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Comparison of high-performance liquid chromatography and capillary zone electrophoresis in penciclovir biodegradation kinetic studies

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Abstract

High-performance liquid chromatography (HPLC) and capillary zone electrophoresis (CZE) were used in biodegradation kinetic studies. This paper describes a rapid penciclovir separation using CZE with detection limits comparable to HPLC. The ionic-strength mediated stacking technique was employed while good resolution was maintained. With a shorter analysis time, comparable detection limits and no organic solvent consumption, CZE is a better method for penciclovir biodegradation studies than conventional reversed-phase HPLC (RP-HPLC).

1. Introduction

Penciclovir, an anti-viral compound, has been shown to have in vitro and in vivo activity against *Herpes simplex* and *Herpes zoster* viruses. Biodegradation studies were recently undertaken as part of the routine environmental assessment of drug substances.

An HPLC method was initially developed to study penciclovir biodegradation kinetics. During the study it was discovered that one of the degradants coeluted with penciclovir. After assessing several different reversed-phase columns and separation optimizations, only YMC AQ reversed-phase columns with 5% organic modifier (methanol) were found to provide sufficient accuracy and precision. One limitation, however, was that the weak solvent strength required for good separation resulted in a comparatively long separation time (22 min) and peaks that were broader than desired. The limit of detection (LOD) for the HPLC method was estimated at 0.5 mg/l. Based on data from the RP-HPLC method [1], the half-life of penciclovir degradation was found to be approximately two hours in activated sludge. A faster separation method was desirable to study the observed rapid biodegradation kinetics so that data could be examined in real time.

Capillary electrophoresis (CE) is generally recognized as a powerful analytical separation technique that brings speed, quantitation, reproducibility and automation to the highly resolving but labor-intensive method of electrophoresis. CE is also becoming an important and widely used technique for routine analytical separations.

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Capillary zone electrophoresis (CZE) is the simplest mode of CE, where separations are performed in homogeneous buffer solutions. A thin band of solute is deposited at the head of a small-diameter capillary and a voltage is applied across the capillary. Under the influence of the applied voltage, charged molecules will migrate away from the starting point and along the capillary. Separation occurs because of differential migration rates of each molecule. The inherent simplicity of CZE combined with its extraordinary resolving power resulted in wide application of this technique [2–5]. CZE is gaining acceptance not only for ionic, ionizable, and macromolecular species related to biotechnology, but for small molecules as well [6–10]. Four fundamental features are required for good separations by CZE [7]. First, the individual mobilities of each solute in the sample must differ from one another. Second, the buffer solution must be homogeneous and the field strength uniformly distributed across the length of the capillary. Third, neither solutes nor sample matrix elements may interact with or bind to the capillary wall. Finally, buffer solution conductivity must substantially exceed the total conductivity of the sample components.

All capillary separation techniques, including CE, are known to have restrictions on the amount of sample that can be injected. However, in CE this can be partially compensated for by using the ionic-strength mediated stacking technique [11,12]. With this technique, larger injections can be made while the precision and accuracy remain satisfactory. In order for the stacking effect to take place, our samples were prepared in HPLC-grade water instead of the mineral salts media (MSM [13]) commonly used in microbial biodegradation studies.

The main purpose of this study was to determine if CZE could be used to monitor penciclovir biodegradation kinetics. We also were interested in determining what advantages might be gained in using this technique. Comparisons of elution order, UV spectra, method sensitivity and precision between RP-HPLC and CZE will be presented in this study.

2. Experimental

2.1. Instrumentation

All measurements were obtained with a Hewlett-Packard 1090 HPLC system and a Hewlett-Packard HP^{3D}CE system (Hewlett-Packard, Palo Alto, CA, USA). Both were equipped with a photodiode array UV detector with the detection wavelength set at 260 nm. A Nelson 900 Series interface and PE Access* Chrom VAX chromatographic software (Perkin-Elmer Nelson System, Cupertino, CA, USA) were used for data collection and analysis. The sampling rates for CE and HPLC were 3 pt/s and 1 pt/s, respectively. The samples were injected in triplicate.

