

Joselito P. Quirino^{a, b) 1)},
Maria T. Dulay^{a)},
Lei Fu^{b)},
Tarak D. Mody^{b)},
Richard N. Zare^{a)}

Capillary electrophoresis separation and native laser-induced fluorescence detection of metallotexaphyrins

^{a)} Department of Chemistry,
Stanford University, Stanford,
CA 94305-5080 USA
^{b)} Pharmacyclics, Inc., 995 East
Arques Avenue, Sunnyvale, CA
94085-4521 USA

Methods based on capillary zone electrophoresis (CZE) and electrokinetic chromatography (EKC) are developed for the separation of motexafin gadolinium (MGd, Xcytrin[®]), a novel redox-active drug, under development for cancer therapy, and two closely related metallotexaphyrin impurities, called PCI-0400 and PCI-0430. The three metallotexaphyrins are baseline resolved using EKC in a separation solution containing 400-mM cetyltrimethyl ammonium bromide, 50-mM phosphoric acid, and 20% methanol. Detection is achieved using native laser-induced fluorescence (LIF) by exciting at 488 nm and collecting emission at 750 nm. Analysis of a representative clinical batch of MGd using EKC combined with native LIF allowed for the detection of PCI-0400 and PCI-0430 along with nine other possible MGd impurities, each of which is present at approximately 0.1% (total weight of the sample).

Key Words: Expanded porphyrins; Metallotexaphyrins; Capillary electrophoresis; Electrokinetic chromatography; Motexafin gadolinium

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1 Introduction

Metallotexaphyrins are metal-coordinated expanded porphyrins that belong to a new class of investigative drugs with diverse clinical applications in radiation and photodynamic therapy [1–6]. Two water-soluble metallotexaphyrin analogues, motexafin gadolinium and motexafin lutetium, are presently undergoing pharmaceutical development and clinical trials [1–6]. Each compound has well-optimized physical characteristics, favorable tissue biocalization features, and a novel mechanism of action [1–3]. Specifically, motexafin gadolinium (MGd, Xcytrin[®]), which is the subject of the present study, is being evaluated as an agent for enhancing the effect of radiation therapy for treatment of cancer [6].

Accurate assessment of the purity of these drug substances is critical to the study of pharmacological, toxicological, and pharmacokinetic profiles of the drugs. Analytical methods that have specificity, accuracy, precision, and robustness are required to assure the quality, strength, identity, and purity of the drug before it goes for marketing approval. Additionally, regulatory agencies, like the U.S. Food and Drug Administration (FDA), require the identification of impurities and degradation products that

occur in the active pharmaceutical ingredient at levels above 0.1% or 0.05% depending on the drug dose [7].

Among the classical tools used for drug purity profiling, including gas chromatography (GC), high-performance liquid chromatography (HPLC) is the most powerful and versatile. Reversed-phase HPLC and GC have been used to separate a variety of metal-free and metal-coordinated porphyrins found in biological and geological samples [8–10]. In the impurity determination of the metallotexaphyrins currently under development, HPLC with photodiode array (PDA) or mass spectrometric (MS) detection has been found useful. HPLC with absorbance detection has also been used for the assay of MGd and motexafin lutetium in human plasma [11].

An attractive complementary (and orthogonal) or alternative separation technique to HPLC is capillary electrophoresis (CE). Since the first reported application of CE to drug analysis [12, 13], well over a hundred papers have been reported on this subject. In fact, CE methods are now listed in the U.S., European, and Japanese Pharmacopoeias [14]. Moreover, pharmaceutical companies have used CE results in FDA regulatory submissions. Such CE methodologies conform to the International Conference on Harmonization (ICH) of technical requirements for the registration of pharmaceuticals for human use [15].

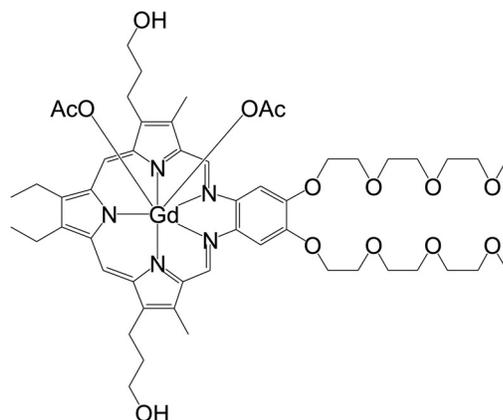
Capillary zone electrophoresis (CZE) and electrokinetic chromatography (EKC) have been shown to be excellent techniques for the separation of anionic and cationic porphyrins [16–28]. CZE and EKC have the advantages

Correspondence: Tarak D. Mody, Pharmacyclics, Inc., 995 East Arques Avenue, Sunnyvale, CA 94085-4521 USA.

