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Capillary electrophoretic separation of anticoagulant rodenticides in aqueous electrolytes modified with organic solvents

Costas Stathakis, Peter L. Warburton and Richard M. Cassidy*

Chemistry Department, University of Saskatchewan, Saskatoon, SK S7N 0W0 (Canada)

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ABSTRACT

The capillary electrophoretic separation of the anticoagulant rodenticides warfarin, chlorophacinone, diphacinone, bromadiolone, dicoumarol and coumatetralyl is described. Simple electrolytes, such as phosphate and borate, as well as phosphate modified with methanol (20–30%, v/v) or acetonitrile (5–30%, v/v) were used in an effort to optimize total analysis time and resolution. Micellar systems in phosphate electrolytes were also studied but with limited success. With the addition of methanol and acetonitrile modifiers it was possible to manipulate electroosmotic mobility, analyte electrophoretic mobility, and separation resolution and efficiency. Optimum resolution and analysis time (6 min) for all rodenticides was achieved in 0.015 mol/l phosphate (pH 7) modified with 22% (v/v) methanol. Separation efficiencies ranged between 459 200–548 800 theoretical plates, analyte migration reproducibility was between 0.1–0.6% R.S.D., and peak area reproducibility was in the range 1.9–9.8% R.S.D.

INTRODUCTION

Anticoagulant rodenticides are used to control mice and rats as well as other mammals such as bats, moles, rabbits and hares [1]. On certain occasions, poisonings of household pets [2] as well as humans [3] have been reported requiring rapid methods of diagnosis followed by administration of suitable antidotes, *i.e.* phytonadione (vitamin K_1). Identification of the presence of these poisons (as well as their metabolites) in animal tissue, blood plasma and urine requires extensive sample pre-treatment, which results in small amounts available for analysis [4]. Consequently, a need exists for sensitive and efficient analytical methods for small amounts of this class of compounds, present either individually or as multicomponent mixtures [4].

Analytical approaches adopted for the analysis of these rodenticides have been based on spectrophotometric [5], fluorometric [6], thin-layer [7,8] or gas chromatographic methods [9–12]. These approaches, however, suffer from a number of drawbacks, and the most suitable approaches appear to be liquid chromatographic (LC) methods [13–15] which have been reviewed by Hunter [4]. Combinations of normal-phase, size-exclusion chromatography [13], and reversed-phase chromatography [14] with post-column pH adjustment and fluorescence detection offer sensitive and selective methods for the analysis of the coumarin-based rodenticides. Ion-exchange chromatography with APS-Hypersil and an acetonitrile–0.04 mol/l Tris–HCl buffer pH 7.5 (75:25, v/v) mobile phase, under isocratic conditions, has been used in the analysis of chlorophacinone and diphacinone residues, but has had limited success in the resolution of diphacinone from warfarin [4]. In spite of the

* Corresponding author.

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success of LC methods there is still a need for analytical methods that can be applied for both diagnostic and quantitative purposes to a wide class of rodenticides. Capillary electrophoresis (CE) offers many features which make it an attractive alternative for the detection and separation of rodenticides. These advantages include speed, efficiency [16], minimal sample requirements and excellent mass detection limits [17]. The CE separation of warfarin and coumachlor has been briefly examined [18], but no other coumarin-based rodenticides have been studied. The aim of this work was to investigate the potential of CE for the determination of coumarin-based rodenticides as well as chlorophacinone and diphacinone. The effect of micellar additives such as sodium dodecyl sulfate (SDS), cetyltrimethylammonium chloride (CTAC), tetraethylammonium chloride (TEAC) and Brij 35, and organic modifiers (CH_3OH 20–30%, CH_3CN 5–30%, v/v) on the separation of a series of six anticoagulant rodenticides was investigated. The effects of these electrolyte systems on migration time, separation efficiency and electroosmotic mobility were examined in an effort to optimize resolution and total analysis time.

EXPERIMENTAL

Instrumentation

The CE instrument used was a Quanta 4000 (Waters Chromatography Division of Millipore, Milford, MA, USA) with a Maxima 820 data station (version 3.30, Dynamic Solutions). Conventional fused-silica capillaries (56 cm × 365 μm O.D. × 75 μm I.D.) were obtained from Polymicro Technologies, Phoenix, AZ, USA. Analyte zones were detected by UV absorbance at 214 nm (Zn lamp). Samples were introduced hydrostatically by elevation of the sample vials to 10 cm for 10 s. All pH values were measured with a Fisher Accumet pH meter (Model 805) calibrated immediately prior to use.

