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Determination of vigabatrin in human plasma and urine by high-performance liquid chromatography with fluorescence detection

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Abstract

A sensitive and specific HPLC method has been developed for the assay of vigabatrin in human plasma and urine. The assay involves derivatization with 4-chloro-7-nitrobenzofurazan, solid-phase extraction on a silica column and isocratic reversed-phase chromatography with fluorescence detection. Aspartam was used as an internal standard. The assay was linear over the concentration range of 0.2–20.0 μg/ml for plasma and 1.0–15.0 μg/ml for urine with a lower limit of detection of 0.1 μg/ml using 0.1 ml of starting volume of the sample. Both the within-day and day-to-day reproducibilities and accuracies were less than 5.46% and 1.6%, respectively. After a single oral dose of 500 mg of vigabatrin, the plasma concentration and the cumulative urinary excretion of the drug were determined.

Keywords: Vigabatrin; 4-chloro-7-nitrobenzofurazan

1. Introduction

A new antiepileptic drug vigabatrin (γ-vinyl-γ-amino butyric acid) is a structural analogue of the inhibitory neurotransmitter γ-amino butyric acid (GABA). Its action is attributed to the irreversible inhibition of GABA-transaminase, an enzyme responsible for the degradation of GABA [1]. By increasing brain concentrations of GABA, vigabatrin appears to decrease propagation of abnormal hypersynchronous discharges, thereby reducing seizure activity [2].

The commercially available drug is a racemic mixture, but only the *S*(+)-enantiomer is pharmacologically active and does not undergo chiral inversion. The absorption characteristics of the enantiomers are similar to those of the racemate. Vigabatrin is rapidly absorbed from the gastrointestinal tract reaching peak plasma concentrations within 1 to 2 h after oral administration. It has a favourable pharmacokinetic profile since it has little protein-binding, is mainly excreted by the kidney and has a long effective elimination half-life (5–7 h) allowing once or twice daily dosing [3].

Measurement of plasma vigabatrin concentrations is useful in assessing compliance and evaluating risks of toxicity [3,4]. Several methods for the assay of vigabatrin in biological fluids have been reported. Grove et al. [5] described the analysis of vigabatrin using an amino acid analyser with microcolumns and fluorimetric detection. This is a time consuming method requiring regeneration of the column after each analysis. Two gas chromatographic methods

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involving the double derivatization step and the use of chiral capillary column have been described. In these methods, enantiomers of the drug were analysed by either the ion monitoring technique in the electron impact mode of ionisation [6] or by thermionic specific detection [7]. Both assays are complex, time consuming and relatively expensive, nevertheless they are sensitive. High-performance liquid chromatographic (HPLC) methods based on rapid and simple derivatization with orthophthaldialdehyde (OPA), have also been used for the analysis of vigabatrin as the racemate [8] or enantiomers [9] in human serum. The major disadvantage of these methods is the instability of the derivatization product.

In this study, a selective and sensitive HPLC method with fluorescence detection is reported for the assay of vigabatrin in human plasma and urine. The method involves derivatization with 4-chloro-7-nitrobenzofurazan (NBD-Cl) which is a specific reagent for primary and secondary aliphatic amines. A solid-phase extraction procedure was used on silica columns to separate the fluorescent NBD-vigabatrin derivative from the endogenous compounds of plasma. The method was tested for applicability in pharmacokinetic studies by assaying plasma and urine samples of two healthy volunteers after a therapeutic dose of vigabatrin.

2. Experimental

2.1. Materials and reagents

Vigabatrin was kindly supplied by Hoechst Marion Roussel (Istanbul, Turkey). The internal standard, aspartam was obtained from Sanecta (Maastricht, Netherlands). NBD-Cl was purchased from Sigma (St. Louis, MO, USA). Acetonitrile and methanol, both HPLC grade, ethyl acetate, hydrochloric acid, potassium chloride, sodium hydroxide, anhydrous sodium sulfate and boric acid, all analytical grade, were from Merck (Darmstadt, Germany). Orthophosphoric acid was from Riedel (Seelze, Germany). Water was doubly distilled.

Sep-Pak silica extraction columns (the sorbent mass of 50 mg in 1 ml cartridge) and filters (0.45 μm, 13 mm for mobile phase filtration; 0.2 μm, 4 mm for sample filtration) were purchased from Waters (Milford, MA, USA).

2.2. Solutions

The stock standard solution of vigabatrin (200 μg/ml) was prepared in water and this was diluted with water to give a standard solution of 20 μg/ml. Plasma and urine calibration samples (0.2, 0.4, 0.8, 1.6, 2.4, 5.0, 10.0, 15.0 and 20.0 μg/ml of plasma and 1.0, 3.0, 6.0, 10.0, 15.0 μg/ml of urine) were prepared daily by spiking 100 μl of drug-free plasma or urine with the appropriate volume of this solution. The total volume of each sample was diluted to 400 μl with water.