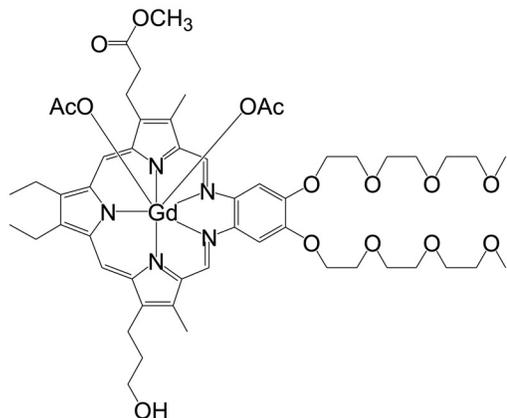
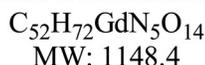
E-mail: T.D. Mody, tmody@pcyc.com

Fax: +1 408 774 0340

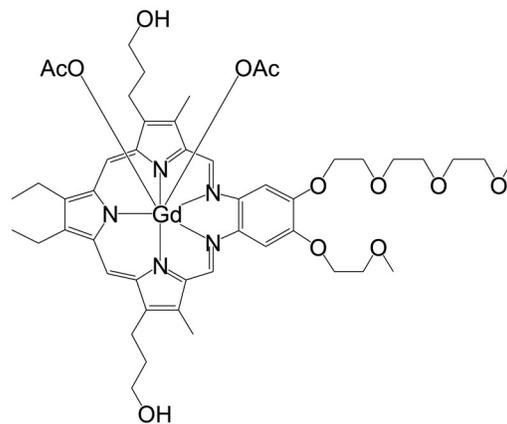
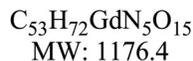
¹⁾ Present address: Scios Inc., 820 West Maude Avenue, Sunnyvale, CA 94085 USA.



Motexafin Gadolinium (MGd)



PCI-0400



PCI-0430

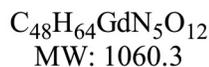


Figure 1. Chemical structures of motexafin gadolinium (MGd), PCI-0400, and PCI-0430.

over HPLC and GC of shorter analysis times, reduced sample and solvent usage, and higher efficiencies, among others. But CE has not been used until now for the separation of expanded porphyrins.

The metallotexaphyrins used in this study each has a net +2 charge arising from the gadolinium(III) central metal ion (upon the loss of two acetate apical ligands). **Figure 1** shows the structure of MGd and two related impurities, PCI-0400 and PCI-0430, which are found in every manufactured batch of MGd. In this paper, we demonstrate the utility of CE, particularly CZE and EKC with absorbance and LIF detection, for the separation of MGd from PCI-0400 and PCI-0430. We also investigate the use of CE for more general impurity profiling of MGd.

2 Experimental

2.1 Apparatus

CZE and EKC experiments were carried out with a Beckman P/ACE System MDQ (Fullerton, CA, USA) equipped with a PDA detector or a LIF detector. Spectra of MGd scanned from 190 nm to 600 nm were obtained with the PDA detector and suggested a maximum absorbance at 472 nm. Emission in the LIF experiments was measured at 750 nm using an emission filter (Andover Corp., Salem, NH, USA) matched to the maximum emission of MGd excited at 488 nm.

Fused-silica capillaries of 50- μ m ID and 375- μ m OD from Polymicro Technologies (Phoenix, AZ, USA) were used.

The effective length (from inlet to detector) was 50.0 cm and the total length was 60.2 cm. The separation voltage was varied from -30 kV to $+30$ kV at the inlet while the outlet was maintained at ground. The capillary liquid coolant temperature was set to 20°C . Pressure injections were carried out at 30 mbar for 10 s.

2.2 Chemicals

Reagents of the highest grade available were purchased from Sigma-Aldrich (Milwaukee, WI, USA) and used as received. The active pharmaceutical ingredient, motexafin gadolinium was manufactured for clinical supplies by Pharmacyclics, Inc., and used without any additional purification [29]. Two MGd-related impurities, called PCI-0400 and PCI-0430, were synthesized at Pharmacyclics by published methods [29, 30]. Stock solutions of these metallotexaphyrins were prepared every week in distilled water (Invitrogen Corporation, Carlsbad, CA, USA) and stored in a cool dark place between experiments. Appropriate amounts of the stock solutions were diluted with distilled water to obtain the sample solutions. Separation solutions used in electrophoresis experiments were prepared daily to minimize possible run-to-run irreproducibility. The separation solution could be a buffer or a solution composed of a buffer, organic solvent, and/or other additives.