2.2. HPCE

The electrophoresis buffer was 23 m*M* potassium phosphate pH 7 buffer. Fused-silica capillaries with bubble configuration were from Hewlett-Packard (Hewlett-Packard). Capillary dimensions were 50 μm I.D. and 64.5 cm length (56.5 cm to detector). Prior to first use, capillaries were treated with 0.5 *M* NaOH for 15 min, followed by a 5-min rinse with deionized water and a 5-min rinse with electrophoresis buffer. Sample injection was done by applying a pressure of 50 mbar for 15 s to the sample valve placed at the grounded end of the capillary. The injected volume was about 17 nl, a voltage of 30 kV was applied, and a temperature of 30°C was maintained throughout. The CE system was operated in the conventional mode with the anode at the injector end of the capillary. The capillary was cleaned after each run by flushing for 2–3 min with 0.5 *M* NaOH followed by deionized water.

2.3. HPLC

YMC-AQ reversed-phase C_{18} columns (250 × 4.6 mm I.D., 5 μm packing) were purchased from YMC (Wilmington, NC, USA) and used as received. The analytes were eluted isocratically with a mobile phase of methanol–water (5:95,

v/v) with 23 m*M* potassium phosphate buffer (pH 7.0). The flow-rate was maintained at 1.0 ml/min, column temperature maintained at 30°C, and the sample injection volume was 20 μl.

2.4. Solute and solvents

Methanol, water (each HPLC grade), potassium phosphate and phosphoric acid were obtained from J.T. Baker (Phillipsburg, NJ, USA) and Fisher Scientific (Pittsburgh, PA, USA). Penciclovir sodium salt (BRL 39123A) was obtained from SmithKline Beecham Pharmaceuticals (King of Prussia, PA, USA). Activated sludge used in the study was obtained from Montgomery County Sewage treatment plant (Oaks, PA, USA).

2.5. Standard solutions

Penciclovir was accurately weighed into a volumetric flask and dissolved in HPLC-grade water to give solution concentrations that ranged from 1 to 1.5 mg/ml. Volumetric dilutions of the stock solution were made to produce lower concentration standards.

2.6. Biodegradation matrix

Penciclovir sodium salt stock solution (2000 mg/l) was mixed with activated sludge to obtain a nominal penciclovir solution concentration of 24.4 mg/l (equivalent to 10 mg carbon per liter as penciclovir). Activated sludge was obtained from a municipal wastewater treatment plant and diluted to a concentration of 1500 mg/l as total suspended solids. After two hours samples were removed from the flask, filtered through a 0.45-μm filter (Millex-HV, Millipore), and placed into vials for analysis by HPLC or CZE.

3. Results and discussions

As shown in Figs. 1 and 2, three biological degradants were observed. In Fig. 1, the chromatogram from the HPLC analysis, the retention order is biodeg no. 1 < biodeg no. 3 < biodeg no. 2 < penciclovir. Fig. 2 shows the electropherogram of the same sample analyzed by CZE where the retention order is penciclovir < biodeg no. 1 < biodeg no. 2 < biodeg no. 3. In HPLC, retention is governed by the distribution of a solute between the stationary phase and the mobile phase [14,15]. In CZE, however, retention is governed by the different rates at which charged molecules will move under the influence of an applied electric field. The unique retention characteristics of CZE opens the door for LC–CZE two-dimensional separations, where one separation mode is followed by a second separation mode in a direction perpendicular to the first. The substantial gain in peak capacity when using two-dimensional separations is especially desirable for plasma protein analysis [16–18]. Figs. 1 and 2 also reveal another CE advantage:

