ELSEVIER

Journal of Chromatography A, 763 (1997) 279–284

JOURNAL OF CHROMATOGRAPHY A

Practical capillary electrophoresis method for the quantitation of the acetate counter-ion in a novel antifungal lipopeptide

L. Zhou, A. Dovletoglou*

Analytical Research, Merck Research Laboratories, Merck and Co., Inc., P.O. Box 2000, Rahway, NJ 07065, USA

Abstract

This is the first report of the validation of a capillary ion electrophoresis method for the quantitative determination of acetate (CH_3COO^-) levels in the acetate salt of a basic lipopeptide. The acetate counter-ion was detected using indirect photometric detection, 4.0 m*M* 4-hydroxybenzoic acid (HOC_6H_4COOH) as the carrier electrolyte and the chromophore mobile phase, and OFM Anion-BT as the electroosmotic flow (EOF) modifier. The EOF modifier is primarily a 20 m*M* myristyltrimethylammonium bromide (TTAB) solution. The apparent pH (pH_{app}) of the electrophoretic buffer was 6.0. The response was linear from 0.9 μg/ml to 46 μg/ml (r^2=0.9997). The capillary electrophoresis (CE) system is very efficient and stable, giving R.S.D. values of about 1.0% for the injection-to-injection precision without using internal standards. The method is accurate as judged by comparison to an ion-exchange HPLC method and by the 99.7% recovery of spiked acetate ion in the aqueous solution of the lipopeptide. The method is rugged with regard to critical method parameters, such as different operational voltages and effective capillary lengths. The overall precision is acceptable with a R.S.D. of 1.5% between 0.2–12% (w/w) acetate ion present in the drug substance without using an internal standard. Comparison to the ion-exchange chromatography and advantages of the CE method are discussed. This CE method has been submitted to and accepted by the regulatory agencies and is now in routine use within our laboratories.

Keywords: Acetate; Lipopeptides; Pneumocandins; Peptides

1. Introduction

Ion chromatography is the most widely used method of analyzing UV-inactive ionizable compounds [1]. Capillary ion electrophoresis is a capillary electrophoretic technique developed for the separation and detection of inorganic and organic species. To date, users of capillary ion electrophoresis have analyzed a variety of sample matrixes with analyte concentrations ranging from mg/l to μg/l. Capillary electrophoresis (CE) has been used recently in the analysis of inorganic metal ions [2]. Detection of these ions is achieved by indirect UV detection [3], where a component is added to the electrophoretic buffer to provide the background UV signal. The method involves introducing a sample containing the ionic species into a narrow bore capillary filled with a carrier electrolyte containing a selected light-absorbing anion. An electric potential is applied across the capillary column causing the ions to elute according to their ionic mobility. Both UV absorbing and UV-transparent ions can be detected and quantitated by UV–visible photometric monitoring.

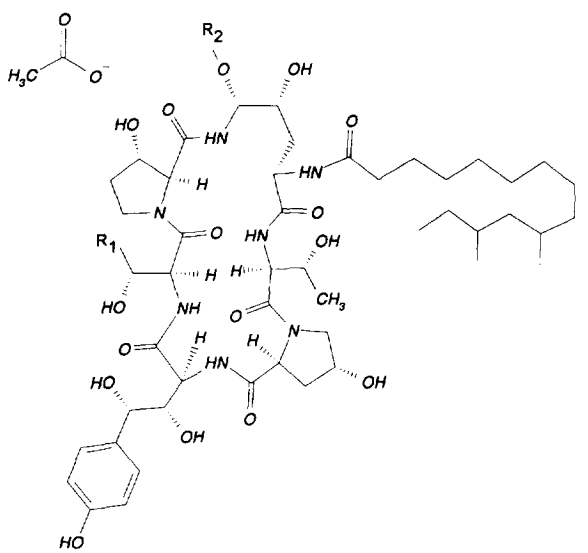
There are many CE applications in the area of pharmaceutical analysis [4–6]. Recently a number of papers have been published that describe the validation of CE methods and the routine use to replace existing HPLC methods [7–10]. These CE methods are used for the determination of drug purity, drug

*Corresponding author.

