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Journal of Chromatography B, 794 (2003) 17–22
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of vigabatrin by capillary electrophoresis with laser-induced fluorescence detection

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Received 7 January 2003; received in revised form 28 April 2003; accepted 28 April 2003

Abstract

A new analytical method for vigabatrin based on capillary electrophoretic separation and laser-induced fluorescence detection has been developed. 5-Carboxytetramethylrhodamine 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) containing 10 m*M* sodium dodecyl sulfate and a green He–Ne laser (excitation at 543.5 nm, emission at 589 nm). The concentration limit of detection in aqueous solution was 24 n*M*. Combined with a simple cleanup procedure, this method can be applied to the determination of vigabatrin in human plasma. A calibration curve ranging from 1.5 to 200 μ*M* was shown to be linear. Both the within-day and day-to-day reproducibilities and accuracies were less then 14.3% and 4.9% respectively. The limit of detection of vigabatrin in plasma was about 0.13 μ*M*.

Keywords: Vigabatrin

1. Introduction

Vigabatrin (γ-vinyl-γ-amino butyric acid) is a structural analogue of the inhibitory neurotransmitter γ-amino butyric acid (GABA). Clinically vigabatrin is used for the treatment of epilepsy [1]. Its action is attributed to the irreversible inhibition of GABA-transaminase, an enzyme responsible for the degradation of GABA [2,3]. By increasing brain concentrations of GABA, vigabatrin appears to decrease propagation of abnormal hypersynchronous discharges. Measurement of vigabatrin concentration in plasma is useful in assessing compliance and evaluating risks of toxicity [4]. Therefore, a simple, sensitive and selective analytical procedure for assay of the drug concentration in plasma is required.

Both gas chromatography (GC) and high-performance liquid chromatography (HPLC) methods for the determination of vigabatrin in biological fluids have been developed. Schramm et al. [5] reported a GC method for vigabatrin enantiomers in plasma involving a double derivatization step. GC in combination with mass spectrometry (MS) for the assay of vigabatrin enantiomers in human body fluids was described by Haegele et al. [6]. In LC methods, since vigabatrin does not have a chromophore or a fluorophore in its structure, it is not easy to detect it with a UV/VIS detector except at low UV wavelength [7], where the detection limits are not favorable. Lengthy extraction and clean-up procedures are required for

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1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(03)00396-9

the analysis of biological samples. HPLC techniques with pre-column derivatization offer more variability. *o*-Phthaldialdehyde (OPA) [8–12], Dns chloride [13] and 4-chloro-7-nitrobenzofurazan (NBD-Cl) [14] have been commonly used as derivatizing agents with fluorescence detection. Chiral separation of vigabatrin enantiomers by HPLC also has been reported [15–17]. Recently, spectrofluorimetric methods were reported for the determination of vigabatrin in urine [18] and tablets [19]. However, copper acetate should be added to eliminate the interference from endogenous amino acids for urine samples.

In recent years, capillary electrophoresis (CE) has been an important separation technique due to its high resolving power, speed and small sample amount required. Derivatization with a suitable fluorophore, followed by capillary electrophoresis-laser-induced fluorescence (CE–LIF) detection, provides sensitivity and selectivity. Despite their widespread applicability to biological and clinical analyses, CE coupled with LIF detection has not been applied to the analysis of vigabatrin yet. In this paper, we describe a novel and sensitive CE–LIF method for the analysis of vigabatrin. 5-Carboxytetramethylrhodamine succinimidyl ester (CTRSE) was used for precolumn derivatization of non-fluorescent drug, followed by detection with a low-cost He–Ne laser-induced fluorescence detection. To our knowledge, this is the first report of a capillary electrophoretic technique employing CTRSE as a derivatization reagent. Linearity, repeatability and detection limit were examined. The applicability of the method to the determination of vigabatrin in human plasma was also demonstrated.

