Determination of Gabapentin in Human Plasma and Urine by Capillary Electrophoresis with Laser-Induced Fluorescence Detection

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A simple and reliable method based on capillary electrophoresis with laser-induced fluorescence detection was developed for the analysis of the antiepileptic drug Gabapentin in human plasma and urine. 4-Chloro-7-nitrobenzofurazan was used for precolumn derivatization of the drug. With an uncoated fused silica capillary (40.0 cm effective length, 50.2 cm total length and 75 μm internal diameter), optimal separation was achieved with 30 mM sodium dodecyl sulfate, 40 mM sodium borate (pH 10.25) and acetonitrile 10% (v/v) as running buffer. The applied voltage was 20 kV and the samples were injected by pressure (3.45 kPa × 3 s). The method was fully validated with regard to linear range, sensitivity, precision, limit of detection and limit of quantification in human plasma and urine samples. Linear ranges were 0.1–15 μg mL−1 for plasma and urine. The intra- and interday precisions were <9.02 and 13.90%, respectively. The recoveries were 96.0–109.3% for plasma and 94.3–98.0% for urine. The method was successfully applied for the determination of Gabapentin in human plasma and urine.

Introduction

Gabapentin (GBP), namely 1-(aminomethyl) cyclohexanecacetic acid, is a synthetic structural analog of γ-aminobutyric acid (GABA), which was originally developed as an add-on therapy for epilepsy. Later researches (1–3) have further approved its pharmacological effects in postherpetic neuralgia, complex regional pain syndrome and neuropathic pains including trigeminal neuralgia. The exact mechanism of action of GBP is not completely understood however, the probable one is that the drug inhibits glutamatergic transmission elicited in the spinal cord by nociceptive stimulation or by direct activation of protein kinase C (4, 5). GBP is well absorbed after a single dose of 300–400 mg and reaches peak plasma concentrations (Cmax) of 2.256–3.230 μg mL−1 within 2–3 h (6, 7). The drug is not metabolized in the liver and mainly excreted by kidney with half-life of 5–8 h. Compared with other older antiepileptic drugs, GBP is generally considered safer, but some patients still suffer some behavioral side effects (8). Recently, several clinical and preclinical studies have further provided evidences of the negative influence of GBP on memory performance (9–11). The results suggest that a repetitive peak-trough pharmacokinetic profile is the underlying cause of memory impairment. Yet, maintenance of stable GBP plasma levels could protect against seizures without causing memory impairment. Consequently, it is essential to establish reliable and accurate analytical methods for the therapeutic drug monitoring of GBP to effectively optimize the treatment outcome and reduce the incidence of side effects.

A variety of chromatographic methods have been proposed for the determination of GBP in human biological fluids, such as gas chromatography (GC) and high-performance liquid chromatography (HPLC). GC separations were coupled to mass spectrometry (MS) (12, 13) or to flame ionization detection (14) after derivatization. With respect to HPLC methods, different derivatizing agents have been used with UV (6, 15–17) or fluorescence detection (7, 18–23). Since GBP does not possess chromophores (what is necessary when UV or fluorescence detection is used), analysis of this drug requires derivatization with derivatizing agents to produce a chromophore. Considering the complexity and dirtiness of the biological samples, most of these methods involved a lengthy liquid–liquid or solid-phase extraction (SPE) sample pretreatment for GBP prior to their analysis to prevent the chromatographic columns from being blocked. Indeed, the multiple extraction procedures increased the overall analysis time and negatively affected the accuracy of the methods. Other LC–MS (24–26), MS–MS (27) and LC–MS–MS (28–31) methods have also been used for the analysis of the drug in human plasma, but the expensive apparatus limit their widespread applicability.

