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Development and Validation of a Capillary Electrophoresis Method for the Determination of Baclofen in Human Plasma

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Abstract: A capillary electrophoretic method (CE) with ultraviolet absorption detection for baclofen in human plasma was developed. The separation was obtained by CE with 50 mM borate buffer at pH 9, followed by detection with an ultraviolet detector at 225 nm. The plasma samples were deproteinized with acetonitrile to remove proteins and to induce sample stacking. With stacking, the limit of detection (LOD) for baclofen in plasma was 0.2 μM. A calibration curve ranging from 1.5 to 300 μM showed to be linear. Both the within-day and day-to-day reproducibility and accuracy were less than 15% and 8%, respectively. The recoveries of baclofen in human plasma were 99.6 – 103.3%.

Keywords: capillary electrophoresis; baclofen; acetonitrile stacking; human plasmas.

1. Introduction

Baclofen(4-amino-3-*p*-chlorophenylbutyric acid) is a *p*-chlorophenyl analogue of γ-aminobutyric acid (GABA) and is widely used as a skeletal muscle relaxant in the treatment of spastic disorders [1]. To obtain the maximum effect and to carry out pharmacokinetic studies, sensitive and selective analytical procedure for assaying the drug concentration in the body fluids is required.

Different methods have been proposed to analyze baclofen in biological fluids. In GC analysis [2, 3], sample derivatization is necessary to enhance the volatility of analyte. GC in combination with mass spectrometry for the assay of baclofen in cerebrospinal fluid and serum was described [4]. LC methods have been used to achieve the separation, followed by detection with an ultraviolet (UV) absorption detector [5-9]. HPLC-fluorescence detection of baclofen in plasma and urine after pre-column derivatization with *o*-Phthaldialdehyde (OPA) [10, 11] and 4-chloro-7-nitrobenzofurazan [12] have been reported. In addition, LC-MS with sample extraction was reported for the determination of balofen in plasma [13, 14]. In CE, naphthalene-2, 3-dicarboxaldehyde (NDA) [15, 16], anthracene-2, 3-dicarboxaldehyde (ADA) [17] and 6-Carboxyfluorescein succinimidyl ester (CFSE) [18] have used as a fluorescent label for baclofen in plasma samples. The derivatized baclofen was separated by CE, followed by detection with laser-induced fluorescence

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(LIF) detector. Chiral separation of baclofen enantiomers by CE also has been reported [19-21].

CE is useful method to quantify drug in biological samples due to its high resolving power, speed while only small sample volume is required. However, low sensitivity has hampered its use in clinical drug monitoring. Recently, several CE/LIF methods with better sensitivity were reported [15-18]. A derivatization step is required for non-fluorescent analytes. Compared with the expensive LIF detector, UV detector is the most common detector for CE. Until now, there is no CE/UV method for the analysis of baclofen. In this study, we developed a CE/UV method for the determination of baclofen in human plasma. The sensitivity could be improved by using acetonitrile stacking. Linearity, reproducibility and detection limits of the developed method were examined.

2. Materials and methods

2.1. Chemicals

Baclofen was purchased from Sigma (St. Louis, MO, U.S.A.). Sodium borate was from Showa (Tokyo, Japan). HCl and acetonitrile were obtained from Merck (Darmstadt, Germany). Water purified with a Barnstead NANOpure system (Dubuque, IA, USA) was used for all solutions. The CE buffer was 50 mM sodium borate at pH 9.0. All solutions were filtered through a 0.45 μm pore-size membrane filter before use.

2.2. CE system

The CE system was assembled in-house. A 0-30 kV power supply (GAMMA high voltage research Inc., Ormond Beach, FL, USA) provided the separation voltage. The capillary used for separation was 50 μm ID x 360 μm OD x 57 cm total length (Polymicro Technologies, Phoenix, AZ, USA). The effective length of capillary is 45 cm. UV absorbance detection was performed at 225 nm with a commercial UV absorbance detector (Model number: SSI 500; Thermo Separation Products, San Jose, CA, USA) equipped with a CE flow cell module. Recording of electropherograms and quantitative measurements of peak area were performed with a computer connected to a SISC data acquisition interface (Scientific Information Service Corporation, Taipei, Taiwan).

