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Journal of Chromatography B, 724 (1999) 101–108

JOURNAL OF CHROMATOGRAPHY B

Chiral high-performance liquid chromatographic analysis of enantiomers of losigamone, a new candidate antiepileptic drug

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Received 10 August 1998; received in revised form 3 December 1998; accepted 3 December 1998

Abstract

An assay based on a single-step liquid–liquid extraction from human plasma followed by high-performance liquid chromatography on a chiral column was developed for the measurement of enantiomers of a racemic new candidate antiepileptic drug. Excellent intra- and inter-assay accuracy and precision and recovery were demonstrated in the desired concentration range of 0.031 to 5.00 μg/ml. The method is free from interferences by other anticonvulsant drugs and their metabolites. The method is being used in a clinical trial of losigamone.

Keywords: Enantiomer separation; Losigamone; Antiepileptic drug

1. Introduction

Epilepsy comprises many diverse disorders and syndromes with heterogeneous etiologies and neuropathologies. It affects an estimated 1% of the world's population (over 50 million people) which makes it one of the most common neurological disorders [1]. A large percentage (20–30%) of patients diagnosed as having epilepsy are not adequately controlled by presently available medications. Losigamone [(±)-5(*R*,*S*)-α(*S*,*R*)-5-(2-chlorophenylhydroxymethyl)-4-methoxy-2(5H)-furanone; AO-33; ADD137022; (±)-LSG] is a new candidate anticonvulsant drug undergoing preclinical and clinical development. It is a racemic tetronic acid derivative (Fig. 1) which is structurally related to a family of naturally occurring substances found in *Piper fadyenii* and *Piper sanctum* [2]. It exhibits anticonvulsant activity in various in vivo animal seizure models, in vitro epileptiform models, and promising results were obtained in the early pilot clinical studies [3,4].

(±)-LSG is a racemic mixture of two enantiomers, AO242 [(+)-LSG; (+)-5(*R*)-α(*S*)-5-(2-chlorophenylhydroxymethyl)-4-methoxy-2(5H)-furanone] and AO294 [(−)-LSG; (−)-5(*S*)-α(*R*)-5-(2-chlorophenyl-

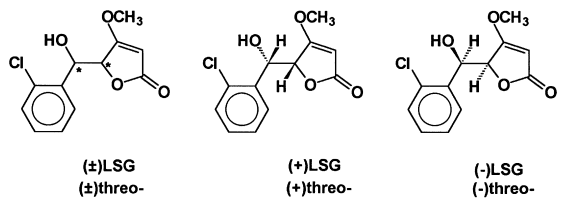

Fig. 1. Chemical structures of racemic (±)-LSG and its enantiomers, (+)-LSG and (−)-LSG.

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0378-4347/99/$ – see front matter
PII: S0378-4347(98)00568-4

hydroxymethyl)-4-methoxy-2(5H)-furanone] (Fig. 1). The two enantiomers were shown to possess different metabolic profiles in human hepatic microsomes [5]. In addition, the two enantiomers exhibited enantiomer–enantiomer interaction under the in vitro human metabolic conditions; AO294 inhibited the metabolism of AO242. The two enantiomers may also differ in their pharmacological activity. The relative activity between the two enantiomers and the racemate differed depending on the test model being employed [3,6].

It is estimated that about 50% of all therapeutic agents have a chiral center [7], yet most of these drugs are administered in the form of a racemic mixture, as opposed to a specific enantiomer [8]. Biological systems are known for their enantioselectivity, and it is becoming more apparent that enantiomers may elicit different pharmacological and toxicological responses (qualitatively and/or quantitatively) due to enantioselectivity in their pharmacokinetics and/or pharmacodynamics [9,10].

With the advent of technological advances in chiral separation and asymmetric synthesis, regulatory agencies of many countries have devoted greater attention on the development of the stereoisomeric drugs. In 1992, the US Food and Drug Administration (FDA) issued a policy statement on the issue {{FDA's Policy Statement for the Development of New Stereoisomeric Drugs 5/1/92, available on the website, http://www.fda.gov/cder/guidance}}. It states: ''When the drug product is a racemate and the pharmacokinetic profiles are different, manufacturers should monitor the enantiomers individually to determine such properties as dose linearity and the effects of altered metabolic or excretory function and drug–drug interactions . . . Therefore, techniques to quantify individual stereoisomers in pharmacokinetic samples should be available early.''