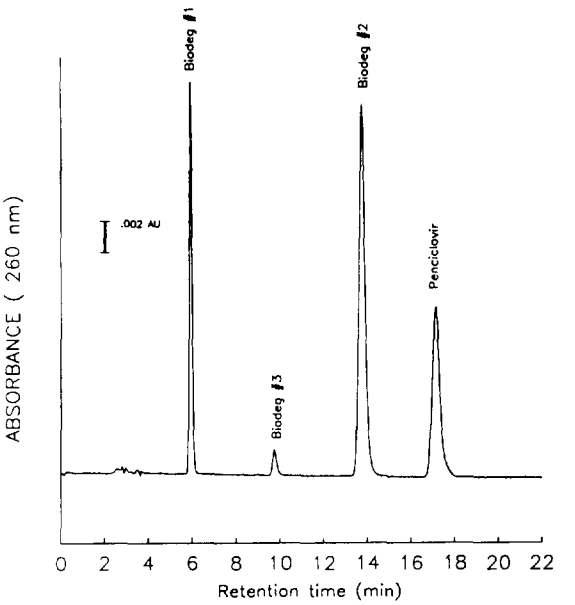

Fig. 1. HPLC chromatogram of penciclovir degradation. Three degradants were observed and are labelled as biodeg #1, biodeg #2 and biodeg #3. Column, YMC AQ-C_{18}, 250 × 4.6 mm I.D.; mobile phase, methanol–23 m*M* potassium phosphate pH 7.0 buffer (5:95, v/v); injection size, 20 μl; flow-rate, 1 ml/min; detection, UV 260 nm; column temperature, 30°C.

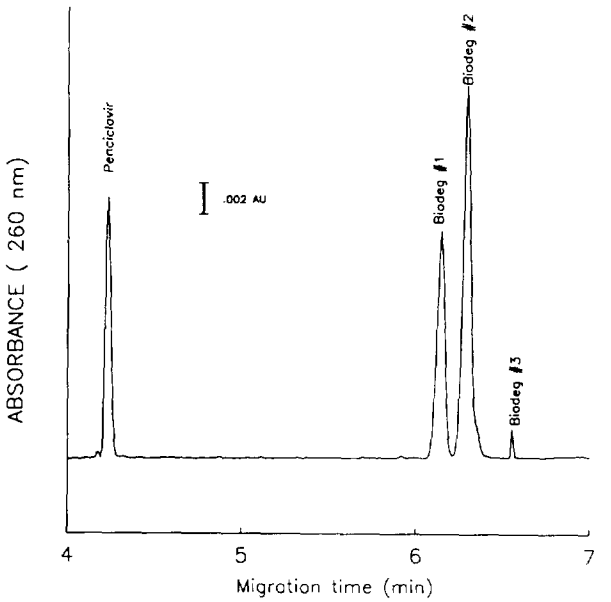

Fig. 2. Electrophoresis of penciclovir degradation; three degradants were observed and are labelled as described in Fig. 1. Capillary, fused-silica with bubble configuration, 56.5 cm (length to detector) × 50 μm I.D.; buffer, 23 m*M* potassium phosphate pH 7.0; injection, pressure (50 mbar) 15 s; detection, UV 260 nm; column temperature, 30°C.

speed. The analysis time is reduced from 22 min using the HPLC method to 7 min using the CZE method without significant loss in resolution. There is yet another advantage to using CE: no organic solvents were used; however, with the HPLC method, a total of 22 ml of organic solvents were needed for each sample analysis, i.e. (22 min × 1 ml/min). With the increasing price for organic solvent disposal, CE is more economical and environmentally safer than conventional HPLC.

Before diode-array detectors (DADs) generally became available for HPLC, peaks were usually identified by their retention times. While retention time remains an important reference point, the use of DADs has reduced the uncertainty of identifying compounds analyzed with different columns or techniques. While retention time is important, it does require that the HPLC systems remain stable from day to day. Great efforts have been made to ensure that the systems remain as constant as possible; DADs, however, remove some of the uncertainty associated with the inevitable changes that do occur. Changes in elution order and retention time are