2.3. Preparation of plasma sample

Blood samples placed on freeze-dried EDTA tubes were immediately centrifuged to obtain the plasma, which was stored –20 ^{o}C until analysis. A 0.5 mL plasma sample was deproteinized by adding 1 mL of acetonitrile. After centrifugation at 6000 x g for 15 min, 90 μL of the supernatant liquid was spiked with 10 μL of baclofen aqueous solution. Plasma samples of various baclofen contents were similarly prepared by spiking the plasma with the different concentration of baclofen aqueous solution (1000、500、100、50、10、5、1μM). The recovery of baclofen in plasma was estimated by spiking 20 μL of $1.0x10^{-3}$ and $1.0x10^{-4}$ M baclofen to 480 μL of plasma sample before deproteinization.

2.4. CE procedure

The CE buffer containing 50 mM sodium borate was prepared with deionized water. The pH of the CE buffer was adjusted to 9.0 by addition of HCl. The capillary was rinsed daily with water for 10 min, followed by a 10 min rinse with CE buffer. The capillary was equilibrated with the CE buffer under electric field of 175 V/cm for 30 min. Samples were injected at the anodic end of the capillary by hydrostatic injection. The sample was injected by raising the anodic end 18 cm above its normal position for 10 – 200 s. After each run, the capillary was washed with water for 5 min, followed by a 5 min rinse with CE buffer. The

capillary was then equilibrated under electric field for 5 min before sample injection.

3. Results and discussion

3.1. Separation of baclofen in plasma sample

Analysis of baclofen in plasma is often problematic due to the complicated matrix.

In order to remove endogenous components in plasma, various solid-phase extraction (SPE) procedures [9, 12, 13, 22] have been developed for cleaning the plasma sample prior to GC or LC analysis. However, the procedure is always tedious and time-consuming. In addition, low recovery of baclofen in plasma has been attributed to loss of baclofen during SPE treatment. On the other hand, with the high resolving power of CE, laborious sample cleanup might not be necessary. In our previous paper [15-17], we have described a simple cleanup procedure for analysis of baclofen in plasma samples. The only pretreatment we performed was to deproteinize the plasma with the addition of acetonitrile, followed by centrifugation. Then the pretreated plasma sample was injected into CE. The applied CE voltage affected the separation of baclofen in plasma. The optimal applied voltage is 10 kV, which provides separation of baclofen from other components in plasma. Typical electropherograms obtained from baclofen-free and spiked plasma samples are shown in Figure 1. Baclofen appears at 9.7 min. The blank plasma shows no significant interferences at the migration time of the baclofen, although the electropherograms contains other extraneous peaks.

3.2. Sample stacking

UV detector is the most common detector for CE. However, the sensitivity is not good enough due to the short optical pathlength of the capillary. An appropriate preconcentration method is needed to improve the sensitivity of the CE analysis. On-line acetonitrile stacking is the simplest technique to solve this problem [23-26]. In our experiment, acetonitrile is used to remove proteins in plasma samples. In addition, plasma samples contain about 150 mM NaCl [27]. After the deproteinization step, the samples contain both acetonitrile and salt which will induce sample stacking in CE. Larger sample volume was injected into CE. Acetonitrile is a low conductivity solvent, and can bring stacking due to the high field strength [27]. The sodium chloride will migrate rapidly early on ahead of the analytes leaving behind an area of higher field strength. The analyte ions in the high field strength accelerate while those in the low field strength slow down leading to a concentration of the analyte ions [27]. Figure 2 compared the electropherograms of different injection volumes. Figure 3 shows that the peak height increased with an increase of sample volume from 10 – 90 s injection. A further increase in injection time did not increase peak height of baclofen. The peak shape of baclofen deteriorated for 120 and 200 s injection time. With 90 s injection time, a factor of 13 in signal enhancement was obtained. The limits of detection (LOD) were estimated based on an S/N ratio of 3 for different injection time (10, 60 and 90 s). Those results were summarized in Table 1. The LODs of 60 s and 90 s injection time were 0.2 μM and 0.2 μM, respectively. The LODs were more than 20 times better than the traditional injection volume. The LOD of 90 s injection time was same to that obtained with 60 s injection time. However, the injection volume was more. This is likely due to the sample loading capacity of the capillary being exceeded at 90 s under the stacking conditions, which is then observed as a loss of separation efficiency and lower plate numbers. Therefore, the optimal injection time is 60 s.