A stock of internal standard solution (500 μg/ml) was prepared in water and appropriate dilutions were made to obtain working solutions (10 μg/ml and 80 μg/ml).

The stock solutions were stored at 4°C and were stable for a month.

NBD-Cl solution (5 mg/ml) was prepared freshly in methanol.

Borate buffer was prepared by dissolving 0.620 g of boric acid and 0.750 g of potassium chloride in 100 ml of water. The pH was adjusted to 10.0 with 0.1 *M* sodium hydroxide solution and the volume was made up to 200 ml with water.

2.3. Instrumentation

The HPLC analysis was carried out on a system consisted of an LC-10AT solvent-delivery system equipped with an injection valve with a 20 μl loop (Shimadzu, Tokyo, Japan). Integrations and system parameters were controlled by CBM-10A software system (Shimadzu). Separation was performed isocratically on a C_{18}, 5 μm Shim-Pack column (250 mm×4.6 mm I.D., Shimadzu) fitted with a guard column (20 mm×3.9 mm I.D., Waters) packed with the same material and maintained at ambient temperature. The column eluate was monitored by a Model 470 scanning fluorescence detector (Waters) set at an excitation wavelength of 460 nm and an emission wavelength of 520 nm. The mobile phase consisted of a mixture of 10 m*M* phosphoric acid–acetonitrile (60:40, v/v) and it was delivered at a flow-rate of 1.0 ml/min.

2.4. Sample preparation

Venous blood samples (2–3 ml) were placed into citrated tubes. Following centrifugation for 10 min at 4500 *g* (room temperature), the plasma obtained and urine samples were stored at −20°C until analysed.

A 100-μl plasma or urine sample was spiked with 100 μl internal standard working solution. Plasma was deproteinized by adding 2 ml of methanol then vortex-mixing for 1 min and centrifugation for 5 min at 4500 *g*, 1 ml of the supernatant was evaporated until its half volume under nitrogen in a block heater at 50°C. The remaining solution or urine sample were reacted with NBD-Cl by adding borate buffer (100 μl) and reagent (100 μl) solutions. The mixture was heated at 70°C for 50 min. After cooling, 0.1 *M* HCl (100 μl) was added and the mixture was extracted with 5 ml of ethyl acetate. The extract was dried on anhydrous sodium sulfate. An aliquot of 1 ml of this extract was evaporated to about 0.2 ml and passed through the solid-phase extraction columns. The extract was eluted with 4 ml of ethyl acetate. After evaporation the residue was dissolved in 400 μl of mobile phase and injected into the HPLC system.

2.5. Linearity

Calibration curves were constructed by analysing a series of standard plasma and urine samples to obtain concentrations of vigabatrin ranging from 0.2 to 2.4 μg/ml (internal standard at 10 μg/ml) and 2.4 to 20.0 μg/ml (internal standard at 80 μg/ml) in plasma and from 1.0 to 15.0 μg/ml (internal standard at 80 μg/ml) in urine. The chromatograms were evaluated on the basis of vigabatrin/internal standard ratios of the peak areas.

2.6. Extraction recovery

The recovery was assessed by using the samples spiked with vigabatrin at concentrations of 0.4, 5.0 and 15.0 μg/ml in plasma and at concentrations of 1.0, 6.0 and 15.0 μg/ml in urine. Triplicate samples for each concentration were derivatized, extracted on silica columns and chromatographed using the procedure outlined above. To determine the recovery aqueous vigabatrin solutions at the same concentration were analysed by using the same procedure except solid-phase extraction and the results were compared with each other.

2.7. Assay validation

The within-day and day-to-day precision and accuracy were determined by analysing samples spiked with vigabatrin at concentrations of 1.6, 5.0 and 15.0 μg/ml in plasma and at concentrations of 3.0, 6.0 and 10.0 μg/ml in urine. Determinations were performed with either six replicates on the same day or ten replicates on separate days.

2.8. Applicability

Two healthy female volunteers, one of them was 23 years old and 50 kg (volunteer A), and the other was 45 years old and 49 kg (volunteer B), were given 500 mg of vigabatrin as commercially available tablets with 200 ml tap water (2 h after breakfast). The volunteers received a standardized diet a day before administration of the drug and during the study period. Venous blood samples were collected in citrated tubes prior to dosage and 30, 45 min, 1, 1.15, 1.30, 2, 3, 4, 6, 8, 12, 16 and 24 h afterwards. The blood specimens were processed to plasma as described above. Urine samples were also collected at intervals for up to 24 h. The samples were stored at −20°C until analysis. Urine was diluted between 1:5 and 1:160 with water depending on its concentration before analysis.