Capillary electrophoresis (CE) is an alternative and effective method for the determination of GBP in biological samples; yet until now, a small number of CE processes for GBP determination in biological samples have been developed. Garcia (32) described a CE-UV method for GBP detection in human plasma after derivatization with fluorescamine, but it suffered from low sensitivity. Laser-induced fluorescence (LIF) detection proves to be more sensitive than UV detection. For the best use of CE-LIF, it is necessary and important to select an appropriate fluorescence derivatization reagent for the analytes. Rada et al. (33), Sarah et al. (34) and Cao et al. (35–37) have built CE-LIF approaches for GBP detection in plasma samples; they used fluorescein isothiocyanate (FITC), 6-carboxyfluorescein succinimidyl ester (CFSE), 5-(4, 6-dichlorotriazinyl) amino fluorescein (DTAF) and 6-oxy-(N-succinimidyacetate)-9-(2’-methoxy carbonyl) fluorescein (SAMF) as derivatization reagents, respectively. The derivatization reaction of FITC with amines is slow and is accompanied with many fluorescent side products. Derivatization reaction of CFSE is simple and convenient but it is limited by its high price. Meanwhile, the electropherogram of its derivatives has many impure peaks and some of them are so enormous.

In research field of CE-LIF, Cao et al have developed numerous CE-LIF methods for determination of GABA and its analogs (including GBP) in human plasma samples (35–37). In Refs (35) and (36), they used DTAF and SAMF as derivatizing reagent, respectively. And they also used FITC, DTAF and SAMF for same analytes for comparative study in reference (37). While the work cited...
above is significant for GBP detection, it is also limited by the IS used, the long reaction times, the use of expensive reagents or run buffer additives, or complex sample preparation methods.

4-Chloro-7-nitrobenzofurazan (NBD-Cl) is a decent and inexpensive fluorescence derivatization reagent and it has been used for derivatization of GBP in serum samples by HPLC (7). In that HPLC process, to prevent the costly chromatographic columns being polluted by filthy biological samples, pretreatment of serum samples included being extracted by organic solvent, evaporated by stream of nitrogen, redissolved by organic solvent and derived by derivatization reagent. It needed a high demand of pretreatment equipment. And there was a guard column as well. Separation of GBP and IS was obtained within 6 min but serum impurity peaks were prominent. Compare with the expensive chromatographic columns used in HPLC, the uncoated fused silica capillaries used in CE are far cheaper and easier to be rinsed. Moreover, NBD-Cl has also been extensively used for the derivatization of amines in CE since it shows obvious advantages such as faster derivatization reaction, clear electropherogram and low cost. However, it has not been applied to derivatization for detection of GBP in biological samples.

In our previous study, a CE-LIF method (using NBD-Cl as the derivatizing agent) for the analysis of GBP in pharmaceutical dosage forms was established and it has been used successfully for the quality assessment of GBP in drug products (38). But when this process was introduced to human plasma, dissatisfactory results were obtained because of the complex matrix interference in biological fluids. On the other hand, since L-phenylalanine (utilized as internal standard (IS) in former research) is endogenous substance that existing in human blood and urine, it would disturb determination of IS and in turn of GBP, so it was not suitable for human plasma and urine samples and baclofen is used as IS in the new work. Some CE processes have been established for GBP determination in serum samples (33–37), while there is not a CE method for urine samples. In this report, in order to expand the application of the CE-LIF process (using NBD-Cl as derivatization reagent) and make it suitable for plasma and urine samples, the process was improved with respect to derivatization and separation conditions.

Our research group has also developed a CE approach for detection of baclofen in pharmaceutical preparation and urine samples and GBP was used as IS (39). The IS and target were exchanged in this work. It was similar to the method in reference (39) while there were some differences. Method in this work presents a real possibility in increasing GBP signal and may be applicable to other complicated biological matrices such as human plasma. Although urine and plasma are both biological samples, they are very different in nature. Diluted urine samples are similar to pharmaceutical preparation that dissolved in water; components contained in plasma are more complex than urine thus there are more matrix effects in plasma. Therefore, plasma samples required more critical temperature and time conditions and also have higher demands for separation conditions. Thus, it seems necessary to re-examine derivatization and separation conditions.

