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Separation of theophylline, caffeine and related drugs by normal-phase capillary electrochromatography

A capillary electrochromatography (CEC) method has been developed for the separation of theophylline, caffeine and five related drugs on a normal-phase column with UV or photodiode array detection. Several binary, ternary and quaternary mobile phase compositions are evaluated for optimal resolution and elution of these drug analytes. The importance of selecting suitable organic solvents, buffer electrolyte, pH and applied voltage is demonstrated by a systematic study. Excellent separation is achieved for the eight drugs using a ternary mobile phase composition of isopropanol/hexane/1 mM Tris (52:40:8, pH 8), with an efficiency of 63 000 theoretical plates per meter at room temperature. Detection limits are typically at the low μg/mL level. The developed method is simple to use and it gives acceptable day-to-day reproducibility.

1 Introduction

Capillary electrochromatography (CEC) is a microseparation technique that uses the electroosmotic flow (EOF) generated by an electric field in the interparticle channels of the packing material to drive the mobile phase and subsequently transport the analytes through a capillary column [1]. Separation is based primarily on differential interaction of the analytes between the mobile and stationary phases [2]. The plug-like flow profile in capillary electrophoresis (CE) is thus combined with the retention selectivity in high performance liquid chromatography (HPLC), providing high efficiencies and resolutions in the separation of complex mixtures. In contrast to the parabolic profile of pressure-driven flows in HPLC, the plug-like profile of EOFs minimizes dispersion in CEC and affords a significant reduction in plate height. Since CEC uses 3 μm particles to generate absolute plate counts as high as 400 000 plates/meter [3], it affords better separation efficiencies than micro-HPLC for closely related molecular species. It also offers improved selectivities relative to micellar electrokinetic chromatography (MEKC) for very hydrophobic compounds, without any use of surfactants.

CEC has become an increasingly important method for chemical analysis. Development and application of CEC methods have been most active in the pharmaceutical industry [4]. Rapid and highly efficient CEC separation of

tipredane from its diastereoisomer was first reported by Euerby *et al.* [5], and CEC separation of various tetracycline mixtures was later achieved by Pesek and Matyska [6]. A large number of pharmaceutical applications (cephalosporin antibiotics, diuretics, macrocyclic lactones, peptides, nucleosides, parabens, phthalates, prostaglandins, purine bases and steroids) have been reviewed by Robson *et al.* [7]. Successful application of CEC has also been demonstrated by Euerby *et al.* [8] in the analysis of a wide range of structurally diverse acidic and neutral pharmaceutical compounds using capillaries packed with reverse-phase materials [8]. Gradient CEC of benzodiazepines, corticosteroids and thiazide diuretic drugs with UV and electrospray ionization mass spectrometric detection has recently been reported by Taylor and Teale [9]. Simultaneous CEC analysis of acids, bases and neutral compounds was first reported by Gillott *et al.* [10] using a mobile phase containing triethanolamine phosphate at pH 2.5. The separation of fifteen acidic, basic and neutral drugs of forensic interest has recently been demonstrated by Lurie *et al.* [11] using CEC with a step gradient. As CEC provides separation results that are often superior to those obtained from HPLC, pharmaceutical companies like Astra Charnwood (Loughborough, Leicestershire, UK) and G. D. Searle (Skokie, IL, USA) use it routinely to obtain the purity profile of drug actives in a single run with UV detection [12].

Theophylline is a bronchodilator that is widely used in the treatment of asthma and bronchospasm in adults. According to a 1992–94 survey published in the American Journal of Hospital Pharmacy, theophylline is one of three drugs most frequently monitored by pharmacokinetics services in the nation's 160 Veterans Affairs medical centers [13]. Theophylline pharmacokinetics has seen a resurgence of

