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Nanomolar derivatizations with 5-carboxyfluorescein succinimidyl ester for fluorescence detection in capillary electrophoresis

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Abstract

The ability of 5-carboxyfluorescein succinimidyl ester (CFSE) to derivatize amino acids at very low concentrations has been investigated. CFSE can derivatize nanomolar concentrations of amino acids, which represents a 1000-fold improvement compared to fluorescein isothiocyanate (FITC). Although the reactive probe is not fluorogenic, few hydrolysis products result from derivatization, leading to relatively clean electropherograms compared to FITC derivatization. Derivatizations of γ-aminobutyric acid suggest that CFSE may be a practical amine-reactive fluorescent probe for the quantification of neurotransmitters in microdialysates.

Keywords: Derivatization, electrophoresis; Carboxyfluorescein succinimidyl ester; Amino acids

1. Introduction

Capillary electrophoresis (CE) is well suited for the separation of biomolecules containing amino acid residues, since the electrophoretic mobility of these analytes can be manipulated simply by controlling the pH of the electrophoresis run buffer. Numerous examples of separations of amino acids, peptides and proteins appear in the scientific literature [1,2]. Detection of non-aromatic amino acids or peptides that contain no aromatic amino acid residue is problematic, however. The ubiquitous UV absorbance detector is incapable of detecting concentrations of these analytes below millimolar levels, due to the absence of a good chromophore. Even if these concentration limits are suitable for a specific application, electromigration dispersion can occur if the mobility of the analyte does not match that of the buffer ion [3].

Derivatization of these analyte can solve these problems. Typically, an amine-reactive fluorogenic probe, such as dansyl chloride, fluorescamine [4] or *o*-phthaldialdehyde (OPA) [5], is used to convert the analyte into a fluorescent derivative. Early work in this area used continuous excitation sources with monochromators to select a narrow range of wavelengths for excitation. Difficulties in focusing light into the small detection volume delineated by the capillary dimensions were experienced. Furthermore, continuous sources, such as the xenon arc lamp, have poor UV emission characteristics and, thus, are not optimal for the excitation of these fluorophores.

Conversely, lasers are excellent excitation sources for fluorescence detection in CE, due to their high intensity and collimation, which allows for easy

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focusing of the beam into the internal diameter of the capillary. A helium–cadmium laser (He–Cd) is suitable for the excitation of a number of different fluorogenic amine-reactive probes. A He–Cd operating at 325 nm can be used to excite OPA derivatives [6]; at 354 nm, the laser can excite fluorescamine derivatives [7] and, presumably, dansyl derivatives; while the 442 nm lasing transition can be used to excite naphthalene-2,3-dicarboxaldehyde (NDA) derivatives [8].

These fluorescent derivatives have relatively weak molar absorptivities and poor fluorescence quantum yields, especially when compared to fluorophores such as fluorescein or tetramethylrhodamine. Zeptomole (10^{-21} mol) mass and picomolar (10^{-12} *M*) concentration detection limits of amino acids derivatized with fluorescein isothiocyanate (FITC) [9] and tetramethylrhodamine isothiocyanate (TRITC) [10] have been reported. Yet these detection limits do not correspond to amounts or concentrations of amino acids, but to diluted derivatives. The chemistry of the isothiocyanate-reactive moiety is sluggish and inefficient [11]. Typically, one is limited to derivatizing the amino acid or peptide at high concentration, 10^{-4} *M* being typical for an effective reaction yield [12].

A comparison of common amine-reactive probes, FITC, fluorescamine, OPA and 9-fluorenylmethyl chloroformate (FMOC), has been performed for amino acid analysis [13]. It was demonstrated that FITC was not the best probe if detection limits were reported with regard to the concentration of amino acid before derivatization and dilution. FITC was outperformed by both OPA and FMOC for this derivatization, the latter by almost an order of magnitude.