3. Results and discussion

Vigabatrin was chromatographically separated from endogenous compounds of plasma and urine using reversed-phase HPLC with fluorescence detection. Derivatization with NBD-Cl was carried out to enhance sensitivity and specificity.

Typical chromatograms of the blank human plasma and plasma samples spiked with vigabatrin and internal standard, aspartam, are shown in Fig. 1A and B, respectively. Fig. 1C represents a chromatogram of plasma sample obtained at 1.5 h after oral administration 500 mg of vigabatrin from a healthy volunteer. The retention times of vigabatrin- and

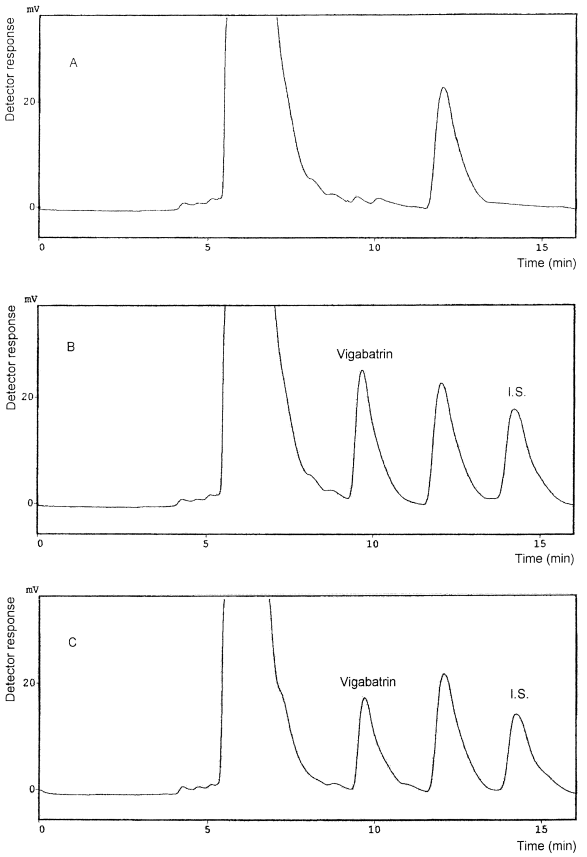

Fig. 1. HPLC chromatograms of (A) a blank human plasma, (B) a human plasma spiked with 20 μg/ml vigabatrin and 80 μg/ml of the internal standard and (C) a plasma sample obtained at 1.5 h after oral administration of 500 mg vigabatrin from a healthy volunteer with 80 μg/ml of the internal standard.

aspartam-NBD derivatives were 9.5 and 14 min, respectively, and the run time was 16 min. The chromatogram of blank plasma showed no interfering peaks having the same retention times as vigabatrin or internal standard derivatives.

A solid-phase extraction procedure seemed to be necessary after derivatization to reduce the interfering products and minimize background signal.

The extraction recoveries of vigabatrin were in the range 70–88% for plasma and 86–91% for urine (Table 1).

The equations of the calibration curves were obtained by the least-squares linear regression analysis of the peak-area ratios of vigabatrin to internal standard versus the concentration. The linearity was

Table 1
Extraction recovery for the assay of vigabatrin in plasma and urine ($n=3$)

Sample	Concentration (μg/ml)		Recovery (%)	RSD (%)
	Added	Found (mean±SD)		
Plasma	0.4	0.28±0.03	70.83	10.71
	5.0	4.18±0.11	83.53	2.63
	15.0	13.24±0.26	88.27	1.97
Urine	1.0	0.86±0.03	86.00	3.49
	6.0	5.30±0.20	88.33	3.79
	15.0	13.71±0.14	91.40	1.02

observed in two concentration ranges between 0.2–2.4 μg/ml and 2.4–20.0 μg/ml in plasma and from 1.0 to 15.0 μg/ml in urine with an excellent correlation coefficients of 0.9999 for all calibration curves. If the vigabatrin plasma levels reported in the literature are considered, the linearity was sufficient in covering the range of concentrations expected to be found in plasma after oral administration of 500 mg of vigabatrin. Under the experimental conditions used, the lower limit of detection (LOD) was 0.1 μg/ml for each sample at a signal-to-noise ratio of 3. The lower limit of quantitation (LOQ) was found to be 0.2 μg/ml in plasma and urine. This value corresponds to 80 pg and 200 pg of vigabatrin in an injection volume of 20 μl for plasma and urine, respectively.