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Abbreviation: PDA, photodiode array

interest in the development of new oral macrolide and fluoroquinolone antibiotics during the past decade. A number of interactions with other drugs have been reported for two new oral macrolides, while some of the fluoroquinolones can inhibit the cytochrome P-450 enzyme system, thereby causing increased serum concentrations of drugs like theophylline and caffeine [14]. Since theophylline is a drug with a narrow therapeutic window (serum concentration = 10–20 mg/L) between efficacy and adverse effects [15], and its concentration at the active sites strongly depends on characteristic parameters of the patient's response during the first 8 h after intravenous administration of the drug, recent arguments have been in favor of an individualization of dosage regimen in patients (particularly neonates, children, aged, or critically ill) receiving theophylline intravenously [16, 17]. While theophylline has been used as a treatment for sleep apnea in infants, caffeine is preferred for treatment of neonatal sleep apnea because it has a wider therapeutic range. HPLC in the reverse-phase mode is used to assay theophylline, caffeine and theobromine in urine, such as samples collected from athletes during doping control [18]. As drugs distribute into body compartments after absorption, samples of tissue [14], serum [19] and plasma [20] (in addition to urine) are used to investigate therapeutic theophylline concentrations in clinical pharmacokinetic experiments [21] and human liver metabolism studies [22].

Currently, CEC is practiced mostly in the reverse-phase mode for the separation of uncharged compounds and compared with the best available technology in HPLC. For organic extracts of drugs from biomedical and clinical samples, it is more appropriate to develop a normal-phase CEC method using chloroform (or hexane/heptane) admixed with isopropanol (or methanol), acetonitrile and a minimal amount of buffer solution as the mobile phase. Wei *et al.* [23] have recently demonstrated the feasibility of normal-phase CEC separations for basic drug compounds. In this work a useful CEC method is demonstrated for the rapid analysis of drug mixtures containing theophylline, caffeine and metabolites in organic extracts. One ultimate goal is to simplify the sample preparation in such analyses by performing one simple solvent extraction or direct dissolution in the mobile phase, thereby saving time and resulting in minimum effort, optimal benefit/ cost ratio, as well as easy timing of blood sampling and laboratory analysis [24].

2 Materials and methods

2.1 Chemicals

Nine different drugs were obtained from Health Canada (Ottawa, ON): acetaminophen, caffeine, hydrochlorothia-

zide, dyphylline, nicotinic acid, phenylbutazone, theobromine, theophylline, β-hydroxyethyltheophylline. All HPLC-grade solvents were purchased from Caledon (Georgetown, ON) and Fisher Scientific (Nepean, ON): acetonitrile, hexane, isopropanol and water. Tris-buffer (50 mM, pH 8) and acetate buffer (50 mM, pH 4.5) were supplied by Beckman Instruments (Palo Alto, CA, USA).

2.2 CEC instrument

CEC experiments were performed on two Beckman P/ ACE systems, model 2100 with a UV detector (set at a wavelength of 206, 214, 254 or 280 nm) and model 5510 with a photodiode array detector (set at a wavelength range of 190–300 nm). A Unimicro Technologies EP-75-20-3-Si column; 75 μm inside diameter, 20 cm length to detector, 27 cm total length, 3 μm particle size, silica, (Pleasanton, CA, USA), was used at a temperature of 25°C. The mobile phases were run under an applied voltage of 3–30 kV at a current of 0.5–2.4 μA. Sample injections were made electrokinetically at 5–10 kV for 1–15 s. For comparison, reverse-phase CEC was performed on a Unimicro Technologies column packed with 3 μm porous C18-bonded silica particles.

