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Short communication

Determination of alkylphosphonic acids using micellar electrokinetic chromatography with laser-induced fluorescence detection and high-salt stacking

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Abstract

Methyl-, ethyl- and propylphosphonic acids (MPA, EPA, and PPA, respectively) were derivatized with panacyl bromide in dry *N,N*-dimethylformamide (DMF). After mixing with a high-salt dilution buffer, the derivatives were separated by micellar electrokinetic chromatography in 35 min and detected by laser-induced fluorescence (He–Cd laser excitation at 325 nm and detection at 500 nm). Baseline resolution was achieved using a separation buffer containing 50 m*M* sodium cholate, 40% (v/v) of acetonitrile and 50 m*M* borate. Addition of 400 m*M* NaCl to the dilution buffer allowed the injection time to be increased to 30 s while still maintaining baseline resolution. Limits of detection for MPA, EPA, and PPA were 0.13 μ*M* (12 ppb), 0.13 μ*M* (14 ppb) and 0.14 μ*M* (17 ppb) injected, respectively. The reproducibility of corrected peak area at 15 μ*M* was 3.7~4.3%.

Keywords: Derivatization, electrophoresis; Sample stacking; Alkylphosphonic acids; Panacyl bromide; Organophosphorus compounds

1. Introduction

Alkylphosphonic acids and their derivatives have found wide applications as herbicides (glyphosate, glufosinate), antibiotics (fosfomycin), and antiviral compounds (foscarnet) [1]. Further, some nerve agents such as sarin, soman and VX hydrolyze to alkylphosphonates and ultimately to methylphosponic acid (MPA) [2]. Separation of alkylphosphonic acids by chromatographic [3–11] and electrophoretic [11–17] methods have been reported in both their native [3–5,7,8,12–17] and derivatized forms [6,9–11].

The first challenge in such determinations is the detection of alkylphosphonic acids because they have no chromophore or fluorophore that would enable direct UV or fluorescence detection. Various methods have been developed to circumvent this problem. Many indirect UV absorbance [12–16] and indirect fluorescence detection methods [17] have been reported for capillary electrophoresis (CE). However, while these indirect detection methods are simple and quick, they lack specificity. Other detection schemes include evaporative light scattering detec-

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tion (ELSD) [4,5], flame photometric detection [8,9] and mass spectrometry (MS) [4,6,7,11]. So far, only one direct fluorescence detection method was reported by Roach et al. using pre-column derivatization by panacyl bromide (Fig. 1) before HPLC separation [10]. However, the resulting products are strongly hydrophobic, making them insoluble in most HPLC mobile phases/CE buffers and the authors had to use strong eluents (60–100% acetonitrile) to elute the alkylphosphonic acid derivatives from a C_{18} column in reasonable time.

The second challenge is how to decrease the detection limits. Some pre-concentration methods have been reported [9,13]. Generally, on-column concentration techniques are less laborious than offline procedures. Recently Landers and co-workers developed a universal stacking technique for micellar electrokinetic chromatography (MEKC) which utilizes higher salt concentration in the sample matrix than in the separation buffer [19–21]. After the separation voltage is applied, the negatively-charged micelles move into the sample zone from the detector side of the sample zone–separation buffer interface. In the higher-conductivity sample zone the electric field strength is lower than in the separation buffer. As a result the micelle migration velocity decreases. The net effect is that micelles accumulate near the detector side of the sample zone–separation buffer interface resulting in a higher phase ratio of micellar phase at the sample–buffer interface. Consequently, the hydrophobic analyte is concentrated in this zone.

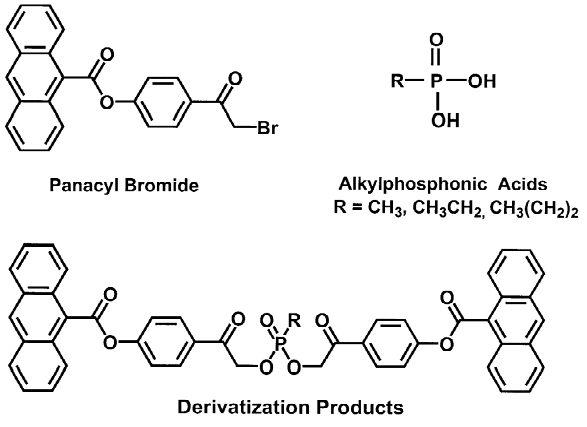

Fig. 1. Structures of panacyl bromide (excitation and emission wavelengths: 362 and 494 nm, respectively), alkylphosphonic acids and their derivatization products.