2.3 Capillary electrophoresis procedure

The capillary was initially flushed (at 1.2 bar) with 1 M NaOH (30 min), followed by methanol (30 min), purified water (30 min), and, finally, with the separation solution (7 min). To ensure repeatability, the capillary was flushed between consecutive runs with 1 M NaOH (3 min), methanol (2 min), purified water (3 min) and, finally, with the separation solution (7 min).

3 Results and discussion

3.1 Separation of metallotexaphyrins

3.1.1 Capillary zone electrophoresis

Separation of charged analytes in CZE is caused by differences in their electrophoretic mobilities [31]. The charge and mass of the analyte as well as the nature of the electrophoretic separation solution affect the electrophoretic mobility. In general, high charge-to-mass ratios provide high electrophoretic velocities. **Figure 2** shows the separation of the three metallotexaphyrins, PCI-0430 (peak 1), MGd (peak 2), and PCI-0400 (peak 3) using CZE with and without organic solvents. Under the conditions stated in Figure 2, the electrophoretic velocity of

each metallotexaphyrin and the velocity of the electroosmotic flow (EOF) are both directed toward the cathode. Analytes with higher electrophoretic mobilities therefore elute faster than lower ones.

CZE runs performed in the absence of organic solvent from the separation solution (100 mM ammonium acetate/water) resulted in poor to no separation of PCI-0430, MGd and PCI-0400 (Figure 2.a). The addition of organic solvents to the separation solution was then investigated in order to alter the electrophoretic mobilities of the test samples. The addition of acetonitrile (30%) was not effective (Figure 2.b). Partial separation of MGd and PCI-0400 was achieved, however, when 30% methanol (Figure 2.c) or 30% ethanol (Figure 2.d) was present in the separation solution. 30% Acetone and 30% isopropyl alcohol also gave similar separation results to those of methanol and ethanol (data not shown). Decreasing or increasing the percentage of organic solvent did not provide any improvement in the resolution of the last two eluting peaks.

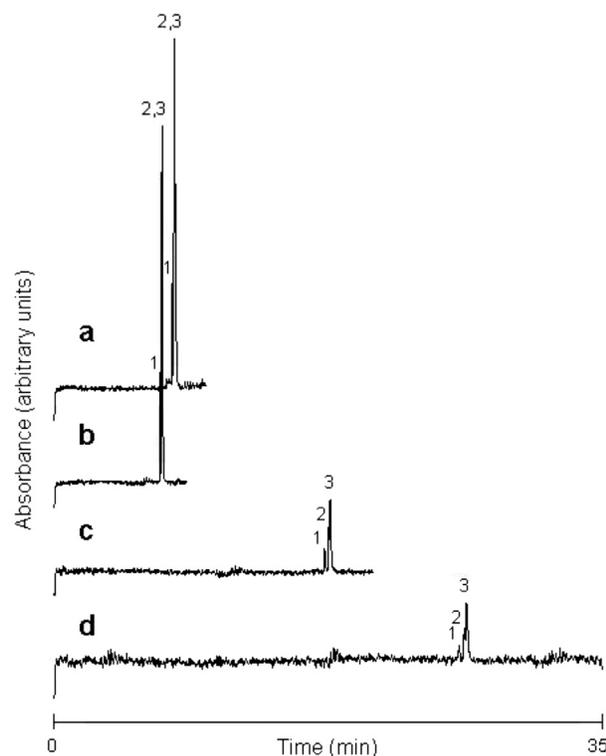


Figure 2. Capillary zone electropherograms of the separation of PCI-0430 (peak 1), MGd (peak 2), and PCI-0400 (peak 3). The separation solutions are composed of different ratios of 100 mM ammonium acetate (pH 4.3), water, and organic solvent. The ratio is (ammonium acetate buffer/water/organic solvent) 5/5/0 in **a**, 5/2/3(acetonitrile) in **b**, 5/2/3(methanol) in **c**, and 5/2/3(ethanol) in **d**. The separation voltage was 25 kV. The concentrations of the samples were around 7–10 $\mu\text{g/mL}$ each. Other conditions are found in the experimental section.