3 Results and discussion

3.1 Mobile phase compositions

The influence of operating parameters on CEC separation was studied, including selectivity-controlling organic solvents, buffer electrolyte and buffer pH. A mobile phase containing a minimal amount of buffer served as a guideline in this study. Basically, the EOF rate increases when the buffer concentration is decreased, down to 1–2 mM [25]. At the lowest buffer concentration of 1 mM, the electrochemical double layer becomes rather thick (Debye length, 5–10 nm). When the double layers overlap seriously in the particle pores, the EOF would be restricted to the interparticle channels, reducing mass transfer and risking a loss of efficiency [26, 35]. A standard binary composition for normal-phase CEC [27], acetonitrile/1 mM Tris (90:10, pH 8) which is best known for its high EOF rate (typically v_{EOF} = 1.7–1.8 mm/s at 20 kV) and reasonable current (I = 2 μA at 20 kV), was first tested with a mixture of caffeine and theophylline. Using an applied voltage of 10 kV, their retention times were 6.00 min and 6.12 min, respectively. This result was good, in contrast to our previous experience with reverse-phase CEC where caffeine could not be retained (k' = O) using acetonitrile/4 mM sodium tetraborate (from 20:80 to 90:10, pH 9.2) as the mobile phase. Resolution was similar among 10 kV, 8 kV, 6 kV and 4 kV, but it became poorer at 2 kV despite the longer retention times of 35.02 min
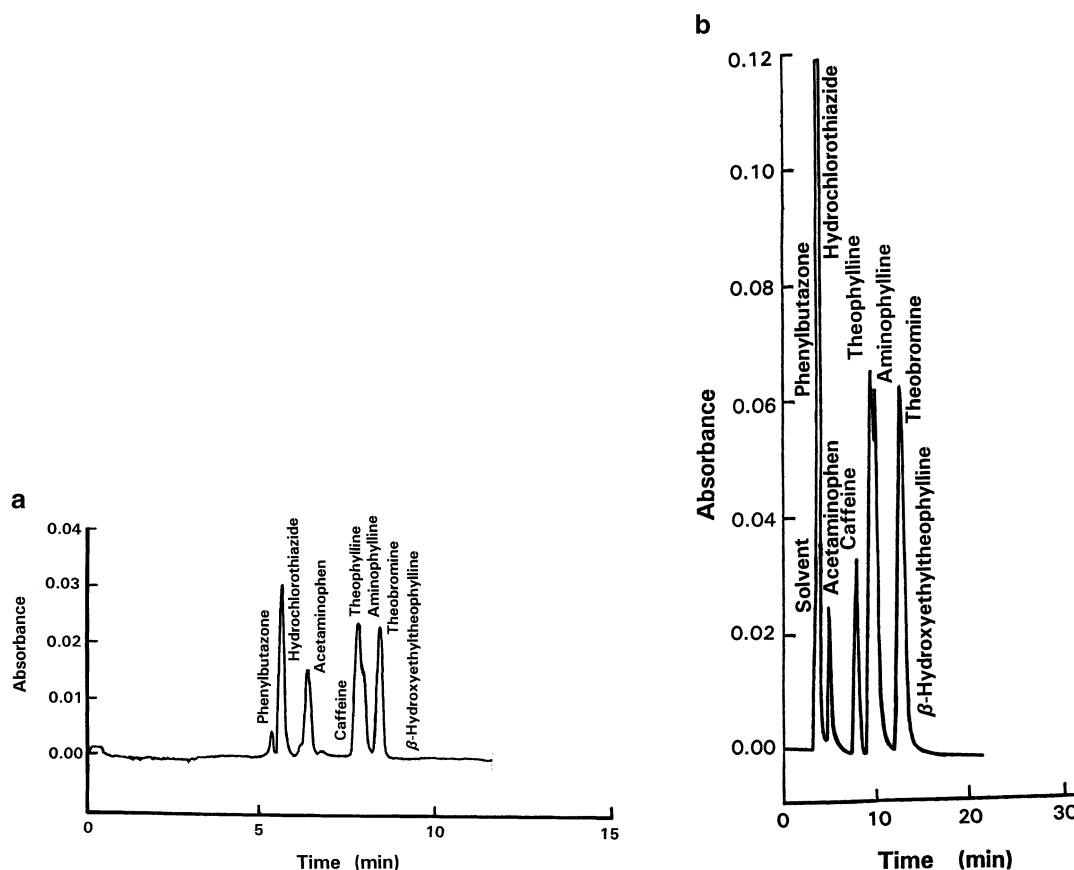
Figure 1. (a) CEC separation of a mixture of caffeine, theophylline, aminophylline and five other drugs at the 5 mg/mL concentration level on Unimicro Technologies EP-75-20-3 μm Si column at 25°C, using a mobile phase of aceto-nitrile/1 mM Tris (90:10, pH 8) at an applied voltage of 8 kV with UV detection at 280 nm. Electrokinetic injection at 5 kV for 5 s. (b) HPLC separation of a mixture of caffeine, theophylline, aminophylline and five other drugs at the 15 μg/mL concentration level on 5 μm Supelcosil LC-Si column, using a mobile phase of acetonitrile at a flow rate of 1.0 mL/min with UV detection at 270 nm. Sample injection volume, 20 μL.

