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Laser-induced fluorescence detection of 9-fluorenylmethyl chloroformate derivatized amino acids in capillary electrophoresis

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ABSTRACT

Laser-induced fluorescence (LIF) was applied to the detection of 9-fluorenylmethyl chloroformate (FMOC-Cl) derivatized amino acids separated by capillary electrophoresis. Fluorescence excitation was provided by a pulsed, KrF laser operating at 248 nm. A limit of detection of $5 \cdot 10^{-10}$ *M* was obtained for FMOC-alanine ($S/N = 2$). Separation of FMOC-derivatized proline, hydroxyproline, and sarcosine was achieved with a 20 m*M* borate buffer (pH 9.2), and the separation of FMOC-derivatized amino acid standard mixture was obtained using a 20 m*M* borate buffer (pH 9.2) containing 25 m*M* sodium dodecyl sulfate.

INTRODUCTION

Capillary electrophoresis (CE) is a rapid and highly efficient separation technique for a large variety of compounds [1]. UV absorption is the most widely used detection mode in CE. To improve sensitivity, fluorescence detection is also used. Detection of native amino acids is difficult because most of them do not possess strong spectrophotometric or fluorogenic properties. Therefore, chemical derivatization is necessary for sensitive determination of amino acids. Fluorescamine, *o*-phthalaldehyde (OPA), 9-fluorenylmethyl chloroformate (FMOC-Cl), and naphthalene-2,3-dicarboxaldehyde (NDA) are some of the common fluorogenic reagents that undergo rapid derivatization reaction with amino acids [2–5], but only FMOC-Cl forms fluorogenic derivatives with both primary and secondary amines. Albin *et al.* [6] have evaluated the applications of fluorescamine, OPA and FMOC-Cl for the fluorescence detection of amino acids in CE using conventional excitation sources. For ultrasensitive detection, laser-induced fluorescence (LIF) detection can be used [7]. LIF detection of fluorescamine-derivatized marine toxins [8], OPA- and NDA-derivatized amino acids [9,10] has been shown with a helium-cadmium laser. To the best of our knowledge, LIF detection of FMOC derivatives has not been done. The goal of this study is to demonstrate that high-sensitivity LIF detection of FMOC-derivatized amino acids in CE can be achieved by utilizing a rugged, affordable, pulsed-laser operating at 248 nm.

EXPERIMENTAL

Apparatus

The home-built CE–LIF system that we used was similar to that described previously [11]. Separations were performed with 70 cm (60 cm to detector) × 50 μm I.D. fused-silica capillaries

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(Polymicro Technologies, Phoenix, AZ, USA). A 1-cm portion of capillary coating was removed by flame for on-column detection. Positive-polarity high voltage for separation was provided by a Glassman power supply (Whitehouse Station, NJ, USA). Gravity injection was carried out by raising the high voltage end of the capillary by 10 cm for 20 s. Fluorescence excitation was provided by a pulsed-UV laser operating at 248 nm (Model GX-500; Potomac Photonics, Lanham, MD, USA). The laser beam was spectroscopically filtered with a line filter (No. 250-S-1D; ARC, Acton, MA, USA) and then focused onto the capillary with a 25 mm f.l., UV-graded bi-convex lens. The laser power after the lens was *ca.* 0.5 mW, which was measured with a power meter (Model 815; Newport Corp., Fountain Valley, CA, USA). Fluorescence emission was collected at a 90° angle to the incident laser beam with a 10×, numerical aperture (NA) = 0.5, UV-graded microscope objective (Model Fluar; Carl Zeiss, Thornwood, NY, USA), or as otherwise specified, with a 20×, NA = 0.4, glass objective (Model 13590; Oriel, Stratford, CT, USA). After passing through a bandpass (FWHM = 60 nm, No. 310-B-1D, ARC) and a cut-off filter (WG-306; Melles Griot, Irvine, CA, USA), the collected emission was detected by a photomultiplier tube (PMT; Model 70680, Oriel). The current output of the PMT was fed into a boxcar averager (Model 4100; EG&G, Princeton, NJ, USA) and its voltage output was displayed in a PS/2 computer via an A/D interfacing module (Model 406; Beckman Instruments, Fullerton, CA, USA).