PII S0021-9673(96)00982-X

potency, impurity content and chiral analysis. Most of the drugs are developed as salts in order to optimize their chemical, physical and pharmaceutical properties. The majority of the drugs are bases in their cationic form (e.g., $-NH_3^+$) and salts containing ions such as Cl^- and CH_3COO^-.

The potential utility of CE for the separation and quantitative determination of acetate ion was investigated. In the past an ion-exchange HPLC method was employed for the acetate determination [11]. In this paper a technique for separating, identifying and measuring ions in solution by capillary ion electrophoresis is described. Typically, the precision of peak areas in CE is poorer than HPLC because of the variability in the injection volumes. The use of an internal standard can reduce this variability. The CE method reported here shows good precision data that is achieved without the use of an internal standard. Also, we report the successful validation of the CE method for the determination of CH_3COO^- content in the acetate salts of basic lipopeptides that belong to a new class of pneumocandins (Fig. 1). Interest in semisynthetic pneumocandins has intensified during the past several years. Recently a number of lipopeptides have been found active against *Pneumocystis carinii* and *Candida* species [12–14].



R_1 = CH_2CONH_2, $(CH_2)_2NH_2$
R_2 = H, $(CH_2)_2NH_2$

Fig. 1. Structures of semi-synthetic pneumocandins.

2. Experimental

2.1. Instrumentation

The CE experiments were carried out using a HP ^{3D}CE (Hewlett-Packard, Piscataway, NJ, USA) system. The HP ^{3D}CE instrument was used with a 56 cm effective length fused-silica capillary (58 cm total length)×75 μm I.D. (Hewlett-Packard). The diode-array detector was set at 450 nm vs. a reference at 220 nm. The capillary temperature was maintained at 25±0.1°C, a hydrodynamic sampling injection model was applied for 3 s and the applied voltage was −20.0 kV. The HPLC experiments were carried out using a Thermo Separations Products (Piscataway, NJ, USA) system equipped with an AS3000 autosampler, UV1000 detector and P4000 pump. Data collection and integration were accomplished using the PE Nelson ACCESS CHROM system (Nelson, Cupertino, CA, USA).

2.2. Reagents

The OFM (Osmotic Flow Modifier) Anion-BT solution (patented buffer) was obtained from Waters (Millipore, Milford, MA, USA). This solution contains primarily a 20 m*M* solution of myristyltrimethylammonium bromide [$CH_3(CH_2)_{13}N(CH_3)_3Br$, TTAB]. The $LiOH \cdot H_2O$, 4-hydroxybenzoic acid (HOC_6H_4COOH), NaH_2PO_4 and CH_3OH were purchased from Aldrich (Milwaukee, WI, USA). The sodium acetate was obtained from Fisher Scientific (Springfield, NJ, USA). All commercial chemicals were used directly without any further purification. Water used in the study was purified with a Milli-Q system (Millipore).

2.3. Electrolyte solution

A 150 mg amount of $LiOH \cdot H_2O$ and 550 mg of 4-hydroxybenzoic acid were placed into a 200 ml volumetric flask and 3 ml MeOH were added onto the floating crystals in order to affect the dissolution. A 20 ml volume of water was added and the solution

was sonicated for few minutes. A 5 ml of OFM Anion-BT (Waters) solution was added into the above 200 ml volumetric flask and diluted to the mark with water. The apparent pH of the solution (pH_{app}) was adjusted to 6.0 with a 20 mM LiOH solution and filtered through a 0.45 μm nylon membrane.

2.4. Sample solutions

The sample diluent had the same composition as the electrolyte solution. The acetate stock solution was prepared by dissolving 100 mg CH_3COONa in 100 ml of the sample diluent and diluting 4.0 ml of that solution with 96.0 ml water. The testing solution was made by weighing 10 mg of the lipopeptide in a 100 ml of water and diluting 10 ml of that solution with 40 ml of water. The solutions used in the linearity test were prepared by varying the concentration of the acetate ion from 0.9 μg/ml to 46 μg/ml (4.5% to 230% of the target concentration of 20 μg/ml acetate ion). A sample solution spiked with the acetate ion stock solution at the 0.35% level was used to test the recovery.