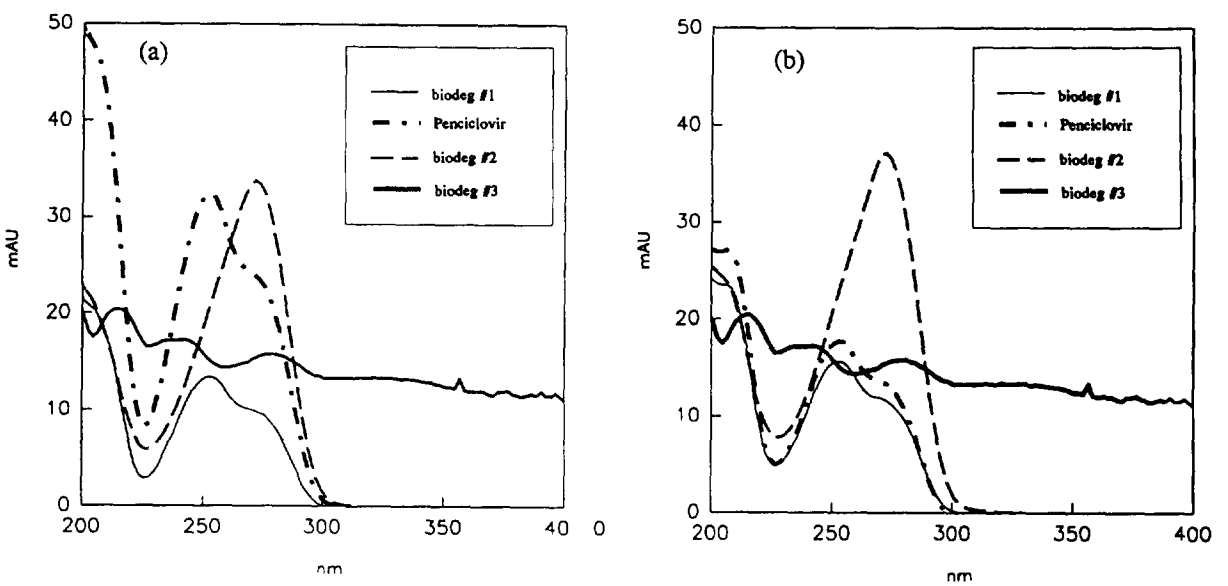

Fig. 3. UV spectra of penciclovir degradants from (a) HPLC, (b) CZE.

especially problematic when working with two techniques which operate under different retention mechanisms, such as HPLC and CE. The lack of standards of the degradation products also makes the correlation between HPLC peaks and CE peaks very difficult. However, by comparing UV spectra obtained from HPLC and CZE, peaks can be more readily assigned even though the retention orders are different. This approach was used in the present study. Fig. 3a,b show the UV spectra of each peak obtained from the HP 1090 and the HP^{3D}CE, respectively. The UV spectra were used to make the peak assignments for the electropherogram shown in Fig. 2.

When compared to HPLC, sensitivity in CZE is limited. However, by increasing the injected amount to about 17 nl, the sensitivity is comparable to that of HPLC (Fig. 4). The contribution of solutes to the total ionic strength of the injection solution can dramatically affect the peak symmetry and the linearity of response. These drawbacks may be partially offset using the ionic-strength mediated stacking technique [8,11,12] where the sample is concentrated during injection. Stacking is possible when the conductivity of the sample matrix is lower than that of the electrophoretic buffer solution. Under these conditions, the electric field strength in the sample matrix is much greater than that of the electrophoretic buffer solution. Because of the higher field strength, the sample