Since (±)-LSG is a new candidate drug currently undergoing development, there is a limited amount of analytical information available in the literature. A high-performance liquid chromatography (HPLC) analysis on a reversed-phase column for the determination of racemic (±)-LSG followed by a chiral column to determine the ratio of enantiomers has been recently described [11]. The main thrust of that published report was concerned with metabolism and pharmacokinetics and the details of the analytical method were not extensively described. The method was applied to samples from human volunteers, not epileptic patients.

The present study was designed to develop a simple HPLC method for the analysis of (±)-LSG enantiomers in plasma which would be suitable for pharmacokinetic studies in epileptic patients.

2. Experimental

2.1. Chemicals

Racemic LSG [(±)-LSG], its two enantiomers [(−)-LSG and (+)-LSG], and the internal standard, α-deshydroxy-5-hydroxy losigamone (AO-368, a structural analog of LSG) were provided by Dr. Wilmar Schwabe, Arzneimittel (Karlsruhe, Germany). Potential LSG metabolites, AO-714 [5′-hydroxy-(±)-LSG], and AO-725 [3′-hydroxy-(±)-LSG] were also obtained from this source. The chemical purity of all reference compounds was >98.5% and the enantiomeric purity of (−)-LSG and (+)-LSG was >99%. Phenytoin (PTH), ethosuximide (ESM), primidone (PRM) and carbamazepine (CBZ) were from U.S.P.C. (Rockville, MD, USA), *p*-hydroxyphenytoin (HPPH) and *p*-hydroxyphenobarbital (PBOH) from Aldrich (Milwaukee, WI, USA), sodium valproate (VPA) from Research Biochemical International (Natick, MA, USA), felbamate (FBM) from Wallace Labs. (Cranbury, NJ, USA), lamotrigine (LTG) from Burroughs Wellcome (Research Triangle, NC, USA), gabapentin (GBP) from Parke-Davis (Ann Arbor, MI. USA), and vigabatrin (VGB) from Merrel Dow (Cincinnati, OH, USA). HPLC solvents were from Burdick & Jackson (Muskegon MI, USA) and the other reagents were of the best commercially available grade. Deionized water was obtained using the Milli-Q reagent-grade water system (Millipore, Bedford, MA, USA).

2.2. Human plasma

Outdated human plasma from the NIH Blood Bank was used for standard curves and quality control samples. Patient plasma was obtained from

epileptic subjects receiving (±)-LSG and either phenytoin and/or carbamazepine.

2.3. Sample preparation

Frozen (−70°C) plasma samples (patient and quality control samples) were allowed to fully equilibrate at ambient temperature (ca. 22°C). LSG enantiomers were extracted from plasma by a simple one-step liquid–liquid extraction. Briefly, 2.5 μg of the internal standard (AO368) in 25 μl of methanol was added to tubes containing 1 ml of plasma, and the contents were mixed. Then, plasma samples were extracted with 5 ml of methyl *tert*.-butyl ether. Following centrifugation, the organic phase was transferred to clean tubes and evaporated to dryness under nitrogen at room temperature. The samples were reconstituted in 50 μl of acetonitrile–water (90:10, v/v) and 2 μl were injected into the HPLC system.

2.4. Extraction recovery

Recoveries were determined following extractions of several different concentrations of (+)-LSG and (−)-LSG. Extraction efficiency was calculated based on the peak height ratio of (extracted analyte/non-extracted internal standard) relative to the corresponding ratio of (non-extracted analyte/non-extracted internal standard). The non-extracted analytes were added to blank plasma extracts in order to avoid any effects of the co-extracted biological material on the adsorption or chromatography.

