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Stereoselective determination of vigabatrin enantiomers in human plasma by high performance liquid chromatography using UV detection

Valentina Franco [a], Iolanda Mazzucchelli [a], Cinzia Fattore [a], Roberto Marchiselli [a], Giuliana Gatti [a], Emilio Perucca [a,b,*]

[a] *Clinical Pharmacology Unit, Department of Internal Medicine and Therapeutics, University of Pavia, Pavia, Italy*
[b] *Institute of Neurology, IRCCS C. Mondino Foundation, Pavia, Italy*

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Abstract

A rapid and simple high-performance liquid chromatographic method for the determination of the R-(−)- and S-(+)-enantiomers of the antiepileptic drug vigabatrin in human plasma is described. After adding the internal standard (1-aminomethyl-cycloheptyl-acetic acid), plasma samples (200 μL) are deproteinized with acetonitrile and the supernatant is derivatized with 2,4,6 trinitrobenzene sulfonic acid (TNBSA). Separation is achieved on a reversed-phase cellulose-based chiral column (Chiralcel-ODR, 250 mm × 4.6 mm i.d.) using 0.05 M potassium hexafluorophosphate (pH 4.5)/acetonitrile/ethanol (50:40:10 vol/vol/vol) as mobile phase at a flow-rate of 0.9 mL/min. Chromatographic selectivity is improved by concentrating the derivatives on High Performance Extraction Disk Cartridges prior to injection. Detection is at 340 nm. Calibration curves are linear ($r^2 \geq 0.999$) over the range of 0.5–40 μg/mL for each enantiomer, with a limit of quantification of 0.5 μg/mL for both analytes. The assay is suitable for therapeutic drug monitoring and for single-dose pharmacokinetic studies in man.
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1. Introduction

Vigabatrin (4-amino-5-hexenoic acid; γ-vinyl-GABA) is an antiepileptic drug introduced in clinical practice in the early 90s [1]. It is a structural analogue of γ-aminobutyric acid (GABA) with a vinyl appendage (Fig. 1), and it exerts its pharmacological activity by increasing brain GABA levels through selective and irreversible inhibition of GABA-transaminase, the enzyme responsible for the degradation of GABA in the central nervous system [1,2]. Vigabatrin was widely used as adjunctive treatment of refractory partial seizures until the discovery, in 1997, of severe irreversible visual field constriction associated with its chronic use [3,4]. Today, vigabatrin is rarely used in the treatment of partial seizures, but it is regarded by many authorities as a drug of choice in infants with West syndrome (infantile spasms), particularly in cases associated with tuberous sclerosis [5–7].

Vigabatrin is a chiral molecule commercialized as the racemate, but only the S-(+)-enantiomer is pharmacologically active. No chiral inversion was detected for the R-(−)-vigabatrin and its presence does not interfere with the action of S-(+)-vigabatrin [1]. Evidence has been provided that the two enantiomers differ not only in pharmacological activity, but also in pharmacokinetic properties [8,9]. This implies that the sum of the concentrations of the two enantiomers in the plasma of patients treated with vigabatrin does not provide a reliable estimate of the concentration of the pharmacologically active S-(+)-enantiomer.

Based on the evidence summarized above, meaningful pharmacokinetic studies with vigabatrin can only be performed by using assays which differentiate between the two enantiomers. Although few such assays have been described [10–16], they are all relatively complex and all involve the use of a mass

* Corresponding author at: Clinical Pharmacology Unit, Department of Internal Medicine and Therapeutics, University of Pavia, Piazza Botta 10, 27100 Pavia, Italy. Tel.: +39 0382 986370; fax: +39 0382 22741.
E-mail address: perucca@unipv.it (E. Perucca).

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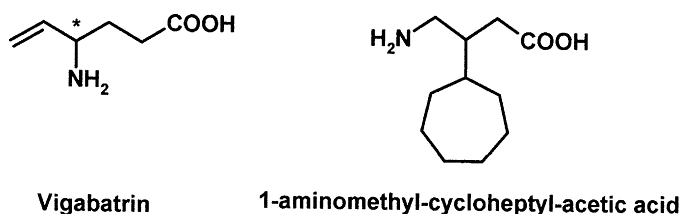

Fig. 1. Chemical structures of vigabatrin and of the internal standard 1-aminomethyl-cycloheptyl-acetic acid. (*) Chiral center.

spectrometer or a fluorimetric detector, which are not available in every laboratory. In this article, we describe a novel enantioselective high-performance liquid chromatographic (HPLC) method which allows the determination of vigabatrin enantiomers in human plasma with UV detection. The method makes use of a Chiralcel-ODR column under reversed-phase conditions, and is sensitive enough for application in single-dose pharmacokinetic studies in humans.