Reagents

Distilled, deionized water (Millipore Milli-Q water purification system, Bedford, MA, USA) was used to prepare all solutions. Glass-distilled acetonitrile, as well as analytical-reagent grade methanol, benzyl alcohol, sodium hydroxide, disodium hydrogenorthophosphate and tetraethylammonium chloride (TEAC) were obtained from BDH (Toronto, Canada). Sodium dihydrogenphosphate was purchased from Fisher Scientific (Nepean, Canada) and SDS, and the rodenticides warfarin and dicoumarol were obtained from Sigma (St. Louis, MO, USA). CTAC, Brij 35 and sodium tetraborate were purchased from Aldrich (Milwaukee, WI, USA). Bromadiolone, coumatetralyl, chlorophacinone and diphacinone (100 μg/ml in methanol) were donated by the Saskatchewan Toxicology Research Center (University of Saskatchewan). Nylon filters (0.2 μm pore) were obtained from Cole-Palmer (Chicago, IL, USA).

Procedures

Sodium hydroxide (0.025 mol/l) was added to dicoumarol and warfarin samples to give a pH of 12. All running electrolyte pH adjustments were made with 0.01 mol/l NaOH. Stock phosphate buffer was prepared by dissolving equal amounts of disodium hydrogenorthophosphate (0.02 mol/l) and sodium phosphate monobasic (0.02 mol/l) and diluting, as needed. Benzyl alcohol or methanol were used as neutral markers. All solutions were filtered through 0.2-μm pore nylon filters immediately prior to use. Capillaries were purged with the separation buffer for 30 min prior to the initial run and for 2 min between runs. If drastic drifts in current and/or retention time were observed the capillary was purged with 0.2 mol/l NaOH for 15 min followed by a 10-min purge with distilled, deionized water. All glassware was rinsed with a saturated KOH–methanol solution followed by rinsing with distilled, deionized water prior to use.

RESULTS AND DISCUSSION

Phosphate and borate electrolytes

Selectivity, in CE, is often manipulated via buffer and/or pH modifications in order to affect the magnitude of the electroosmotic flow (μ_{eos})

[19–21] as well as the size/charge ratio of the analytes, and thus their respective electrophoretic mobilities [22,23]. The rodenticides studied are shown in Fig 1. These compounds possess weakly acidic protons (underlined on each individual structure), and the dissociation of these acidic protons was examined for separations in phosphate and borate buffers. The acid dissociation constant (pK_a) for warfarin is 5.05 [24]; values for the other rodenticides are expected to be in the same range, but could not be found in the literature. Studies in 0.015 mol/l phosphate (pH 7) and 0.01 mol/l borate (pH 11) resulted, respectively, in incomplete resolution (only four peaks obtained) and baseline resolution of all six rodenticides but at a relatively long analysis time (16 min).

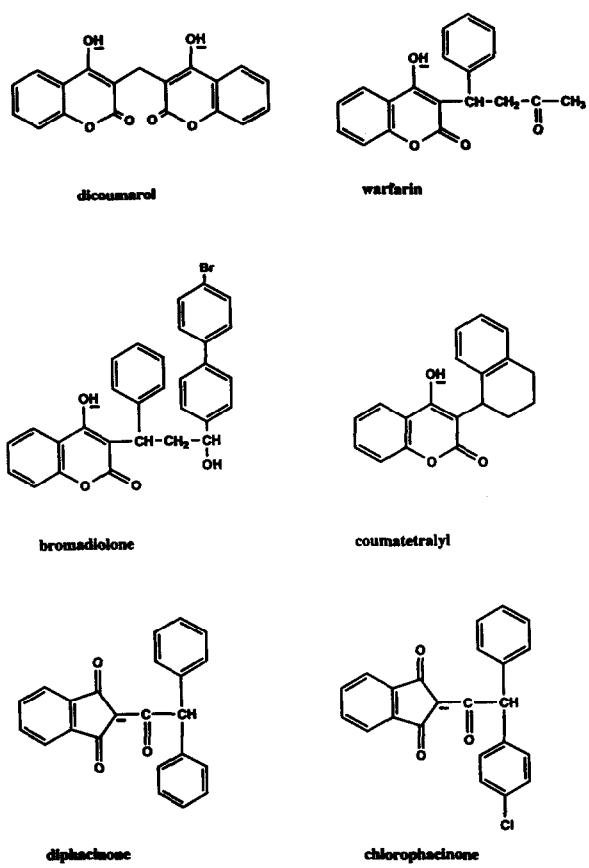

Fig. 1. Structures of the six anticoagulant rodenticides. Underlining indicates acidic proton.