2.5. Chromatographic conditions

Chromatographic analysis was performed with a Hewlett-Packard (Waldbronn, Germany) series 1100 HPLC system with diode array detector and autosampler. The separation was achieved using a 5 μm Chiradex LiChroCart (250 mm×4 mm; Hewlett-Packard, Waldbronn, Germany) analytical column and a Chiradex LiChroCart (4 mm×4 mm; EM Science, Gibbstown, NJ, USA) guard column. In addition, a C_{18} saturator column (30 mm×4.6 mm; Phenomenex, Torrance, CA, USA) was placed between the pump and the injector to help extend the lifetime of the analytical column. The mobile phase was composed of 0.01 *M* sodium dihydrogenphosphate, pH 4.2–methanol–acetonitrile (89:7:4). The flow-rate was 0.7 ml/min and the column temperature was 47°C. The UV signal was monitored at 230 nm which corresponds to an absorption maximum for LSG under these conditions.

2.6. Quantitation

For each analysis, a standard curve was generated by adding known, varying amounts of (−)-LSG and (+)-LSG and a constant amount of the internal standard to blank plasma. The standards (0.03125 to 5.0 μg/ml) were selected to bracket the anticipated range of experimental values. For calculation purposes, these standards were divided into two groups to improve the quantitation at the lower concentration end. Standards 0.03125 to 0.50 μg/ml and 0.50 to 5.0 μg/ml were used for the low and high concentration standard curves, respectively. Quantitation was achieved with Hewlett-Packard ChemStation software (Rev. A.04.02 on Windows 95 and Rev. A.05.02 on Windows NT 4.0) by using peak height ratios of (−)-LSG and (+)-LSG to the internal standard. Linear least-squares (unweighted) analysis was performed to obtain the best fit for the standard curve data and to calculate the experimental values.

2.7. Assay validation

Quality control samples were prepared in blank human plasma to contain 0.080, 0.20, 0.80, 1.60 and 4.00 μg/ml nominal concentrations of (−)-LSG and (+)-LSG. They were stored frozen along with experimental samples and used to ensure day to day accuracy of the method. Reproducibility and accuracy of the method were evaluated in triplicate on three different days. Relative standard deviation (RSD) and relative mean error (RME) were used as a measure for precision and accuracy, respectively. RSD is defined as 100×(SD/mean), and RME as 100×[(absolute value of the difference between the nominal and measured analyte concentrations)/(nominal analyte concentration)].

2.8. Sample stability

Plasma samples spiked with varying concentrations of (−)-LSG and (+)-LSG were prepared. One set was analyzed immediately. Other sets were allowed to remain at room temperature for 24 hrs, −70°C for 49 days, 6°C for 7 days, 40°C for 20 hrs, or undergo three freeze–thaw cycles. The freeze–thaw procedures were performed on three consecutive days. A cycle consisted of freezing for about 23 h at −70°C and thawing at room temperature (22°C) until samples equilibrated at ambient temperature (about 1 h).

3. Results and discussion

3.1. Chromatography

This method was developed for the on-going National Institute of Neurological Disorders and Stroke (NINDS)-sponsored clinical trial of (±)-LSG. It was designed to measure the concentration of LSG enantiomers after the addition of LSG to the antiepileptic regimen of phenytoin and/or carbamazepine and discontinuation of the latter two drugs. Therefore, the goals of the method were to separate the enantiomers and to avoid interferences from possible LSG metabolites and phenytoin and carbamazepine and their metabolites. Several cyclodextrin-based chiral HPLC columns were evaluated as well as reversed-phase columns with chiral modifiers (various cyclodextrins). The Chiradex column was found optimum for chiral separation of the compounds of interest. Different mobile phase compositions were evaluated, including various combinations of acetonitrile, methanol, different pH phosphate buffers, ammonium acetate, triethylamine and acetic acid. The optimum composition was found to consist of 0.01 *M* sodium dihydrogen phosphate pH 4.2, methanol, and acetonitrile. Fine tuning was accomplished by varying the percentage of methanol and acetonitrile and column temperature. Representative chromatograms of patient samples before and after a single oral dose of (±)-LSG and a spiked blank plasma are shown in Fig. 2. The elution time for LSG enantiomers was less than 15 min. Carbamazepine epoxide (CBZE), a CBZ metabolite was resolved from (−)-LSG. CBZ itself and PHT and its *p*-hydroxy metabolite (HPPH) eluted considerably later (enantiomeric *p*-hydroxy phenytoin metabolites were resolved). The entire run time under the isocratic conditions was nearly 60 min in order to allow elution of CBZ, PHT and HPPH. Because these compounds were not quantitated, a gradient ramp was established after elution of internal standard (I.S.) in order to decrease the run time and thereby increase the assay throughput. Attempts to differentially extract CBZ, CBZE, PHT and HPPH from the LSG compounds using liquid–liquid or solid-phase extraction prior to HPLC were not successful. With a gradient, it was possible to comfortably decrease the run time to 35 min. In addition, other anticonvulsant drugs and their metabolites (the two major in vitro metabolites of LSG, AO714 and AO725; ESM; PB; PHB; PRM; VPA; FBM; GBP; topiramate; LTG; VGB) were also shown not to interfere with the analysis of (+)-LSG and (−)-LSG.