The molecular mass of the metallotexaphyrins increases from peak 1 to peak 3. The molecular masses are 1060.3 amu for PCI-0430 (peak 1), 1148.4 amu for MGd (peak 2), and 1176.4 amu for PCI-0400 (peak 3). Each metallotexaphyrin studied contains a gadolinium(III) cation (i.e., net charge is +2) and each has a similar macrocyclic framework. Thus, the electrophoretic mobility change between each metallotexaphyrin is essentially caused by the differences in molecular mass.

Nonaqueous CE was also investigated using acetonitrile or methanol containing increasing concentrations of ammonium acetate (50 mM to 100 mM). The three metallotexaphyrins eluted as two broad peaks. Under the present experimental conditions, CZE and nonaqueous CE were not successful for the separation of the test metallotexaphyrins.

3.1.2 Electrokinetic chromatography

Separation of charged analytes in EKC is caused by the combined effects of analyte electrophoretic mobility and analyte partitioning to a pseudostationary phase (PS) that is added to the separation solution [32]. Neutral and charged PSs were used in the separation of the metallotexaphyrins. The neutral PSs were γ -cyclodextrin, β -cyclodextrin, Brij 56 (polyoxyethylene(10)cetyl ether), Brij 98 (polyoxyethylene(20)oleyl ether), and PEG1000 (polyethylene glycol, MW = 1000 amu). Baseline separations were achieved with Brij 56 or PEG 1000 in the separation solution.

The charged PSs used were the anionic micelles of sodium dodecyl sulfate (SDS), sodium taurocholate, and sodium taurodeoxycholate, and the cationic micelle of cetyltrimethylammonium bromide (CTAB). The anionic microemulsion of SDS, butanol, and *n*-heptane was also studied. The anionic PSs proved to be unsuitable owing to strong electrostatic attraction between the positively charged analytes and the negatively charged PSs.

The cationic PS, CTAB, does not have a high affinity for the metallotexaphyrins because of electrostatic repulsion, but CTAB does allow for hydrophobic interaction with the metallotexaphyrins. The results are good separations with efficiencies greater than 200,000 plates/m. Experimental parameters were adjusted to obtain the optimum separation conditions. The pH of the buffers were varied from pH 2 to 8, the concentration of CTAB was changed from 25 mM to 400 mM, and the organic solvent content was altered from 5 to 35% v/v. **Figure 3** presents our best result, which uses 400 mM CTAB, 20% methanol, and 50 mM phosphoric acid (pH \approx 2). The migration order is different from that in CZE because of the difference in separation mechanism. Partitioning may be the primary cause of separation because the electrophoretic mobili-

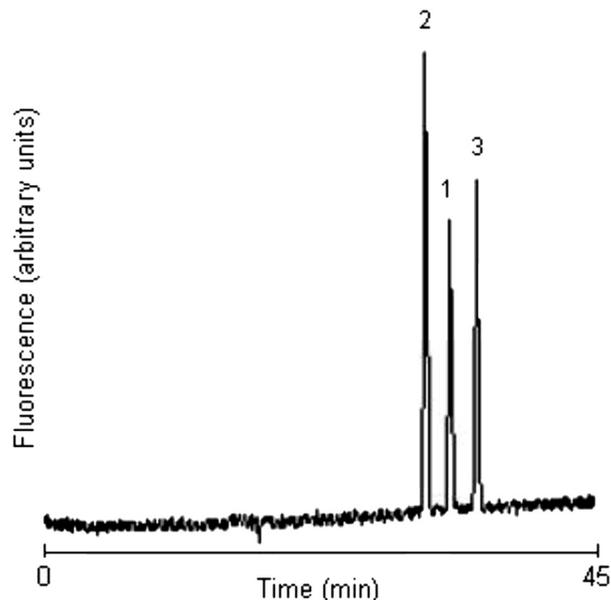


Figure 3. Electrokinetic chromatogram of the separation of MGd and two related impurities, PCI-0430 and PCI-0400 are depicted. The separation solution is a mixture of 400 mM cetyltrimethyl ammonium bromide, 50 mM phosphoric acid, and 20% methanol. The separation voltage was -20 kV at the inlet. The concentrations of the samples were around $45 \mu\text{g/mL}$ each. Identity of the peaks is the same as in Figure 2.

ties do not differ significantly from those observed in the CZE separation. It was found that addition of methanol was necessary to solubilize CTAB in the separation solution. Acetonitrile was also a good solubilizing agent; however, the current was ≈ 1.5 times larger than that in methanol. Typical currents ranged from 70 to 73 μA under these conditions. Lower operating currents are desirable to prevent Joule heating.