Materials and methods

Chemicals and solutions

GBP and baclofen were purchased from National Institute for Control of Pharmaceutical and Biological Products (Beijing). The capsules of GBP (100 mg GBP per capsule) were provided by Hengrui Medicine Co., Ltd. (Jiangsu, China). NBD-Cl was obtained from Acros (Geel, Belgium). Sodium borate was from Sigma (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS) was purchased from Kelong Chemical Reagent Ltd. (Chengdu, China). All the reagents were of analytical grade. Deionized water was purified by a synergy UV water system from Millipore (Milford, MA, USA).

Stock solutions of GBP and IS were both 1 mg mL−1 in water. NBD-Cl solution (100 mM) was prepared and diluted with acetonitrile (ACN) to required concentrations. Stock solutions of sodium borate (100 mM) and SDS (100 mM) were both prepared with water. All the stock solutions were prepared weekly and stored at 4°C.

The derivatization buffer of 30 mM sodium borate was adjusted to pH 8.9 with 1 M HCl. The running buffer was composed of sodium borate (40 mM, pH 10.25), SDS (30 mM) and ACN (10%, v/v); the pH of this solution was adjusted to 10.25 using 1 M NaOH. All solutions were filtered through a 0.45-μm pore-size membrane filter before use.

Instrumentation

A P/ACE MDQ CE system (Beckman Coulter Instrument, Fullerton, CA, USA) equipped with an LIF detector (excitation at 488 nm and emission at 520 nm) was employed throughout the experiment. A 32 Karat Software (version 8.0) equipped with a computer was used for system control and data analysis. All separations were performed using an uncoated fused silica capillary (Hebei, China) with 75 μm inner diameter, 375 μm outer diameter, 50.2 cm total length and 40 cm effective length. The capillary was thermostatted at 25°C. Samples were injected by applying a pressure of 3.45 kPa for 3 s at the anodic end of the capillary and separations were carried out on a 20 kV voltage.

New capillary was washed consecutively with methanol for 5 min, 1 M NaOH for 10 min, and water for 10 min. At the beginning of each working day, the capillary was rinsed with 1 M HCl for 3 min, 0.5 M NaOH for 5 min, water for 5 min and finally with the running buffer for 5 min. Between runs, the capillary was rinsed with 0.5 M NaOH for 0.5 min, water for 2 min and running buffer for 3 min.

Sample pretreatment

A statement of human subjects protocol approval

The plasma and urine samples used in this research came from West China hospital of Sichuan province and all the human subjects declare that they volunteered for the experiment. A document about 'Ethical Approval' had been brought forth by Sichuan University and scanning copy was submitted as Supplementary data.

Plasma samples

Blood sample from healthy volunteer was collected into the tube containing disodium EDTA and centrifuged immediately at 3,000 rpm for 10 min to obtain the plasma, which was stored at −20°C until analysis. A 50 μL aliquot of plasma sample was spiked with 10 μL solution of GBP, 10 μL solution of IS and 100 μL ACN. After mixing for 10 s on a vortex mixer and...
centrifugation for 5 min at 5,000 rpm, the supernatant liquid was transferred into an eppendorf tube, to which 1 mL of ethyl acetate was added. The mixture obtained was vortexed for 5 s and then centrifuged for 3 min at 3,000 rpm. After removing the supernatant organic layer, the underlayer liquid was derivatized as described below. Blood sample from patient was not spiked with GBP and 10 μL water was added instead of it. Other procedures were the same as with blank blood sample.

**Urine samples**

Urine sample from healthy volunteer was diluted 1 : 9 with water, and then the solution was filtered through a 0.45 μM filter to remove the solid material. A 50 μL filtered urine solution was transferred to a vial, and 10 μL of GBP, 10 μL of IS were added to prepare as the spiked urine sample, which was derivatized as described below. Sample from patient was not spiked with GBP and 10 μL water was added instead of it. Other procedures were the same as with blank urine sample.

