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Journal of Chromatography A, 877 (2000) 233–237

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Short communication

Analysis of baclofen by capillary electrophoresis with laser-induced fluorescence detection

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Received 9 December 1999; received in revised form 26 January 2000; accepted 2 February 2000

Abstract

A new analytical method for baclofen (4-amino-3-*p*-chlorophenylbutyric acid) based on capillary electrophoretic separation and laser-induced fluorescence detection has been developed. Naphthalene-2,3-dicarboxaldehyde 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 a He–Cd laser (excitation at 442 nm, emission at 500 nm). Linearity ($r \geq 0.99$) over three orders of magnitude was generally obtained and the concentration limit of detection was in the nanomolar level. Coupled with a simple cleanup procedure, the method was successfully applied to the analysis of baclofen in human plasma. Recovery of spiked baclofen in plasma was 98%. The relative standard deviation values on peak size and migration time were 7.9% and 0.4%, respectively. The limit of detection of baclofen in plasma was 10 ng/ml.

Keywords: Pharmaceutical analysis; Baclofen; Naphthalenedicarboxaldehyde; Aminochlorophenylbutyric acid

1. Introduction

Baclofen (4-amino-3-*p*-chlorophenylbutyric acid) is a *p*-chlorophenyl analogue of γ-aminobutyric acid (GABA) which, unlike the natural amino acid, is capable of passing the blood–brain barrier. Clinically baclofen is widely used as a skeletal muscle relaxant in the treatment of spastic disorders [1,2]. For the achievement of an optimal pharmacotherapy with baclofen, sensitive and selective analytical procedure for assay of the drug concentration in the body fluids is required.

Both gas chromatographic (GC) and high-performance liquid chromatographic (HPLC) methods for the determination of baclofen in biological fluids have been developed. Degen and Riess [2] reported a GC with electron-capture detection method for baclofen involving extraction and derivatization. GC in combination with mass spectrometry (MS) for the assay of baclofen in cerebrospinal fluid and serum was described by Swahn et al. [3]. Sioufi et al. [4] employed chiral capillary column GC to the separation of baclofen enantiomers in human plasma and urine. Harrison et al. [5] and Wuis et al. [6] determined baclofen in biological fluids using HPLC with UV detection. HPLC–fluorescence detection of baclofen in plasma and urine after precolumn derivatization with *o*-phthaldialdehyde [7] and 4-chloro-7-nitrobenzofurazan [8] have been reported. Recently, Flärdh et al. [9] described an HPLC–tandem

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0021-9673/00/$ – see front matter
PII: S0021-9673(00)00168-0

MS method for the determination of baclofen in plasma. Chiral separation of baclofen enantiomers by HPLC using cyclodextrin as a mobile phase additive also has been reported [10,11].

In recent years, capillary electrophoresis (CE) has been an important separation method as a result of its high resolving power and speed. Fluorescence detection, particularly the laser-induced fluorometry (LIF), also has become popular in CE mainly because of its extremely high sensitivity. Despite their widespread applicability to biological and clinical analyses, CE coupled with LIF detection has not been applied to the analysis of baclofen yet. In this paper, we describe a novel and sensitive CE–LIF method for the analysis of baclofen. Naphthalene-2,3-dicarboxaldehyde (NDA) was used for pre-column derivatization of the non-fluorescent drug. Linearity, repeatability and detection limit were examined. The applicability of the method to the analysis of baclofen in human plasma was also demonstrated.

2. Experimental

2.1. Apparatus

The CE system was assembled in the laboratory. A high-voltage power supply (Model PS/MJ30P0400-11; Glassman High Voltage, Whitehouse Station, NJ, USA) was used to provide the separation voltage. The electrophoretic capillary was 50 cm total length×50 μm I.D.×360 μm O.D. The polymer coating was burned off 5 cm from the cathodic end of the capillary to form a detection window. Samples were injected by electromigration.