2. Experimental

2.1. Standard, reagents and solvents

Vigabatrin and its R-(−)- and S-(+)-enantiomers were gifts from Sanofi-Aventis (Paris, France). The internal standard 1-aminomethyl-cycloheptyl-acetic acid, a gabapentin analogue (Fig. 1), was obtained from Parke-Davis (Ann Arbour, Michigan). HPLC grade methanol, acetonitrile, acetic acid were from Sigma (Milan, Italy). Potassium hexafluorophosphate (98%) and the derivatizing agent picryl sulfonic acid (2,4,6 trinitrobenzene sulfonic acid, TNBSA, as a 5% wt/vol aqueous solution) were purchased from Sigma (Milan, Italy).

Ultrapure water was prepared using the Millipore-Q-plus water purification system (Millipore, Milan, Italy). Saturated borate solution (in water) was prepared with sodium tetraborate from Sigma (Milan, Italy). High Performance Extraction Disk Cartridges (4 mm/1 mL) consisting in a 0.75 mm-thick membrane loaded with bonded silica C18 particles (standard density) were from 3 M Center (St. Paul, MN, USA). Drug-free human plasma from healthy volunteers for preparation of calibration curves and quality control samples was obtained from a local blood bank (Immunohaematology and Transfusion Service, IRCCS Policlinico S. Matteo, Pavia, Italy).

2.2. Instrumentation and chromatographic conditions

Assays were performed on a Shimadzu liquid chromatograph (Shimadzu Scientific Instrument Inc., Columbia, Maryland, USA) consisting of a System Controller SCL-10Avp, an LC-10ADvp solvent delivery module, an LP-10ADvp pump with a Low-Pressure Gradient Flow Control Valve FCV-10Alvp, an on-line Degasser DGU-14A, a SIL-10ADvp auto injector and a LaChrom L-7400 variable wavelength detector (Merck, Darmstadt, Germany) set at 340 nm. The analytical column was a Chiralcel-ODR (250 mm × 4.6 mm i.d., 10 μm, Daicel Inc., Schilling, Milan, Italy) heated at 25 °C by a model T-6300 thermostate (Merck, Darmstadt, Germany), with an ODR cartridge as a pre-column (10 mm × 4 mm i.d., 10 μm, Daicel Inc., Schilling, Milan, Italy).

The mobile phase consisted of potassium hexafluorophosphate (0.05 M, pH 4.5)/acetonitrile/ethanol. Analyses were performed with a gradient elution starting with 50/40/10 (vol/vol/vol) for 13 min, then modified to 40/45/15 (vol/vol/vol) over 1 min, and returned to 50/40/10 (vol/vol/vol) after 26 min from the time of injection. Flow rate was 0.9 mL/min and the total duration of the chromatographic run was of 40 min.

2.3. Stock solutions and standards

Stock solutions of racemic (R,S)-vigabatrin (1 mg/mL) and internal standard (1-aminomethyl-cycloheptyl-acetic acid, 0.5 mg/mL) were prepared in water and methanol, respectively. Working standard solutions were prepared by diluting stock solutions in acetonitrile. Calibrators were prepared by spiking drug-free human plasma with appropriate amounts of (R,S)-vigabatrin. Quality control samples containing 1, 15, and 35 μg/mL for each enantiomer were prepared and processed in the same manner as the calibrators. Solutions of pure R-(−)- and S-(+)-vigabatrin enantiomers, 1 mg/mL, were used for peak identification.

2.4. Calibration curves and determination of unknowns

Calibrators containing 0.5, 1.25, 2.5, 5, 12.5, 25, 30, 40 μg/mL of each enantiomer were used for the preparation of calibration curves. Calibration curves were constructed by plotting the R-(−)- and S-(+)-vigabatrin enantiomers to internal standard peak area ratio as a function of the enantiomers concentrations in the calibrators. The concentration of each enantiomer in the unknowns was calculated from the least-squares linear regression equation of the calibration curve.