**Derivatization**

The following derivatization procedure was applied to both plasma and urine samples obtained after the sample pretreatment step: to the sample solution obtained, 100 μL of derivatization buffer and 100 μL of NBD-Cl solution (40 mM) were added. The resulting mixture was heated at 60°C for 20 min. And then the derivative solution was injected into the CE apparatus.

**Results**

An accurate and reliable CE-LIF method was developed to determine the presence of GBP in biological fluids, which used NBD-Cl as precolumn derivatizing agent. Since the biological fluid is more complex than the pharmaceutical formulations, the previous CE-LIF means (36) was not appropriate for biological samples. Based on it, the separation and derivatization conditions were readjusted and the modified approach resolved it ultimately. The established method was successfully applied to the analysis of GBP in real samples of patients’ plasma and urine samples; clear electropherograms could be obtained without complex pretreatment.

**Discussion**

**Optimization of the derivatization conditions**

Allowing for the biological sample matrix effect on the derivatization reaction, several parameters such as reaction temperature and time, buffer pH and concentration, and concentration of the derivatizing agent on the fluorescence intensity and efficiency of derivatization were re-investigated.

**Effect of reaction temperature and time**

The effect of reaction temperature on the fluorescence intensity was investigated over the range of 30–80°C with a 10°C increment and keeping the reaction time at 30 min. As shown in Figure 1, higher temperature was favorable for the derivatization reaction and the fluorescence intensity reached a maximum at 60°C. By further increasing the temperature, an apparent decrease on the fluorescence intensity was observed due to unstable status of the derivatives at high temperatures. Reaction times in the range of 10–50 min were then investigated at 60°C. The results suggested that the fluorescence intensity enhanced sharply with extending time up to 20 min, after which it increased very slowly. On the basis of these results derivatization was performed at 60°C for 20 min throughout the study. Compared with pharmaceutical formulations, the reaction temperature and time were discrepant. This result further confirmed the influence of matrix effects of biological samples on the derivatization efficiency.

**Effect of buffer pH and concentration**

Alkaline buffer would promote the reaction since the derivatization reaction of GBP with NBD-Cl is a typical nucleophilic reaction. The effect of sodium borate buffer pH was investigated in the range of 7.9–9.9 by keeping the buffer concentration constant at 30 mM. The fluorescence intensity of the derivatives was highest at pH 8.9. Then sodium borates at pH 8.9 with different concentrations in the range of 10–50 mM were studied.
shows that the fluorescence intensity of the derivatives enhanced with increasing buffer concentrations, and a plateau was achieved when the concentration was >30 mM. Thus, a derivatization buffer condition of 30 mM sodium borate at pH 8.9 was chosen.

**Effect of NBD-Cl concentration**

The effect of NBD-Cl concentration on the fluorescence intensity was investigated in the range of 10–50 mM. Improvement of derivatives fluorescence intensity was observed with increasing the NBD-Cl concentration up to 40 mM, but there was no significant improvement with further increasing the concentration. A plateau was observed with NBD-Cl concentration in the range of 40–50 mM. Thus, a 40 mM NBD-Cl concentration was used subsequently.

According to above results, an optimum derivatization condition of 30 mM sodium borate at pH 8.9, 40 mM NBD-Cl and reaction for 20 min at 60°C was obtained.

**Optimization of the separation conditions**

The prior separation condition (10 mM SDS, 10 mM sodium borate, pH 9.75) was firstly applied to the plasma and urine samples but peak efficiency was poor. Taking into account the complexity of biological samples, it was essential to readjust the buffer conditions. A number of modifications to the running buffer including pH, species, concentration and choice of organic solvent were tested, before selecting the buffer that offered the best resolution for GBP and IS along with the miscellaneous components in the biological samples.