and 35.97 min. Under 8 kV separation for optimal resolution and analysis time, a mixture of caffeine, theophylline, aminophylline and five other drugs produced the chromatogram shown in Fig. 1a. Inadequate resolution was observed between caffeine at 7.70 min and theophylline at 7.85 min, and aminophylline constituted total interference at a retention time of 7.90 min. The molecular structures of these drugs are illustrated in Fig. 2 to show both similarities and differences, except for aminophylline which consists of two moles of theophylline with one mole of ethylenediamine. A comparison was made with normal-phase HPLC separation of the same drug mixture using acetonitrile as the mobile phase, as shown in Fig. 1b. Despite the longer retention times, this HPLC analysis provided sufficient resolution for caffeine from theophylline and aminophylline. However, the N values afforded by HPLC (1200 for acetaminophen and 1300 for theobromine) were significantly lower that those afforded by CEC

(4400 for acetaminophen and 10600 for theobromine). For this reason, it was desirable to develop a better CEC mobile phase for the separation of these drugs.

Second, a ternary mobile-phase composition established for normal-phase HPLC of phospholipids on silica gel columns, isopropanol/hexane/water (52:40:8) [28], was modified to contain 1 mM Tris (pH 8) for CEC operation. At 30 kV, elution and good separation of theophylline (at 20.56 min), caffeine (at 26.10 min), and four other drugs could be achieved. It clearly demonstrated the separation capability of this mobile-phase composition, which can potentially be suitable for all kinds of samples prepared in organic solvents ranging from acetonitrile to chloroform. Interestingly, the elution order for theophylline and caffeine was different from that obtained using the binary mobile phase of acetonitrile/1 mM Tris (90:10, pH 8). CEC could also be performed with photodiode array (PDA)
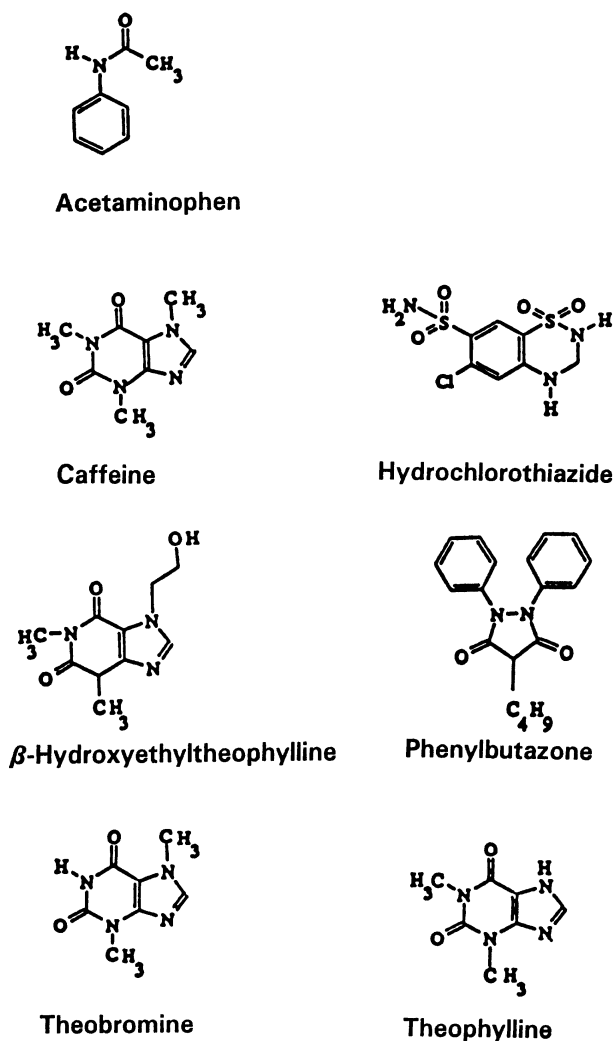
Acetaminophen

Caffeine

Hydrochlorothiazide

β-Hydroxyethyltheophylline

Phenylbutazone

Theobromine

Theophylline

Figure 2. Molecular structures of theophylline, caffeine and related drugs.