2.5. Procedures

Every new capillary was washed with 1 *M* NaOH solution for 30 min, flushed with deionized water for 10 min and 0.2 *M* NaOH solution for 30 min. Between each injection, the capillary was rinsed with the electrophoretic buffer solution for 5 min.

3. Results and discussion

3.1. Electroosmotic flow modifier

CE is a rapid separation technique which operates on the basis of the differential migration of charged and uncharged species in an electric field. The application of an electric field induces a flow within the capillary which is called electroosmotic flow (EOF). Control of the EOF can affect the migration times and the separation of the analytes. The reversal of EOF is achieved by the addition of a cationic surfactant, such as TTAB to the electrophoretic buffer. This reversal of the flow is caused by the formation of a bilayer at the walls of the capillary, making the wall charge positive. An excellent discussion on the characterization of the cationic surfactant induced reversal of the EOF in CE has appeared recently [15].

3.2. Indirect photometric detection

Most of the small ions do not absorb in the UV or visible regions of the spectrum. This detection problem has been solved by using indirect photometric detection, in which a UV-absorbing solute serves as the additive to the background electrolyte. This additive, known as a visualizing reagent, elevates the baseline. The visualizing reagent in our method should have the same charge and ionic strength as the analyte, in order to migrate along with the analyte within the electrophoretic buffer (pH_{app}=6.0). Based on these principles 4-hydroxybenzoic acid was chosen and its UV spectra was recorded. When the acetate ions are present, they displace the 4-hydroxybenzoic acid ions as required by the principle of the electroneutrality. As the separated ions migrate past the detector window, they are measured as negative peaks relative to the high baseline. By reversing the signal and reference wavelength at the diode-array a positive signal is obtained. For that reason the diode-array detector was set at 450 nm vs. a reference at 220 nm.

3.3. Capillary ion electrophoresis

Capillary ion electrophoresis utilizes the principles of CE in combination with an EOF modifier and indirect UV detection mode for the rapid detection and quantification of ions. The electrophoretic buffer solution was prepared by mixing 4-hydroxybenzoic acid and OFM Anion-BT that reverses the EOF. A −20 kV reversed voltage was applied on a 58 cm× 75 μm fused-silica capillary, and an indirect detection mode was used.

3.4. Linearity

A linearity was tested from duplicate injections on series dilutions over concentrations from 0.9 μg/ml to 46 μg/ml. The detector response of the acetate peak is linear over the concentration 0.0009 to

0.0460 mg/ml, which corresponds to 4.5 to 230% of the target concentration (0.02 mg/ml acetate ion, equivalent to 0.04 mg/ml sodium acetate). The linearity coefficient (R^2) over the entire range is greater than 0.999. The precision of duplicate injections at 1.4 μg/ml and 5.0 μg/ml is excellent (% error less than 0.5%).

3.5. LOD and LOQ

The signal-to-noise ratio for the 1 μg/ml standard is >20, which indicates the method yields excellent limits of detection and quantitation for this ion. The LOQ of the CE method is 0.9 μg/ml.

3.6. Precision

The area counts of six consecutive injections of an acetate solution showed a R.S.D. of 1.0% Three weighings of the lipopeptide and sodium acetate standard were made on three different days and each solution was injected in duplicate each day. The