The amino acid standard mixture containing 17 amino acids (100 pmole/μl each) was obtained from Hewlett-Packard (Palo Alto, CA, USA). Sodium dodecyl sulfate (SDS; Kodak, Rochester, NY, USA) was washed with ether before use. FMOC-Cl (Sigma, St. Louis, MO, USA) was prepared in acetonitrile as a 5 m*M* solution. All other chemicals were obtained from Sigma. Electrophoresis buffers were prepared in high purity water generated with a NANOpure water system (Barnstead, Dubuque, IA, USA) and filtered through a 0.2-μm membrane filter before use. The amino acids were derivatized by mixing 500 μl of dilute sample, 100 μl of 0.4 *M* boric acid-sodium hydroxide (pH 9.3), and 400 μl of FMOC-Cl for 1 min. Excess FMOC-Cl and its hydrolyzed products were extracted twice from the reaction mixture with 2 ml pentane. The aqueous portion was diluted to the appropriate concentration with water before CE analysis. A filtered urine sample was diluted 10-fold with water and the diluted sample was then subjected to the FMOC derivatization described above. The aqueous portion was diluted 100-fold before CE analysis.

RESULTS AND DISCUSSION

FMOC-Cl reacts rapidly (*ca.* 1 min) with primary and secondary amines to form highly fluorescent, stable derivatives [12]. FMOC-Cl and its hydrolyzed products fluoresce, but they can be readily extracted with pentane. Sensitive fluorescence detection of FMOC-derivatized amino acids in CE were achieved using conventional excitation sources such as xenon or deuterium lamps [6]. To further improve sensitivity, LIF detection could be used. The absorption maximum of FMOC derivatives is at approximately 265 nm; thus, the UV laser (248 nm) should provide adequate excitation for these molecules. Since the emission of FMOC is at approximately 315 nm, a UV-graded microscope objective from Carl Zeiss (10×, NA = 0.5) was used for emission collection. We found that this objective provided at least a 5-fold increase in sensitivity as compared to a conventional glass objective (*e.g.* 20×, NA = 0.4 from Oriel). This enhancement was mainly due to the higher transmission of light in the 300 nm region. Fig. 1 shows the high-sensitivity LIF detection of $1.2 \cdot 10^{-8}$ *M* FMOC-alanine ($2 \cdot 10^{-5}$ *M* before derivatization). The limit of detection (LOD) is approximately $5 \cdot 10^{-10}$ *M* (*S*/*N* = 2) which is *ca.* 100-fold more sensitive than that obtained using a xenon lamp (260 nm) as the excitation source [6]. This level of detection is comparable to that of NDA and about 40-fold more sensitive than that of OPA in other CE–LIF studies [9,10]. Derivatization at a low sample concentration is subject to the possibility of an inefficient derivatization reaction and sample loss. A 100-fold less concentrated alanine solution (*i.e.* $2 \cdot 10^{-7}$

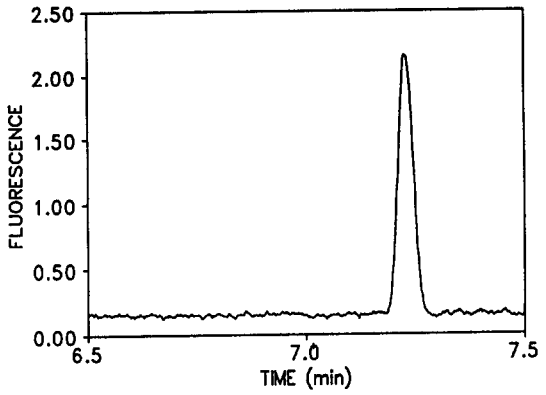

Fig. 1. Electropherogram of $1.2 \cdot 10^{-8}$ *M* FMOC-alanine. Capillary: 70 cm × 50 μm; running buffer: 20 m*M* borate (pH 9.2); voltage: 20 kV; gravity injection: 10 cm, 20 s.

M) was derivatized and then diluted to $1.2 \cdot 10^{-8}$ *M* for injection (Fig. 2). As a comparison, the blank peak is also shown in Fig. 2. The LOD obtained in this case agrees with that obtained from Fig. 1; this suggests that the problems mentioned above did not occur for sample levels of at least 10^{-7} *M*. A 5-point peak-height calibration curve of FMOC-alanine was constructed between $1.3 \cdot 10^{-9}$ and $1.3 \cdot 10^{-7}$ *M*. The response of the LIF detection was linear for at least 2 orders of magnitude ($r^2 = 0.9996$).