detection, using the same column and mobile phase composition for the analysis of theophylline, caffeine, and the four other drugs. The results in Fig. 3 illustrate that the PDA afforded UV detection at multiple wavelengths (*e.g.*, 214, 254 and 280 nm), thereby providing spectral information to assist the identification of individual chromatographic peaks (most notably for acetaminophen which absorbs very strongly at 254 nm). The signal-to-noise ratio was excellent in these chromatograms, as well as a good column efficiency of 63 000 plates/m calculated for the caffeine peak. This efficiency was not as high as typical CEC performance in the reverse-phase mode, partly due to peak tailing for basic drugs using normal-phase CEC. In comparison, the concentration sensitivity of CEC-PDA was slightly lower than that for HPLC-UV, being at the 4 µg/mL level for caffeine when sample injection was made at 10 kV for 15 s. Similar detection limits

(5–10 µg/mL) had been reported earlier for the separation of various tetracycline mixtures by CEC on a C_{18} capillary column with UV detection at 254 nm, using electrokinetic sample injection for 5–10 s at 10 kV [6]. One analogous comparison of HPLC with CE for the determination of caffeine had also been reported previously: in HPLC the LOD was 0.1 µg/mL for caffeine whereas in CE it was 2 µg/mL [29].

Third, a quaternary mobile-phase composition of acetonitrile/isopropanol/hexane/50 mM ammonium acetate (56:38:5:1, pH 4.5) previously used by Sandra *et al.* [30] for the separation of triglycerides was evaluated. CEC of theophylline, caffeine and the five other drugs using this composition at 25 kV yielded rapid electrochromatographic elution of all seven drugs within 6 min, as shown in Fig. 4. Unfortunately, incomplete resolution is evident. One major advantage of using quaternary mobile phases in CEC, nonetheless, is their flexibility in the variation of elution strength at constant v_{EOF}. Acetonitrile is efficient in the generation of EOF [31], so its percentage in the mobile phase composition should be decided first. Hexane is essential for the solubility of chloroform in the mobile phase, and its low elution strength only justifies a small percentage in the mobile phase composition. Isopropanol is added to ensure compatibility of hexane with acetonitrile and the aqueous buffer, and its elution strength is moderate. The acetate buffer provides a constant pH environment for the generation of EOF, and it contains water that has great elution strength in normal-phase chromatography on a silica column. Obviously, the best way to adjust elution times without changing the flow rate drastically is by varying the relative percentages of isopropanol and acetate buffer in the mobile-phase composition. For hypothesis verification purposes, a stronger quaternary mobile phase composition of acetonitrile/isopropanol/hexane/1 mM acetate (56:31:5:8, pH 4.5) was next tested for fast separation of the drug mixture by normal-phase CEC. Using 25 kV, a fast electrochromatogram was produced in that the last peak for β-hydroxyethyltheophylline coeluted with the caffeine peak at 4.04 min. Resolution of β-hydroxyethyltheophylline from caffeine could not be obtained when the applied voltage was decreased to 20 kV or 15 kV to lower the EOF rate. These results show that resolution cannot always be enhanced by allowing more time for CEC separation.

