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Determination of gabapentin in human plasma by capillary electrophoresis with laser-induced fluorescence detection and acetonitrile stacking technique

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Abstract

A sensitive analytical method for gabapentin [1-(aminomethyl) cyclohexaneacetic acid] (GBP) in human plasma based on capillary electrophoretic separation and laser-induced fluorescence (LIF) detection has been developed. 6-Carboxyfluorescein succinimidyl ester (CFSE) was used for precolumn derivatization of the non-fluorescent drug in plasma. Optimal separation and detection were obtained with an electrophoretic buffer of 50 mM sodium borate (pH 9.5) and an air-cooled argon-ion laser (excitation at 488 nm, emission at 520 nm). A calibration curve ranging from 0.3 to 150 μM was shown to be linear. The concentration limit of detection (LOD) in plasma was 60 nM. We also demonstrate how the detection limit can be enhanced by using acetonitrile stacking technique. With stacking, the limit of detection for gabapentin in plasma was 4.8 nM. A calibration curve ranging from 0.03 to 15 μM was shown to be linear. Both the within-day and day-to-day reproducibility and accuracy were ≤10.8 and 6.0%, respectively.

Keywords: Acetonitrile stacking technique; Gabapentin

1. Introduction

Gabapentin [1-(aminomethyl) cyclohexaneacetic acid] (GBP) is a new antiepileptic drug currently being introduced in therapy worldwide [1–3]. As shown in Fig. 1, GBP is a structural analogue of the inhibitory neurotransmitter γ-amino butyric acid (GABA). GBP crosses the blood–brain barrier. However, GBP is neither a GABA agonist nor an inhibitor of GABA uptake or degradation [4,5]. The mechanism of GBP's anticonvulsant properties remains unknown. Measurement of GBP concentration in plasma is useful for furthering studies of its efficacy, dose-related side effects, and action mechanism. Therefore, a simple, sensitive, and selective analytical procedure for assay of the drug concentration in plasma is required.

Different methods have been proposed for analyzing GBP in biological fluids. In gas chromatography (GC) analysis [6,7], sample derivatization was necessary to enhance the volatility of the analyte. GC combined with mass spectrometry has been described for therapeutic drug monitoring [8]. In liquid chromatography (LC) methods, since GBP does not have a chromophore or a fluorophore in their structure, it is not easy to detect it with UV-Vis detector except at low UV wavelengths, where the detection limits are not favorable. Lengthy extraction and clean-up procedures are required for the analysis of real sample. HPLC techniques with pre-column derivatization offer more variability. 2,4,6-Trinitrobenzenesulfonic acid (TNBS) [9,10] and phenylisothiocyanate [11] have been used as derivatizing agent with UV detection. *o*-Phthaldialdehyde (OPA) was the most common derivatizing agent for fluorescence detection [12–19], but the OPA-derivative was only stable for 25 min. LC-tandem MS was also reported for the quantitation of GBP in human plasma [20]. In capillary electrophoresis (CE), fluorescamine [21] and fluorescein isothiocyanate (FITC) [22] were used as a derivatizing agent for GBP. A laser-induced fluorescence (LIF) detector was used to detect FITC-derivatized GBP in rats. Recently, spectrofluorimetric methods were reported for the determination of GBP in dosage forms, urine and plasma. However, copper acetate or

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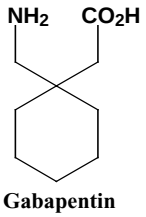

Fig. 1. Chemical structure of GBP.

copper hydroxide should be added to eliminate the interference from endogenous amino acids for biological samples [23,24].

CE is a useful method to quantify drugs in biological samples. However, complicated extractions and low sensitivity have hampered its use in clinical drug monitoring. To overcome this limitation and increase the sensitivity, we combined the acetonitrile stacking technique and laser-induced fluorescence detection. Acetonitrile was used to remove proteins in plasma sample and induce sample stacking in CE. 6-Carboxyfluorescein succinimidyl ester (CFSE) was used for precolumn derivatization of GBP, followed by detection with an air-cooled argon-ion laser-induced fluorescence detector. To our knowledge, CFSE has not been employed in the derivatization of GBP yet.