2. Experimental

2.1. Chemicals

Vigabatrin was purchased from Tocris Cookson Inc. (Bristol, UK). CTRSE was obtained from Molecular Probes (Eugene, OR, USA). The CTRSE reagent for derivatization of vigabatrin was prepared in acetonitrile. Sodium borate was from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS) was from Aldrich (Milwaukee, WI, USA). All other chemicals were of reagent grade. Water purified with a Barnstead NANOpure system (Dubuque, IA, USA) was used for all solutions. The CE buffer was 50 m*M* sodium borate at pH 9.5, containing 10 to 50 m*M* SDS. 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. 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 25 cm above its normal position for 5 s. The 543.5 nm beam (1.5 mW) from a He–Ne laser (Uniphase, San Jose, CA, USA) was used for excitation. The laser light was focused into 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 589 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. CTRSE derivatization procedure

The stock standard solution of vigabatrin (1 m*M*) was prepared in water and diluted with water to achieve the final desired concentration. First, 100 μl of vigabatrin standards was mixed with 80 μl of 50 m*M* borate buffer (pH 9.5). Then 20 μl of CTRSE (1 m*M* in acetonitrile) was added and thoroughly mixed. The resulting solution was allowed to stand at room temperature for 30 min. 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 for at least 3 h at room temperature.

2.4. Preparation of plasma sample

The blood sample was centrifuged immediately to obtain the plasma, which was stored at −20 °C until analysis. A 0.5 ml plasma sample was deproteinized by adding 0.5 ml of acetonitrile. After centrifugation at 8800 *g* for 15 min, 90 μl of the supernatant liquid was spiked with 10 μl of vigabatrin. Plasma samples of various vigabatrin contents were similarly prepared by spiking the plasma with the desired amount of vigabatrin. Then 100 μl of vigabatrin-spiked plasma was reacted with 20 μl of 1 m*M* CTRSE and 80 μl of 50 m*M* borate buffer (pH 9.5), following the procedure described above. The recovery of baclofen in plasma was estimated by spiking 20 μl of vigabatrin standards to 480 μl of plasma sample before deproteinization.

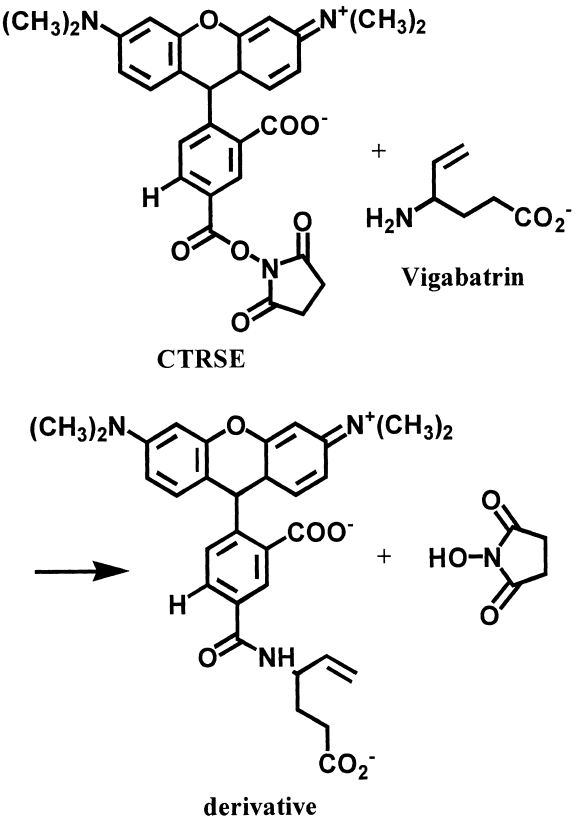

Fig. 1. Derivatization scheme of vigabatrin with CTRSE.

3. Results and discussion

3.1. Derivatization and separation

CTRSE 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 [20]. The proposed derivatization scheme is shown in Fig. 1. The excitation maxima of CRTSE coincides closely with the 543.5 nm output wavelength of the green He–Ne laser. Green He–Ne laser has features of stability, low-cost and long lifetime. The excitation wavelength is preferable in bioanalysis due to the fact that excitation in this region is relatively free from interferences. Results from our preliminary work indicated that CTRSE reacted rapidly with vigabatrin in basic media to form a fluorescent adduct. This makes it amenable to LIF detection with high sensitivity.