**Effect of SDS concentration**

It was reported that the addition of SDS in the running buffer could both improve the separation efficiency and enhance the sensitivity; our previous research also confirmed it. Thus, SDS was added to the running buffer and effect of SDS content on separation efficiency and sensitivity was investigated. Buffers containing SDS in the range of 10–50 mM were explored, respectively. Results showed that sensitivity of GBP derivant enhanced as SDS content increased. However, with augment of SDS content, resolution increased to maximum at 30 mM SDS and then decreased; also migration time extended. Considering about sensitivity, resolution and migration time, 30 mM SDS was chosen to be the optimum SDS concentration.

**Effect of running buffer pH and concentration**

To find the best separation conditions of biologic samples, running buffers consisting of 10 mM sodium borate, 30 mM SDS at different pH were applied, in particular in the 8.75–10.75 pH range. It was found that in a running buffer of pH <10.25, the drug could not be separated from the endogenous components in plasma and urine samples. Better results were obtained at pH 10.25 and 10.75 however, the migration time was extended greatly and the sensitivity was weakened at pH 10.75. Considering both sensitivity and efficiency, pH 10.25 was selected as the optimum pH value of the running buffer.

Different sodium borate concentrations in the range of 10–50 mM were examined. The results demonstrated that the sodium borate concentration ameliorated the resolution significantly, but high concentrations resulted in excessive Joule heat. Therefore, a concentration of 40 mM borate buffer at pH 10.25 was chosen as an optimum sodium borate concentration for the running buffer, although complete resolution was not obtained yet.

**Effect of organic modifier**

After all the factors mentioned above have been optimized, there was not sufficient resolution in biological samples. It was reported that organic solvent influenced separation efficiency signal. In an attempt to enhance the separation, the addition of organic modifiers to the running buffer was investigated. Several organic modifiers such as methanol, ACN and isopropanol were studied. The results showed that the addition of ACN could achieve better resolution of the analytes. Then, the effect of ACN concentration on the separation was inspected in the range of 5–20% (v/v). And finally, a baseline resolution was obtained by adding 10% ACN as organic modifier.

The optimum separation conditions were chosen as: running buffer containing 30 mM SDS, 40 mM sodium borate (pH 10.25) and 10% (v/v) ACN. Typical electropherograms obtained from the analysis of GBP in plasma and urine are represented in Figures 3a and 4a, respectively. Compared with the electropherograms of blank plasma and urine (Figures 3b and 4b), it

![Figure 3. Typical electropherograms of plasma samples. (a) Human blank plasma spiked with 200 µg mL⁻¹ GBP and 100 µg mL⁻¹ IS. (b) Human blank plasma. Derivatization conditions: 30 mM sodium borate (pH 8.9), 40 mM NBD-Cl, reaction time of 20 min at 60°C. Separation conditions see Figure 1.](https://academic.oup.com/chromsci/article-abstract/53/6/986/591722)

![Figure 4. Typical electropherograms of urine samples. (a) Human blank urine spiked with 4 µg mL⁻¹ GBP and 4 µg mL⁻¹ IS. (b) Human blank urine. Derivatization conditions see Figure 4. Separation conditions see Figure 1.](https://academic.oup.com/chromsci/article-abstract/53/6/986/591722)
could be seen that GBP was well separated from all other sample constituents and the IS within 15 min.

Comparison with the previous method
For comparison, the previous and current CE-LIF methods were applied to plasma and urine samples from epileptic patients who were undergoing therapy with GBP, severally. With regard to urine samples, both of the two approaches (methods in Ref. (38) and this work) showed good separating ability but the latter required longer separation time. But when applied to plasma samples, the current one revealed better capability than the former. Electropherograms of spiked plasma samples with the two CE-LIF methods are shown in Figure 5. Under the prior separating condition, base line separation could not be achieved. This phenomenon testified to the necessity of improvement. After a series of melioration, the subsequent process was less susceptible to the interference by endogenous amino acids and base line separation was obtained, GBP and the IS were separated from the many interfering peaks as seen Figure 5b. There was a slight overlap at the beginning of the GBP peak with the previously published method and this was mostly eliminated with the new. Though the improvement of the separation of GBP was rather modest, it was significant since IS with the older method could no longer be used as it was endogenous substance. Meanwhile, the overlap of the previous IS was serious.