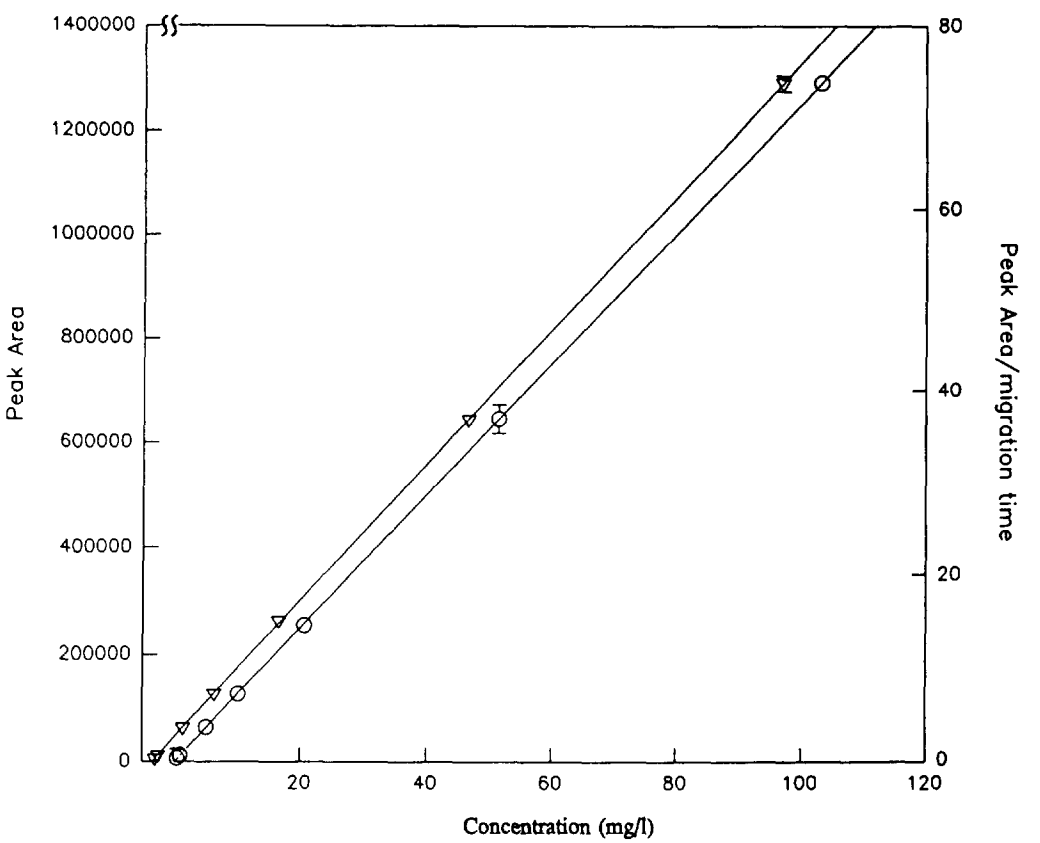

Fig. 4. UV-absorbance detector response for penciclovir with 99% confidence interval. (▽) Reversed-phase liquid chromatography (left y-axis). (○) Capillary zone electrophoresis (right y-axis).

