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Journal of Chromatography A, 972 (2002) 289–293

JOURNAL OF CHROMATOGRAPHY A

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Short communication

Capillary electrophoresis of baclofen with argon-ion laser-induced fluorescence detection

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Received 8 May 2002; received in revised form 16 July 2002; accepted 16 July 2002

Abstract

A capillary electrophoretic method with laser-induced fluorescence detection for baclofen (4-amino-3-*p*-chlorophenylbutyric acid) has been developed. 6-Carboxyfluorescein succinimidyl ester was used for precolumn derivatization of the non-fluorescent drug. Optimal separation and detection were obtained with an electrophoretic buffer of 50 m*M* sodium borate (pH 9.5) and an air-cooled argon-ion laser (excitation at 488 nm, emission at 520 nm). Linearity ($r \geq 0.99$) over three orders of magnitude was generally obtained and the lowest derivatizable concentration limit for baclofen in aqueous solution was 10 n*M* (2 ng baclofen/ml). Coupled with a simple clean up procedure, the method can be applied to the analysis of baclofen in human plasma at micromolar level. Recovery of spiked baclofen in plasma was 95%. The relative standard deviation values on peak size (0.5 μ*M* level) and migration time were 8.2 and 1.0% ($n=7$), respectively. The limit of detection of baclofen in plasma was 0.1 μ*M* (21 ng/ml).

Keywords: Pharmaceutical analysis; Baclofen; Carboxyfluorescein succinimidyl ester

1. Introduction

Baclofen (4-amino-3-*p*-chlorophenylbutyric acid), a skeletal muscle relaxant widely used in the treatment of spastic disorders [1], is a *p*-chlorophenyl analogue of γ-aminobutyric acid (GABA). Unlike the natural amino acid, baclofen is capable of passing the blood–brain barrier. For the achievement of an optimal pharmacotherapy with baclofen, a sensitive and selective analytical procedure for assay of the drug concentration in the body fluids is required.

Many gas chromatographic (GC) and high-performance liquid chromatographic (HPLC) methods have been reported for the determination of baclofen in biological fluids [2–12]. Recently, a capillary electrophoretic (CE) method coupled with laser-induced fluorometry (LIF) also has been developed by us for the analysis of baclofen [13,14]. Naphthalene-2,3-dicarboxaldehyde (NDA) was used as the fluorescent label and a He–Cd laser with a 442-nm line was used as the excitation source.

In this paper we describe an alternate CE–LIF method for baclofen using 6-carboxyfluorescein succinimidyl ester (CFSE) as the fluorescent labeling reagent and an air-cooled argon-ion laser as the excitation source. CFSE is an important dye for oligonucleotide labeling [15–17] and rapid DNA sequencing [18–20] applications. CFSE also has

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)01083-X

been shown to be far more superior to fluorescein isothiocyanate (FITC), the most popular amine-reactive fluorescent probe, for derivatizing nanomolar concentrations of amino acids [21,22]. To our knowledge, CFSE has not been employed in the derivatization of baclofen yet.

2. Experimental

2.1. Apparatus

The CE–LIF system was assembled in the laboratory and has been described previously [13]. Briefly, a high-voltage power supply (Glassman High Voltage, Whitehouse Station, NJ, USA) was used to provide the separation voltage. The electrophoretic capillary (Polymicro Technologies, Phoenix, AZ, USA) was 50 cm (45 cm to the window)×50 μm I.D.×360 μm O.D. Samples were injected by hydrodynamic injection. LIF detection was performed using the 488-nm line from a 20 mW air-cooled argon ion laser (Uniphase, San Jose, CA, USA). The laser beam was focused onto the fused-silica capillary with a 1-cm focal length lens. A linear-graded neutral density filter (Edmund Scientific, Barrington, NJ, USA) was used to select optimal excitation laser powers. The fluorescence emission was collected with a 10× microscope objective and passed through a 520-nm interference filter (effective bandwidth 10 nm, Edmund Scientific). The fluorescent image was focused onto a photomultiplier tube (PMT, Model R928, Hamamatsu, Hamamatsu City, Japan). The PMT current was amplified by a picoammeter (Keithley Instruments, Cleveland, OH, USA), converted to voltage and recorded using a Macintosh computer equipped with a MacLab/4 data acquisition interface (Analog Digital Instruments, NSW, Australia). The whole CE–LIF system was held in a large light-tight box constructed from black Plexiglas to exclude stray light.