Micellar–phosphate electrolytes

Micellar electrokinetic chromatography (MEKC), first introduced by Terabe *et al.* in 1984 [25], has been successful for the separation of neutral and ionic compounds [26]. Since the rodenticides should interact with micellar aggregates, this approach was examined. The four surfactant systems, above their respective critical micellar concentration, briefly examined in this study were: SDS, CTAB, TEAC and the mixed micellar system Brij 35/SDS. Studies with SDS (0.05 mol/l) in 0.01 mol/l phosphate at pH 7 gave prolonged retention times with no substantial improvement in separation resolution as compared to the pure phosphate electrolyte. A cationic surfactant (CTAC plus phosphate buffer) was then used in an attempt to reverse the direction of the electroosmotic flow [27] and decrease total analysis time of the negatively charged analytes, but this system gave extremely poor resolution as a result of strong association between the micellar aggregates and analytes. TEAC (0.025 mol/l), in 0.01 mol/l phosphate at pH 7, was also examined, but no improvement in separation efficiency was observed. In an effort to moderate strong micelle–analyte interactions observed with the cationic and anionic micelles, a more polar mixed micellar system consisting of Brij 35 and SDS (in phosphate) was used. This attempt also proved to be futile, and gave poor overall resolution and efficiency.

Mixed phosphate–organic electrolyte

The addition of organic modifiers to aqueous buffers is capable of inducing changes in the magnitude of the electroosmotic flow [23,28,29] as well as changes in the relative electrophoretic mobility of analytes [22,23]. Consequently, the effect of addition of CH_3OH and CH_3CN to the separation electrolyte was investigated. Electroosmotic flow decreased almost linearly, by 21 and 22%, respectively, with increasing % CH_3OH (20–30%, v/v) or % CH_3CN (5–30%, v/v). Such decreases have been attributed to a decrease in the dielectric constant (ϵ) and zeta electrokinetic potential (ζ) [22,28]. The relatively small decrease in medium viscosity (η) [28] is expected to be of minor importance to the observed changes in the magnitude of the elec-

troosmotic flow [22], and these effects would also be offset by the influence of decreases in buffer ionic strength due to the presence of the organic solvent, which would tend to increase the electroosmotic flow [21]. An overall decrease in analyte electrophoretic mobility (μ_{ep}), as a function of % CH_3OH and CH_3CN was observed, (see Fig. 2 for CH_3OH), which is in agreement with previously reported results for the analysis of a series of tricyclic amines [22]. The decrease in analyte μ_{ep} can also be ascribed to a decrease in the medium dielectric constant (ϵ) with organic solvent content. In this instance a decrease in ϵ results in a shift of the acid–base equilibrium towards the neutral species which decreases the effective [30] analyte electrophoretic mobility. The observed levelling effect of μ_{ep} at higher percentages of organic solvent, observed for both methanol and acetonitrile, may be due to the fact that higher concentrations of solvent can begin to stabilize the charged form of the analyte via ion–dipole interactions [31]. This effect would compete with the charge dissipation process described above, resulting in modest decreases and/or stabilization of analyte electrophoretic mobility at higher concentrations of organic modifier. Methanol was found to provide good separations in the concentration range 25–30% (v/v) whereas acetonitrile modifier could not resolve chlorophacinone and diphacinone in the concentration range 5–15% (v/v) and coumatetralyl and dicoumarol in the range 15–30% (v/v). This is most likely due to the higher μ_{eos} observed in the presence of acetonitrile as compared to methanol given the higher dielectric constant (ϵ) of the former [29]. With methanol in the electrolyte composition range of 25–30% (v/v) no significant improvement in resolution was observed at higher concentrations of methanol, and this higher concentration also gave prolonged migration times. Since separation currents were relatively low (approximately 40 μA) the field strength was increased to 536 V/cm and this gave optimum analysis time and resolution; optimum conditions were achieved in 0.015 mol/l phosphate (pH 7) with 22% methanol (see Fig. 3). Separation efficiencies were found to range between 459 200 and 548 800 theoretical plates. The reproducibility (R.S.D.) of overall migration mobility (μ_{mig}), was found to range between 0.1–0.6%, and peak area reproducibility was 1.9–9.8% (R.S.D.). The detection limit for dicoumarol at a signal-to-noise ratio 3 (peak-to-peak noise) was $4 \cdot 10^{-6}$ mol/l corresponding to 18 pg of analyte injected on to the capillary. Least squares calibration curves were determined for dicoumarol ($R^2 = 0.994$) and warfarin