2. Experimental

2.1. Chemicals

GBP was purchased from Tocris Cookson Inc. (Bristol, UK). CFSE was obtained from Molecular Probes (Eugene, OR, USA). The CFSE reagent for derivatization of GBP was prepared in dimethylformamide (DMF) and was used immediately to avoid possible degradation. Sodium borate was from Showa (Tokyo, Japan). Phenylpropanolamine (PPA) was obtained from Sigma (St. Louis, MO, USA) and used as an internal standard (IS). The purities of GBP, CFSE, and PPA were 98, 97, and 99%, respectively. All other chemicals were of reagent grade. Water purified with a Barnstead NANOpure system (Dubuque, IA, USA) was used for all solutions. All solutions were filtered through a 0.45 μm pore-size membrane filter before use.

2.2. CE–LIF system

The CE–LIF system was assembled in-house and has been described previously [25,26]. 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 i.d. × 360 μm o.d. × 60 cm total length (Polymicro Technologies, Phoenix, AZ, USA). The effective length of capillary is 40 cm. Samples were injected by raising the anodic end 18 cm above its normal position for 10–60 s. The 488 nm beam (10 mW) from an air-cooled argon-ion laser (Uniphase, San Jose, CA, USA) was used for excitation. The laser light was focused onto the capillary with a 1.4 cm focal length lens. The fluorescence emission was collected with a 10× microscope objective (0.25 N.A.; Edmund Scientific, Barrington, NJ, USA) and passed through a 520 nm interference filter (10 nm effective bandwidth; Edmund Scientific, Barrington, NJ, USA). The fluorescence image was focused onto a photomultiplier tube (PMT, Model R928; Hamamatsu Corp., Bridgewater, NJ, USA). The PMT current was converted to voltage by a 10 kΩ resistor. 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). The LIF detection system was held in a large light-tight box constructed from black Plexiglas to exclude stray light.

2.3. CFSE derivatization procedure

The 0.025–10 μM GBP solution containing 10 μM PPA was prepared in water. The 50 μl of GBP and PPA standards were mixed with 40 μl of 50mM borate buffer (pH 9.5). The 10 μl of CFSE (10 mM in DMF) was then added and thoroughly mixed. The resulting solution was shaken for 30 min using a Vortex shaker. We found that the fluorescence intensity of the derivatized product increases with increasing time of derivatization reaction. After 30 min, the fluorescence intensity became stable. The derivatized product was stable at least 4 h at room temperature.

2.4. Preparation of plasma sample

Blood sample was centrifuged immediately to obtain the plasma, which was stored −20 °C until analysis. A 0.5 ml plasma sample was deproteinized by adding 1 ml of acetonitrile. After centrifugation at 8800 × *g* for 15 min, 90 μl of the supernatant liquid was spiked with 10 μl of GBP solution containing 15 μM PPA. Plasma samples of various GBP contents were similarly prepared by spiking the plasma with the desired amount of GBP. The 50 μl of GBP-spiked plasma was reacted with 10 μl of 10 mM CFSE and 40 μl of 50 mM borate buffer (pH 9.5), following the procedure described above. The recovery of GBP in plasma was estimated by spiking 20 μl of GBP and PPA standards to 480 μl of plasma sample before deproteinization.