Proline (Pro), hydroxyproline (Hyp), and sarcosine (Sar) are secondary amino acids that show important pathological and biological properties [13–16]. Determination of these compounds has been achieved using thin-layer chromatography, gas chromatography or high-performance liquid

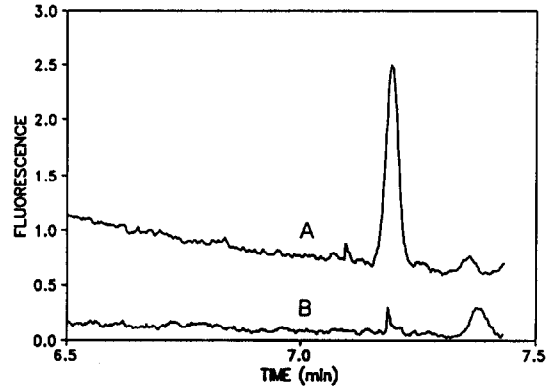

Fig. 2. Electropherograms of (A) $1.2 \cdot 10^{-8}$ *M* FMOC-alanine, and (B) blank. Running conditions as in Fig. 1.

chromatography with various spectroscopic, radiometric, or electrochemical detection methods [17–22]. Most of these methods are time-consuming. Recently, Guzman *et al.* [23] used CE to separate fluorescamine-derivatized Pro and Hyp. Because fluorescamine does not form fluorogenic derivatives with secondary amino acids, absorption detection (214 nm) was used in their study. These authors obtained a detection limit at the 10^{-5} *M* level, which is typical for absorption detection in CE. Fig. 3 shows the electropherogram for the separation of FMOC-derivatized Pro, Hyp and Sar. With LIF detection, an LOD of each amino acid was obtained at the 10^{-9} *M* level. With a 75 μm capillary, Pro and Hyp were partially resolved at the indicated running conditions, but were baseline separated when a 50 μm capillary was used. A borate buffer containing SDS greatly enhanced the separation of Pro and Hyp (below).

Determination of amino acids in biological samples [12] and protein hydrolysates [24] has been performed with precolumn derivatization using FMOC-Cl, and subsequent analysis of the derivatives by reversed-phase HPLC with gradient elution. Alternately, efficient separation of FMOC-derivatized amino acids can be achieved by CE using an SDS micellar buffer [6,25]. Fig. 4 is the electropherogram for the separation and high sensitivity LIF detection of amino acid

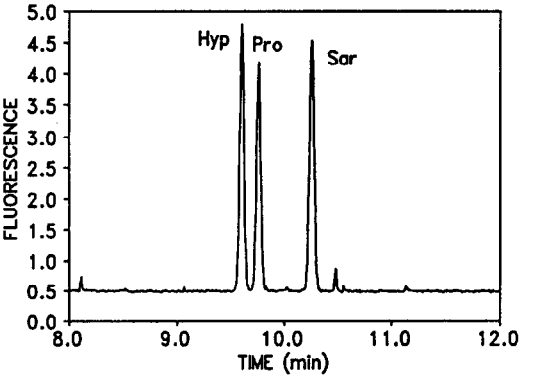

Fig. 3. Electropherogram for the separation of proline (Pro), hydroxyproline (Hyp) and sarcosine (Sar). Capillary: 70 cm × 50 μm; running buffer: 10 m*M* phosphate (pH 7.0); voltage: 25 kV; gravity injection: 10 cm, 20 s. The concentration of each amino acid is $4 \cdot 10^{-8}$ *M*. A glass microscope objective was used.

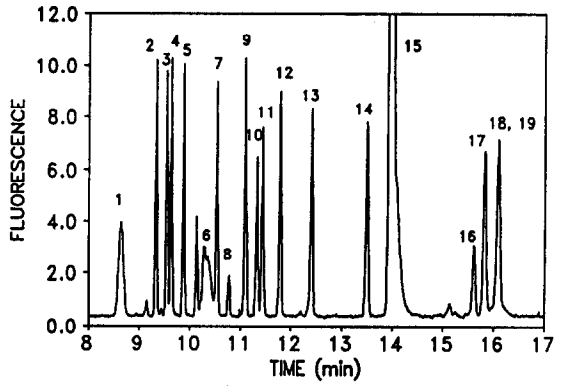

Fig. 4. Electropherogram for the separation of FMOC-derivatized amino acid standard mixture + Hyp. Capillary: 70 cm × 50 μm. Running buffer: 20 m*M* borate (pH 9.2)–25 m*M* SDS; voltage: 20 kV; gravity injection: 10 cm, 20 s. The concentration of each amino acid is $3 \cdot 10^{-8}$ *M*. Peaks: 1 = hydroxyproline; 2 = serine; 3 = threonine; 4 = alanine; 5 = glycine; 6 = proline; 7 = valine; 8 = tyrosine; 9 = glutamic acid; 10 = methionine; 11 = aspartic acid; 12 = isoleucine; 13 = leucine; 14 = phenylalanine; 15 = FMOC background; 16 = cystine; 17 = arginine; 18 = lysine; 19 = histidine.

