Separation of porphyrin-based photosensitizer isomers by laser-induced fluorescence capillary electrophoresis

Methods for the separation of photosensitizer isomers, such as benzoporphyrin derivative monoacid, benzoporphyrin ethyl monoacid, 2-[1-hexyloxyethyl]-2-devinylpyropheophorbide-a, diethyleneglycol diester benzoporphyrin derivative, tin ethyl etiopurpurin, and phthalocyanine tetrasulfonate, have been systematically developed by CE. Detection was accomplished by UV absorption at 214 nm or by LIF with excitation at 442/488 nm and emission at 690 nm. The effects of three major experimental parameters of buffer types, organic solvents, and surfactant additives are described. The optimized separation conditions were determined so as to provide satisfactory separation efficiency and analysis time. The methods are shown to be suitable for the separation and determination of porphyrin and phthalocyanines regioisomers, diastereoisomers, and enantiomers.

Keywords: Chiral additives; Enantiomers; Fluorescence detection; Phthalocyanines; Porphyrins; Regioisomers

DOI 10.1002/elps.200500183

1 Introduction

Photosensitizers are molecules that have the special property of absorbing photonic energy and using this energy, by a number of pathways, to carry out chemical reactions in cells and body tissues [1]. They have been widely applied in the field of photodynamic therapy (PDT). In PDT, a photosensitizer first selectively accumulates in certain cells or tissues, this is followed by irradiation with various doses of light in the visible region. The resultant generation of excited state species can then produce cytotoxic singlet oxygen or other radical species. This process eventually damages the proteins, lipids, nucleic acids, and other cellular components of the target cells or tissues and can lead, for instance, to tumor necrosis and ablation [2]. An ideal photosensitizer should exhibit absorbance in the red region of spectrum with high molar absorption coefficients. These properties allow more penetration to deeper tissue resulting in more damage to deep seated sites.

The first photosensitizer used in clinical PDT for bladder carcinoma, esophageal, and lung cancer was hematoporphyrin derivative and its purified fraction, Photofrin® [3]. The prolonged and generalized photosensitivity in the skin was its primary side effect. Benzoporphyrin derivative monoacid ring A (BPDMA, 1, also known as verteporfin®) is the active component of Visudyne®. This second generation photosensitizing drug exhibits less skin photosensitivity. It is utilized in the treatment of certain forms of age-related macular degeneration [4, 5]. Other potential photosensitizers have been developed with improved photophysical properties and tumor selectivity, such as tin etiopurpurin (SnET2) [6], motexafin lutetium [7], meta-tetrahydroxyphenyl chlorin [8], mono-i-aspartyl chlorin e6 [9], 5-aminolevulinic acid (ALA), which is a metabolic precursor in the biosynthesis of protoporphyrin IX [10], phthalocyanines [11], verdins [12], psoralens [13], anthracycline [14], and other nonporphyrin compounds [15].

Isomers usually have different physicochemical properties and their differing configurations can also strongly influence their pharmacological properties. Regulatory guidelines on “stereoisomers in New Chemical Entities” have been in place in most jurisdictions around the world since the early 1990s, but such guidelines still need to be harmonized. In general, the regulatory agencies acknowledge that developing a single enantiomer, a racemate, or a more complex mixture of regioisomers is up to the pharmaceutical company. However, it is absolutely clear that each stereoisomer must be studied.
separately, which means that methods for the separation and analysis of each compound must be developed. Thus the need for analytical methods with high-resolution power and efficiency is critically important for control in manufacturing, pharmacokinetic, and pharmacodynamic studies. In addition, the separation of isomers plays crucial roles for addressing their safety and efficiency. HPLC has proved to be a powerful tool for the separation of photosensitizer [16–21], but the close physical and chemical properties of photosensitizer isomers, aggregation, ionic charge, solubility, and strong adsorption on the column can limit the usefulness of HPLC for their separation. Due to significant improvements in separation resolution and low sample cost of CE, it has become increasingly popular and has been recognized as one of the most effective techniques for porphyrin isomer separation. For example, the successful separation of coproporphyrin and uroporphyrin isomers [22–26], polyhematoporphyrin oligomers [27], photofrin oligomers [28, 29], cationic porphyrin atropisomers [30], and benzoporphyrin mono- and diacid enantiomers and regiosomers [31] shows the effectiveness of this technique.

In the present study, several CE methods have been developed for the separation of porphyrin and phthalocyanines isomers. Different running buffers and additive systems were evaluated and optimized in order to obtain good separation with efficiency and acceptable time profiles.