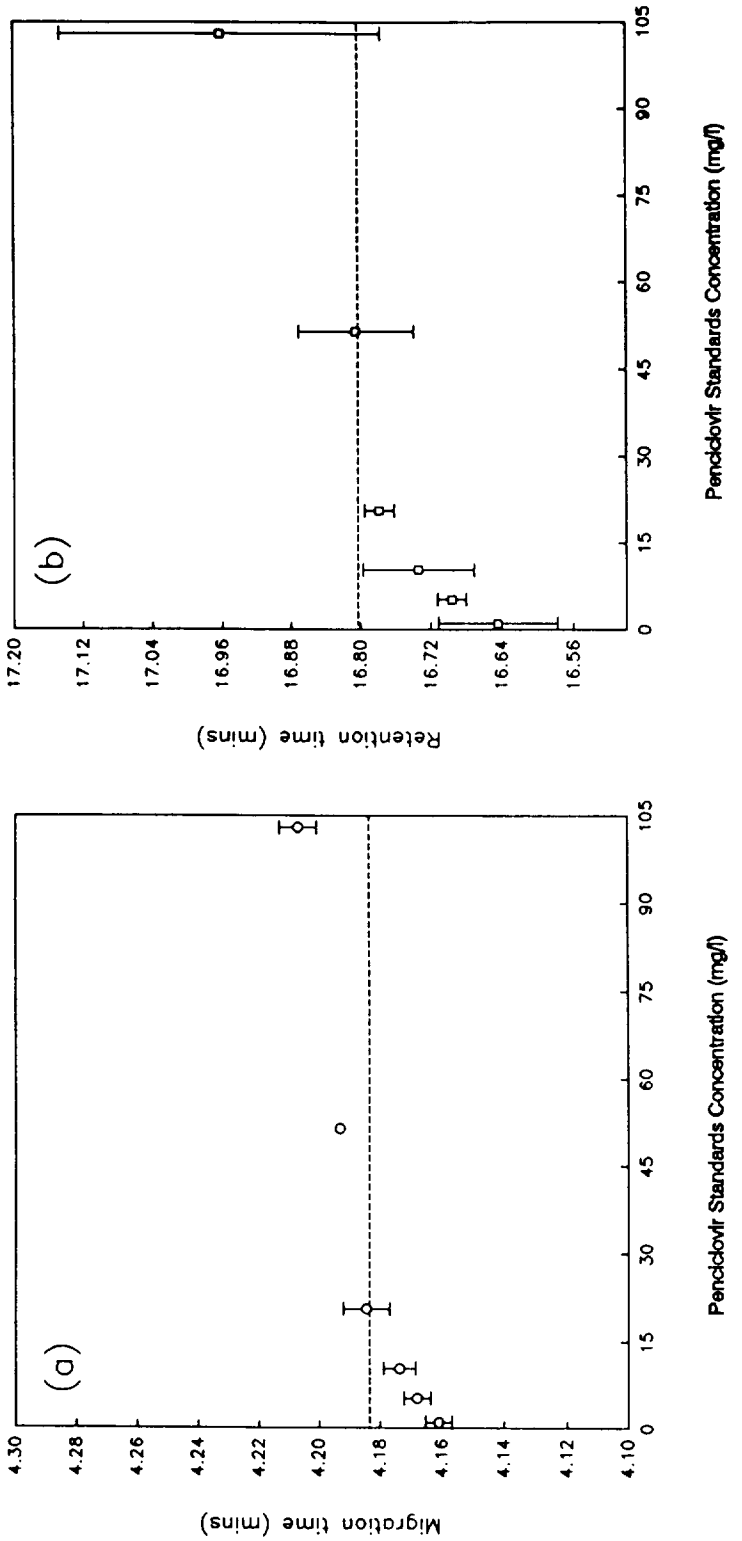

Fig. 5. Precision of (a) CZE migration time and (b) HPLC retention time. All penciclovir standards were injected in triplicate: The precision of CZE is better than that of HPLC (0.42 %R.S.D. vs. 0.84 %R.S.D.): The error bar and the dotted line are the 99% confidence interval and the averaged value, respectively.

ions will rapidly migrate until they encounter the zone of higher conductivity (lower field strength) associated with the buffer solution. The sample ions "stack" until the solute zone conductivity approaches the conductivity of the buffer solution. In this way, the sample zone can be concentrated to a very narrow band, providing very sharp solute peaks. Using this technique, larger injection volumes can be used to increase the overall detection sensitivity of CZE. In the present study, a large 17-nl injection was used without adverse effects. Peak shapes were acceptable, and method sensitivity was comparable to that of HPLC with the limit of detection (LOD) for CZE estimated at 0.5 mg/l.

For CZE to be a useful analytical technique, migration-time reproducibility must be comparable to that of established techniques such as HPLC. It is desirable, although not always possible, to keep relative standard deviations smaller than 0.1% [where %R.S.D. = (standard deviation (σ) of the migration time divided by the average migration time) · 100] [8,19]. Hydrodynamic injection, where the sample is injected by applying pressure to the sample vial and forcing the sample into the capillary, has been shown to affect electroosmotic flow [20]. Hydrodynamic injections, because they affect the electroosmotic flow, have been shown to be the predominant factor affecting migration-time precision in CZE [21]. Migration-time variability is expected to increase with long injection time (15 s).

Since the injection time for the CZE used for this study was 15 s, potentially high migration-time variability was anticipated. In spite of this potential disadvantage, Fig. 5 shows that the migration-time precision for CZE in this study is found to be better than that of HPLC (0.42 %R.S.D. vs. 0.84 %R.S.D.). We attribute the low CZE migration-time variability to the ion stacking effect. For HPLC, Hsu and Cooper [22] have demonstrated that when using silica-based columns at low organic modifier content ($< 15\%$), the "brush like" stationary phase behaves abnormally. With only 5% methanol as modifier in the current HPLC method, a lower precision of retention time is expected.

4. Conclusions

Today, CZE is no longer in its infancy. Analysts are now beginning to develop CZE as an analytical tool for everyday use. This study has shown the feasibility and desirability of using CZE to monitor a rapid biodegradation kinetic study instead of using conventional reversed-phase HPLC. With larger numbers of theoretical plates, shorter separation times, adequate detection limits and essentially no organic mobile phase consumption, CZE can be a reliable and environmentally friendly analytical technique. Based on different retention mechanisms, a combination of CZE with HPLC (especially, microcolumn) can provide the necessary separation of complex samples consisting of numerous components (for example, > 100). For such samples, linear columns generally have inadequate power to cleanly resolve the components; this is more pronounced with an increasing number of components [23–27]. CZE should be useful in those cases where fast, highly efficient separations are required.

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