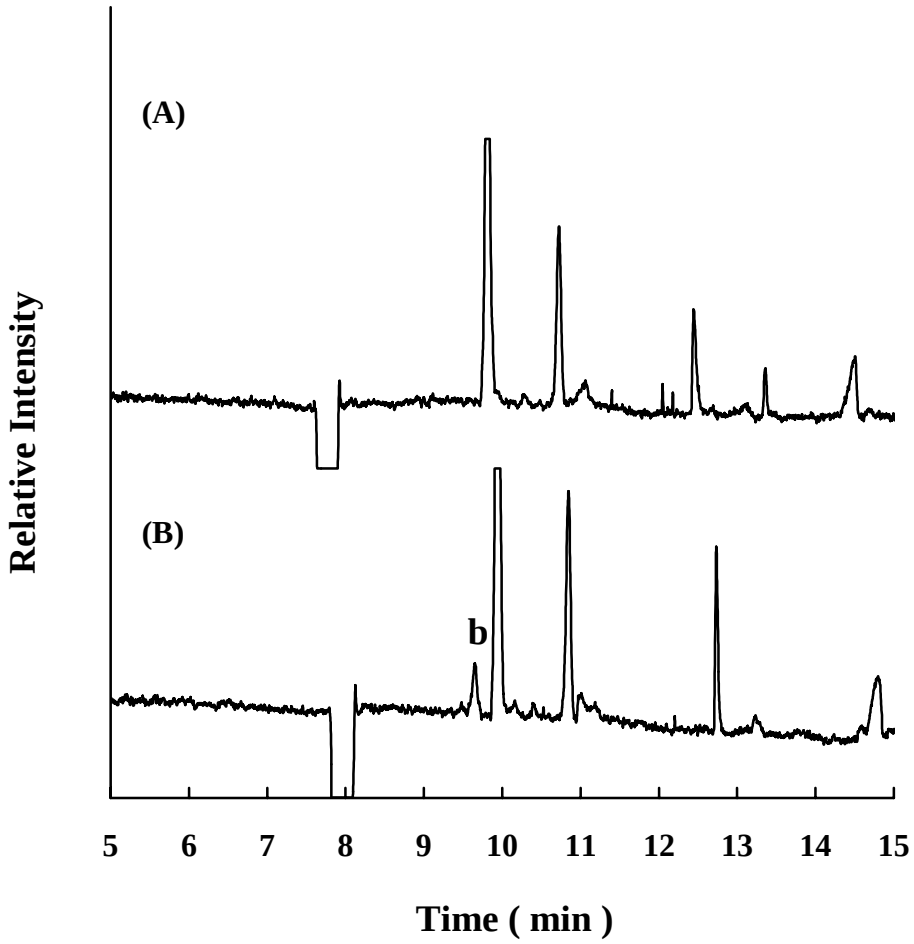

Figure 1. Electropherograms of plasma samples. (A) plasma blank; (B) plasma spiked with 15 μM baclofen. b = baclofen. Separation capillary, 57 cm total length (45 cm to the window) x 50 μm I.D. x 360 μm O. D.; electrophoretic buffer, 50 mM sodium borate, pH 9.0; separation voltage, 10 kV; hydrodynamic injection, 10 s at 18 cm height; UV absorption detection, λ = 225 nm

3.3. Method validation

The developed method was validated by hydrodynamic injection at 18 cm height for 60 s. The electropherogram of 1.5 μM baclofen in plasma was shown in Figure 4. A calibration curve was constructed for the baclofen in plasma in the concentration range 1.5 – 300 μM. The linear equation was y = 2.25 (±0.03) x –2.82 (±4.48) with an r = 0.9996 (n = 6). The results of the assay validation study were summarized in Table 2. The within-day and day-to-day reproducibility expressed as relative standard deviation (RSD) were found to be less than 15 % and 8 %, respectively. The accuracy of the method expressed as relative mean error (RME) was ≤ 5.0 %. According to *Guidance for Industry, Bioanalytical Method Validation* [28], precision should be less than 15 % and accuracy

should be within 85 % and 115 %. In our experiments, the results of precision and accuracy fulfilled the requirements. The recoveries of baclofen from plasma were determined by spiking 480 μL of plasma with 20 μL of $1x10^{-4}$ M and $1x10^{-3}$ M baclofen standard prior to deproteinization. The recoveries of baclofen were in the range 99.6 – 103.3 % (Table 3). At a signal-to-noise (S/N) ratio of 3, the LOD for baclofen in plasma was calculated to be 0.2 μM. With an injection volume of 28.3 nL, this value corresponds to 5.6 fmol of baclofen. Based on a signal-to-noise ratio of 10, the theoretical limit of quantitation (LOQ) was calculated to be 0.8 μM. The effective LOQ of the assay, defined as the lowest quantifiable concentration with the variation of precision and accuracy < 20%, was found to be 1.5 μM. However, the detection limit is not sufficient for the normal drug level encountered in plasma (0.04 – 1.4 μM) following clinical administration of 20 mg of baclofen to a healthy adult [10, 12]. CE analysis coupled with other stacking techniques such as sweeping is probably a solution for improving the detection limit.