By changing the reactive moiety present in a fluorescein-based probe, one may presumably improve the derivatization chemistry, thereby lowering the concentration of biomolecule that can be derivatized. We have demonstrated in this laboratory that derivatizations with the succinimidyl ester amine-reactive moiety are more than an order of magnitude faster than their isothiocyanate counterpart [14]. This work investigates the use of 5-carboxyfluorescein succinimidyl ester (CFSE) for the derivatization and analysis of small biomolecules.

2. Experimental

2.1. Apparatus

The CE-laser-induced fluorescence (LIF) detection system has been described previously [15]. Briefly, the system used a Spellman (Plainview, NY, USA) Model CZE1000R high-voltage power supply. Positive potentials were applied to the inlet end of the separation capillary for both injection and electrophoresis. The outlet end of the capillary was immersed in a vial containing the separation buffer, which was grounded. A window was burned in the polyimide coating of the capillary, approximately 10 cm from the outlet end of the capillary. The capillaries used (50 μm I.D.×186 μm O.D.) were obtained from Polymicro Technologies (Phoenix, AZ, USA).

The laser used in this study was a Uniphase (San Jose, CA, USA), air-cooled, light-controlled 4 mW Ar^+ laser (Model 2012-4SLL). The output beam was focused onto the window of the capillary using a 6.3× microscope objective [working distance (WD), 21 mm; numerical aperture (NA), 0.20]. Fluorescence and light scatter were collected at 90° to the excitation beam using a 16× microscope objective (WD, 3.7 mm; NA, 0.32). The collection objective was fastened to a light-tight box using a standard threaded microscope objective holder. The light-tight box contained all of the remaining optical components necessary for measurement of the fluorescence signal. Collected fluorescence was imaged onto an iris diaphragm, to spatially filter the fluorescence signal. Two Omega Optical (Brattleboro, VT, USA) bandpass filters (525BP15 and 535DF35) were used to spectrally filter both Raman and Rayleigh light scatter. Fluorescence was detected using a Hamamatsu (Bridgewater, NJ, USA) Model R1477 photomultiplier tube (PMT). The PMT was always powered to −1000 V in this study, as this setting was optimum for the signal-to-noise ratio. The current produced by the PMT was filtered and converted to a voltage by a low pass filter (10 MΩ resistor, 0.1 μF capacitor) and collected at 10 Hz using a National Instruments (Austin, TX, USA) NB-MIO-16X-H (16 bit resolution) data acquisition board that was plugged into a NuBus expansion slot

of an Apple (Cupertino, CA, USA) Model Quadra 650 personal computer.

The detection limit for fluorescein using this CE–LIF system was approximately 10^{-12} *M*.

2.2. Derivatization

In all derivatization reactions, the concentration of amine-reactive fluorescent probe (FITC or CFSE) was $1 \cdot 10^{-3}$ *M* at the start of the reaction. The use of lower concentrations of probe was investigated to determine if hydrolysis products could be minimized relative to the derivative, but optimal derivatization was found to occur with a large excess of probe. Fresh stock solutions of amine-reactive fluorescent probe at 0.1 *M* were made in *N*,*N*-dimethylformamide (DMF) and were used immediately. The derivatization buffer used for both amine-reactive fluorescent probes was 0.1 *M* borate, pH 9.28. Derivatization was allowed to proceed overnight in the dark.

2.3. Electrophoresis

After derivatization, all solutions were diluted by 10^2 in run buffer, which was identical to that used for derivatizations. Dilution ensured that amine-reactive fluorescent probe concentrations were less than 10^{-5} *M* during analysis, so that the PMT was not damaged by excessive fluorescence. Thus, at the lowest concentration of amino acid that was derivatized, 10^{-9} *M* γ-aminobutyric acid (GABA), the product was diluted to 10^{-11} *M* after derivatization. This diluted concentration is about an order of magnitude greater than the detection limit for fluorescein and, thus, should be detectable, provided that the derivatization efficiency is significant (i.e. >10%) and the fluorescent quantum yield of fluorescein is not reduced by derivatization. Injections of all derivatization reaction mixtures were performed electrokinetically at 1 kV for 5 s. It is estimated that the injection volume represents approximately 0.1% of the total capillary volume. Separations were accomplished at 25 kV, which yielded an electric field of approximately 500 V cm^{-1}. Peak identification was performed by spiking with standards or by high-performance liquid chromatography (HPLC)–electrospray mass spectrometry.