In the present report, a series of linear alkylphosphonic acids, methyl-, ethyl- and propylphosphonic acids were derivatized with the fluorescent dye panacyl bromide. The resultant products were separated by MEKC using cholate–acetonitrile–borate as separation buffer followed by laser-induced fluorescence (LIF) detection. The recently introduced MEKC stacking procedure of Landers and co-workers was successfully used to enhance the sensitivity of MEKC procedure [19–21].

2. Experimental

2.1. Apparatus

All MEKC experiments were performed on a P/ACE 2100 capillary electrophoresis system equipped with an LIF detector and P/ACE Station software for instrument control and data acquisition (Beckman Instruments, Fullerton, CA, USA). A 325 nm He–Cd laser (Omnichrome, Chino, CA, USA) with an output power of 5 mW was used as the excitation source. The laser was coupled to the LIF detector through an SMA fiber optic receptacle (Model 3056-8M, Omnichrome), a 1 m multimode fiber optic patchcord with a 100/140-μm (core/cladding) diameter and SMA 906 connectors (Polymicro Technologies, Phoenix, AZ, USA). An XB84-500DF25 band pass filter (Omega Optical, Brattleboro, VT, USA) was used to collect the fluorescence signal at 500±12.5 nm. Data were collected at 10 Hz with a detector response time of 0.5 s. The capillary was thermostated at 25 °C.

2.2. Reagents

Methylphosphonic acid (98%, MPA), ethylphosphonic acid (98%, EPA), propylphosphonic acid (95%, PPA), *N,N*-diisopropylethylamine (99.5%, redistilled), cholic acid (98%, sodium salt monohydrate), *N,N*-dimethylformamide (99.8%, DMF), calcium hydride (95%), and molecular sieve 3A (8–12 mesh) were purchased from Aldrich (Milwaukee, WI, USA). Panacyl bromide was from Molecular Probes (Eugene, OR, USA). Acetonitrile (HPLC grade) was

from Fisher Scientific (Fair Lawn, NJ, USA). Sodium tetraborate and sodium chloride were analytical grade from BDH (Toronto, Canada).

Commercially available DMF was refluxed over CaH_2 (5%, w/v) for 5 h at 60 °C, and then distilled onto molecular sieve 3A at 56 °C and 20 mmHg (1 mmHg=133.322 Pa). The dry DMF was stored under N_2 for later use. Stock solutions of 5 m*M* alkylphosphonic acids, 120 m*M* *N,N*-diisopropylethylamine and 10 m*M* panacyl bromide were prepared using dry DMF and kept strictly airtight. All the other solutions were prepared with Nanopure 18 MΩ water (Barnstead, Chicago, IL, USA). The separation buffer was 50 m*M* borate, 50 m*M* sodium cholate and 40% (v/v) of acetonitrile (pH 10.6, without further pH adjustment) prepared every other day and degassed prior to use unless stated otherwise.

2.3. Derivatization reaction

The derivatization procedure was adapted from Roach et al.'s method [10]. The optimized procedure was as follows: 60 μl of the alkylphosphonic acids in DMF was mixed with 50 μl of 120 m*M* *N,N*-diisopropylethylamine solution, 100 μl of 10 m*M* panacyl bromide solution and 190 μl of dry DMF in a 4-ml capped borosilicate glass vial. The vial was heated in an 80 °C water bath for 30 min. Then 200 μl of the reaction mixture was added to 200 μl of the dilution buffer containing 100 m*M* borate and 400 m*M* sodium chloride. Finally, the sample was vortexed for 10 s to ensure thorough mixing prior to injection.

2.4. CE parameters

MEKC separation was performed at 30 kV (normal polarity) in a 57 cm (50 cm to the detection window)×75 μm I.D. fused-silica capillary (Polymicro Technologies). Injections were 30 s hydrodynamic at 3.45 kPa (145 nl) unless otherwise stated. Before use each new capillary was conditioned by flushing at 138 kPa with 1 *M* NaOH for 10 min, distilled water for 10 min, 0.1 *M* NaOH for 5 min and distilled water for 10 min. Between runs the capillary was washed at 138 kPa with 0.1 *M* NaOH for 2 min, distilled water for 2 min and running buffer for 5 min.

3. Results and discussion

3.1. CE separation parameters

Sodium dodecyl sulfate (SDS) is typically used as the pseudo-stationary phase in MEKC. However, SDS is not efficient for strongly hydrophobic analytes because these analytes exhibit high partitioning even at low concentrations of SDS or after addition of organic modifiers [18]. Our preliminary studies showed that the derivatization products could not be separated from unreacted dye using SDS. Alternatively, natural surfactants such as sodium cholate, sodium deoxycholate and their taurine conjugates, have been reported to be suitable for the separation of very hydrophobic compounds [18]. Unlike the typical SDS micelles, the cores of such bile salt micelles contain both hydrophobic and hydrophilic regions [18]. The different structure properties of cholate micelles result in much lower partitioning than SDS micelles.