2.5. CE procedure

The CE buffer containing 50 mM sodium borate was prepared with deionized water. The pH of the CE buffer was adjusted to 9.5 by addition of NaOH. 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 417 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 10–90 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. Derivatization and separation

FITC is the most popular amine-reactive fluorescent probe. However, the chemistry of the isothiocyanate reactive moiety is slow and inefficient. FITC derivatization reaction was usually carried out overnight for completeness. By changing the reactive moiety present in a fluorescein-based probe, one may improve the derivatization chemistry, thereby lowering the concentration that can be derivatized. CFSE has been shown to be far more superior to FITC for derivatizing nanomolecular concentrations of amine-containing analyte [27,28]. CFSE has a succinimidyl ester functionality suitable for precolumn derivatization of primary amine-containing analytes. Formation of the derivative is made possible by the succinimidyl ester undergoing nucleophilic attack by the primary amine functionality of analytes. Fig. 2 shows the electropherogram of 0.5 μM GBP derivatized by CFSE in 50 mM sodium borate solution (pH 9.5). A single peak appears at 12.9 min (peak "G", Fig. 2A). PPA was included in the sample as an internal standard (peak "IS") for improvement of precision and accuracy. Compared with the blank electropherogram (Fig. 2B), there are few hydrolysis products generated by CFSE derivatization. The derivatization was relatively fast (30 min). We found that the peak size of both GBP and PPA did not change significantly after 30 min derivatization time.

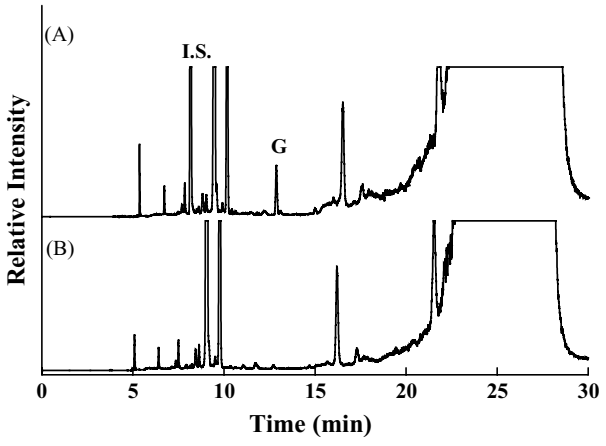

Fig. 2. Electropherograms of CFSE-labeled GBP with LIF detection. (A) A 250 nM CFSE–GBP derivative; (B) plasma blank. G: GBP and IS: PPA. Separation capillary, 60 cm total length (40 cm to the window) × 50 μm i.d. × 360 μm o.d.; electrophoretic buffer, 50 mM sodium borate, pH 9.5; separation voltage, 18 kV; hydrodynamic injection, 10 s at 18 cm height; LIF detection, $\lambda_{ex} = 488$ nm, $\lambda_{em} = 520$ nm.

A calibration curve for GBP in aqueous solution was constructed over the concentration range 0.025–10 μM. The peak area ratio of GBP/PPA versus GBP's molar concentration plot showed a good linearity with a correlation coefficient (r) of 0.9975 ($n = 7$). The slope was found to be 0.1198 (±0.0038) and the y-intercept was calculated to be −0.0032 (±0.0159). At a concentration level of 0.5 μM GBP, the relative standard deviation (R.S.D.) values on peak area and migration time were 4.5 and 0.9% ($n = 7$), respectively. Based on a signal-to-noise (S/N) ratio of three, the lowest derivatizable concentration limit for GBP in aqueous solution was calculated to be 3.7 nM. For an injection volume of 4.5 nl, this value corresponds to 17.6 amol of GBP.

3.2. GBP in plasma sample

In order to evaluate the applicability of the method for biological and clinical analysis, human plasma spiked with GBP was used as the test sample. Analysis of GBP in plasmas is often problematic. Since CFSE can react with primary amines and amino acids as well, the derivatization reaction between GBP and CFSE may be affected by the endogenous plasma components. On the other hand, with the high resolving power of CE, laborious sample cleanup might not be necessary. As described in the experimental section, the only pretreatment we performed was to deproteinize the plasma with the addition of acetonitrile, followed by centrifugation. Typical electropherograms obtained from GBP-free and spiked plasma samples are shown in Fig. 3. There were no peaks observed across the window for the CFSE–GBP derivative and CFSE-PPA derivative in the electropherogram of blank plasma (Fig. 3B). Although the electropherogram contains many extraneous peaks, they do not interfere with the GBP and IS due to the high resolving power of CE. It is possible that amino acids content in human plasma may differ from one individual to another. We have used plasma samples from six different healthy volunteers.