Many porphyrins are naturally fluorescent. We examined the potential for a sensitive and selective detection scheme without derivatization using LIF. It is known that the test metallotexaphyrins have a characteristic fluorescence emission at around 750 nm after excitation at 488 nm with an argon ion laser. As shown in Figure 3, a clean baseline separation with only one small system peak (before 20 min) was obtained. In contrast, when using absorbance detection at 472 nm (data not shown), several positive and negative system peaks were observed.

The reproducibility of migration times has been examined quantitatively. The %RSD ($n = 3$) of migration time using MGd as the internal standard is 0.4% and 0.5% for PCI-0430 and PCI-0400, respectively. These values suggest good reproducibility of the method.

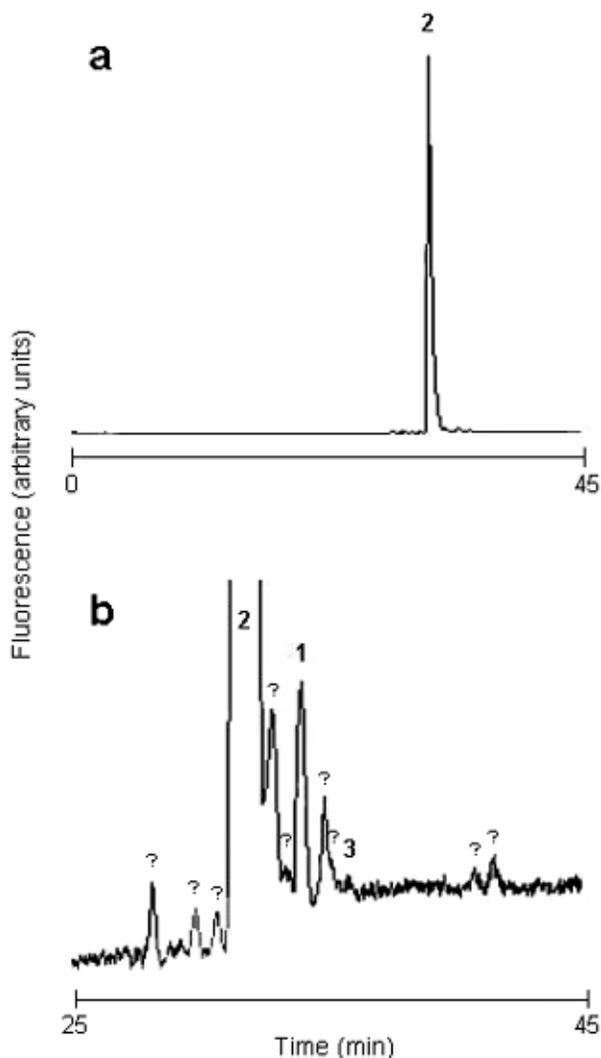


Figure 4. Electrokinetic chromatograms illustrating the separation of MGd, PCI-0430, and PCI-0400: (a) full electrokinetic chromatogram; and (b) 50 × magnification of (a) with respect to the y-axis. All conditions and identity of peaks are the same as in Figure 3. The amount of MGd weighed and dissolved in 0.4 mL water was 1 mg.

3.2 Impurity profiling of motexafin gadolinium

To assess the utility of CE for the impurity profiling of an MGd sample, a concentrated solution of MGd from a clinical batch was injected into the capillary under the present EKC conditions. **Figure 4.a** shows the electrokinetic chromatogram of this sample, which is magnified 50 times in **Figure 4.b**. In addition to PCI-0430 and PCI-0400, nine impurity peaks are observed (marked as '?') [33]. The analysis of MGd by HPLC takes approximately 90 minutes (gradient condition), whereas in CE the time required is significantly shortened to less than 40 minutes (isocratic condition). The presence of PCI-0430 and PCI-0400 were confirmed by spiking experiments. The concentration of

these impurities, based on peak heights, were approximately 0.1% to 0.3% of the MGd peak; the contents are all approximately 0.1% when based on peak areas. The limit of detection is at the sub-ppm level, which is comparable to HPLC results [33]. The present results clearly indicate that the sensitivity of the method is sufficient for the impurity determination of MGd as an investigative pharmaceutical product.

4 Concluding remarks

Capillary electrophoresis in the electrokinetic chromatography mode with native laser-induced fluorescence detection was used to separate and quantitate the investigative drug, motexafin gadolinium and its related impurities. Conditions were optimized to afford a detection limit for impurities present in the expanded porphyrin motexafin gadolinium at approximately 0.1% of the total sample.

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