2.5. Sample preparation and derivatization

A 200 μL aliquot of plasma was mixed with 200 μL of internal standard working solution (12.5 μg/mL) and with 200 μL of acetonitrile. After vortexing for 15 s and centrifuging for 10 min at 1400 × *g*, 200 μL of supernatant were transferred into glass tubes. To each tube were added 70 μL of saturated sodium borate solution and 5 μL of the derivatizing agent (5% TNBSA), resulting in an amber-orange colored solution. The tubes were then tightly capped, incubated at 50 °C for 10 min, and vortexed for 10 s. The caps were then removed and the derivatization was stopped by adding 250 μL of 0.25 M acetic acid, which resulted in a color change from amber-orange to yellow. The trinitrobenzene derivatives were then concentrated on High Performance Extraction Disk Cartridges and interfering substances were washed away. The solid phase membrane was activated with 250 μL of methanol, followed by 200 μL of water. The mixture was then passed through the conditioned membrane by vacuum and eluted with acetonitrile (150 μL) and water (150 μL). The eluates were mixed and transferred to a glass autosampler vial. A 25 μL volume was injected into the column by using the autosampler.

2.6. Assay performance characteristics

Recovery was determined by comparing concentration from extracted QCs ($n = 6$ for each QC) with those obtained after injection of known volumes of stock solution prepared and processed in a similar manner as the QCs.

Reproducibility (precision) and accuracy were evaluated at three concentrations. Replicates of samples spiked with 1, 15, 35 μg/mL of each enantiomer were processed according to the procedure used for unknowns and the measured concentrations were used to calculate mean values and coefficients of variation (CV%) for within-day and between-day variability. The deviation of the mean measured value from the spiked value [(Measured/Spiked) × 100] was used as a measure of accuracy.

The lowest limit of detection (LOD) was defined as the concentration yielding a signal to noise ratio of 3.3, whereas the limit of quantitation (LOQ) was considered as the lowest tested concentration (value of the lowest calibrator) at which CV% was <20% and accuracy >80%.

Specificity was evaluated by injecting solutions containing potentially co-prescribed drugs, prepared and processed in the same way as the calibrators.

Stability of the analytes in extracted samples was assessed by comparing assay values in fresh extracts and in extracts stored in mobile phase for 24 h and for 4 days in autosampler vials at room temperature.

2.7. Calculations and statistical analysis

Chromatograms were analysed using a Class-VP Chromatography Data System (Shimadzu Corporation, Duisburg, Germany). Concentration results are reported as μg/mL. The enantioselective factor was evaluated as the ratio between the capacity factor (K') of R-(−)-vigabatrin and that of S-(+)-vigabatrin. K' values were calculated as $(T_r - T_0)/T_0$, where T_r and T_0 are the retention times (RTs) for the analyte and for a non-retained substance (injection solvent), respectively. In stability studies, comparisons of assay values were made by Student's t-test for paired data. A two-tailed P-value < 0.05 was considered significant. Statistical parameters were calculated using Excel Microsoft Office.

3. Results

3.1. Chromatographic separation

Under the chromatographic conditions described above, optimal separation of the enantiomers was obtained, with peak RT of 24.9 min for the internal standard, 10.3 min for S-(+)-vigabatrin and 11.3 min for R-(−)-vigabatrin. The enantioselectivity factor was 1.14.

Representative chromatograms of a blank plasma sample, a calibration sample and a sample from a patient receiving vigabatrin therapy are shown in Fig. 2.

3.2. Linearity

Calibration curves for each enantiomer were linear over the concentration range investigated (0.5–40 μg/mL), with mean slopes of 15.29 for S-(+)-vigabatrin and 15.07 for R-(−)-vigabatrin ($n = 5$). Correlation coefficients were ≥0.999 for all calibration curves (Table 1).

3.3. Recovery, precision, accuracy, and sensitivity

Recoveries were 96 ± 7% for S-(+)-vigabatrin and 94 ± 3% for R-(−)-vigabatrin.