One of the drawbacks of chiral cyclodextrin-based columns is, by manufacturer's admission, the relative lack of column ruggedness in comparison with reversed-phase columns. Even with relatively clean samples and the inclusion of guard and saturator columns, it was our experience that a significant loss of resolution became noticeable after 300 samples.

3.2. Extraction

A simple one-step liquid–liquid extraction was employed. The extraction efficiency was determined at several concentrations of (−)-LSG (0.125–3.0 μg/ml) and (+)-LSG (0.25–3.0 μg/ml) and was found to be greater than 90% in all instances.

Initially, we have developed a solid-phase extraction method using Oasis HLB solid-phase cartridges (Waters, Milford, MA, USA). This extraction method also yielded greater than 90% recovery of the analytes. However, we subsequently developed and selected the above simple liquid–liquid extraction as the preferred method because it gave visually and chromatographically ''cleaner'' samples.

3.3. Quantitation

The lower limit of detection (LOD) for non-extracted (−)-LSG and (+)-LSG was found to be 0.0078 μg/ml which yielded a signal/noise ratio of

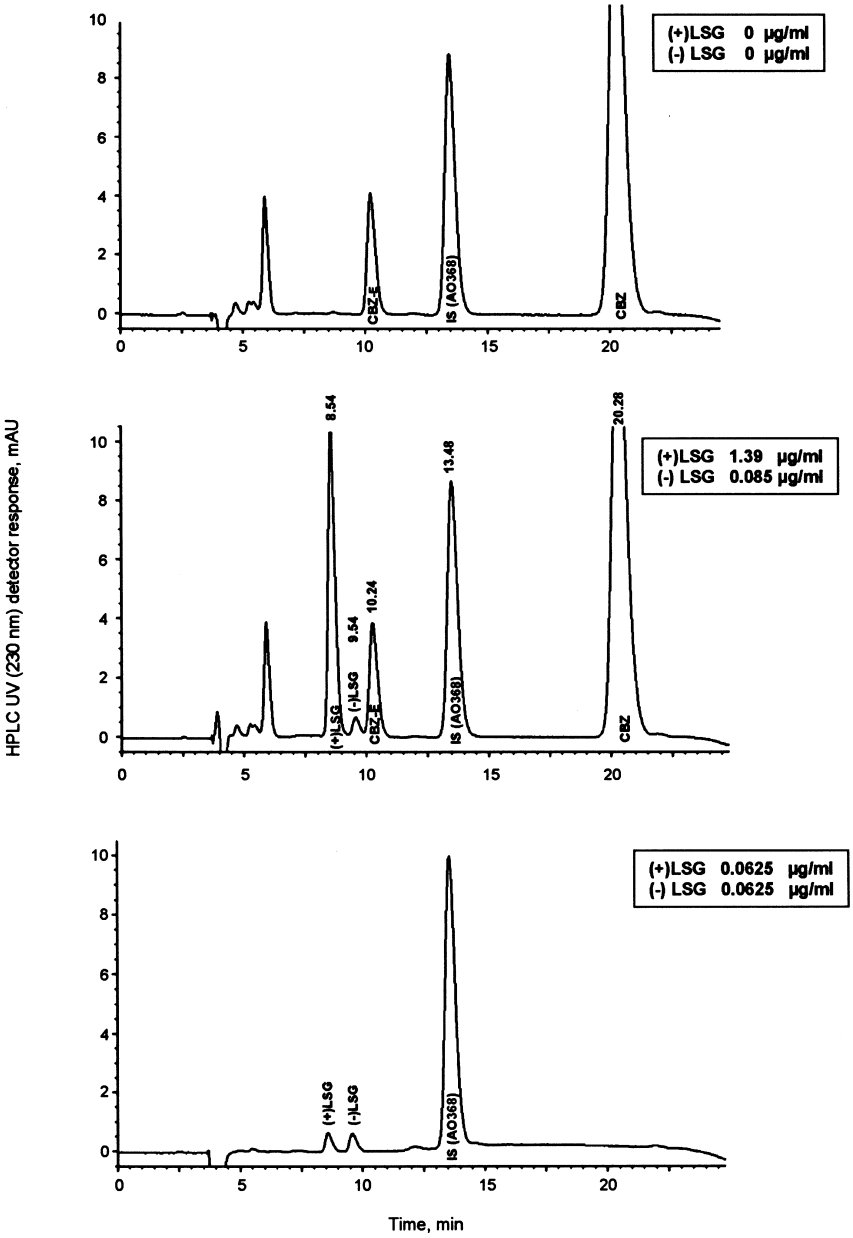

Fig. 2. HPLC chromatograms of patient blank plasma, prior to receiving (±)-LSG (top panel), patient plasma sample 1.5 h after a single oral dose of 500 mg (±)-LSG (middle panel), and a blank plasma spiked with (+)-LSG and (−)-LSG.

about 4 for both enantiomers. The lower limit of quantitation (LOQ) was 0.030 μg/ml for each enantiomer. Standard calibration curves were designed in a concentration range of 0.03125 to 5.0 μg/ml of (−)-LSG and (+)-LSG to bracket the anticipated concentration range. The lowest