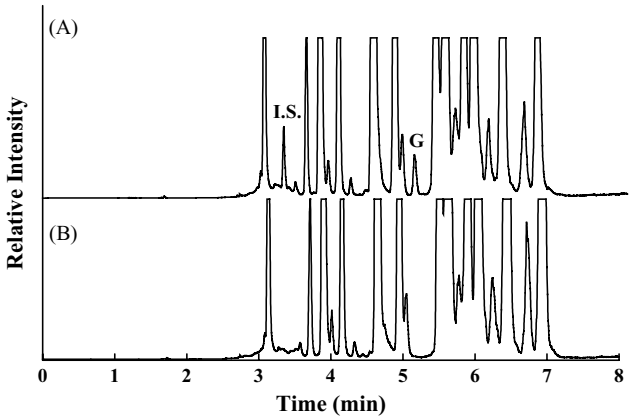

Fig. 3. Electropherograms of plasma samples. (A) Plasma spiked with 3 μM GBP and 15 μM PPA; (B) blank. G: GBP and IS: PPA. Electrophoretic buffer, 50 mM sodium borate, pH 9.5; separation voltage, 25 kV; other conditions as in Fig. 2.

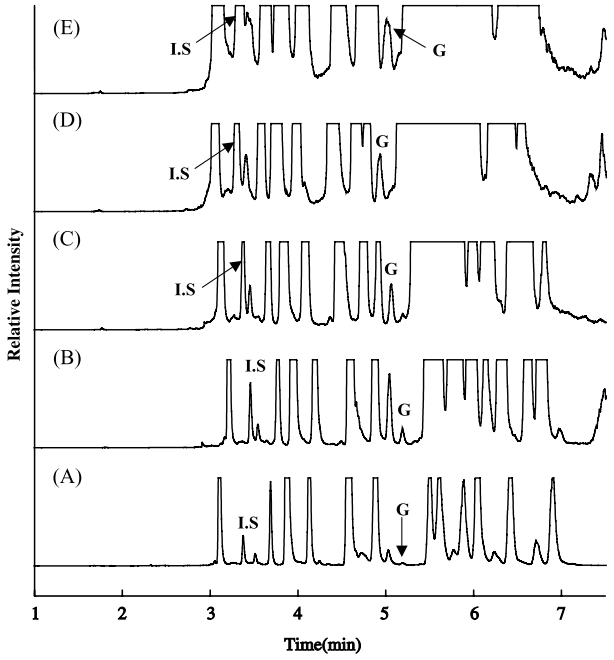

Fig. 4. Electropherograms of GBP (3 μM) in plasma with different injection time. (A) 10 s; (B) 20 s; (C) 40 s; (D) 60 s; (E) 90 s. G: GBP and IS: PPA. Hydrodynamic injection, at 18 cm height; electrophoretic buffer, 50 mM sodium borate, pH 9.5; separation voltage, 25 kV; other conditions as in Fig. 2.

The peak pattern was all similar to that in Fig. 3, although the relative peak size varied among different samples. There was no interfering peaks across the window of the GBP and IS peak in blank plasma samples ($n = 6$ sources).