2.4. Reagents

CFSE and FITC were obtained from Molecular Probes (Eugene, OR, USA). All other reagents were of the highest purity offered by Sigma (Oakville, Canada).

3. Results and discussion

3.1. Fluorescein isothiocyanate derivatizations

A CE separation of the derivatization products of a reaction between 10^{-6} *M* ε-*tert*.-butoxycarbonyl (Boc)–L-lysine and FITC is shown in Fig. 1. The label depicting the migration time of the α-fluorescein thiocarbamyl (FTC)–ε-Boc–L-lysine derivative is based on relative migration times determined at higher concentrations of ε-Boc–L-lysine derivatized with FITC. At this concentration, it is difficult to determine whether the amino acid has been derivatized or not by comparisons to a blank with any degree of certainty. The reasons for this are twofold. First, the derivatization chemistry is inefficient, limiting the concentration of amino acid that can be derivatized. Second, a large number of fluorescent hydrolysis products and/or impurities can mask any signal attributable to a fluorescent derivative, especially at lower amino acid concentrations.

The derivatization chemistry is slow and requires that the amino acid primary amine be deprotonated, which explains why FITC derivatizations are carried out at high pH (i.e. >9). According to Satchell and Satchell [16], in the case of an amine derivatization with an excess of isothiocyanate in an aqueous medium, the rate of reaction is second order with respect to amine, or, in this case, amino acid, and isothiocyanate. Yet, hydrolysis of FITC is base catalyzed, thus, hydrolysis becomes more significant at higher pH values. Compromise conditions for satisfactory reaction rates minimizing hydrolysis are typically found at about pH 9–9.5. It has been shown that the reaction of isothiocyanates with OH^- is also