3.2 Applied voltage

The influence of applied voltage on CEC current, retention time and resolution was further evaluated with theophylline and caffeine, using acetonitrile/isopropanol/hexane/1 mM acetate (56:31:5:8, pH 4.5) as the mobile phase. Over the range of 3–30 kV, the current varied be-
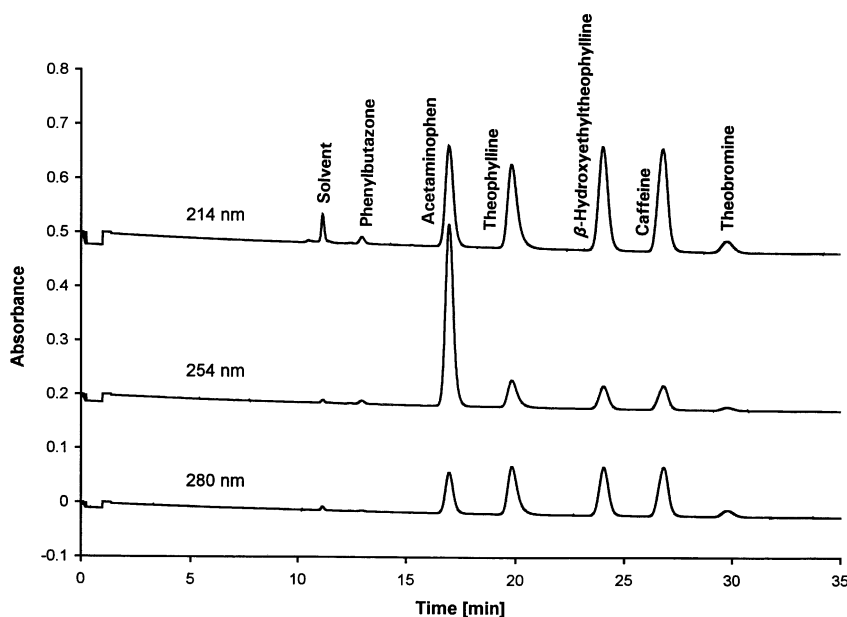
Figure 3. CEC separation of a mixture of theophylline, caffeine and four other drugs at the 1.0 mg/mL concentration level on Unimicro Technologies EP-75-20-3 μm Si column at 25°C, using a mobile phase of isopropanol/hexane/ 1 mM Tris (52:40:8, pH 8) at an applied voltage of 30 kV with PDA detection at 214, 254 and 280 nm. Electrokinetic injection at 10 kV for 15 s.
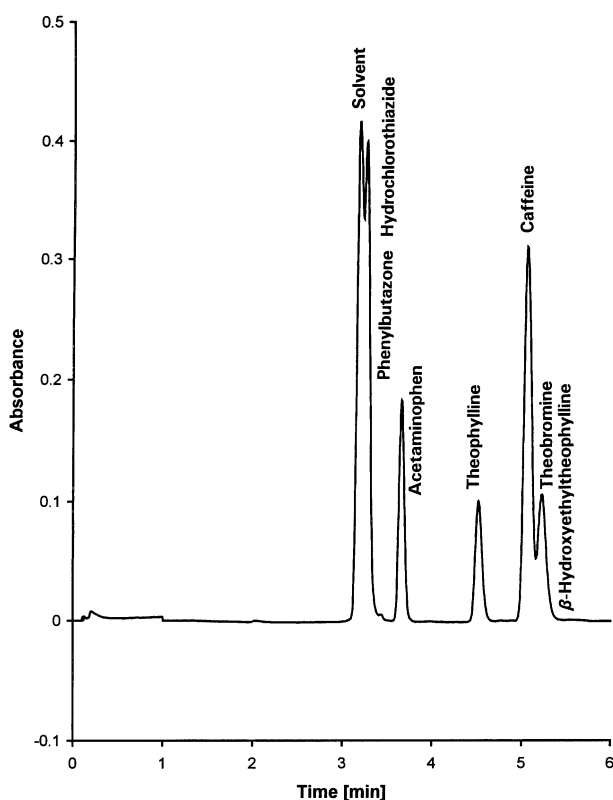

Figure 4. CEC separation of a mixture of theophylline, caffeine and five other drugs at the 1.0 mg/mL concentration level on Unimicro Technologies EP-75-20-3 μm Si column at 25°C, using a mobile phase of acetonitrile / isopropanol / hexane / 1 mM acetate (56:38:5:1, pH 4.5) at an applied voltage of 25 kV with PDA detection at 214 nm. Electrokinetic injection at 10 kV for 2 s.

tween 0.5 μA and 2.4 μA, showing a linear relationship with a correlation coefficient of $r^2 = 0.9941$. This indicated that the influence of Joule heating was not negligible. The resolution between theophylline and caffeine was maximal at 3 kV, but the analysis time of 45 min was almost unacceptable. Higher voltages up to 10 kV could be employed to reduce the analysis time down to 12 min and still provide baseline resolution. A further increase in applied voltage decreased the resolution significantly due to a large EOF rate as well as Joule heating. Figure 5 shows that a linear relationship exists between retention time and the inverse of applied voltage for both theophylline and caffeine. This can be understood in terms of CEC migration rate (v_{CEC} in units of mm/s), which is the sum of EOF rate (v_{EOF}) and electrophoretic migration rate (v_{EP}) multiplied by the retardation factor $1/(1+k')$ [32–34]

$$v_{CEC} = (v_{EOF} + v_{EP})/(1 + k') = (aE + bE)/(1 + k') = (a + b)\ V/L\ (1 + k') \quad (1)$$