standard represents an injection of 1.25 ng of LSG enantiomer on column. Because this constituted a large concentration range, the calibration standards were divided to cover two standard curves, 0.03125 to 0.50 and 0.50 to 5.0 μg/ml. This led to an improvement in the accuracy and precision at the lower

Table 1
Intra-assay accuracy and precision data for determination of (−)-LSG and (+)-LSG in human plasma

[Nominal] (μg/ml)	[Measured (−)-LSG] (μg/ml)			[Measured (+)-LSG] (μg/ml)		
	Mean±SD (*n*)	RSD (%)	RME (%)	Mean±SD (*n*)	RSD (%)	RME (%)
0.080	0.081±0 (3)	0	1.3	0.081±0.003 (3)	3.7	1.3
0.20	0.21±0.01 (3)	4.8	5.0	0.21±0 (3)	0	5.0
0.80	0.81±0.01 (3)	1.2	1.3	0.81±0.01 (3)	1.2	1.3
1.60	1.60±0.02 (3)	1.3	0	1.59±0.02 (3)	1.3	0.6
4.00	3.97±0.05 (3)	1.3	0.8	3.95±0.04 (3)	1.0	1.3

concentration range and was used throughout the study. The linear regression coefficients (r^2) for both standard curves were routinely >0.99. Quality control samples were prepared to contain 0.08, 0.20, 0.80, 1.60, and 4.00 μg/ml of (−)-LSG and (+)-LSG in blank human plasma. Assay validation was performed in triplicate with these quality control samples on three different occasions. The intra- and inter-assay data are summarized in Tables 1 and 2, respectively. Good accuracy and reproducibility were evident throughout the concentration range for both enantiomers. An injection volume of 2 μl was used to minimize any effects on chromatography and column lifetime while maintaining the desired sensitivity. It should be possible to further decrease the lower limit of quantitation by using a larger proportion of the final sample volume for injection.

Consistent with another report [11], there was no evidence of racemization.