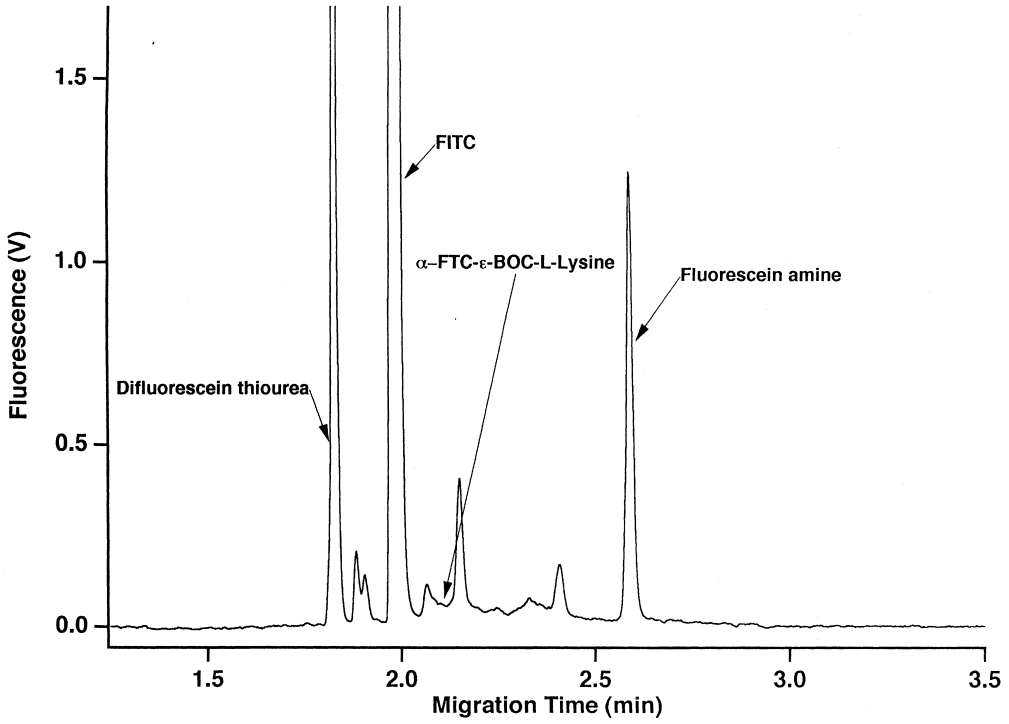

Fig. 1. Electropherogram of a 100-fold dilution of a derivatization reaction between 10^{-6} *M* ε-Boc–L-lysine and 10^{-3} *M* FITC.

second order [11], thus, the rate of FITC depletion in a derivatization reaction can be shown to be:

$$-\frac{\mathrm{d[FITC]}}{\mathrm{d}t} = k_D[\mathrm{H_2N\text{-}CHR\text{-}COO^-}][\mathrm{FITC}] + k_H[\mathrm{OH^-}][\mathrm{FITC}]$$

where k_D is the rate constant of amino acid derivatization and k_H is the rate constant of FITC hydrolysis.

The rate constants k_D and k_H are quite similar for the amino acid glycine and a large number of aryl isothiocyanates [11]. Thus, it becomes apparent why the derivatization of low concentrations of amino acids is problematic with FITC. At pH 9, the OH^- concentration is 10^{-5} *M*. If k_D and k_H are similar, then, for amino acid concentrations below 10^{-5} *M*, hydrolysis of FITC becomes the dominant mechanism for FITC depletion. For our case of 10^{-6} *M* ε-Boc–L-lysine, hydrolysis may be occurring ten-times faster than derivatization.

With a vast excess of amine-reactive fluorescent probe, however, one would still expect significant derivatization of the amino acid. This is no doubt occurring, but the derivative is lost in the large number of hydrolysis products and/or impurities in the dye. The production of fluorescein amine through hydrolysis, followed by its reaction with FITC to produce difluorescein thiourea is well documented [11,17] and the reactions are presented in Fig. 2. These two products are evident in Fig. 1. There are, however, seven additional peaks in the electropherogram that also appear in the blank and are due to either hydrolysis or to impurities present in the purchased FITC. It is apparent that their presence can mask derivative peaks, as in this case.

3.2. Carboxyfluorescein succinimidyl ester derivatizations

While FITC is the most commonly used fluorescein-based amine-reactive fluorescent probe, it is not the best choice for derivatizing amino acids at low concentrations. The performance of three different

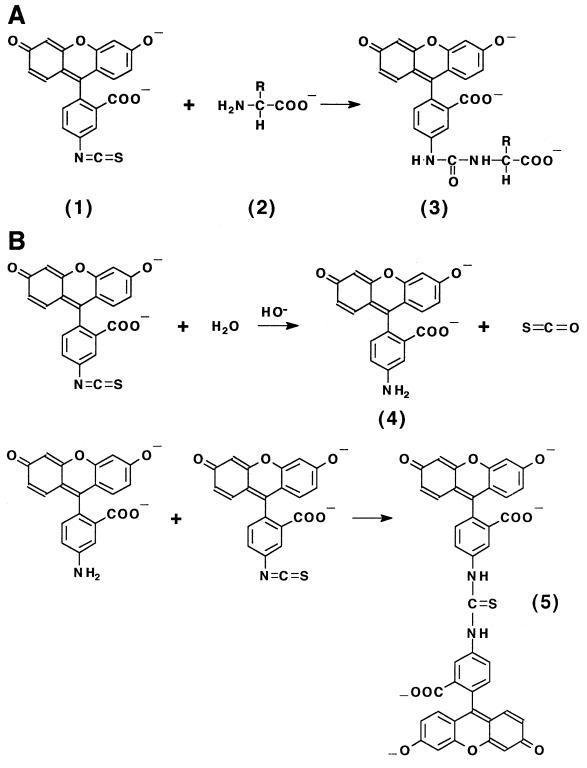

Fig. 2. FITC reactions: (A) amino acid derivatization: (1) FITC, (2) generic amino acid, (3) FTC–amino acid derivative; (B), FITC hydrolysis: (4) fluorescein amine and (5) difluorescein thiourea.