When the reaction mixture was injected into the CE system with 50 m*M* borate buffer at pH 9.5, the derivative peak could not separate from excess labeling reagent. In order to separate the derivative from unresolved peak, micellar electrokinetic capillary chromatography (MEKC) was investigated. The micellar phase corresponds to the stationary phase in conventional chromatography and moves with a velocity that is different from the surrounding aqueous phase during electrophoresis. Solutes which partition into the micellar phase can be separated from more hydrophilic solutes. The micellar phase was formed by adding 10–50 m*M* SDS to borate buffer. Separation was achieved with a buffer of 50 m*M* borate buffer (pH 9.5) containing 10 m*M* SDS. The applied field strength for the separation was 350 V/cm. Fig. 2 shows the electropherogram of 125 n*M* CTRSE-labeled vigabatrin obtained with LIF detection. The peak of CTRSE-labeled vigabatrin appears at 7.6 min. Its full width at half height is

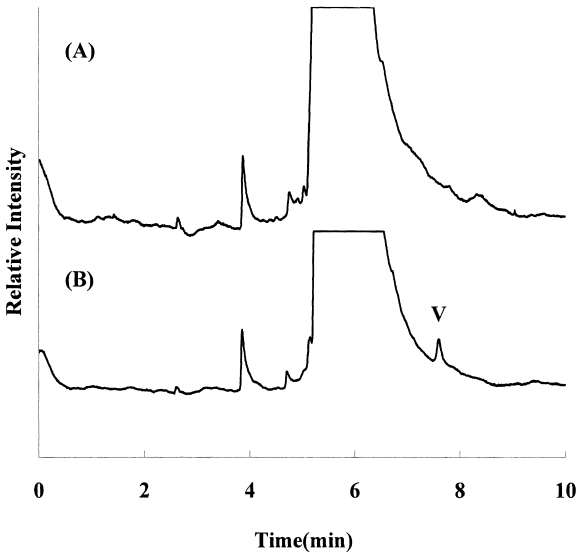

Fig. 2. Electropherograms of CTRSE-labeled vigabatrin with LIF detection. (A) 125 n*M* CTRSE-vigabatrin derivative; (B) blank. v=vigabatrin. Separation capillary, 60 cm total length (40 cm to the window)×50 μm I.D.×360 μm O. D.; electrophoretic buffer, 50 m*M* sodium borate+10 m*M* SDS, pH 9.5; separation voltage, 21 kV; hydrodynamic injection, 5 s at 25 cm height; LIF detection, λ_{ex}=543.5 nm, λ_{em}=589 nm.

about 4.7 s, corresponding to ca. 37 975 theoretical plates.

A calibration curve for vigabatrin was constructed over the concentration range 2.5×10^{-7} to 10^{-4} *M*. The peak area versus concentration plot showed a good linearity ($y=7.3\times10^{9}x+101$) with a correlation coefficient (r) of 0.9951 ($n=5$). At a concentration level of 0.5 μ*M* vigabatrin, the relative standard deviation (RSD) values on peak area and migration time were 9.5% and 0.9% ($n=7$), respectively. At a signal-to-noise (S/N) ratio of 3, the concentration limit of detection for vigabatrin in aqueous solution was calculated to be 24 n*M*. With an injection volume of 0.96 nl, this value corresponds to 23 amol (or 3 fg) of vigabatrin, which is the smallest detection limit of vigabatrin ever reported.

3.2. Vigabatrin in plasma sample

In order to evaluate the applicability of the method for biological and clinical analyses, human plasma spiked with vigabatrin was used as the test sample. Analysis of vigabatrin in body fluids is often problematic. Since CTRSE can react with primary amines and amino acids as well, the derivatization reaction between vigabatrin and CTRSE 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. The only pretreatment we performed was deproteinizing the plasma with the addition of acetonitrile, followed by centrifugation. In order to separate vigabatrin from other components in plasma, a 50 m*M* borate buffer (pH 9.5) containing 50 m*M* SDS was used as the electrophoretic buffer. The samples were injected by raising the anodic end of capillary 19 cm above its normal position for 10 s. Typical electropherograms obtained from vigabatrin-free and spiked plasma samples are shown in Fig. 3. There were no peaks observed across the elution time for the CTRSE-vigabatrin derivative in the electropherogram of blank plasma (Fig. 3A). Although the electropherogram contains many extraneous peaks, they do not interfere with the vigabatrin 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. The peak pattern was all similar to that in Fig. 3, although the relative peak size varied among different samples. There were no