Method validation
The developed method was validated in terms of linearity, limit of quantitation (LOQ), limit of detection (LOD), extraction yield and precision.

Figure 5. Electropherograms of patient’s plasma samples spiked with 4 µg mL⁻¹ GBP. Derivatization conditions see Figure 4. Separation conditions: (a) 10 mM SDS, 10 mM sodium borate (pH 9.75), applied voltage of 20 kV and temperature of 25°C; (b) see Figure 1.

Linearity
The linearity was developed in blank plasma and urine, which were separately spiked with GBP standard solutions to achieve final concentrations ranging from 0.1 to 15 µg mL⁻¹ for plasma and urine. And the IS was maintained constant at 100 µg mL⁻¹ for plasma and 10 µg mL⁻¹ for urine. Calibration curves were obtained by linear least-squares regression analysis plotting of peak-area ratios (GBP/IS) versus the drug concentrations. Spiked blank plasma samples and spiked blank urine samples containing GBP at nine different concentrations were analyzed to construct the calibration curves. The linear equations were $y = 0.0144x - 0.0015$ ($r^2 = 0.9989$) for plasma, and $y = 0.1601x + 0.0005$ ($r^2 = 0.9998$) for urine samples.

Limit of quantitation and limit of detection
The LOQ was determined as the lowest concentration of the plasma and urine spiked with GBP in the calibration curve. The LOD was derived as the lowest concentration which gives a signal-to-noise ratio of 3 for GBP. The LOQ values were 0.1 µg mL⁻¹ for both plasma and urine samples. Nanogram-level LOD values were obtained by combination with ACN stacking (34) or SPE (35) techniques. LOD values of this method, based on a signal-to-noise ratio of 3, were 0.03 µg mL⁻¹ for plasma and 0.02 µg mL⁻¹ for urine, without complicated pretreatment. It was reported that patients who respond to GBP had serum GBP concentrations of ≥2 µg mL⁻¹ (42). Garcia (32) got a mean serum level for dozens of patients on GBP was 5.2 µg mL⁻¹ with a range of 0–12 µg mL⁻¹. Moreover, it is claimed that the LOQ should be at least in the range 1/10–1/20 of the $C_{max}$. These values of the experiments indicated that this handy method could meet the requirement of samples analysis.

Extraction yield
Extraction yields were evaluated at three different concentration levels (0.2, 6 and 12 µg mL⁻¹) by comparing the peak areas of GBP in the test solutions with the un-extracted standard solutions at the same concentration levels. Data are summarized in Tables I and II for plasma and urine, respectively. As a result, the average extraction yield values were 72.4% for plasma and 92.9% for urine (the extraction yields of IS were 94.0% for both samples).

Precision
The precision of the method was determined on spiked plasma and urine samples by analyzing them five times within the same day (to obtain repeatability data) and over 5 different days (to obtain intermediate precision data), respectively. Each assay was carried out at three different GBP concentrations (0.2, 6 and 12 µg mL⁻¹). The relative standard deviation percentage (RSD %) of migration time and peak areas of GBP are depicted in Table I for plasma and Table II for urine. According to

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<th>Concentration (µg/mL)</th>
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<th>Interday (RSD %) Migration time</th>
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shown in Figure 6. As one could note, GBP could be detected given in Table III, which were in good agreement with those identified by adding the standard solution into the samples. Without interference from the matrix. The peaks of GBP were plotted by adding standard solutions to blank plasma. Perhaps it operation process slightly. The standard curve of the method is plotted by adding standard solutions to blank plasma. Perhaps it could eliminate matrix distraction to some extent hence more accurate quantitative result could be achieved. The repeatable and accurate determination results illustrated that the described CE-LIF method is accurate, practical and it seems to be suitable for the routine analysis of GBP in human plasma and urine samples. It is expected to state a scope of operation for clinical analysis.

Supplementary Material


References

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