fluorescein-based amine-reactive probes, based on the rate of reaction, the degree of substitution, hydrolysis and derivative stability, have been compared in our laboratory. CFSE was determined to be a superior derivatization reagent in these categories. Electrophoretic separation of the derivatization products of a reaction between 10^{-7} *M* ε-Boc–L-lysine and CFSE is shown in Fig. 3. It is evident that an easily recognizable derivative peak is present, despite the fact that the amino acid concentration is an order of magnitude lower than that for the FITC derivatization. It is also evident that there is only one substantial hydrolysis product, 5-carboxyfluorescein, two minor (1.5 V > peak height >0.5 V) and perhaps eight trace (0.1 V > peak height >0.01 V) hydrolysis products and/or CFSE impurities. Derivatization and hydrolysis reactions are presented in Fig. 4.

It, therefore, appears that the hydrolysis of CFSE is less complex than that for FITC, minimizing the number of significant hydrolysis peaks that would appear in an electropherogram. It is also likely that the rate constant for amino acid derivatization by CFSE is significantly larger than that for hydrolysis, which may explain a derivative peak with a signal-to-noise ratio of about 10^3 at this concentration. Based on this S/N ratio, concentrations of 0.3 n*M* could be recognizably derivatized, if a detection limit of 3σ is used.

This result compares favorably to a previous study where four different amine-reactive probes were investigated for low concentration amino acid derivatization [13], and it is marginally better than that found on derivatization with NDA [8]. If commercial preparations of OPA are carefully purified, it is possible to reduce the lowest derivatizable concentration of analyte considerably with this probe. This has been performed for the HPLC–LIF analysis of amino acids and γ-glutamyl peptides from rat brain microdialysates [18]. Table 1 compares these studies to our own. Both OPA and NDA may be superior probes to CFSE in some applications, however, since they are fluorogenic and the derivatization process produces few fluorescent byproducts if the chemistry is carefully controlled. Thus, electropherograms display few peaks that are not attributable to OPA- or NDA–amino acid derivatives.

Some problems do exist with the use of these probes, however. There is a need to use concentrated sodium cyanide solutions to provide the necessary nucleophile for NDA derivatization. Furthermore, NDA derivatives require the use of a He–Cd- (442 nm) [8] or an Ar^+ laser operating at 457.9 nm [19] for efficient excitation. Problems with OPA derivatization include the production of relatively unstable derivatives [20], which may prove problematic for pre-column labeling strategies. Also, OPA excitation requires a source of UV light. Since laser excitation must be used to obtain optimal sensitivity, the choices are either a He–Cd laser (325 nm) or an expensive, water-cooled Ar^+ laser operating in the UV region.

Conversely, the use of CFSE has no serious toxic component and can thus be regarded as being relatively benign. The derivatization links fluorophore to analyte through an amide bond that is