LIF detection in CE experiments was performed using the 442 nm line from a He–Cd laser (Model IK 4153R-C; Kimmon Electric, Tokyo, Japan). An interference filter centered at 442 nm with a 10 nm effective bandwidth (Edmund Scientific, Barrington, NJ, USA) was used to reject plasma discharge emission light. 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) was used to select optimal excitation laser powers. The fluorescence emission was collected with a 10× microscope objective and passed through a 500 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 (Model 485; Keithley Instruments, Cleveland, OH, USA), converted to voltage and recorded using an integrator (Chromatocorder 21; System Instruments, Tokyo, Japan). A *RC* low-pass filter with a 1 s time constant was connected between the picoammeter and the integrator to reduce the background noise. The whole CE–LIF detection 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). NDA was purchased from Fluka (Buchs, Switzerland). Potassium cyanide was obtained from E. Merck (Darmstadt, Germany). The CE buffer was 50 m*M* aqueous sodium tetraborate (pH 9.5). 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. NDA derivatization procedure

Aliquots of the baclofen standards (in borate buffer, pH 9.5) or human plasma were mixed with a 100-fold excess of cyanide. A 100-fold excess of NDA was then added and thoroughly mixed. The resulting solution was allowed to stand at room temperature for 30 min before diluting with 50 m*M* borate buffer to achieve the final desired concentration. The diluted 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 0.5 ml plasma sample was deproteinized by adding 0.5 ml of acetonitrile. After centrifugation at 9500 *g* for 10

min, 0.1 ml of the supernatant liquid was reacted with CN^- and NDA in a borate buffer at pH 9.5 following the procedure described above. The final sample volume was 0.5 ml. The recovery of baclofen from plasma was examined by spiking the plasma sample with baclofen standard before deproteinization.

3. Results and discussion

NDA reacts with primary amines in the presence of CN^- to form 1-cyanobenz[*f*]isoindole derivatives, which possess excellent stability and high quantum efficiency, even in aqueous buffer solution [12]. Another advantage of these compounds is that the excitation maxima coincide closely with the 442 nm output wavelength of the He–Cd laser. Results from our preliminary work also indicated that NDA reacted rapidly with baclofen in basic media to form a fluorescent adduct. The fluorescence spectral properties of the NDA–baclofen derivative ($\lambda_{ex\ max}$=443 nm, $\lambda_{em\ max}$=490 nm) make it amenable to LIF detection with high sensitivity.

Fig. 1A shows the electropherogram of 300 n*M* NDA-labeled baclofen obtained with LIF detection. The electrophoretic buffer contained 50 m*M* sodium borate at pH 9.5 and the laser power at 442 nm was 1 mW. A single peak appears at 8.1 min. Its full width at half height is about 4 s, corresponding to *ca.* 82 000 theoretical plates. A few extraneous peaks also appear in Fig. 1A, which may be due to the trace impurities in the derivatizing agent and/or the background buffer, as evidenced by the blank electropherogram (Fig. 1B). The peak shape of NDA-labeled baclofen is slightly tailed, probably caused by its interaction with the capillary wall.

A calibration curve for baclofen was constructed over the concentration range 10^{-8} to 10^{-5}*M*. The peak area versus concentration plot showed a good linearity ($y = 1.1 \cdot 10^7 x - 2.1$) with a correlation coefficient (r) of 0.999 (n=10). At a concentration level of 0.5 μ*M* baclofen, the relative standard deviation (RSD) values on peak area, peak height and migration time were 5.1%, 4.7% and 0.6% (n=7), respectively. The electropherogram of a 10 n*M* NDA-labeled baclofen is illustrated in Fig. 1C. At a signal-to-noise (S/N) ratio of 3, the concentration limit of detection for NDA–baclofen derivative in aqueous solution was calculated to be 5 n*M* (or 1.1 ng/ml). With an injection volume of 5.5 nl, this value corresponds to 27 amol (or 6 fg) of baclofen, which is the smallest detection limit of baclofen ever reported.

