Fig. 3 above when electroosmotic flow is more important than k' interactions. The conditions for the tetracycline separations are not optimized in these examples but are used to illustrate the influence of the stationary phase on the separation. Optimization of various tetracycline separations will be the subject of another report [29].

The stability of the capillary and reproducibility of the data were tested by first making a series of 31 consecutive injections for lysozyme. This was followed by an identical series of 31 injections for ribonuclease A. No discernible increase or decrease in retention times for either protein is observed. The reproducibility of each result is ±1.5%. The test was conducted after the capillary had been mounted and demounted several times including washing with methanol followed by dry storage, and subjected to more than 100 injections of other samples.

Finally, several neutral compounds were tested in order to measure k' values in the absence of electrophoretic migration. In all cases small but measurable

k' values were obtained for a variety of aromatic compounds. For example, the compounds 1- and 2-naphthol had k' values of 0.17 and 0.11, respectively, in the pH 3.7 buffer with 30% acetonitrile. This result is similar to that obtained in another study [30] using a pH 3.5 buffer with 25% acetonitrile for a 10-μm capillary coated internally with poly(vinylsiloxane). In the presence of 100 μM cetyltrimethylammonium bromide, the k' values for the two compounds were 0.058 and 0.043.

In conclusion, the possibility of electrochromatographic experiments in this new capillary format looks promising. Etching and subsequent modification of the capillaries overcomes many of the problems encountered in packed capillaries. The increase in surface area as well as the extension of the surface toward the center of the capillary apparently provides a geometry which is compatible with sufficient solute–bonded phase interactions to promote the degree of retention required in order to convert from electrophoresis to electrochromatography. Under such conditions a variety of separations such as enantiomeric resolution or analyses of neutral compounds without mobile phase additives should be possible. These and other potential applications are under current investigation. In addition, some of the fundamental aspects of the capillary system need to be studied such as the effect of various etching conditions, different experimental parameters in both the silation and hydrosilation reactions, other organic modifiers in the mobile phase and a broad range of organic moieties attached to the etched surface.

Acknowledgments

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