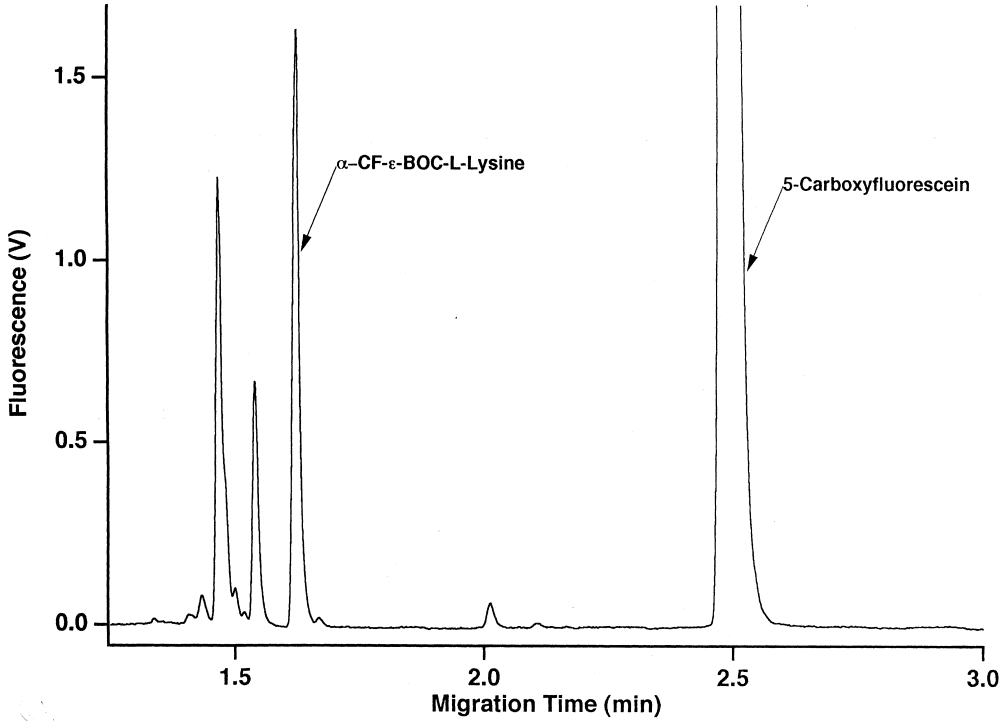

Fig. 3. Electropherogram of a 100-fold dilution of a derivatization reaction between 10^{-7} *M* ε-Boc–L-lysine and 10^{-3} *M* CFSE.

extremely stable [14]. CFSE is fluorescein-based and, thus, an ubiquitous air-cooled Ar^{+} laser operating at 488 nm can be used as an excitation source. Furthermore, Fig. 3 demonstrates that there are few hydrolysis products produced by CFSE derivatization that can seriously interfere with amino acid quantification, if the concentrations of amino acid are kept

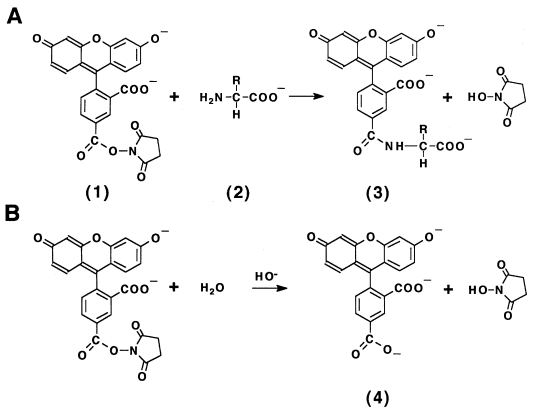

Fig. 4. CFSE reactions: (A) amino acid derivatization: (1) CFSE, (2) generic amino acid, (3) CF–amino acid derivative; (B) CFSE hydrolysis and (4) 5-carboxyfluorescein.

Table 1
Comparison of amino acid detection limits: lowest derivatizable concentrations

Reagent	Concentration[a] (n*M*)	Reference
FITC	1600	[13]
FITC	1000	[c]
Fluorescamine	7200	[13]
OPA	1000	[13]
OPA	0.1	[18]
OPA[b]	0.04–0.4	[18]
FMOC–Cl	200	[13]
NDA	0.4	[8]
CFSE	0.3	[c]

[a] Lowest derivatizable concentrations are based on a signal-to-noise ratio of three, extrapolated from higher concentration derivatizations.
[b] OPA derivatization of peptides from rat brain dialysates.
[c] This study.

above 10^{-7} *M*. Run buffer conditions could be manipulated presumably to alleviate any overlap between analyte peaks and hydrolysis products if the sample is not too complex.