In MEKC, the analyte partitioning between the micelle phase and aqueous phase can be adjusted to an optimal value by simply changing the surfactant concentration [18]. The effect of cholate concentration was examined by adding 30–60 m*M* sodium cholate to a separation buffer containing 50 m*M* borate and 40% (v/v) of acetonitrile. The resulting electropherograms are shown in Fig. 2. Unreacted dye eluted prior to MPA, EPA and PPA derivatization products because these products are more hydrophobic than the unreacted dye. Other by-product peaks (not shown in Fig. 2, but indicated as B in Fig. 3) eluted after the analyte peaks. As shown in Fig. 2, the migration time of the analyte peaks increased with the increasing cholate concentration. Also, the resolution between MPA and EPA (the critical pair since these are more difficult to separate than the EPA/PPA pair) increased from 0.80 at 30 m*M* cholate to 1.64 at 60 m*M* cholate. Due to the limited solubility of sodium cholate in 40% acetonitrile and concerns associated with Joule heating, 50 m*M* sodium cholate was used in all further experiments.

Another advantage of bile salts is that they can

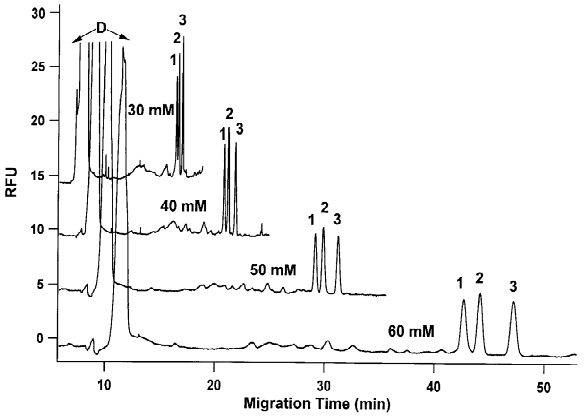

Fig. 2. Effect of cholate concentration on the separation. MEKC separation buffer: cholate–40% (v/v) acetonitrile–50 m*M* borate; voltage: 30 kV; injection: 30 s at 3.45 kPa; dilution buffer: 100 m*M* borate–400 m*M* NaCl; dilution ratio: 50:50 (v/v, reaction mixture vs. dilution buffer); sample injected: 2.5 μ*M*; peak designation: (D) unreacted dye, (1) MPA, (2) EPA, (3) PPA.

tolerate higher concentrations of organic modifiers such as acetonitrile (up to 50%) [18]. More acetonitrile results in a slower electroosmotic flow (EOF). Generally a lower EOF leads to an extended elution time window and thus achieving better resolution. Fig. 3 illustrates the effect of varying acetonitrile concentration in a separation buffer containing 50 m*M* borate and 50 m*M* of sodium cholate. The unknown peaks eluting after the analytes were evident in blank experiments and are believed to be

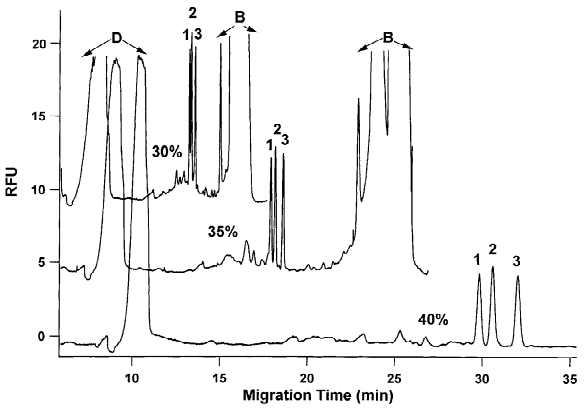

Fig. 3. Effect of % acetonitrile on the separation. MEKC separation buffer: 50 m*M* cholate–acetonitrile–50 m*M* borate; peak designation: (B) byproduct peaks; other conditions and peak designation are as in Fig. 2.

degradation products of panacyl bromide. A number of small byproduct peaks were also observed between the unreacted dye and analyte product peaks. These byproduct peaks are due to side reactions, presumably hydrolysis since these peaks were lower and simpler when freshly-prepared dry DMF was used. As can be seen in Fig. 3, the resolution between MPA and EPA increased from 0.94 to 1.64 as the acetonitrile concentration was increased from 30% to 40%. The hydrolysis products were also better resolved from the analyte product peaks at higher acetonitrile concentrations and therefore caused no interference. When the acetonitrile concentration was increased to 45%, MPA, EPA and PPA eluted after 90 min (not shown). As a compromise between separation time and resolution, 40% acetonitrile was used in subsequent experiments.