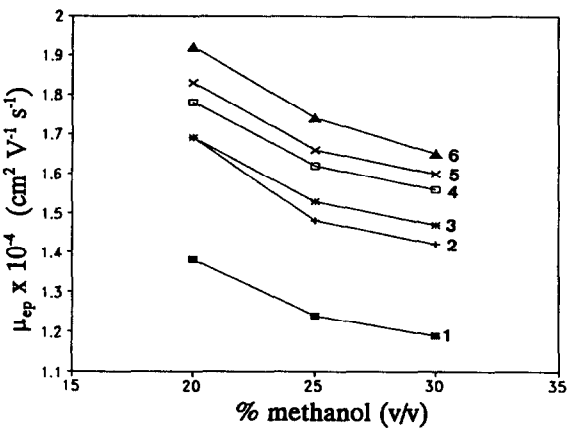

Fig. 2. Plot of electrophoretic mobility (μ_{ep}) *versus* % CH_3OH. Experimental conditions: 0.015 mol/l phosphate (pH 7) modified with methanol; neutral marker, benzyl alcohol; field strength 379 V/cm; hydrostatic injection from 10 cm for 10 s. Plots: 1 = bromadiolone; 2 = chlorophacinone; 3 = diphacinone; 4 = warfarin; 5 = coumatetralyl; 6 = dicoumarol.

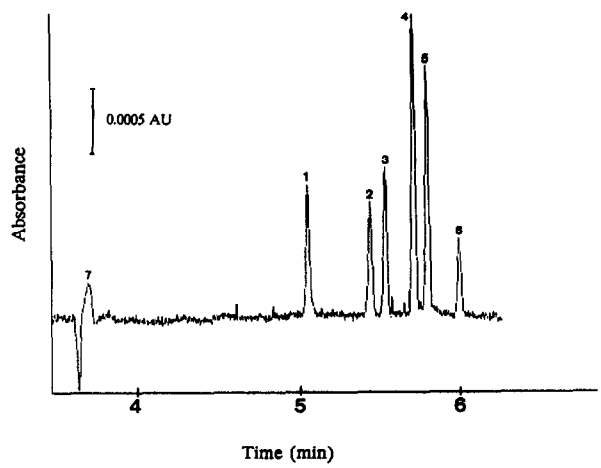

Fig. 3. Electropherogram for six anticoagulant rodenticides in 22% (v/v) CH_3OH. Experimental conditions: 0.015 mol/l phosphate (pH 7); field strength 536 V/cm; see Fig. 2 for compound identification and other experimental conditions. 7 = Neutral marker (methanol).

($R^2 = 0.995$), in the concentration range $3 \cdot 10^{-5}$–$1.1 \cdot 10^{-3}$ mol/l. Calibration linearity, in the concentration range $6.7 \cdot 10^{-5}$–$1.1 \cdot 10^{-3}$ mol/l, was evaluated by plotting sensitivity, corrected for non-zero intercept, *versus* concentration (five concentration points) as described elsewhere [32]. The maximum difference between the values of sensitivity, observed in this concentration range, was 18% for dicoumarol and 12% for warfarin.

CONCLUSIONS

CE offers a rapid and simple means of identifying and separating multi-component mixtures of anticoagulant rodenticides. In fully establishing CE as a preferred method of analysis, however, additional work is required to determine the performance of the technique with real samples (*i.e.* animal tissue extracts). The use of organic modifiers appears to be promising in analyzing structurally similar and relatively water-insoluble compounds as well as in inducing changes in the electroosmotic flow and analyte electrophoretic mobility.