3.3. Acetonitrile stacking for plasma sample

Due to matrix effect, the lowest derivatizable concentration limit for GBP in plasma was calculated to be 60 nM, about 16 times higher than that in aqueous solution. Attempts to enhance the analyte signal by further increasing the concentration of CFSE during derivatization was unsuccessful. Therefore, a technique involving on-line concentration was needed to improve sensitivity. In CE, a unique type of stacking occurs when mixtures of acetonitrile and salt are present in the sample [29–31]. Acetonitrile offers an effective method to remove proteins in plasma samples. In addition, plasma samples contain high concentration of salt [32]. With regard to simplicity and ease of operation, acetonitrile stacking is a suitable method for on-line concentration of plasma sample in CE. Larger sample volume was injected into CE. Fig. 4 compared the electropherograms of different injection volumes. Fig. 5 shows that the peak height increased with an increase of sample volume from 10 to 60 s injection. A further increase in injection time did not increase the peak height of GBP. The peak shape of GBP deteriorated for 90 s injection time. In addition, the IS peak was interfered by other components of plasma sample (Fig. 4A). With 60 s injection time, a factor of 26 in signal enhancement was obtained. However, the theoretical plate number N dropped from 35,000 to 25,000. The limits of detection (LOD) were estimated based on an S/N ratio of three for different injection time (10, 20, and 60 s). Those results were summarized in Table 1. The LODs of 20 and 60 s injection time were 4.8 and 3.6 nM, respectively. The LODs

Table 1
LODs of GBP in plasma for different injection time

Injection time (s)	LOD (nM)	Injection volume[a] (nl)
10	60.0	4.5
20	4.8	8.9
60	3.6	26.8

[a] Injection volume $= \rho g\, \Delta h d^4 \pi t / 128 \eta L$, where ρ is the buffer density; g is the gravitational constant; Δh is the height differential of the reservoirs; d is the capillary inside diameter; t is the time; η is the buffer viscosity; and L is the total capillary length [35].

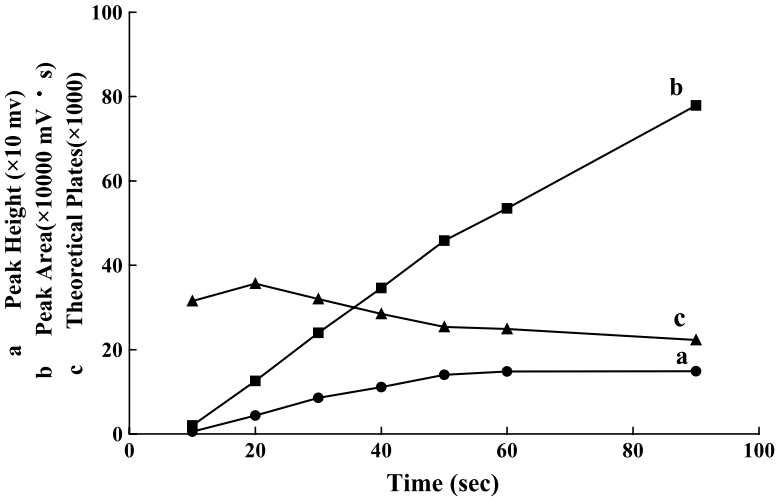

Fig. 5. Effect of injection time on peak height, peak area, and theoretical plate. Plasma samples spiked with 3 μM GBP were injected at different periods of time.

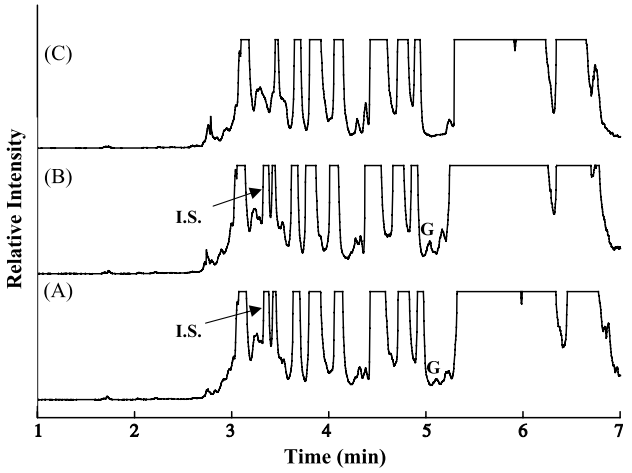

Fig. 6. Electropherograms of GBP in plasma. (A) 30 nM; (B) 150 nM; (C) plasma blank. G: GBP and IS: PPA. Electrophoretic buffer, 50 mM sodium borate, pH 9.5; separation voltage, 25 kV; hydrodynamic injection, 20 s at 18 cm height; other conditions as in Fig. 2.