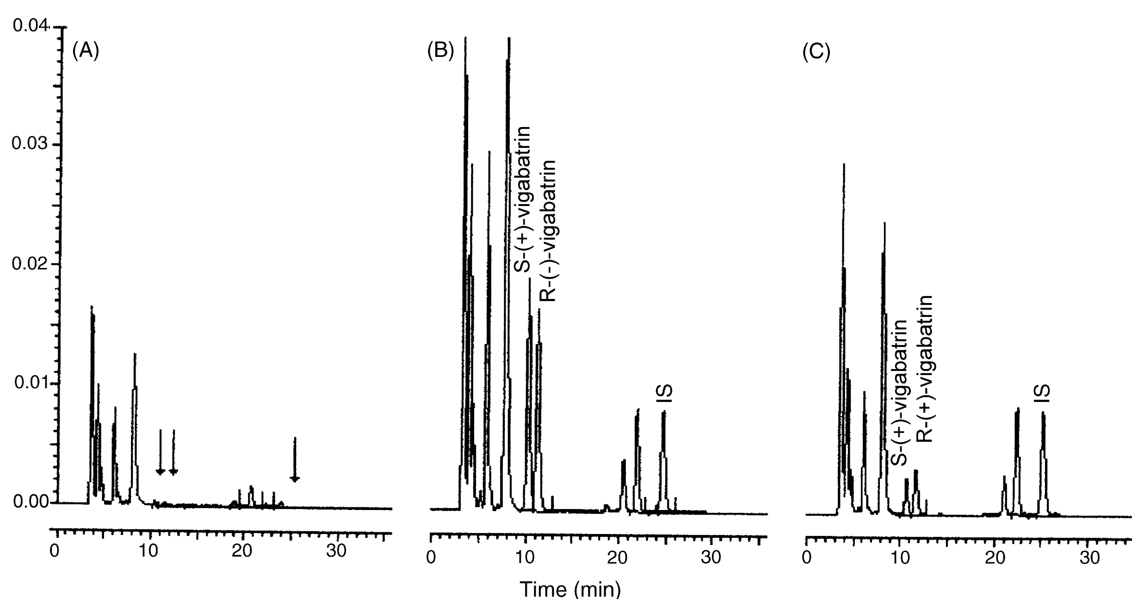

Fig. 2. Representative chromatograms of a blank plasma sample (A), a calibration sample (B, 15 μg/mL of each enantiomer) and a sample of a 31-year female patient receiving vigabatrin therapy (2000 mg/day, estimated R-(−) and S-(+)-vigabatrin concentrations of 3.1 and 2.3 μg/mL respectively). The arrows in (A) correspond to the retention times of S-(+)-vigabatrin, R-(−)-vigabatrin and the internal standard (IS) respectively.

Table 1
Performance characteristics of the assay of the S-(+)- and R-(−)-enantiomers of VGB

Parameters	S-(+)-VGB	R-(−)-VGB
Calibration curves ($n = 5$)		
Slope (mean ± S.D.)	15.29 ± 3.37	15.07 ± 3.28
Intercept (mean ± S.D.)	0.21 ± 0.27	−0.02 ± 0.33
Correlation coefficient (mean ± S.D.)	0.999 ± 0.001	0.999 ± 0.001
Within-day variation		
Spiked value ($n = 7$)	1.0	1.0
Measured value (mean ± S.D.)	1.0 ± 0.1	1.0 ± 0.1
Precision (CV%)	9.1	7.3
Accuracy (%)	97	101
Spiked value ($n = 7$)	15.0	15.0
Measured value (mean ± S.D.)	14.3 ± 0.4	14.1 ± 0.7
Precision (CV%)	2.5	5.1
Accuracy (%)	95	94
Spiked value ($n = 7$)	35.0	35.0
Measured value (mean ± S.D.)	36.0 ± 2.8	36.1 ± 2.8
Precision (CV%)	7.8	7.8
Accuracy (%)	103	103
Between-day variation		
Spiked value ($n = 14$)	1.0	1.0
Measured value (mean ± S.D.)	1.0 ± 0.1	1.0 ± 0.1
Precision (CV%)	10.5	7.6
Accuracy (%)	96	102
Spiked value ($n = 14$)	15.0	15.0
Measured value (mean ± S.D.)	14.6 ± 0.9	14.3 ± 0.9
Precision (CV%)	6.4	6.3
Accuracy (%)	98	96
Spiked value ($n = 14$)	35.0	35.0
Measured value (mean ± S.D.)	35.9 ± 2.1	35.5 ± 2.2
Precision (CV%)	5.9	6.2
Accuracy (%)	103	102

The concentration range of calibration curves was 0.5–40 μg/mL. All concentration values are expressed in μg/mL.

Within- and between-day accuracy and precision are reported in Table 1. For both analytes and for all concentrations tested, within-day and between-day CV% were ≤10.5%. Accuracy ranged from 94% to 103% for both S-(+)-vigabatrin and R-(−)-vigabatrin.

3.4. Sensitivity and performance characteristics at the LOQ

Between-day precision at the LOQ (0.5 μg/mL) was 16% for S-(+)-vigabatrin and 8% for R-(−)-vigabatrin. Between-day accuracy at the LOQ (0.5 μg/mL) was 82% for S-(+)-vigabatrin and 86% for R-(−)-vigabatrin.

3.5. Specificity

The following antiepileptic drugs were carried through the assay by injection of 25 μL of solutions containing 1 mg/mL of each compound, and were not detected under the chromatographic conditions used: ethosuximide, valproic acid, phenobarbital, primidone, lamotrigine, levetiracetam, lorazepam, and prednisolone. The antiepileptic drugs gabapentin and pregabalin produced peaks that did not interfere with the assay.