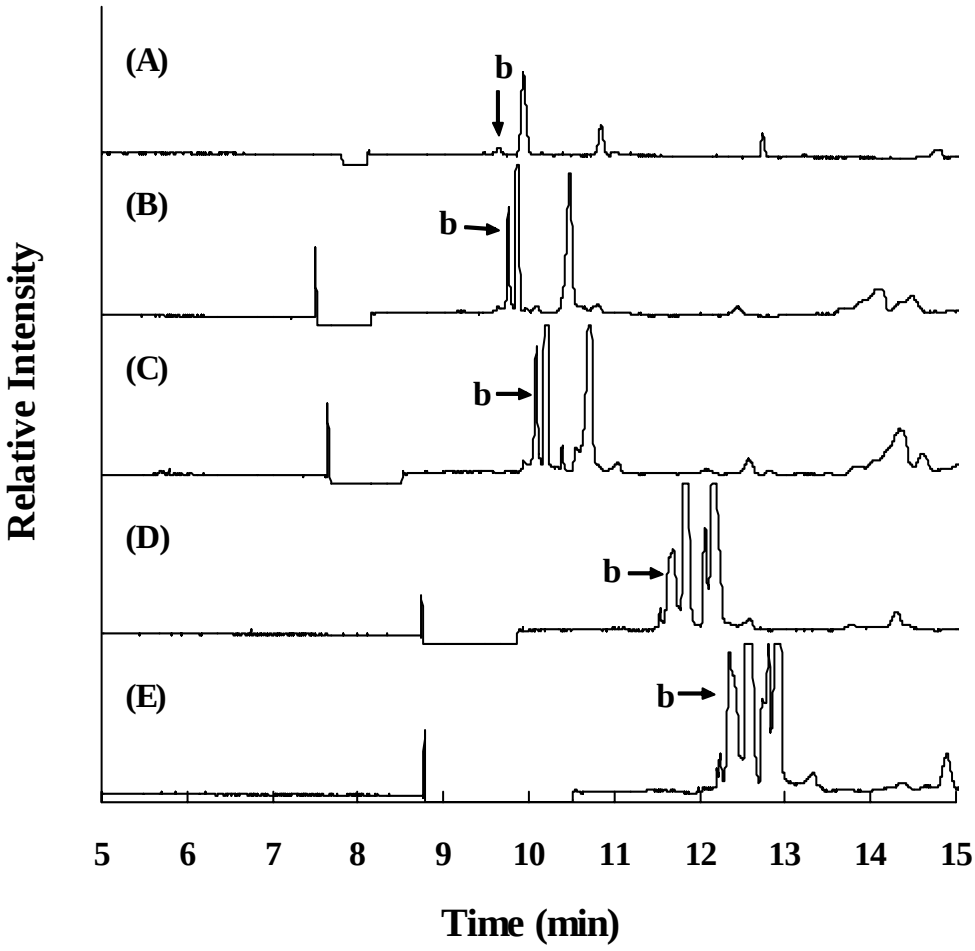

Figure 2. Electropherograms of baclofen (15 μM) in plasma with different injection time. (A) 10 s; (B) 60 s; (C) 90 s; (D) 120 s; (E) 200 s. b = baclofen. Other conditions as in figure 1

Table 1. LODs of baclofen in plasma for different injection time

Injection time	LOD (μM)	Injection volume[a]
10 s	6.4	4.7 nL
60 s	0.2	28.3 nL
90 s	0.2	42.4 nL

a Injection volume = $\frac{\rho g \Delta h d^4 \pi t}{128 \eta L}$; ρ = buffer density; g = gravitational constant; Δh = height differential of the reservoirs; d = capillary inside diameter; t = time; η = buffer viscosity; L = total capillary length

