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Sensitive Absorption-Based Wave-Mixing Detector for Anthracycline Drugs Separated By Capillary Electrophoresis

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Abstract

Sensitive absorption-based detection of anthracycline antibiotics, daunorubicin and doxorubicin is demonstrated using a capillary electrophoresis system interfaced to a nonlinear wave-mixing detection system. Unlike conventional absorption methods, this nonlinear absorption method can detect very thin analytes (50 μm) efficiently. At the same peak height, the wave-mixing CE peak is narrower than a conventional CE peak, and hence, compared to other laser-based or non-laser-based CE on-column detection methods, our wave-mixing detection method offers intrinsically enhanced separation resolution even when using identical CE separation conditions. In this unusually sensitive “absorbance” detection method, two input laser beams interact to produce a thermally induced grating from which coherent laser-like wave-mixing signal beams are created. Using our sensitive “absorbance” on-column CE detector, we report a preliminary concentration detection limit of 9.9×10^{-10} M using a 50 μm i.d. capillary column. The corresponding “injected” mass detection limit is 9.1×10^{-18} mol using an injection volume of 9.2 nL. The corresponding preliminary “detected” mass detection limit inside the 12-pL detector probe volume is 1.2×10^{-20} M.

Keywords

Nonlinear laser spectroscopy; sensitive absorption detection; capillary electrophoresis; anthracycline

Introduction

Capillary electrophoresis (CE) is an effective analytical tool for many applications including analysis of metabolites and drugs, analysis of natural products and proteins, and DNA sequencing. It offers high separation efficiency, short analysis time, small sample and buffer requirements, and convenient direct on-column detection. Due to small detector volumes (<nL) and short light absorption path lengths (<50 μm) available, on-column detection in capillary electrophoresis demands sensitive detection methods that can use short light path lengths efficiently. The most commonly used on-column detection methods for capillary electrophoresis are UV-visible absorption, conventional fluorescence and laser-induced fluorescence methods. Conventional UV-visible absorption detectors offer good linearity but they lack detection sensitivity. Laser-induced fluorescence offers good detection sensitivity,

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but they are applicable only to compounds that can fluoresce or can be labeled with fluorescing tags.

An unusually sensitive laser-based “absorbance” detection method based on forward-scattering wave mixing offers excellent detection sensitivity for small absorbance measurements while using short absorption path lengths (50 μm). In this nonlinear laser detection method, two coherent input laser beams are focused and mixed inside an absorbing analyte. The resulting thermal gratings scatter off incoming photons from the two input beams to produce wave-mixing signal beams. The signal has important nonlinear characteristics including its cubic dependence on laser power and its quadratic dependence on solute absorption coefficient. The coherent laser-like properties of the signal beam allow virtually 100% optical signal collection efficiency with excellent S/N. Since the signal is visible to the naked eye even at low concentration levels, optical alignment is simple and convenient.

We have demonstrated effective use of nonlinear wave-mixing methods in various applications for both gas- and liquid-phase analytes using a wide range of lasers including low-power diode lasers. In gas-phase media, the backward-scattering wave-mixing optical arrangement yields sub-Doppler spectral resolution and it allows hyperfine structure measurements and isotope ratio analyses in various atomizers including hollow-cathode discharge plasmas, flames and graphite furnaces (1–4). For continuously flowing liquid analytes, we have reported attomole-level detection sensitivity (5) that is comparable or better to those of laser-based fluorescence detection methods, and yet our detection system is applicable to both fluorescing and non-fluorescing analytes. In addition, we demonstrated circular dichroism and optical activity measurements at trace-concentration levels using nonlinear wave mixing (6–8).