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REFERENCES

1 W.E. Howard, in S. Parker (Editor), *McGraw-Hill Encyclopedia of Science and Technology*, Vol. 15, McGraw-Hill, New York, 1987, p. 39.
2 G.D. Osweiler, *Clinical and Diagnostic Veterinary Toxicology*, Kendall/Hunt Publ. Co., Dubuque, IA, 1985, pp. 334–335.
3 R.W. Holmes and J. Love, *J. Am. Med. Ass.*, 148 (1952) 935–937.
4 K. Hunter, *J. Chromatogr.*, 321 (1985) 255–272.
5 R.A. O'Reilly, P.M. Aggeler and L.S. Leong, *J. Clin. Invest.*, 42 (1963) 1542–1551.
6 M. Corn and R. Berberich, *Clin. Chem.*, 13 (1967) 126–131.
7 K. Opong-Mensah and W.R. Porter, *J. Chromatogr.*, 455 (1988) 439–443.
8 P. Owen, A. Pendlebury and A.C. Moffat, *J. Chromatogr.*, 161 (1978) 187–193.
9 H.S. Funnell and N. Platonow, *J. Forensic Sci.*, 15 (1970) 601–604.
10 S. Hanna, M. Rosen, P. Eisenberger, L. Rasero and L. Lachman, *J. Pharm. Sci.*, 67 (1978) 84–86.
11 R.W. Bullard, G. Holguin and J.E. Peterson, *J. Agr. Food Chem.*, 23 (1975) 72–74.
12 R.W. Bullard, R.D. Thompson and G. Holguin, *J. Agr. Food Chem.*, 24 (1976) 261–263.
13 D.E. Mundy and A.F. Machin, *J. Chromatogr.*, 234 (1982) 427–435.
14 K. Hunter, *J. Chromatogr.*, 270 (1983) 267–276.
15 K. Hunter, *J. Chromatogr.*, 270 (1983) 277–283.
16 R.A. Wallingford and A.G. Ewing, *Adv. Chromatogr.*, 29 (1989) 1–76.
17 S.W. Compton and R.G. Brownlee, *BioTechniques*, 6 (1988) 432–439.
18 K.J. Potter, R.J.B. Allington and J. Algaier, *J. Chromatogr.*, 652 (1993) 427–429.
19 K.D. Altria and C.F. Simpson, *Chromatographia*, 24 (1987) 527–532.
20 H.J. Issaq, I.Z. Atamna, G.M. Muschik and G.M. Janini, *Chromatographia*, 32 (1991) 155–161.
21 J. Vindevogel and P. Sandra, *J. Chromatogr.*, 541 (1991) 483–488.
22 K. Salomon, D.S. Burgi and J.C. Helmer, *J. Chromatogr.*, 549 (1991) 375–385.
23 S. Fujiwara and S. Honda, *Anal. Chem.*, 59 (1987) 487–490.
24 K.A. Connors, G.L. Amidon and V.J. Stella, *Chemical Stability of Pharmaceuticals*, Wiley, New York, 1986, p. 804.
25 S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya and T. Ando, *Anal. Chem.*, 56 (1984) 111–113.
26 A.S. Cohen, S. Terabe, J.A. Smith and B.L. Karger, *Anal. Chem.*, 59 (1987) 1021–1027.
27 K.D. Altria and C.F. Simpson, *Anal. Proc.*, 25 (1988) 85.
28 J.C. Reijenga, G.V.A. Aben, Th.P.E.M. Verheggen and F.M. Everaerts, *J. Chromatogr.*, 260 (1983) 241–254.
29 T. Tsuda, K. Nomura and G. Nakagawa, *J. Chromatogr.*, 248 (1982) 241–247.
30 J. Vacík, in Z. Deyl (Editor), *Electrophoresis: A Survey of Techniques and Applications, Part A: Techniques*, Elsevier, Amsterdam, New York, 1979, Ch. 1, p. 11.
31 R.G. Bates, in J.F. Coetzee and C.D. Ritchie (Editors), *Solute–Solvent Interactions*, Marcel Dekker, New York, 1969, Ch. 2, pp. 74–78.
32 M. Janoski and R.M. Cassidy, *LC·GC*, 10 (1992) 692–696.