Derivatization of 10^{-7} *M* concentrations of GABA and taurine are evident in the electropherograms presented in Fig. 5. These compounds are important neurotransmitters that are extremely difficult to detect, as neither compound is electroactive nor contains any chromophore. In each case, the derivative signal produced possesses a S/N ratio $>10^3$, which suggests the ability to derivatize nanomolar concentrations, which is necessary for the practical quantification of neurotransmitters collected by microdialysis [21]. A calibration curve for GABA was constructed over the concentration range 10^{-7} to 10^{-9} *M*. The log(peak area) vs. log [GABA] plot gave a straight line slope of 0.99 and a correlation coefficient (r) of 0.99, indicative of a straight line relationship, however, the S/N ratio of the derivative for $1 \cdot 10^{-9}$ *M* GABA was below ten. Thus, quantitative determinations at this concentration would tend to be imprecise [22].

4. Conclusions

It has been demonstrated that CFSE can quantitatively derivatize nanomolar levels of amino acids, which is a concentration that is three orders of magnitude lower than that obtainable with FITC. This ability is due to the reactivity of the succinimidyl ester moiety with primary amines. Presumably, this reactivity is sustained when linked with other fluorescent probes that are commercially available, such as 5-carboxytetramethylrhodamine succinimidyl ester, the family of BODIPY dyes with succinimidyl ester moieties and a number of others available from Molecular Probes. Derivatization of neurotransmitters such as GABA and taurine at nanomolar levels holds promise for their analysis in

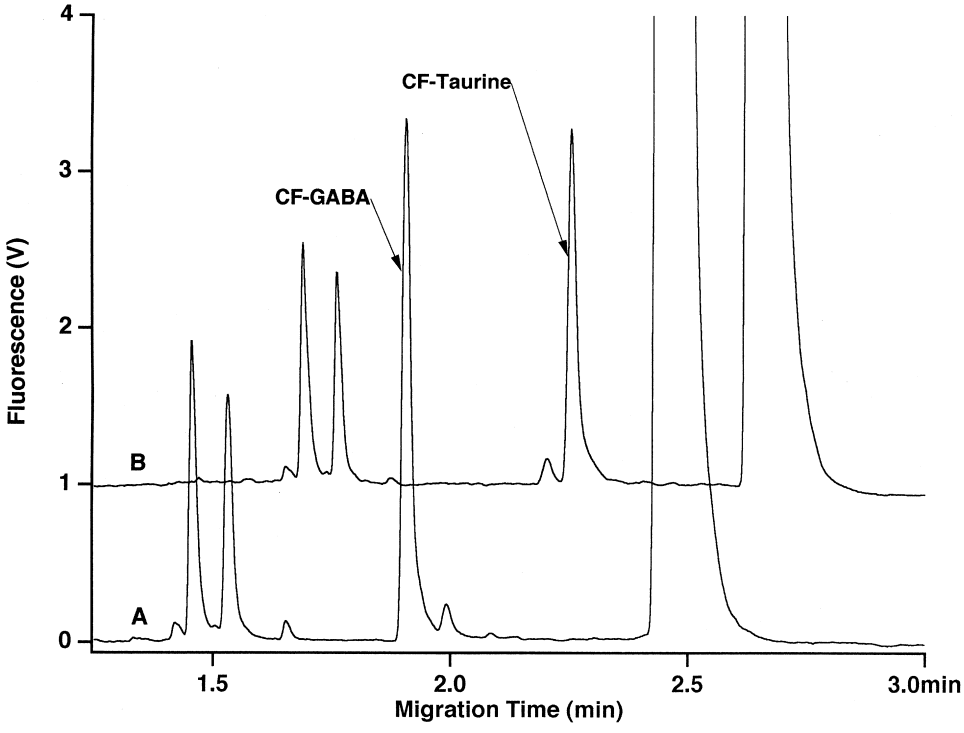

Fig. 5. Electropherograms of 100-fold dilutions of derivatization reactions between (A) 10^{-7} *M* GABA; (B) 10^{-7} *M* taurine and 10^{-3} *M* CFSE. (B) is offset in both fluorescence and time axes.

microdialysate samples. Our group will investigate this application in the near future.

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