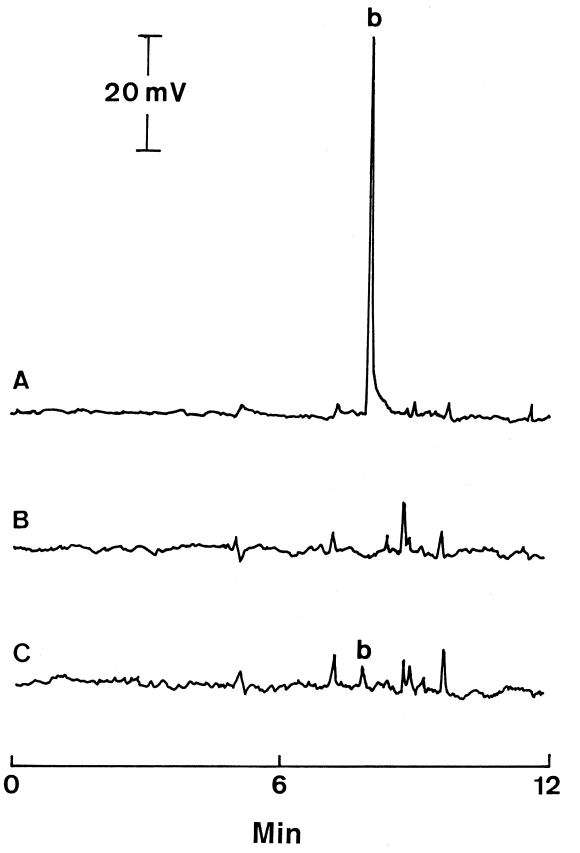

Fig. 1. Electropherograms of NDA-labeled baclofen with LIF detection. (A) 300 n*M* baclofen; (B) blank; (C) 10 n*M* baclofen. b=baclofen. Separation capillary, 50 cm total length (45 cm to the window)×50 μm I.D.×360 μm O.D.; electrophoretic buffer, 50 m*M* sodium borate (pH 9.5); separation voltage, 12 kV (55 μA); electrokinetic injection, 3 s at 12 kV; laser power, 1 mW, except 2 mW in (C).

To evaluate the applicability of the method for biological and clinical analyses, baclofen-spiked human plasma was chosen as the test sample. Analysis of baclofen in body fluids is often problematic due to its amino acid structure. Since NDA can react with primary amines and amino acids as well, the derivatization reaction between baclofen and

NDA may be affected by the endogenous plasma components. For the measurement of baclofen concentrations in the nanogram per milliliter range, as needed for pharmacokinetic studies, removal of the endogenous amino acids is often a necessity. Various solid-phase extraction (SPE) procedures employing C_{18} [4,5,9], silica gel [8], or ion-exchange [3,7] columns have been developed for cleaning the biological fluid samples prior to GC or HPLC analysis. Although SPE is generally effective in reducing the interferences caused by endogenous plasma components, its procedure is always tedious and time-consuming. Besides, low recovery of baclofen in plasma has been attributed to loss of baclofen during SPE treatment [5]. On the other hand, with the high resolving power of CE, we found that sample cleanup by SPE might not be necessary. As described in the Experimental section, the only sample pretreatment we performed was deproteinizing the plasma with acetonitrile, followed by centrifugation. Typical electropherograms obtained from baclofen-free and spiked plasma samples subjected to this simple pretreatment are shown in Fig. 2. The blank plasma (Fig. 2A) shows no interferences at the migration time of the NDA–baclofen derivative, although the electropherogram contains many extraneous peaks. It was not possible to remove these endogenous plasma components by a single deproteinization pretreatment. Similar problem was also found even after laborious sample cleanup with SPE [4–9].

By keeping the derivatizing agents (CN^- and NDA) in large (100-fold) excess in the reaction mixture and injecting the sample at a fixed time, in the present case 30 min after starting the derivatization reaction, the RSD values on peak area,