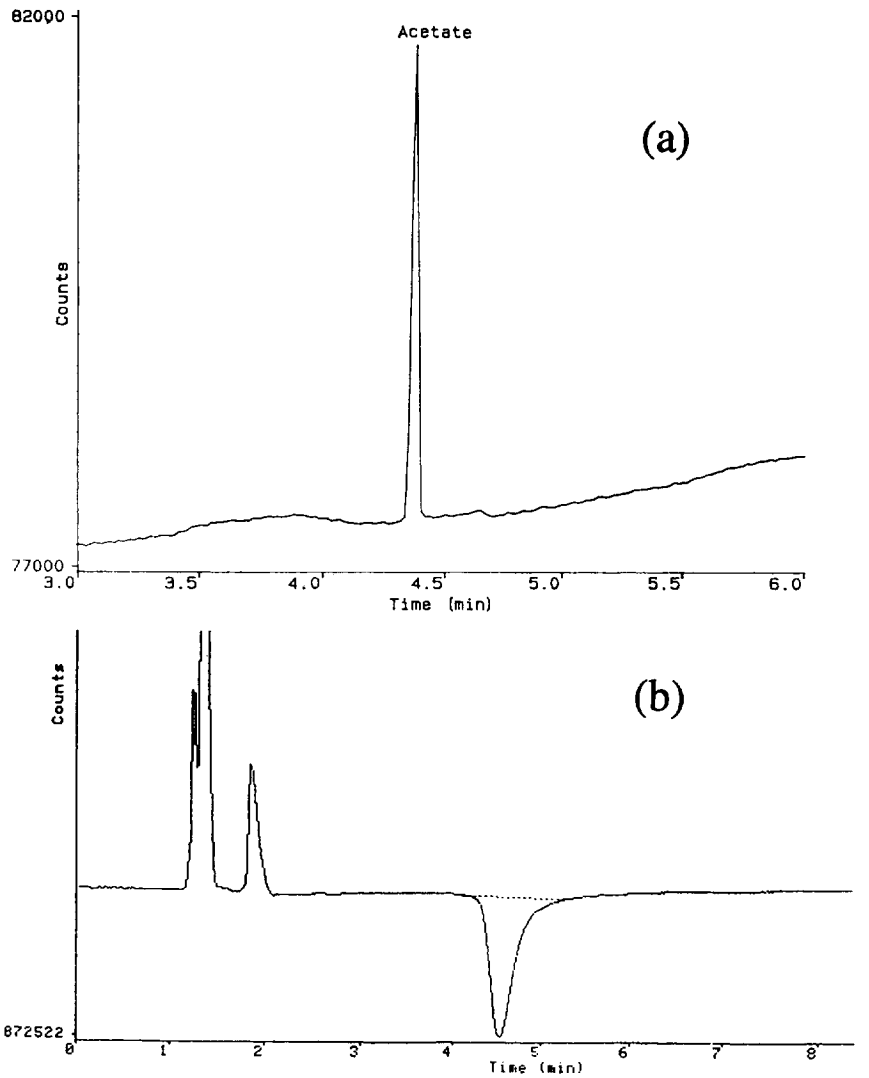


Fig. 2. (a) Electropherogram of acetate ion standard (0.04 mg/ml), (b) chromatogram of acetate ion standard (1 mg/ml). Experimental conditions are described in Section 2 and in Table 1.

results range from 8.4% (w/w) to 8.8% (w/w) of acetate in the drug substance with an overall average of 8.6% (w/w).

3.7. Accuracy

The CE method is accurate as shown by spiking a standard solution of sodium acetate into a sample of the lipopeptide at the 18 μg/ml acetate level and recovering 99.7%. Also, the accuracy of this method was assessed by comparing the % (w/w) of acetate found by the CE assay to the % (w/w) found using an HPLC ion-exchange method. The two methods found the level of acetate in the drug substance to be 8.70% (w/w) (CE) and 8.75% (w/w) (HPLC).

3.8. Ruggedness

The ruggedness was evaluated by comparing the R.S.D. of six consecutive injections of the lipopeptide and the sodium acetate standard using different capillary effective lengths (51 and 58 cm) or operation voltages (−18 and −20 kV). The R.S.D. varied from 1.0% to 1.2% without using internal standards. The recommended system suitability requirements for this CE method based on our experience using several capillaries are that the precision should be less than 5.0% R.S.D. for the response factor for the standards and samples at the 2 to 12% (w/w) range, and the peak tailing factor should be between 0.5 and 2.5.

3.9. CE vs. HPLC

In comparison with the current HPLC ion-exchange method [11] (using PRP-X100 ion-exchange column), the CE method has several obvious advantages (Fig. 2). First, the fused-silica capillary costs US$ 10–50 vs. US$ 500 for the PRP-X100. Second, the equilibrium analysis time for CE is ~20 min. vs. at least a few hours for the HPLC method. Third, the CE method provides much higher separation efficiency and peak shape. Fourth, the LOD for the acetate ion using the CE method is <0.1 μg/ml vs. 1–2 μg/ml using the HPLC. Finally, the CE exhibits much better injection precision than the HPLC method (Table 1).