2 Materials and methods

2.1 CE system

All experiments were performed using a Beckman P/ACE 5500 system consisting of an autosampler, a vacuum injection system, a thermostated capillary compartment (5–50°C), and a UV-Vis detector, diode array detector (DAD), or LIF detector with a 3 mW argon laser module (488 nm) (Beckman Coulter, Mississauga, ON, Canada), a Liconix helium-cadmium laser (442 nm, 6.8 mW), and band pass filters 690 ± 5 nm (1 in. ID, Oriel, Stratford, CT, USA). Uncoated fused-silica capillary columns were obtained from Polymicro Technologies (Phoenix, AZ, USA). Unless otherwise specified, dimensions were typically 50 μm ID, 355 μm OD, and 37 cm total length (30 cm to the detector). The new capillary was treated with 1.0 M NaOH for 15 min followed by a 15 min rinse with deionized distilled water and equilibrated with the electrophoresis running buffer overnight before use. Data collection, processing, and analysis were performed using the P/ACE® Station software package (Beckman).

2.2 Instruments

Fluorescence spectra were recorded on an AMINCO-Bowman series 2 luminescence spectrometer (SLM-Aminco, Urbana, IL, USA) with a high power xenon lamp. Absorption spectra were recorded on an 8452A diode array spectrophotometer (Hewlett Packard, Palo Alto, CA, USA) or a Cary 50 UV-Vis spectrometer (Varian, Harbor City, CA, USA) using a fused-silica cuvette with a 1 cm path length. A GLC-1 general laboratory centrifuge, a Microfuge E” (Beckman), and a Millex®-GV filter unit, 0.22 μm pore size (Millipore), were also employed.

2.3 Chemicals

Hydroxypropyl-β-CD (HP-β-CD), sulfated-β-CD, and β-CD were from Beckman, CTAB from Sigma (St. Louis, MO, USA), and SDS from Fisher (Springfield, NJ, USA). Taurocholic acid, sodium salt hydrate, deoxytaurocholic acid, sodium salt, deoxycholic acid, sodium salt, and Brij 35 were obtained from Aldrich (Milwaukee, WI, USA). Cholic acid and taurodeoxycholic acid, sodium salts were purchased from Sigma. The buffer solutions were pH 11.04, 100 mM CAPS, pH 9.2, 100 mM Na₂B₄O₇·10H₂O (borax), pH 8.1, Tris, pH 6.8, 100 mM ammonium acetate, and pH 2.8, 100 mM citric acid (Sigma). These were diluted to the appropriate concentration before use. All solutions were filtered through a 0.22 μm filter prior to use. Water was distilled and deionized with a NANO pure II ultra water system (Barnstead/Thermolyne, Dubuque, IA, USA), DMSO, ACN, 1-propanol, iso-propanol, and methanol were HPLC-grade (Fisher Scientific), and all other chemicals were of analytical grade and used without further purification.

2.4 Samples

Benzoporphyrin derivative monoacid (BPDMA, 1; see Scheme 1) was synthesized via the Diels-Alder adducts of dimethyl propophorphyrin and dimethyl acetylene dicarboxylate [4]. The time of hydrolysis of the propionic dimethyl esters with 25% hydrochloric acid dictates the final ratio between monoacid and diacid that are formed. The separation of the diacids from the monoacid analogs is achieved by chromatography [32]. Benzoporphyrin ethyl monoacid (BPDMAe, 2) was synthesized from the Diels-Alder adducts of protoporphyrin and dimethyl acetylene dicarboxylate, and transesterified to the diethyl ester. This was hydrolyzed to make the monoacid ethyl ester. Compounds 1 and 2 were dissolved in DMSO at a concentration of 1.0 mg/mL. The stock solutions were stored frozen below 0°C in the dark. Immediately before use, a fraction of the stock solution was diluted with
DMSO to a concentration of $2.0 \times 10^{-2}$ mg/mL. Diethyleneglycol diester benzoporphyrin derivative (DGE-BPD, 3) was synthesized as follows [33]: To 2.0 g benzoporphyrin derivative diacid (B-ring) in 50 mL ethylene glycol and 100 mL dichloromethane was added 1.0 mL sulfuric acid. The reaction was stirred for 18 h at room temperature. Then the reaction was added to a stirring mixture of 100 mL 5% aqueous ammonium acetate and 100 mL dichloromethane. The organic layer was isolated and then washed twice with 50 mL water. The solvent was removed by rotary evaporation. The dark green residue was then chromatographed on 75 g alumina (deactivated with 5% water) and eluted with a gradient of 0.5–5% methanol in dichloromethane. The solvent from the fractions containing product was then removed by rotary evaporation. The residue was dried in vacuo overnight to provide 2.02 g of the analytical pure green solid product. Stock solutions 3 were prepared in DMSO at a concentration of 1.0 mg/mL and stored in the fridge in the dark. Immediately before use, a fraction of the stock solution was diluted with DMSO to a concentration of $2.0 \times 10^{-2}$ mg/mL.