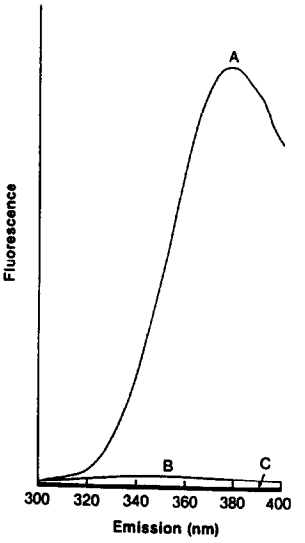

Fig. 5. Fluorescence spectra of (A) SDS, (B) SDS washed with diethyl ether, and (C) water. The SDS concentration was about 10 m*M*.

standard mixture + Hyp that was derivatized with FMOC-Cl. The concentration of each amino acid injected was $3 \cdot 10^{-8}$ *M*. Milofsky and Yeung [26] have pointed out that fluorescence of the impurities associated with SDS degraded detection at 300–400 nm significantly, and the authors purified the SDS by recrystallization before use. We found that most of the SDS impurities were also efficiently removed by washing with diethyl ether (Fig. 5). Background fluorescence was further reduced by the 310-nm bandpass filter. As a result, our detection was degraded by only a factor of about 2 with the addition of 25 m*M* SDS in the running buffer. In Fig. 4, the peaks are identified by spiking of individual derivatized amino acids. With the indicated running conditions, almost all of the amino acids were resolved in under 17 min. McLaughlin *et al.* [25] have show the separation of 21 amino acids with a higher concentration SDS buffer; this highlights the potential application of CE as an alternative separation tool to HPLC in the analysis of FMOC-derivatized amino acids. Since the Hyp peak eluted prior to all of the other tested amino acids, the CE system may facilitate the determination of Hyp in certain biological applications [23]. As an example to show the analysis of a real sample, FMOC-derivatized human urine (1:1000 dilution) was subjected to CE–LIF analysis, as shown in Fig. 6. Identification of the individual peaks was not performed because this was not the goal of this study; rather, it demonstrates the potential application of this CE–LIF system for the high-sensitivity determination of biological amino compounds.

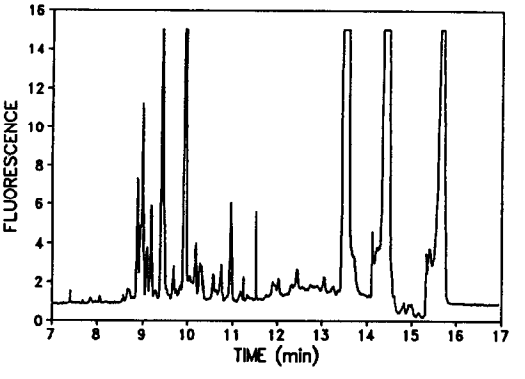

Fig. 6. Electropherogram for the separation of FMOC-derivatized urine (1:1000 dilution). Running conditions as in Fig. 4.

CONCLUSIONS

This study demonstrates that high-sensitivity LIF detection of FMOC-derivatized amino acids separated in CE could be achieved by using a pulsed-laser operating at 248 nm. An LOD of $5 \cdot 10^{-10}$ *M* for FMOC-alanine was obtained, which was comparable to the LOD obtained with NDA and better than those obtained with OPA in other LIF studies. Although not as sensitive as the FMOC derivatives, we could detect OPA derivatized amino acids at the 10^{-8} *M* level with the 248-nm laser. The combination of efficient separation of CE and high-sensitive detection of LIF should be very useful for the analysis of FMOC-labeled amino acids in protein hydrolysates and the amino compounds in biological samples. The use of the combined OPA-FMOC derivatization for the selective detection of secondary amines [17] in CE with LIF detection is currently being studied in this laboratory.

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