where both rates are proportional to the electric field strength E (in units of V/cm) and hence the applied voltage V across the capillary column of length L [35]. This linear relationship between v_{CEC} and E had previously been noted [26]. Hence,

$$t_R = L/v_{CEC} = L^2\ (1+ k')/(a+b)\ V \quad (2)$$

Such a linear relationship between retention time and the inverse of applied voltage had been found to exist in a previous ion-exchange CEC study [36]. While a (= μ_{EOF} = $\varepsilon_r, \varepsilon_o \zeta/\eta$) is a constant for the same column and mobile
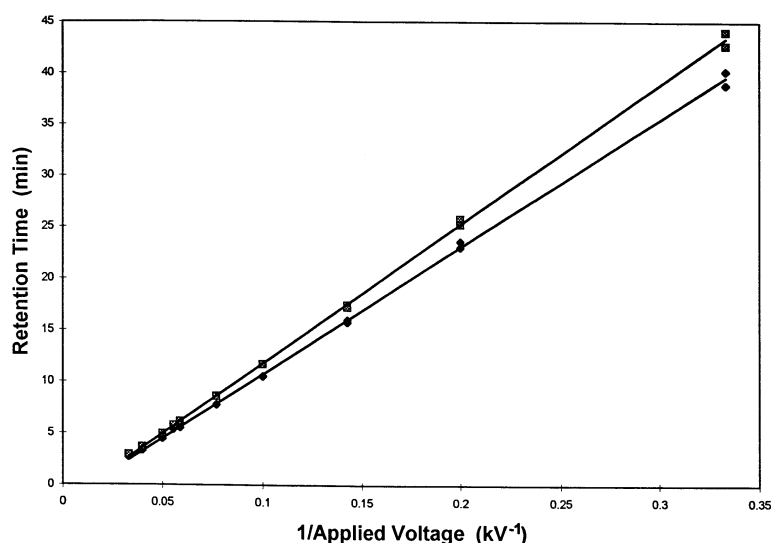
Figure 5. Linear relationship between retention time and the inverse of applied voltage for theophylline (◆) and caffeine (□), under the same CEC conditions as in Fig. 4.

phase, k' and b (= μ_{ep} = $q/6\pi\eta r$) are characteristic of each analyte. For this reason, the slopes of the theophylline and caffeine plots in Fig. 5 are different.

4 Concluding remarks

This work has demonstrated that normal-phase CEC is a valuable alternative for the traditional HPLC (and MEKC) analysis of theophylline, caffeine and related drugs when it can be carried out on a commercial CE instrument without pressurization. These results confirm that CEC can now be extended to cover the normal-phase chromatographic separation mode for the determination of a broad range of pharmaceutical compounds. Once all separation parameters are optimized, the normal-phase CEC technique is not too difficult to use. Choosing the ternary mobile-phase composition of isopropanol/hexane/1 mM Tris (52:40:8, pH 8), a mixture of theophylline, caffeine, aminophylline and five other drugs can be separated with good resolution.

5 References

[1] Colón, L. A., Reynolds, K. J., Alicea-Maldonado, R. Fermier, A. M., *Electrophoresis* 1997, *18*, 2162–2174.

[2] Colón, L. A., Guo, Y., Fermier, A., *Anal. Chem.* 1997, *69*, 461A–467A.

[3] Schure, M. R., Murphy, R. E., Klotz, W. L., *214th ACS National Meeting* 1997, Las Vegas, NV (ANYL 181).

[4] Majors, R. E., *LC.GC* 1998, *16*, 96–110.

[5] Euerby, M. R., Johnson, C. M., Bartle, K. D., Myers, P., Roulin, S. C. P., *Anal. Commun.* 1996, *33*, 403–405.

[6] Pesek, J. J., Matyska, M. T., *J. Chromatogr. A* 1996, *736*, 313–320.

[7] Robson, M. M., Cikalo, M. G., Myers, P., Euerby, M. R., Bartle, K. D., *J. Microcol. Sep.* 1997, *9*, 357–372.

[8] Euerby, M. R., Gilligan, D., Johnson, C. M., Roulin, S. C. P., Myers, P., Bartle, K. D., *J. Microcol. Sep.* 1997, *9*, 373–387.