2.5 CE separation procedures

The instrument was programmed to rinse the capillary at high pressure with 0.2 M NaOH for 5 min, pure water for 5 min, and then the separation buffer for 3 min. Samples were then introduced with pressure (0.5 psi) injection mode for 2.0 s. The separation was conducted in a constant-voltage mode by applying 0–30 kV across the ends of the capillary with normal or reversed polarity. The analytical signal was monitored either using a UV detection wavelength of 214 nm or with the laser excitation wavelength at 488 nm/442 nm and fluorescence detection at 690 nm. The detector cell window was made by burning and removing the polyimide coating layer on the capillary. The temperature of the capillary was held at 20°C. Data were collected at 5 Hz. Between runs, the capillary was cleaned and equilibrated with successive 5 min rinses of 1.0 M NaOH and pure water.

3 Results and discussion

The identical physical properties of enantiomers and the close physical properties of the regioisomers of the tetrapyrrolic systems described here make their separation a special challenge. In addition, their relatively poor solubility profiles coupled with a tendency to aggregate in aqueous buffer adds to these challenges.

To overcome these later problems, we have used DMSO to dissolve all of the isomer analytes. Furthermore, differing pH and ionic running buffer systems, nonpolar, polar,
and neutral organic solvents, and anionic, cationic, and neutral surfactants were optimized in order to prevent analyte aggregation and obtain optimal selectivity with acceptable detection signal peaks.

3.1 BPDMA (1) and BPDMAe (2)

The chlorin-like BPDMA (1) consists of an equal mixture of two regioisomers, each regioisomer consists of a pair of enantiomers whose separation has been previously reported [31]. BPDMA has a characteristic major broad absorption peak in the Soret region (420 nm) with an absorption coefficient of 61 000/M×cm and several other peaks at longer wavelengths. Its emission spectrum is around 692 nm and is close to the red Q-band, with a Stokes shift of ~4 nm for fluorescence. BPDMAe (2), one of the byproducts produced during the production of BPDMA (1), also consists of two regioisomers, their only difference is that the methyl group in BPDMA (1) is replaced with an ethyl group in BPDMAe (2). Compound 2 shows similar chemical and physical properties to BPDMA (1). It was necessary to establish a method and separate it from BPDMA (1).

It is well known that the buffer pH and ionic strength, organic solvent, and surfactant additive have dramatic influences on the electrolyte viscosity, dielectric constant, EOF mobility, and analyte electrophoretic mobility in CE. Thus when 30% ACN was added to an electrolyte of pH 9.2, 240 mM borate buffer solution containing 10 mM SDS, BPDMA (1) and BPDMAe (2) were successfully separated (Figs. 1a and b). By comparing their migration times with that of EOF, it could be concluded that both BPDMA and BPDMAe existed in negative charged states in electrolyte. In this example both the ACN and SDS prevent aggregation of the analytes, and influence their migration behavior in the capillary. This approach indicates the feasibility of utilizing the same experimental conditions for more complex mixture. We further optimized the separation parameters of buffer and SDS concentration, organic solvent percentage in the electrolyte, and the separation voltage, and found baseline separation when the separation voltage was lowered from +25 to +10 kV at 120 mM borate, 28% ACN, and 15 mM SDS (Fig. 1c). The resulting current also dramatically dropped from 118 to 23 to achieve better peak shape. A low electrolyte ion strength also minimizes its fluorescence quenching to the analytes. A similar separation was obtained using

![Figure 1. Separation of BPDMA (1) and BPDMAe (2). Ex/Em 442 nm/690 nm. BPDMA 7.5 × 10⁻⁵ mg/mL and BPDMAe 2.5 × 10⁻⁵ mg/mL except (a) where both BPDMA and BPDMAe were 1.0 × 10⁻⁴ mg/mL. (a and b) +25 kV, pH 9.2, 240 mM borate buffer containing 30% ACN and 10 mM SDS. (c) +10 kV, pH 9.2, 120 mM borate buffer containing 28% ACN and 15 mM SDS. (d) +10 kV, pH 9.2, 120 mM borate buffer containing 24% ACN, 4% DMF, and 16 mM SDS. (e) +10 kV, pH 7.12, 30 mM PBS buffer containing 30% ACN and 20 mM SDS. (f) +20 kV, pH 9.2, 240 mM borate buffer containing 30% ACN and 10 mM SDS.](image-url)
different electrolyte compositions, i.e., SDS concentration, ACN amount, buffer types, or buffer ionic strength (Figs. 1d and f). The separation efficiency dramatically improved when these two organic additives, ACN and DMF, were used in the running buffer (Fig. 1d). Methanol or propanol did not provide any improvement for these separations.