were more than 10 times better than the traditional injection volume. Compared with the previously described HPLC [11,19] and CE [21] methods, the sensitivity of the present study was better. The LOD of 60 s injection time was similar to that obtained with 20 s injection time. However, the injection volume was three times more. This is likely due to the sample loading capacity of the capillary being exceeded at 60 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 20 s.

3.4. Method validation

The developed method was validated by hydrodynamic injection at 18 cm height for 20 s. The electropherogram of a 30 nM GBP in plasma is illustrated in Fig. 6A. At a signal-to-noise ratio of three, the LOD for GBP in plasma was calculated to be 4.8 nM. With an injection volume of 8.9 nl, this value corresponds to 42.7 amol of GBP. A calibration curve was constructed for the GBP in plasma in the concentration range 0.03–30 μM. The linear equation was $y = (0.3553 \pm 0.0033)x - (0.0882 \pm 0.0425)$ with an $r = 0.9998$ ($n = 7$). This range sufficiently covers the normal drug level encountered in plasma (0.14–26 μM) following clinical administration of 400 mg of GBP to a healthy adult [11,33]. 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 were found to be ≤8.0 and 10.8%, respectively. The accuracy of the method expressed as relative mean error (R.M.E.) was ≤6.0%. According to *Guidance for Industry, Bioanalytical Method Validation* [34], 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. Based on a signal-to-noise ratio of 10, the theoretical limit of quantitation (LOQ) was calculated to be 17 nM. The effective LOQ of the assay, defined as the lowest quantifiable concentration with the variation of precision and accuracy ≤20%, was found to be 0.15 μM. The electropherogram of 0.15 μM GBP in plasma was shown in Fig. 6B. The recoveries of GBP from plasma were determined by spiking 480 μl of plasma with 20 μl of 5×10^{-4} and 5×10^{-6} M GBP standard prior to deproteinization. The recoveries of GBP were in the range 91.2–99.7% (Table 3).

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

Concentration added (μM)	Found (mean ± S.D.)	R.S.D. (%)	R.M.E. (%)
Within-day ($n = 5$)			
15.00	14.97 ± 1.16	7.7	−0.2
1.50	1.41 ± 0.11	8.0	−6.0
0.15	0.15 ± 0.01	7.9	−4.0
Day-to-day ($n = 10$)			
15.00	14.13 ± 1.16	4.8	−5.8
1.50	1.46 ± 0.11	8.9	−2.9
0.15	0.14 ± 0.02	10.8	−4.0

Hydrodynamic injection, 20 s at 18 cm.

Table 3
Recovery of GBP in plasma ($n = 3$)

Concentration added (μM)	Found (mean ± S.D.)	Recovery (%)	R.S.D. (%)
20.00	19.94 ± 2.74	99.7	13.6
0.20	0.19 ± 0.02	91.2	12.9

Hydrodynamic injection, 20 s at 18 cm.

4. Conclusions

A sensitive and selective CE–LIF method for the determination of GBP in human plasma was developed. CFSE has been utilized for derivatization of GBP, followed by detection with an argon-ion LIF detector. We also demonstrate how the detection limit can be enhanced by using acetonitrile stacking technique. A detection limit of 4.8 nM GBP in plasma was achieved. This developed CE–LIF method, with its simplicity, sensitivity and ease of operation, is suitable for routine analysis of GBP in human plasma samples.

Acknowledgements

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