2.2. Chemicals

Baclofen was obtained from Sigma (St. Louis, MO, USA). A stock solution of 10 m*M* baclofen was prepared in sodium borate buffer (50 m*M*, pH 9.5). CFSE was purchased from Molecular Probes (Eugene, OR, USA). The CFSE reagent for derivatization of baclofen was prepared in dimethylformamide (DMF) and was used immediately to avoid possible degradation. Phenylpropanolamine (PPA) was obtained from Sigma and was used as an internal standard (I.S.). All other chemicals were of analytical-reagent grade. Distilled water was further purified by passing it through a NANOpure II deionization system (Barnstead/Thermolyne, Dubuque, IA, USA). All solutions were filtered through a 0.45-μm pore-size membrane filter before use.

2.3. CFSE derivatization procedure

An aqueous standard mixture (or human plasma) containing 1~10 μ*M* baclofen, 1~10 μ*M* PPA (I.S.) and 1 m*M* CFSE was prepared in a 50 m*M* borate buffer (pH 9.5). The final volume of the mixture was 100 μl. The resulting solution was shaken for 15 min using a Vortex shaker, followed by standing at room temperature for 45 min in the dark. The solution was filtered through a 0.45-μm pore-size membrane filter prior to injection.

2.4. Preparation of plasma sample

Blood samples placed in freeze–dried EDTA tubes were centrifuged immediately to obtain the plasma, which was stored −20 °C until analysis. A 200-μl plasma sample was deproteinized by adding 200 μl of acetonitrile. This 1:1 (v/v) ratio of acetonitrile–plasma was sufficient for protein precipitation. After centrifugation at 8500 *g* for 15 min, 40 μl of the supernatant liquid was spiked with internal standard and reacted with CFSE following the procedure described above. The final sample volume was 100 μl.

3. Results and discussion

In our previous research, NDA was used as a fluorescent probe for baclofen [13,14]. Although NDA provides good sensitivity and high stability for primary amines, its main drawback is the need to use concentrated CN^- solution to provide the necessary nucleophile for NDA derivatization. Furthermore,

NDA derivatives require the use of a He–Cd laser (442 nm) for efficient excitation, which is not a ubiquitous laser source.

Among other amine-reactive fluorescent derivatizing agents, fluorescein derivatives are the most widely used. In addition to their high absorptivity, near unity fluorescence quantum yield and good water solubility, fluoresceins (including CFSE) have an excitation maximum (ϵ=88 000 cm^{-1} M^{-1} at 490 nm, pH>6 [23]) that closely matches the 488 nm spectral line of the easily obtainable argon-ion laser. Besides, the use of CFSE involves no serious toxic component and can be regarded as being relatively benign. Fig. 1A shows the electropherogram of 0.5 μ*M* baclofen (peak ''B'') derivatized by CFSE in 50 m*M* sodium borate solution (pH 9.5). PPA was included in the sample as an internal standard (peak ''I.S.'') for improvement of precision and accuracy. An air-cooled argon-ion laser with 2-mW power at 488-nm line was used for LIF detection. In comparison with the blank electropherogram (Fig. 1B), there are few hydrolysis products generated by CFSE derivatization that may interfere with baclofen quantification. In order to ensure a complete derivatization, the concentration ratio of CFSE to baclofen in the reaction mixture was 100:1 or higher. The two large peaks appearing at 5 and 13 min in Fig. 1 were due to hydrolysis products of excess CFSE in the mixture.

The yield of CFSE-baclofen derivative was found to be pH dependent, as shown in Fig. 2. In a 50 m*M* borate buffer, the optimal pH for derivatization was pH 9.5. While the FITC derivatization reaction was usually carried out overnight for completeness, the CFSE derivatization was relatively fast (1 h). We found that the peak size of both baclofen and PPA (i. s.) did not change significantly after 60 min derivatization time (data not shown). The CFSE-baclofen derivative was also found stable for at least 24 h if stored at 4 °C in the dark.