In this report, we demonstrate separation and detection of anthracycline antibiotics, daunorubicin (DAU) and doxorubicin (DOX), using a capillary electrophoresis system interfaced to an absorption-based nonlinear wave-mixing detector. The studies of anthracyclines and the determination of DAU and DOX mostly involve HPLC and capillary electrophoresis (9–14) and excellent detection limits have been reported using fluorescence-based detection methods. Using our sensitive “absorbance” on-column CE detector, we report a preliminary concentration detection limit of 9.9×10^{-10} M at S/N of 2 using a 50- μm i.d. capillary column. The corresponding preliminary “injected” mass detection limit is 9.1×10^{-18} mol using an injection volume of 9.2 nL. The corresponding preliminary “detected” mass detection limit inside the 12-pL laser probe volume is 1.2×10^{-20} M.

Experimental

Figure 1 shows a simple and easy-to-align forward-scattering wave-mixing on-column detector for CE. The laser source is a continuous-wave argon ion laser operating at 476.5 nm. The laser passes through a polarizer and then a 70:30 beam splitter to form the pump beam and the probe beam. In order to maximize wave-mixing grating contrast, the path length difference between the pump and the probe is adjusted so that it is shorter than the coherence length of the laser. These two input beams are then focused on the capillary using a 7-cm focal length lens. The capillary is positioned in a donut-shaped mount and controlled by a XYZ translational stage for precise optical alignment. An aperture is placed right after the capillary to allow only the signal beam to pass through. The coherent laser-like signal beam is then filtered by a polarizer and an aperture and then focused by a 20-cm focal length lens on a photodiode. The photodiode signal is processed by a lock-in amplifier (Stanford Research Systems, Inc., Sunnyvale, CA, Model SR810 DSP) which is referenced to a

mechanical chopper modulating the probe beam at 200 Hz. The signal is finally digitized by a computer.

Our custom-built CE system consists of a power supply (Glassman High Voltage, Inc., Whitehouse Station, NJ, Model PS/MJ30P0400-11) with voltage and current monitors, two electrodes, two buffer vials, and a piece of capillary. For safety considerations, the anode is kept into a Plexi-Glass box. The cathode is placed on the side arm of the box in order to maintain the same height for the two buffer vials and to reduce the total length of the capillary. The laser beams are interfaced to the uncoated fused silica capillary (Polymicro Technologies, Inc., Phoenix, AZ, 75 cm total length, 45 cm effective length). Capillary tubes with different dimensions are used in this study including those with 50 μm i.d./363 μm o.d., 75 μm i.d./363 μm o.d., 100 μm i.d./363 μm o.d., and 180 μm i.d./340 μm o.d. A 1-cm wide detection window is created for on-column detection by burning off the coating and cleaning with methanol. The capillary is back filled with an alignment solution for optical alignment using a vacuum pump (Barnant Company, Barrington, IL, Model 400–1901). Before CE separation runs, the capillary is flushed with 0.1 M NaOH, DI water, and the running buffer for 20 minutes each, followed by the buffer at separation voltage for about 30 min. The analytes are injected electrokinetically at the anodic end at 12 kV for 5 s.

The running buffer consists of acetonitrile and 100 mM, pH 4.2, sodium dihydrogen phosphate buffer (70/30, v/v). It is filtered with a Nylon membrane filter (Phenomenex, Torrance, CA) and degassed with a sonicator. The analyte stock solutions are prepared by dissolving solid DAU and DOX analytes (Calbiochem, La Jolla, CA) in DI water. Different analyte concentrations are prepared by diluting the stock solution with the running buffer, followed by filtration with a Nylon membrane filter and degassing with a sonicator.

Results and Discussion

The basic anthracycline structure consists of a tetracyclic quinoid moiety that yields optical absorption around 250 nm and 480 nm when coupled to an amino-sugar (15). Although DAU and DOX exhibit stronger absorption at 250 nm, this UV wavelength is not convenient for trace analysis of these two drugs in real-time drug monitoring sessions due to the presence of strong interferences from other entities in the biological matrix (15) and the intrinsically higher background noise levels associated with this wavelength range.