[9] Taylor, M. R., Teale, P., *J. Chromatogr. A* 1997, *768*, 89–95.

[10] Gillott, N. C., Euerby, M. R., Johnson, C. M., Barrett, D. A., Shaw, P. N., *Anal. Commun.* 1998, *35*, 217–220.

[11] Lurie, I. S., Conver, T. S., Ford, V. L., *Anal. Chem.* 1998, *70*, 4563–4569.

[12] Stevenson, R., *Amer. Lab.* 1998, *January*, 20H–20M.

[13] Howard, C. E., Capers, C. C., Bess, D. T., Anderson, R. J., *Am. J. Hosp. Pharmacy* 1994, *51*, 1672–1675.

[14] Rodvold, K. A., Piscitelli, S. C., *Clin. Infect. Dis.* 1993, *17*, S192–S199.

[15] Holford, N., Hashimoto, Y., Sheiner, L. B., *Clin. Pharmacokinet.* 1993, *25*, 506–515.

[16] Aarons, L., *Eur. J. Drug Metabolism Pharmacokinet.* 1993, *18*, 97–100.

[17] Nikiforidis, G. C., Argyropoulos, C. P., Kassimatis, T. I., Ithakissios, D. S., *Eur. J. Drugs Metabolism Pharmacokinet.* 1997, *22*, 265–276.

[18] Delbeke, F. T., Debacker, P., *J. Chromatogr. B* 1996, *687*, 247–252.

[19] Li, Z., Chen, G., *Acta Pharmacol. Sin.* 1994, *15*, 267–270.

[20] Eldesoky, E., Meinshausen, J., Buhl, K., Engel, G., Haringskaim, A., Drewelow, B., Klotz, U., *Ther. Drug Monit.* 1993, *15*, 281–288.

[21] Schulz, H. U., Hartmann, M., Steinijans, V. W., Huber, R., Luhrmann, B., Bliesath, H., Wurst, W., *Int. J. Clin. Pharmacol. Therapeutics* 1996, *34*, S51–S57.

[22] Nagai, N., Furuhata, M., Ogata, H., *Biol. Pharmaceut. Bull.* 1995, *18*, 1610–1613.

[23] Wei, W., Luo, G. A., Hua, G. Y., Yan, C., *J. Chromatogr. A* 1998, *817*, 65–74.

[24] Tanigawara, Y., Yano, I., Kawakatsu, K., Nishimura, K., Yasuhara, M., Hori, R., *J. Pharmacokinet. Biopharmaceut.* 1994, *22*, 59–71.

[25] van den Bosch, S. E., Heemstra, S., Kraak, J. C., Poppe, H., *J. Chromatogr. A* 1996, *755*, 165–177.

[26] Li, D., Remcho, V. T., *J. Microcol. Sep.* 1997, *9*, 389–397.

[27] Unimicro Technologies, Inc. *http://www.unimicrotech.com* application notes APR8L1.

[28] Letter, W. S., *J. Liq. Chromatogr.* 1992, *15*, 253–266.

[29] Jimidar, M., Hamoir, T. P., Foriers, A., Massart, D. L., *J. Chromatogr.* 1993, *636*, 179–186.

[30] Sandra, P., Dermaux, A., Ferraz, V., Dittmann, M. M., Rozing, G., *J. Microcol. Sep.* 1997, *9*, 409–419.

[31] Wright, P. B., Lister, A. S., Dorsey, J. G., *Anal. Chem.* 1997, *69*, 3251–3259.

[32] Rathore, A. S., Horváth, C., *J. Chromatogr. A* 1996, *743*, 231–246.

[33] Wu, J. T., Huang, P., Li, M. X., Lubman, D. M., *Anal. Chem.* 1997, *69*, 2908–2913.

[34] Wei, W., Luo, G., Yan, C., *Amer. Lab.* 1998, *January*, 20C–20D.

[35] Ericson, C., Liao, J. L., Nakazato, K., Hjertén, S., *J. Chromatogr. A* 1997, *767*, 33–41.

[36] Wei, W., Hu, P., Wang, Y. M., Wang, R. J., Luo, G. A., *Chin. Anal. Chem.* 1997, *25*, 1132–1137.