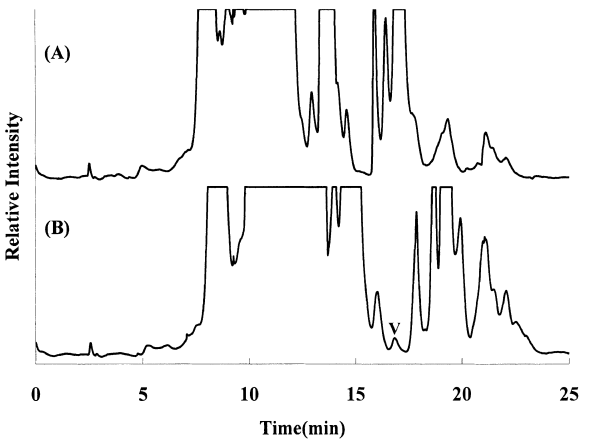

Fig. 3. Electropherograms of plasma samples. (A) plasma blank; (B) plasma spiked with 10 μ*M* vigabatrin. v=vigabatrin. Electrophoretic buffer, 50 m*M* sodium borate+50 m*M* SDS, pH 9.5; other conditions as in Fig. 2.

Table 1
Within-day and day-to-day precision and accuracy of vigabatrin in plasma

Concentration added (μM)	Found (mean±SD)	RSD (%)	RME (%)
Within-day ($n=5$)			
8.0	7.8±0.9	11.2	−2.9
100.0	95.1±11.2	11.8	−4.9
Day-to-day ($n=10$)			
8.0	7.7±1.0	13.0	−3.6
100.0	95.5±13.7	14.3	−4.5

interfering peaks across the elution window of the vigabatrin peak in blank plasma samples ($n=6$ sources).

3.3. Method validation

The detection limit of vigabatrin in plasma was estimated to be about 0.13 μM ($S/N=3$). The limit of quantitation (LOQ) was calculated to be 0.69 μM. This value corresponds to 0.69 fmol and 3.66 fmol of vigabatrin in an injection volume of 5.3 nl for plasma, respectively. Compared with the previously described HPLC methods [14], the sensitivity of the present study was better. A calibration curve was constructed for the vigabatrin in plasma in the concentration range 1.5–200 μM. The linear equation was $y=5.8\times10^{8}x+3813$ with an $r=0.9997$ ($n=4$). This range sufficiently covers the normal drug level encountered in plasma (1.5 μM–150 μM) following clinical administration of 500 mg of vigabatrin to a healthy adult [4,14]. The results of the assay validation study were summarized in Table 1. The within-day and day-to-day reproducibility expressed as relative standard deviation (RSD) were found to be less than 11.8% and 14.3%, respectively. The accuracy of the method expressed as relative mean error (RME) was less than 4.9%. According to *Guidance for Industry, Bioanalytical Method Validation* [21], 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 vigabatrin from plasma were determined by spiking 480 μl of plasma with 20 μl of 2×10^{-4} M and 2.5×10^{-3} M vigabatrin standard prior to deproteinization. The recoveries of vigabatrin were in the range 95.2–100.3% (Table 2).

4. Conclusions

A sensitive and selective CE–LIF method for the determination of vigabatrin was developed. CTRSE has been utilized for derivatization of vigabatrin, followed by detection with a He–Ne LIF detector. This work demonstrates the first application of CTRSE for the analysis of an amine-containing drug by CE–LIF. A detection limit of 24 nM vigabatrin was achieved. This highly sensitive CE–LIF method, combined with the simple clean-up procedure, can be applied to the determination of vigabatrin in human plasma. The convenience of the developed method is facilitated by its simplicity, sensitivity, and the use of a low cost LIF detector.

Acknowledgements

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

Table 2
Recovery of vigabatrin in plasma ($n=3$)

Concentration added (μM)	Found (mean±SD)	Recovery (%)	RSD (%)
8.0	7.6±0.9	95.2	12.0
100.0	100.3±5.1	100.3	5.1

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