Compared with the previously described HPLC methods, the sensitivity of the present study was lower than that of the method reported by Tsanaclis et al. [8] or similar to the method reported by Vermeij and Edelbroek [9]. On the other hand, the quantitation limit of the present study was lower than those of the GC methods [6,7].

The results of the assay validation study are presented in Table 2. The within-day and day-to-day reproducibilities expressed as relative standard deviation (RSD) were found to be 0.95–3.64% and 0.89–5.46%, respectively, indicating good precision. The accuracy of the method expressed as relative mean error (RME) was always below 1.6% which was shown to be satisfactory.

The stability of the extracts was verified over a 72 h period. Additionally, the stability studies carried out directly in plasma indicated that samples were stable for at least 6 months when stored at −20°C.

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

Sample	Concentration (μg/ml)		RSD (%)	RME (%)
	Added	Found (mean±SD)		
Plasma	Within-day (n=6)			
	1.6	1.58±0.057	3.64	−1.08
	5.0	5.01±0.074	1.47	0.23
	15.0	15.11±0.144	0.95	0.75
	Day-to-day (n=10)			
	1.6	1.58±0.086	5.46	−1.28
	5.0	4.99±0.112	2.24	−0.14
	15.0	15.18±0.286	1.88	1.22
Urine	Within-day (n=6)			
	3.0	3.02±0.070	2.33	1.6
	6.0	6.06±0.200	3.30	1.01
	10.0	10.02±0.275	2.74	0.25
	Day-to-day (n=10)			
	3.0	3.04±0.083	2.74	1.30
	6.0	6.07±0.054	0.89	1.32
	10.0	10.07±0.291	2.89	0.77

To test the applicability of the presented HPLC method in pharmacokinetic studies two pilot experiments were performed with healthy volunteers. In this study, plasma concentrations were calculated from the regression equations of the calibration curves. After a single oral dose of 500 mg of vigabatrin, the plasma concentration–time profile shown in Fig. 2 was obtained. Maximum plasma concentrations (C_{max}) of the drug from two volunteers were 15.6 and 14.9 μg/ml at 0.75 and 1 h (t_{max}), respectively. The elimination half-life of the drug ($t_{1/2}$) and area under the curve (AUC) were found to be 7.91 and 8.15 h; 62.04 and 79.53 μg h/ml, respectively. The cumulative urinary excretions of the drug are shown in Fig. 3; 85–90% of vigabatrin was excreted unchanged within 24 h after administration. These pharmacokinetic parameters are in good agreement with those found previously [3].

It can be concluded from this study that the proposed HPLC method which combines solid-phase extraction and fluorescence detection reaches the

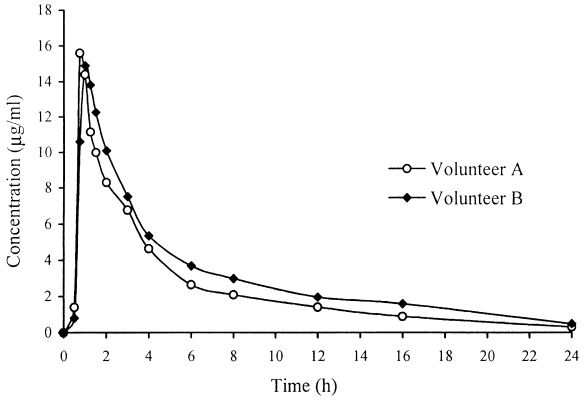

Fig. 2. Plasma concentration–time profiles of vigabatrin in two healthy volunteers after a single 500 mg oral dose.

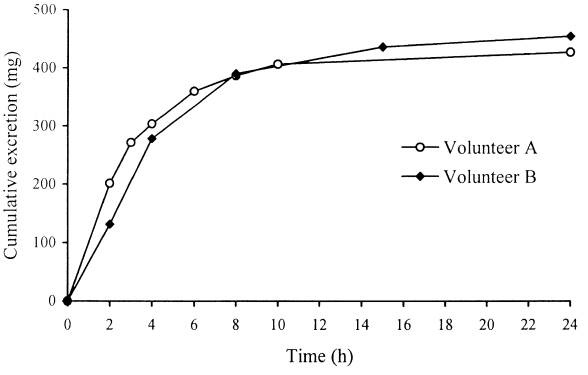

Fig. 3. Cumulative excretion of vigabatrin in urine of two healthy volunteers after a single 500 mg oral dose.

optimum performance in terms of sensitivity, selectivity, precision and accuracy for pharmacokinetic studies of vigabatrin. This method will be utilized clinically to monitor mid-dose plasma vigabatrin levels which may be useful in guiding dosage, determining compliance and confirming toxicity.

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