4. Conclusions

In this paper a technique for separating, identifying and measuring ions in solution by capillary ion electrophoresis was described which provides improved sensitivity and resolution of anionic species. It is concluded that charge-reversed CE in concert with indirect photometric (UV) detection is superior to ion-exchange chromatography in the determination of acetate ion with regard to simplicity, sensitivity, accuracy and speed of analysis. The results generated by the CE method compared well with the results obtained from the HPLC method. An electrolyte system using indirect UV detection using 4-hydroxybenzoic acid as the chromophore and as

Table 1
Comparison of the CE and ion-exchange HPLC methods

CE	HPLC[a]
Capillary: fused-silica (58 cm×75 μm)	Column: PRX×100 (250×4.6 mm)
Electrophoretic buffer: 4-hydroxybenzoic acid and OFM Anion-BT, pH=6.0	Eluent: 0.5 m*M* trimesic acid, pH=4.6
Voltage: −20 kV	Flow-rate: 2.0 ml/min
Injection: 3 s (50 mbar)	Injection volume: 10 μl
Detection: 450 nm vs. 220 nm	256 nm vs. 264 nm
Injection precision: 1.0% R.S.D.	2.0% R.S.D.
Method precision: 1.5% R.S.D.	
Accuracy: 8.70% acetate (R.S.D.=0.8%)	8.75% acetate
Recovery: 99.7%	92.3%
LOD: <0.1 μg/ml	1–2 μg/ml
LOQ: 0.9 μg/ml	10 μg/ml

[a] Conditions from Ref. [11].

the carrier electrolyte and OFM Anion-BT solution as the EOF modifier was developed to determine and quantitate acetate ions in basic lipopeptides. In comparison with other electrolyte systems it was found to be the most favorable for the analysis of organic acids with low mobility. Similar CE methods or the same method can be used for the analysis of many other organic and inorganic ions ($H_2PO_4^-$, HPO_4^{2-}, formate, thiocyanate, methanesulfonate, Br^-, Cl^-, citrate and different alkyl sulfonate ions, etc.). Using a cationic additive, inorganic anions with high mobility could also be separated. Similar methods that combine CE with indirect photometric detection could become practical means of establishing drug substance and/or drug product stoichiometry because they are suitable for virtually all ionic analytes lacking chromophores.

Acknowledgments

The authors would like to thank Dr. R.S. Egan for his support.

References

[1] B. Rossner and G. Schwedt, Fresenius' Z. Anal. Chem., 320 (1985) 566.
[2] K. Altria, T. Wood, R. Kitscha and A. Roberts-McIntosh, J. Pharm. Biomed. Anal., 13 (1995) 33.
[3] J. Morawaki, P. Alden and A. Sims, J. Chromatogr., 640 (1993) 359.
[4] A. Aumatell and A. Guttman, J. Chromatogr. A, 717 (1995) 229.
[5] B.R. Thomas, X.G. Fang, R.J. Tyrrell and S. Ghodbane, J. Chromatogr. B, 657 (1994) 383.
[6] J. Luksa and Dj. Josic, J. Chromatogr. B, 667 (1995) 321.
[7] K.D. Altria and C.F. Simpson, Chromatographia, 24 (1987) 527.
[8] P. Jandik and W.R. Jones, J. Chromatogr., 546 (1991) 445.
[9] G.W. Tindal and R.L. Perry, J. Chromatogr., 533 (1993) 227.
[10] R. Stahl, J. Chromatogr. A, 686 (1994) 143.
[11] M.M. See, R. Thompson, N. Grinberg, H.J. Perpall, G. Bicker and P. Tway, J. Liq. Chromatogr., 18 (1995) 137.
[12] J.M. Balkovec, Expert Opin. Invest. Drugs, 3 (1994) 65.
[13] D.M. Schmatz, M.A. Powles, D. McFadden, K. Nollstadt, F.A. Bouffard, J.F. Dropinski, P. Liberator and J. Andersen, Antimicrob. Agents Chemother., 39 (1995) 1320.
[14] F.A. Bouffard, M.L. Hammond and B.H. Arison, Tetrahedron Lett., 36 (1995) 1405.
[15] C.A. Lucy and R.S. Underhill, Anal. Chem., 68 (1996) 300.