A calibration curve for baclofen in aqueous solution was constructed over the concentration range 0.01 to 10 μ*M*. The ratio of peak area of baclofen/PPA versus baclofen's molar concentration plot showed a good linearity ($y=3.3\cdot10^{5}x-2.5\cdot10^{-2}$) with a correlation coefficient (r) of 0.999 (n=7). At a concentration level of 0.5 μ*M* baclofen, the relative standard deviation (RSD) values on peak area and migration time were 5.3 and 0.6% (n=7), respectively. Based on a signal-to-noise (S/N) ratio of 3, the lowest derivatizable concentration limit for baclofen in aqueous solution was calculated to be 10 n*M* (or 2 ng baclofen/ml). This value is similar to that obtained in our previous work with NDA derivatization and LIF detection using a He–Cd laser [13].

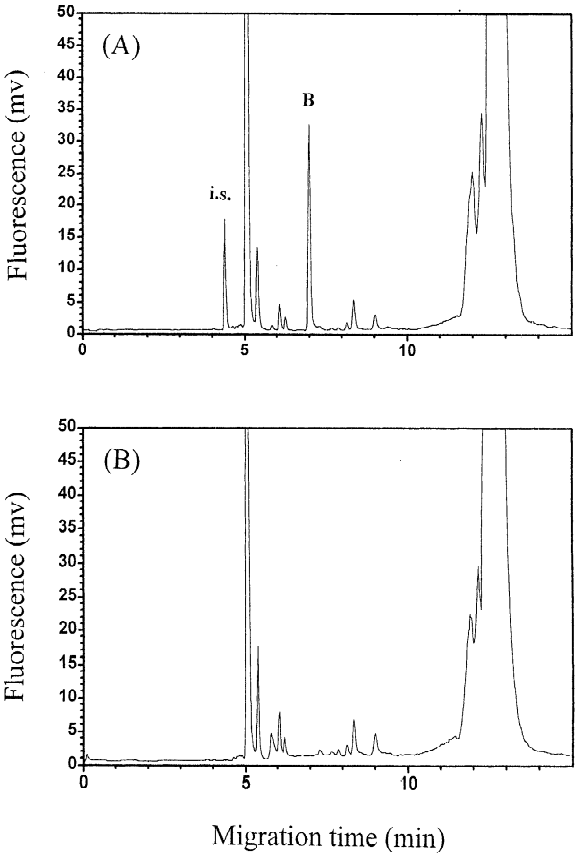

Fig. 1. Electropherograms of CFSE-derivatized (A) baclofen and (B) blank with LIF detection. Peak identities: B=baclofen (0.5 μ*M*); I.S.=internal standard (0.5 μ*M*). Separation capillary, 50 cm (45 cm to the window)×50 μm I.D.×360 μm O.D.; electrophoretic buffer, 50 m*M* sodium borate (pH 9.5); separation voltage, 21 kV; hydrodynamic injection, 3 s at 25 cm height; laser power, 2 mW.

To evaluate the applicability of the method for biological and clinical analyses, baclofen-spiked human plasma was used as the test sample. Preparation of plasma sample was described in the Experimental section. Typical electropherograms obtained from spiked and baclofen-free plasma samples

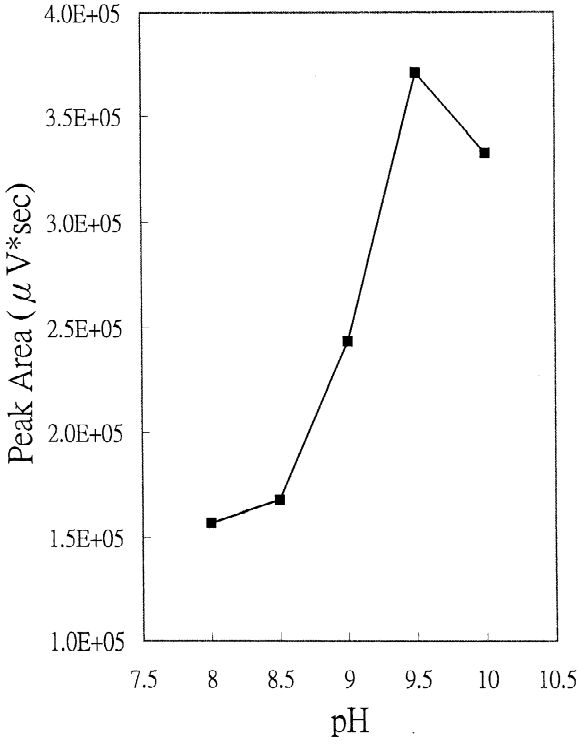

Fig. 2. Effect of pH on the yield of CFSE-derivatized baclofen. 50 m*M* sodium borate containing 1 μ*M* baclofen and 0.1 m*M* CFSE; derivatization time, 1 h; CE–LIF conditions as in Fig. 1.