3.2. Optimization of the derivatization reaction

The reaction conditions of Roach et al.'s method [10] were used as a starting point for optimization of the derivatization conditions. The effect of dye concentration was studied by mixing different amounts of 10 m*M* panacyl bromide with 60 μl of 0.2 m*M* mixed sample solution, 50 μl of 120 m*M* *N,N*-diisopropylethylamine solution and then diluting to a total volume of 400 μl with dry DMF. The other conditions were as described in Section 2.3. The response of each phosphonic acid increased significantly with increasing dye concentration used and reached a plateau approximately at 2.5 m*M*. This concentration of panacyl bromide was used in all further experiments.

The concentration of *N,N*-diisopropylethylamine in the reaction mixture is critical as it consumes the HCl produced and thus pulls the whole reaction to completion. However, too much base leads to hydrolysis of the dye and can cause severe interferences with detection [10]. Different amounts of 120 m*M* *N,N*-diisopropylethylamine solution were added to 60 μl of 0.2 m*M* mixed sample solution and 100 μl of 10 m*M* panacyl bromide, and then diluted to a total volume of 400 μl with dry DMF. The other conditions were the same as above. Each phosphonic acid peak area increased as the concentration of *N,N*-diisopropylethylamine was increased up to 15

m*M*. Higher concentration of base yielded no further increase in response.

Thus, 2.5 m*M* panacyl bromide and 15 m*M* *N,N*-diisopropylethylamine were chosen as the optimal reaction conditions, as described in Section 2.3. Under these reaction conditions the derivatization reaction is more than 2/3 complete within 5 min and complete by 20 min. No degradation of the products was observed up to 40 min, the maximum reaction time studied. A reaction time of 30 min was used in our work.

3.3. High-salt stacking

Palmer and Landers have demonstrated that two requirements are critical to successful high-salt stacking in MEKC [20]. First, the conductivity of sample matrix must exceed that of the separation buffer so that the electric field decreases significantly inside the sample zone. Second, the sample matrix must contain a co-ion (chloride in our work) with a higher intrinsic electrophoretic mobility than cholate. The second condition guarantees the formation of a pseudo-steady-state boundary between the micelle and co-ion component in the sample matrix. The effect of NaCl concentration added to the sample was studied from 0 to 700 m*M*. The resolution for the MPA/EPA pair initially improved from 1.02 at 0 m*M* to 1.67 over a broad optimum at 200–400 m*M*, as expected based on the stacking mechanism above. The resolution then decreased as the peak shape degraded at higher salt concentrations. Similar behavior was observed when the peak efficiency was monitored (plot not shown). This behavior is consistent with that of Palmer et al. [19]. The degradation of resolution at higher salt concentrations is probably due to the disappearance of the pseudo-steady-state boundary between the micelle and co-ion component. That is, as the conductivity of the sample matrix is further increased, the electrophoretic movement of co-ion against EOF is further slowed, causing poor stacking (destacking).

3.4. Calibration, reproducibility and detection limit

Quantitative studies showed a linear response in the concentration range studied (0.0033–0.33 m*M* mixed sample, corresponding to 0.25–25 μ*M* injected). Correlation coefficients (R) were greater than 0.996 with intercepts equal to zero within the 95% confidence interval.

The reproducibility ($n=11$) of migration time and corrected peak area (defined as the ratio of peak area to migration time) were studied using 0.2 m*M* mixed sample (corresponding to 15 μ*M* injected). Migration time reproducibility was 2.9–3.6% and the corrected peak area reproducibility was 3.7–4.3%.

Based on a S/N of 3, the limits of detection (LODs) were 0.13 μ*M* (12 ppb) MPA, 0.13 μ*M* (14 ppb) EPA and 0.14 μ*M* (17 ppb) PPA injected. These are better than most previous reports [3,5,10–13,15,16].

4. Conclusions

A fluorometric determination of three linear alkylphosphonic acids is reported. After derivatization with panacyl bromide in dry DMF, baseline resolution was achieved using MEKC with cholate micelles and 40% acetonitrile. The addition of an optimal concentration of salt resulted in high-salt stacking which decreased the detection limit ten-fold while maintaining baseline resolution.

Acknowledgements

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