Figure 2 shows the UV-visible absorption spectra of DAU and DOX in a 100 mM phosphate buffer - ACN solvent (30:70, v/v). The wavelength maximum in the visible range for both drugs is 476.5 nm. Our argon ion laser provides several wavelengths (457.9 nm, 476.5 nm, 488 nm, 515 nm, etc.) and we use the 476.5 nm argon ion line to probe both anthracyclines. At this wavelength, the absorption coefficients of DAU and DOX in the selected binary buffer system are $1.3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ and $1.2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, respectively. These two clinically important anticancer drugs exhibit substantial native absorption in the visible wavelength range, and therefore, they are used as analytes in our nonlinear wave-mixing “absorption-based” detection system.

Since the migration of analyte species is based on electrophoresis and electroosmosis effects, aqueous electrolyte buffer systems are usually used in CE systems. However, organic or mixed organic-aqueous buffers offer some advantages in CE including increased solubility for solutes that show poor solubility in water, reduced Joule heating, and enhanced separation resolution. It has been demonstrated that the use of ACN organic modifier in an aqueous phosphate buffer reduces the interaction of anthracycline analytes with the capillary wall (16), reduces Joule heating and enhances separation efficiency. Hence, a mixture of

aqueous phosphate buffer and ACN organic solvent (30:70, v/v) is used as a binary buffer electrolyte system in our CE system to separate DAU and DOX.

Since the wave-mixing signal has a quadratic dependence on the refractive index change with temperature (dn/dT) of the solvent, a solvent with good thermo-optical properties should be used, when possible. The dn/dT value for water is low ($0.14 \times 10^{-4} \text{ mW}^{-1} \text{ cm}$ at 20° C) and organic solvents have higher dn/dT values, ranging from $1.9 \times 10^{-4} \text{ mW}^{-1} \text{ cm}$ (methanol) to $5.9 \times 10^{-4} \text{ mW}^{-1} \text{ cm}$ (carbon tetrachloride). Hence, the addition of an organic modifier in an aqueous buffer enhances the dn/dT value, resulting in a stronger wave-mixing signal. Furthermore, the absorption coefficients of the two anthracyclines under study increase as the ratio of organic solvent to water in the buffer system is increased, resulting in a stronger wave-mixing signal.

As shown in Figure 3, the absorption coefficient of DAU increases from $6.43 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ in a pure aqueous phosphate buffer to $1.29 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ in a binary buffer containing 70 % ACN. Hence, the use of a higher percentage of ACN organic modifier in the CE buffer not only improves CE separation of DAU and DOX, it also enhances the buffer thermo-optical properties, resulting in a stronger wave-mixing signal.

Figure 4 compares theoretical and experimental wave-mixing CE peak profiles to that expected from a conventional CE system. Since the multi-photon wave-mixing signal has a quadratic dependence on analyte concentration, the theoretical wave-mixing peak profile has a squared Gaussian profile. As shown in Figure 4, our experimental wave-mixing peak profile for DAU closely matches to the expected squared Gaussian profile. The conventional CE absorption or fluorescence signal, resulting from one-photon excitation, has a linear dependence on analyte concentration, and hence, the conventional CE peak follows a normal Gaussian profile, as shown in Figure 4. At the same peak height, the wave-mixing CE peak is narrower than a conventional CE peak, and hence, compared to other laser-based or non-laser-based CE on-column detection methods, our wave-mixing detection method offers intrinsically enhanced separation resolution even when using identical CE separation conditions.

Figure 5 shows an electropherogram of a mixture of $6.7 \times 10^{-8} \text{ M}$ DAU and $7.0 \times 10^{-8} \text{ M}$ DOX using a phosphate buffer-ACN mixture (30:70, v/v). The amounts of DAU and DOX injected into the CE system are 0.60 femtomole and 0.63 femtomole, respectively, and the two drugs are well resolved.