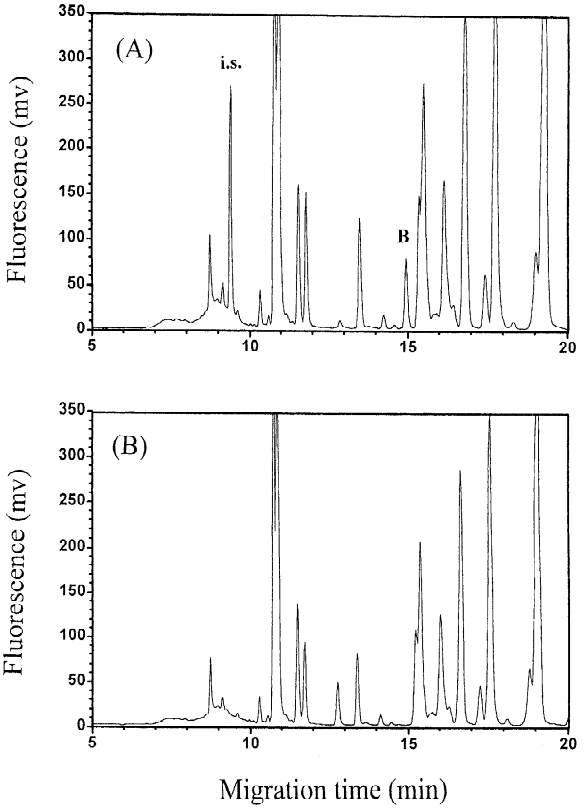

Fig. 3. Electropherograms of plasma samples. (A) Plasma spiked with 2 μ*M* baclofen; (B) plasma blank. Separation voltage, 15 kV; other conditions as in Fig. 1.

are shown in Fig. 3. Spiked baclofen (peak ''B'') and internal standard (peak ''I.S.'') can be clearly identified in Fig. 3A. In the blank, both baclofen and internal standard were not included. The blank plasma (Fig. 3B) shows no interferences at the migration times of the CFSE-baclofen derivative and the I.S., although the electropherogram contains many extraneous peaks, probably from the endogenous plasma components. In comparison with Fig. 1, the migration times of baclofen and I.S. in Fig. 3 shift significantly. In Fig. 1, a separation voltage of 21 kV was used for the aqueous baclofen standard. However, this condition was not optimal for the resolution of many extraneous peaks in plasma sample. Therefore, a separation voltage of 15 kV was used in Fig. 3 for better resolution.

The RSD values on peak area and migration time of a plasma sample containing 0.5 μ*M* baclofen were 8.2 and 1.0% ($n=7$), respectively. Recovery of baclofen from plasma was determined by spiking 0.5 ml of plasma with 80 ng (i.e., 0.75 μ*M*) of baclofen standard prior to deproteinization, followed by derivatization and CE–LIF analysis. Based on triplicate measurements, the mean recovery was found to be 95%. Since baclofen is not the only species that may react with CFSE during derivatization, the fraction of baclofen as CFSE-labeled form may be lower in the presence of other endogenous plasma components. We have examined several concentrations (0.5~3 m*M*) of CFSE in the solution mixture during plasma derivatization, and 1 m*M* CFSE was found to be the optimum. Even if this concentration level were not sufficiently excess to label all baclofen in plasma, the error would be minimal because an internal standard was also included in the plasma during derivatization.

Due to matrix effect, the lowest derivatizable concentration limit for baclofen in plasma was found

to be 0.1 μ*M* (or 21 ng baclofen/ml), about 10 times higher than that in aqueous solution. Attempts to enhance the analyte signal by further increasing the concentration of CFSE during derivatization was unsuccessful because the background signals due to the plasma matrix also increased significantly. A more complete sample preparation procedure for plasma is probably required before nanomolar detection sensitivity can be achieved.

Acknowledgements

Financial support from the National Science Council of Taiwan is gratefully acknowledged.

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