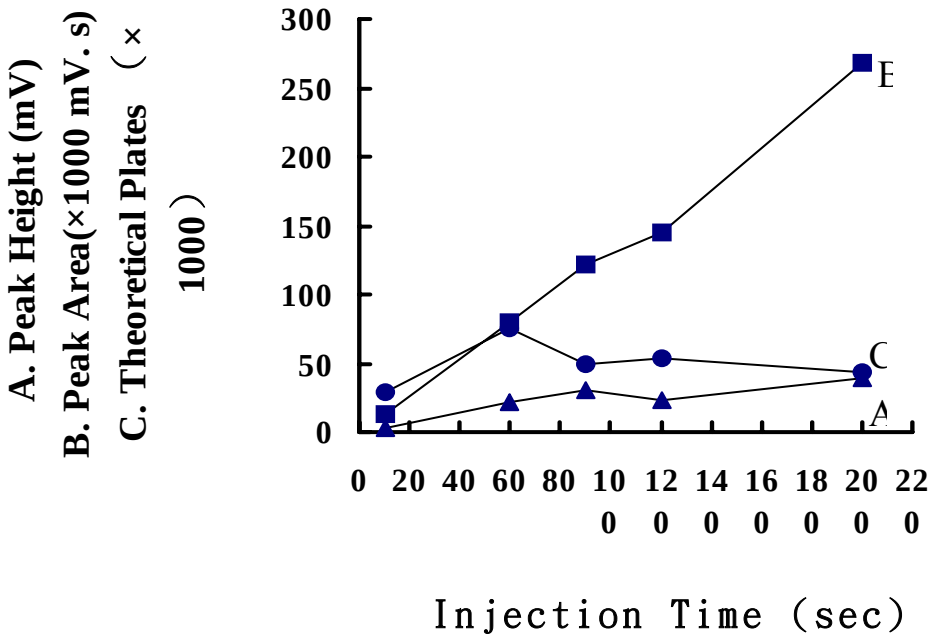

Figure 3. Effect of injection time on peak height, peak area and theoretical plate. Plasma samples spiked with 15 μM baclofen were injected at different periods of time

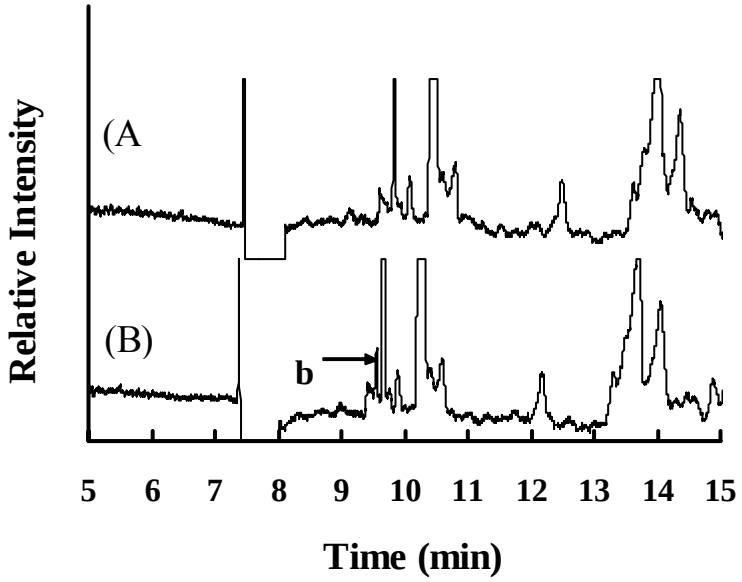

Figure 4. Electropherograms of (A) plasma blank; (B) plasma spiked with 1.5 μM baclofen. b = baclofen. Injection time = 60 s at 18 cm height. Other conditions as in Figure 1

Table 2. Within-day and day-to-day precision and accuracy of baclofen in plasma

Concentration added (μM)	Found (mean ± SD)	RSD (%)	RME (%)
Within-day (n = 5)			
150	152.9 ± 9.4	6.1	1.9
15	14.9 ± 2.2	14.5	-0.3
1.5	1.6 ± 0.2	12.8	4.1
Day-to-day (n = 5)			
150	149.9 ± 2.2	1.5	-0.0_2
15	15.0 ± 0.4	2.4	0.0_3
1.5	1.5 ± 0.1	5.2	1.4

Hydrodynamic injection, 60 s at 18 cm.

Table 3. Recovery of baclofen in plasma (n = 3)

Concentration added (μM)	Found (mean ± SD)	Recovery (%)	RSD (%)
40	39.8 ± 1.6	99.6	3.9
4	4.1 ± 0.6	103.3	13.5

Hydrodynamic injection, 60 s at 18 cm.

4. Conclusions

A CE-UV method for the determination of baclofen in human plasma was developed and validated. The plasma samples were deproteinized with acetonitrile, then directly injected into CE. The sensitivity could be enhanced by acetonitrile stacking. A detection limit of 0.2 μM baclofen in plasma was achieved. The developed method, with its simplicity, short analysis time, and ease of operation, is suitable for routine analysis of baclofen in human plasma samples.

Acknowledgements

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