3.6. Stability

The analytes were stable in samples stored in extraction solvent for 24 h ($n = 8$) and for 4 days ($n = 4$) in autosampler vials at room temperature.

4. Discussion

Compared with most other antiepileptic drugs, vigabatrin is freely soluble in water and therefore its quantification in biological fluids cannot be based on the extraction procedures typically used for other anticonvulsants [10]. Only a limited number of assays for vigabatrin have been reported in the literature. Grove et al [11] described a non-enantioselective method which made use of an amino acid analyser, microcolumns, and fluorimetric detection. This method involved a long analysis time, with column regeneration after each run. An improved non-enantioselective HPLC assay with fluorimetric detection, developed by Smithers et al. [12], requires a complex derivatization procedure in which the cupric ion is used to bind the plasma α-amino acids before vigabatrin and the internal standard are reacted with dansyl-chloride.

The earliest enantioselective methods reported were a gas chromatography–mass spectrometry (GC–MS) assay using a chiral capillary column [8], and a reversed-phase HPLC assay which involves a diastereomeric derivative formation with tert-butyloxy-L-leucine *N*-hydroxy-succinimide ester [13]. The latter, however, does not appear to have been applied to biological matrices. More recently, Bruckner et al. [14] demonstrated that the enantioselective separation of mixtures of D,L-aminoacids can be accomplished by HPLC after converting each enantiomer to the corresponding diastereomeric isoindolyl derivative using *o*-phtaldialdehyde (OPA) and chiral *N*-acylated cysteines. This procedure has been applied to the separation and quantification of the enantiomers of vigabatrin in serum [15]. The OPA derivatization of samples previously deproteinized with either methanol or acetonitrile is advantageous because it allows full automation of the derivatization step and the simultaneous determination of vigabatrin and gabapentin, another antiepileptic drug [16]. The enantiomers of vigabatrin have been also analyzed as their *N*-trifluoroacetyl-*o*-propylester derivatives using a selected ion monitoring technique in the chemical ionization mode [17].

The time consuming nature of the assays described above, together with their need for specialized equipment, prompted us to investigate the possibility of developing a simpler enantioselective method suitable for human pharmacokinetic studies and compatible for use with an UV detector, which is commonly available in most laboratories. Development of the method started with the realization that the vigabatrin molecule is not detectable in the UV–vis spectrum. Vigabatrin, however, has a primary amino group which is a target for standard derivatizing agents such as TNBSA [18,19]. Although the chemical details of the reaction between TNBSA and vigabatrin has not been described, analogy with reactions involving other substrates with similar structure suggests that the final product is a trinitrobenzene derivative [20,21]. In addition to permitting UV detection,

the derivatization step by involving the amino group attached to the chiral centre of the analyte may also improve the chromatographic separation. Our method differs from current assays in many ways: (i) detection is done by UV, with high sensitivity due to strong absorbance at 340 nm; (ii) evaporation/concentration steps are not necessary; (iii) sample preparation/derivatization is rapid and simple; (iv) the derivatives are eluted in a small volume of solvent and consequently can be injected directly into the HPLC system; (v) trinitrobenzene derivatives are very stable and can be stored at room temperature for at least 4 days without degradation.

The present assay shows favourable performance characteristics in terms of recovery, accuracy and precision. No interference is observed with commonly co-prescribed antiepileptic drugs and with steroids, which are frequently used in the management of infantile spasms.

Because vigabatrin acts as an irreversible suicide-inhibitor of GABA transaminase, its duration of action outlasts its presence in plasma and no clear-cut relationship has been identified between its concentration in plasma and therapeutic and toxic effects [22]. Therefore, routine monitoring of plasma vigabatrin concentration is generally not recommended, except for a check for compliance or for assessing the possibility of unusual pharmacokinetic patterns in patients showing an unexpected drug response [22]. Measurement of plasma vigabatrin concentrations can also be valuable for research purposes to investigate its pharmacokinetics in special patients groups, and in the presence of potentially interacting drugs. Since the two enantiomers differ in pharmacodynamic and pharmacokinetic properties, these studies can only be meaningfully conducted by using an enantioselective assay. The method described in this article offers significant advantages in terms of simplicity and ease of use, and it has sufficient sensitivity to allow quantification of the concentrations of each enantiomer which are observed after administration of single oral doses of the racemate in infants [17] and in adults [9]. Therefore, this method can provide a useful tool not only for therapeutic drug monitoring purposes, but also for the conduction of pharmacokinetic studies in a variety of clinical settings. In our laboratory, the assay is currently being applied to the investigation of R-(−)- and S-(+)-vigabatrin pharmacokinetics during pregnancy in women with epilepsy.

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