Figure 6 shows an electropherogram of DAU at a trace concentration level. The preliminary “injected” concentration detection limit for DAU is determined to be $9.9 \times 10^{-10} \text{ M}$ at S/N of 2, which corresponds to an injected mass detection limit of $9.1 \times 10^{-18} \text{ mol}$. As illustrated in Table 1, our wave-mixing detection sensitivity level, especially the mass detection sensitivity, is comparable or better than those previously reported for laser-based absorbance or fluorescence determination of anthracyclines separated by high-performance liquid chromatography (HPLC) or CE.

Conclusions

Our absorption-based wave-mixing detector offers fluorescence-like detection sensitivity levels in a CE system and it can be used to detect a wider range of fluorescing and non-fluorescing analytes. Unlike conventional absorption methods, this nonlinear absorption method can detect very thin analytes ($50 \mu\text{m}$) efficiently. At the same peak height, the wave-mixing CE peak is narrower than a conventional CE peak, and hence, compared to other laser-based or non-laser-based CE on-column detection methods, our wave-mixing detection method offers intrinsically enhanced separation resolution even when using identical CE

separation conditions. Hence, this nonlinear laser-based technique offers important advantages for sensitive absorption detection of biochemical and biomedical analytes in a wide range of applications.

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Figure 1. Experimental setup for capillary electrophoresis wave-mixing detection of anthracycline drugs. P1, P2, polarizers; L1, L2, focusing lenses; A1, A2, apertures; F, filter.

Figure 2. Absorption spectra of (a) 2.7×10^{-5} M DAU and (b) 2.8×10^{-5} M DOX in a 100 mM phosphate buffer/acetonitrile solvent binary mixture (30:70, v/v) at pH 4.2.

Figure 3. Absorption spectra of 2.7×10^{-5} M DAU in a phosphate CE buffer modified with (a) 0 % acetonitrile, (b) 30 % acetonitrile and (c) 70 % acetonitrile.

Figure 4. Comparison of CE peak profiles: (a) theoretical Gaussian profile, (b) theoretical squared Gaussian profile and (c) experimental wave-mixing signal profile.

Figure 5.

Capillary electropherogram of a mixture of (a) 6.7×10^{-8} M DAU and (b) 7.0×10^{-8} M DOX separated in a 100 mM phosphate buffer/acetonitrile solvent binary mixture (30:70, v/v) at pH 4.2. Injection, 5 s at 12 kV; separation, 24 kV, 10.6 μ A; capillary dimensions, 72 cm total length, 50 μ m i.d., 45 cm effective length.

Figure 6.

Capillary electropherogram of 3.9×10^{-9} M DAU in a 100 mM phosphate buffer/ acetonitrile solvent binary mixture (30:70, v/v) at pH 4.2. Injection, 5 s at 12 kV; separation, 30 kV, 13 μ A; capillary dimensions, 75 cm total length, 50 μ m i.d., 45 cm effective length.

Table 1

Comparison of laser-based detection methods for Anthracycline.

Analytical Method	Injection Volume (μL)	Analyte	Molar Detection Limit (injected) (mol/L)	Mass Detection Limit (injected) (mole)	Ref.
HPLC-Fluorescence 474 nm	1000	DOX	8.6×10^{-10}	8.6×10^{-13}	17
HPLC-Fluorescence 480 nm	400	DOX	8.6×10^{-10}	3.4×10^{-13}	18
HPLC-Fluorescence 480 nm	30	DOX	2.4×10^{-10}	7.2×10^{-15}	10
HPLC-Absorbance 490 nm	200	DAU	1.7×10^{-8}	3.5×10^{-12}	19
CE-Fluorescence 476.5 nm	0.014	DAU	8.9×10^{-11}	1.2×10^{-18}	16
CE-Wave Mixing 476.5 nm	0.0092	DAU	9.9×10^{-10}	9.1×10^{-18}	This Work