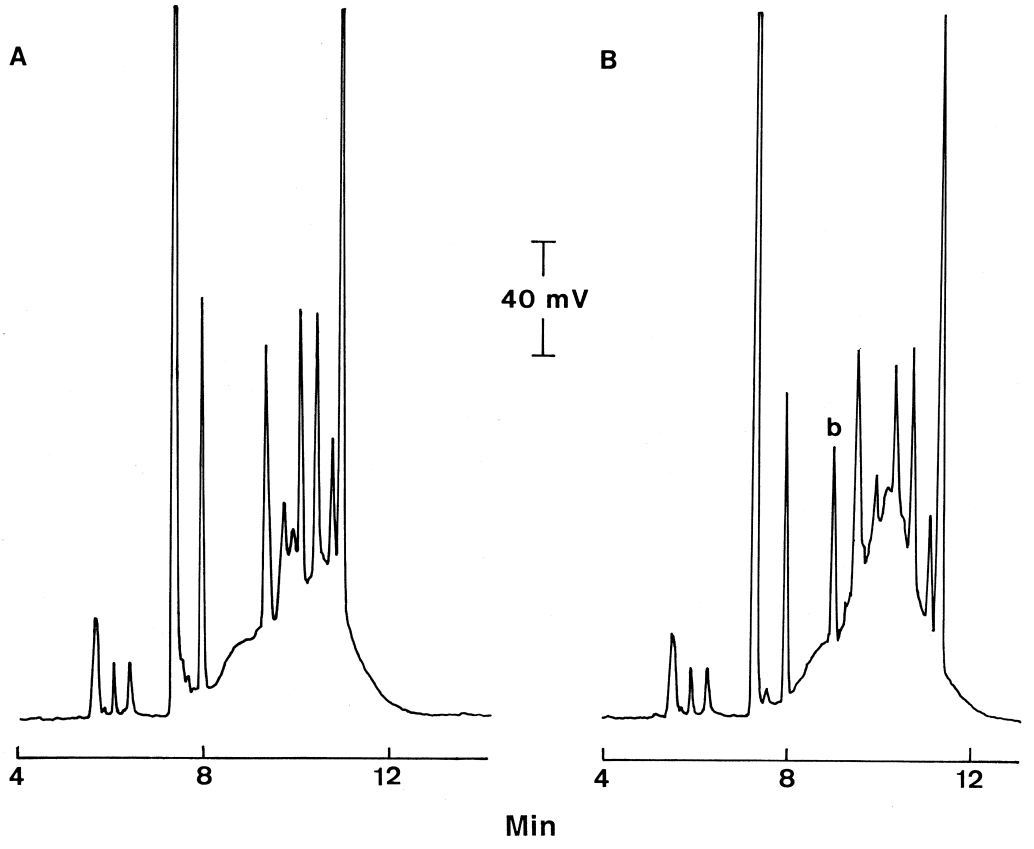

Fig. 2. Electropherograms of plasma samples. (A) Plasma blank; (B) plasma spiked with 200 ng/ml baclofen. b = baclofen. Conditions as in Fig. 1.

peak height and migration time of a plasma containing 0.5 μ*M* (107 ng/ml) baclofen were 9.3%, 7.9% and 0.4% ($n=7$), respectively. A calibration graph was prepared for the baclofen in plasma in the concentration range 0.1−2.0 μ*M*. The linear equation was $y=3.4\cdot10^{6}x+3.0\cdot10^{3}$ with a $r=0.997$ ($n=5$). The slope of this calibration line is about three times smaller than that obtained with standard solutions, which indicates the presence of matrix effect in plasma samples. Since baclofen is not the only species which may react with NDA during derivatization, the fraction of baclofen as NDA-derivatized form may be lower in the presence of other endogenous plasma components. Attempts to increase the fraction of NDA-derivatized baclofen by increasing the concentrations (200-fold excess) of derivatizing agents was unsuccessful because the background signals due to the plasma matrix also increased significantly. The recovery of baclofen from plasma was determined by spiking 0.5 ml of plasma with 50 ng of baclofen standard prior to deproteinization. Quantitation was performed with the method of standards addition. Based on triplicate measurements, the mean recovery was found to be 98%. The detection limit of baclofen in plasma was estimated to be about 10 ng/ml ($S/N=3$).

4. Conclusion

A new analytical method for baclofen based on CE separation and LIF detection was developed. NDA was used to derivatize the non-fluorescent baclofen at low concentrations. This method has high sensitivity and good reproducibility. Coupled with a simple deproteinization pretreatment, this CE−LIF method can be applied to the analysis of baclofen at sub-micromolar level in human plasma.

Acknowledgements

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

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