3.2 DGE-BPD

DGE-BPD (3) is also a chlorin-like derivative, it contains two chiral centers but, due to the exclusive trans-relationship on the exocyclic ring it exists as only a pair of enantiomers. Its photophysical properties are congruent with 1 and 2. As with other BPD isomers, using an He–Cd laser excitation at 442 nm or an argon laser excitation at 488 nm results in a strong emission at 695 nm, near red (Q-band), with a Stokes shift of −3 nm for fluorescence.

Problems that limit the separation are that DGE-BPD (3) is a hydrophobic neutral chiral compound and separation of the enantiomers depends on their abilities to interact with chiral additives along with the other factors that will influence their mobility behavior. Two types of buffer systems of borate and phosphate, as the running buffer, were utilized and examined for the DGE-BPD (3) enantiomer separation. Borate buffer was found to be better since it also interacts with these porphyrin analogs and assists in the enantiomer separation [31]. Due to the aggregation properties of DGE-BPD (3), greater than 30% ACN was required in the electrolyte since aggregation both interferes with the separation pattern and quenches the fluorescence signal. Further increasing the amount of ACN suppressed the analyte signal and made the migration time too long. Sodium cholate, which had previously been found to be an effective chiral additive to distinguish between both optical isomers as well as highly hydrophobic compounds [25, 31], was evaluated here. DGE-BPD (3) enantiomers began to separate when the cholate concentration was above 70 mM. The baseline DGE-BPD (3) enantiomer separation was achieved when cholate was above 100 mM. We obtained the best separation efficiency by adding 150 mM sodium cholate into pH 9.2, 80 mM borate buffer containing 30% ACN and sodium cholate. Sodium cholate: (a) 100 mM, (b) 125 mM, and (c) 150 mM.

3.3 HPPH

Pheophorbides and pyropheophorbides are effective photosensitizers against tumors grown in mice and rats [36, 37]. Their derivatives have been found to possess both rapid clearance from skin, high in vitro cytotoxicity and high in vivo antitumor efficacy in a murine tumor model [38]. Because the HPPH (4) starting materials are derived from a natural source, the isomeric center at the D-ring is enantiomerically pure. However the isomeric center located alpha to the A-ring is racemic which results in a diastereoisomer mixture for HPPH. At pH 9.2 in a 240 mM borate buffer solution that contains 30% ACN and 1.5 mM SDS, HPPH (4) has a maximum absorbance, Soret band, at 420 nm with the major long wavelength absorbance Q-band in the red at 660 nm, with \( \varepsilon = 47,500/ \text{M} \times \text{cm} \). The differences in the physiological effects of the HPPH (4) diastereoisomers have not been examined because no preparative scale separation has yet been achieved.

Like most porphyrin derivatives HPPH (4) also undergoes aggregation in aqueous solution. We found that the most extensive aggregation of HPPH (4) occurred when no organic solvent was used. This resulted in a “fluorescence signal”, which was below the detection limit.

© 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
However, increasing the ACN from 0 to 20%, gave a good signal peak, albeit with severe tailing. By increasing the organic solvent to 30%, the peak occurred as a sharp and narrow signal that indicated HPPH (4) was in its completely monomeric form. Exceeding 30% organic solvent in the electrolyte was avoided since, as above, this resulted in capillary clogging. Organic solvent plays a role in altering both the polarity and viscosity of the electrolyte. As a consequence both the EOF and the electrophoretic mobility of the analytes were affected. Many noise spikes appeared when using methanol and no HPPH (4) signal appeared within 40 min when using DMSO. DMF, isopropanol, and 1-propanol were also successfully used for the separation, but ACN was found to be the best organic solvent. HPPH (4) could not be separated using CAPS buffer. Ammonium acetate caused noise spikes that lead to a current breakdown and interruption of the separation process, phosphate buffer helped in the separation but resulted in a high current due to the high ionic strength. Borate buffer was found to be the best electrolyte with high buffer capacity which did not generate significant current. The nonionic Brij 35 added into the running buffer did not improve the separation. Sodium cholate only partially separated HPPH diastereoisomers and the capillary was clogged when using high concentrations. SDS, which is the most frequently used additive in micellar EKC, was found to be the best additive in this study when used below its CMC. The difference in mobility of the HPPH (4) diastereoisomers was evident even at very low concentration of 1.5 mM SDS. An ideal baseline separation was obtained by increasing the SDS concentration to 2.0 mM (Fig. 3), further increasing the SDS concentration caused longer migration and poor separation resolution. The apparent formation constant of HPPH (4) with monomeric SDS was calculated to have a high K value of 719.24 and 711.88, respectively [31, 39]. The K value indicates the possibility of separation along with a strong complexing ability and large capacity factor when changing the SDS concentration.