3.4. Metabolites

There was no evidence of metabolites in non-hydrolyzed patient samples. However, after glucuronidase/arylsulfatase hydrolysis, small

Table 2
Inter-assay accuracy and precision data for determination of (−)-LSG and (+)-LSG in human plasma[a]

[Nominal] (μg/ml)	[Measured (−)-LSG] (μg/ml)			[Measured (+)-LSG] (μg/ml)		
	Mean±SD (*n*)	RSD (%)	RME (%)	Mean±SD (*n*)	RSD (%)	RME (%)
0.080	0.080± 0.004 (9)	5.0	0	0.078±0.005 (9)	6.4	2.5
0.20	0.21±0.01 (9)	5.0	5.0	0.21±0.01 (9)	5.0	5.0
0.80	0.80±0.04 (9)	5.0	0	0.80±0.04 (9)	5.0	0
1.60	1.57±0.04 (9)	2.5	1.9	1.58±0.04 (9)	2.5	1.3
4.00	3.86±0.11 (9)	2.8	3.5	3.90±0.10 (9)	2.6	2.5

[a] Combined data for three assays performed on three different days.

amounts of AO-714 and AO-725 could be detected and quantitated.

3.5. Stability

The analytes were shown to be stable in plasma under most storage conditions, including seven weeks at −70°C, seven days at 6°C, 24 h at room temperature, and during three freeze–thaw cycles. The only exception was storage at 40°C for 20 h which resulted in significant losses of the LSG enantiomers (Table 3).

3.6. Applicability of the assay to clinical samples

The assay is currently being used in an on-going NINDS-sponsored clinical trial of LSG. It has been shown suitable for single dose pharmacokinetic studies of (±)-LSG even in the presence of phenytoin and/or carbamazepine co-medication. Representative plasma concentration–time profiles for AO242 and AO294 following a single does of (±)-LSG are depicted in Fig. 3. It can be clearly seen that the two enantiomers exhibit vastly different pharmacokinetic behavior as was observed in human volunteers [11]. The apparent plasma clearance of (+)-LSG was more than 10-fold greater than that of (−)-LSG.

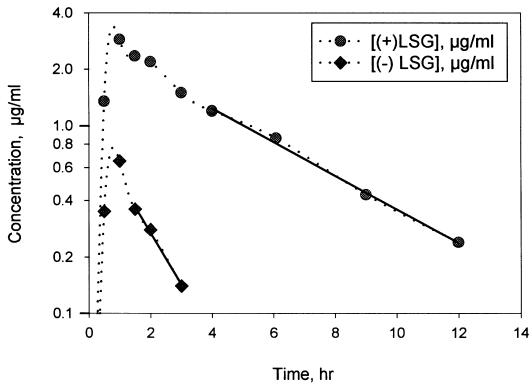

Fig. 3. Plasma concentration–time profile for (+)-LSG and (−)-LSG in a patient after a single oral dose of 500 mg (±)-LSG. Symbols depict actual data and the dashed lines are fitted values. The terminal elimination phase for the two enantiomers is depicted by linear least-squares regression generated solid lines.

4. Conclusions

A simple method consisting of a single-step liquid–liquid extraction and a HPLC analysis using a chiral column and UV detection was developed and validated for enantiomers of (±)-LSG in human

Table 3
Stability data (values are expressed as percentages after normalization to the corresponding nominal concentrations and represent a mean±SD for triplicate samples)

Storage conditions	Enantiomer	Nominal concentrations (μg/ml)				
		4.00	1.60	0.80	0.20	0.08
Fresh	(+)-LSG	103±3	109±2	104±1	105±5	
	(−)-LSG	103±4	107±1	100±1	110±5	
7 weeks	(+)-LSG	99±3	105±3	95±4	95±5	
−70°C	(−)-LSG	99±6	104±3	96±4	90±0	
7 days	(+)-LSG	96±2	98±3	106±0	105±5	98±1
6°C	(−)-LSG	97±2	99±3	105±0	105±5	95±1
24 h	(+)-LSG	97±2	99±2	105±3	105±5	99±3
22°C	(−)-LSG	98±2	100±1	104±3	105±0	91±3
3 Freeze–	(+)-LSG	95±2	97±1	99±3	105±0	98±1
thaw cycles	(−)-LSG	96±1	98±1	99±3	105±0	94±0
20 h	(+)-LSG	74±9	81±3	85±5	85±5	76±3
40°C	(−)-LSG	73±9	79±4	83±5	80±5	68±3

plasma. The method is presently being employed in a clinical trial for this new candidate anticonvulsant drug.

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