3.4 SnET2

Tin ethyl etiopurpurin dichloride (SnET2, 5) is also a hydrophobic chlorin-like analog. It has a chiral center located at the A-ring resulting in a racemic mixture. The use of it as a photosensitizer has demonstrated significant effects on canine prostate and human immunodeficiency [40, 41]. PHOTREX™ (rsotaporphin, SnET2) has been issued an approval letter by the FDA, to Miravant Medical Technologies on September 30, 2004, for the treatment of wet age-related macular degeneration. It has a major absorption peak around 660 nm and several small peaks between 582 and 734 nm in the red region of the visible spectrum and absorption between 404 and 436 nm.

Only marginally mobility of the enantiomers was achieved when using the conventional phosphate, acetate, Tris, and citrate buffer systems. Enantioselectivity was dramatically diminished at decreasing buffer pH and reduced ionic strength when using cholate in the electrolyte. Completely eliminating the buffer system resulted in a shorter analyte migration time. Further increasing the pH and borate ionic strength to 120 mM and keeping the ACN concentration at 30% and cholate at 15 mM resulted in enantiomers separation which depended upon the chiral additive in the electrolyte. Partial separation of the SnET2 (5) enantiomers was achieved within a relative short migration time of 4 min at a cholate concentration of 15 mM (Fig. 4a). The effects of other types of bile salts such as taurocholate, deoxytaurocholate, and deoxycholate and chiral additives of β-CD, HP-β-CD, and sulfated-β-CD did not achieve any separation. This failure
Separation of porphyrin-based photosensitizer isomers

3.5 Pcs

Sulfonation of the phthalocyanine nucleus places a sulfonato group on each of the benzo rings, however, since either of the β-positions can be substituted many regioisomers can be formed (6). These isomers all exhibit absorption peaks at 218, 280, and 332 nm and a 638 nm peak in the Q-band region. Metallophthalocyanines have shown promise for PDT [42, 43]. Pcs (6) are prone to considerable aggregation particularly in aqueous solution. When dissolved in DMSO (6) the isomers exist principally in their monomeric form.

The choice of an appropriate amount of organic solvent was essential for the isomer separation since it determined the degree of aggregation in the electrolyte. No signal was observed in the absence of organic solvent. However, when adding 10% ACN into the electrolyte, aggregation was completely prevented and the mixture of isomers presented as one major sharp peak. Up to eight isomers could be separated by increasing the amount of ACN from 10 to 60%. The same behavior was observed with increasing amounts of other additives in the running buffer. Brij 35 and cholate did not provide any significant assistance for the separation. However, SDS was found to be the best additive for the separation. Figure 5 shows the high-resolution electropherogram of Pcs (6) where more than nine isomers were separated. The multipeak profile in Fig. 5 results from contamination with trisulfonated isomers found in the commercial samples.
4 Concluding remarks

Porphyrins and related “pyrrolic” macrocycles all suffer from severe aggregation properties and low solubilities. These problems can be overcome by the appropriate use of organic solvents in the running buffer. Once these macrocycles are disaggregated their optical properties, strong absorption, and fluorescence in the visible region facilitate their detection at low concentration. Further use of surfactants and chiral additives has allowed us to couple the high resolving power of CE and reversibility of LIF to develop short separation times for a variety of optical and region isomers of porphyrins, chlorines, and phthalocyanines. We anticipate that the methods described here will serve as a general guide for the separation of porphyrin and phthalocyanine based photosensitizers where such separation is essential for an understanding of the pharmacological effects of each separate isomer [31, 44].

The authors acknowledge the support by QLT PhotoTherapeutics and the Natural Sciences and Engineering Research Council of Canada.

Received March 2, 2005
Revised May 25, 2005
Accepted May 30, 2005

5 References