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Chiral separation of Frovatriptan isomers by HPLC using amylose based chiral stationary phase

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Abstract

A stereospecific HPLC method for separation of Frovatriptan enantiomers in bulk drug and pharmaceutical formulations was developed and validated on a normal-phase amylose derivertized chiral column. The effects of the organic modifiers namely 2-propanol, ethanol and diethyl amine (DEA) in the mobile phase were optimized to obtain the best enantiomeric separation. Calibration curves were linear over the range of 200–6150 ng/mL, with a regression coefficient (R^2) of 0.9998. The limit of detection (LOD) and limit of quantification (LOQ) were 65 ng/mL and 200 ng/mL, respectively. The method was accurate and precise and suitable for the intended purpose. Analysis results were compared with the results obtained by using a validated chiral CE method and found to be in very good agreement. This method can be successfully applied to the enantiomeric purity analysis of Frovatriptan in pharmaceutical bulk drug samples and formulations.

Keywords: Frovatriptan; Enantiomeric separation; HPLC; Amylose based stationary phase and validation

1. Introduction

Separation of enantiomers has become very important in analytical chemistry, particularly in the pharmaceutical and biological fields, because some enantiomers of racemic drugs have relatively different pharmacokinetic properties and diverse pharmacological or toxicological effects [1–4]. This is one of the most vital reasons why the regulatory authorities insist more on stringent investigation for evaluating the safety and the effectiveness of drugs containing chiral centers. Enantiomeric separations have acquired importance in all the stages of drug development and the commercialization process. Therefore, the development of new methods for efficient chiral separations mainly based on HPLC, capillary electrophoresis (CE) or gas chromatography (GC) is more than necessary. Among the chromatographic methods so far developed, HPLC methods based on chiral stationary phases are widely employed for the assays of drug isomers in pharmaceutical preparations and biological fluids [5–7].

Frovatriptan, administered as a single enantiomer (*R*)-(+)-3-(methylamino)-1,2,3,4-tetrahydro-9H-carbazole-6-carboxamide (Fig. 1) is a potent 5-hydroxytryptamine receptor agonist. Frovatriptan reverses cerebral vasodilation by activating 5-HT_{1B}, and it prevents neurogenic inflammation by activating 5-HT_{1D} [8]. Frovatriptan is more potent than other tryptamine derivative with a half-life of 26 h, which means its active ingredient remains in the blood for at least 20 h, longer than any other triptan. Compared with sumatriptan and naratriptan, Frovatriptan has a four-fold higher affinity to 5-HT_{1B}.

The yield of (*R*)-Frovatriptan is dependant on the configuration of pyroglutamic acid, which is a commercially available reagent and (*R*)-Frovatriptan is predominantly the major product in the presence of L-pyroglutamic acid. We have earlier separated the enantiomers of Frovatriptan using cyclodextrin mediated capillary electrophoretic method [9]. A well-known limitation of capillary electrophoresis is limited sensitivity caused by small injection volumes and detector optics owing to online detection [10].

The present HPLC method is comparatively more sensitive with detection limits of 65 ng/mL. A survey of literature shows that no chiral HPLC method has been reported for separating the enantiomers of Frovatriptan. In this study, a simple and efficient

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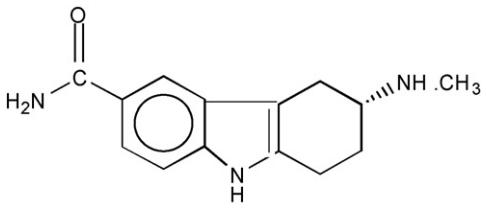

Fig. 1. Structure of (*R*)-Frovatriptan.

chiral HPLC was developed and validated for the separation of enantiomers of Frovatriptan with commercially available chiral stationary phase (Chiralpak AD-H). The developed method is also applied to the bulk drug and pharmaceutical formulations for evaluating enantiomeric purity.

2. Experimental

2.1. Reagents and materials

Chiralpak AD, AD-H and AD-RH, Chiralcel OD, OD-H and OD-RH, Chiralcel OJ and OB columns were procured from Diacel Chemical Industries (Tokyo, Japan). *n*-Hexane (HPLC grade) was purchased from S.D. Fine Chemical Ltd. (Mumbai, India). 2-Propanol was purchased from Merck (Mumbai, India). Ethanol (Absolute) was obtained from Tedia Company Inc. (Fairfield, OH, USA) and Diethyl amine (DEA; 99.5%, redistilled) was purchased from Aldrich Chemicals Co., Inc. (Milwaukee, WI, USA). Racemic mixture and various batches of bulk drug of Frovatriptan were obtained from Bulk Actives-III, Dr. Reddy's (Bollaram, Hyderabad, India). (*S*)-Frovatriptan standard (chemical purity: 99.81%; chiral purity: 99.97%) and (*R*)-Frovatriptan standard (chemical purity: 99.90%; chiral purity: 100%) were obtained by purifying the racemic mixture and bulk drug, respectively on a preparative HPLC. Frovatriptan tablets (Migard 2.5 mg film coated tablets) were obtained from A. Menarini Pharmaceuticals UK Ltd.

2.2. Chromatographic equipment and operating conditions

The equipment consisted of Waters 510 HPLC pump, Waters 717 plus auto sampler and Waters 2996 PDA detector (Waters Corp., Milford, MA, USA). The output signal was monitored and integrated using Waters Empower software (Build 1154). The analysis and validation was performed on Chiralpak AD-H (250×4.6) mm column, preceded with a guard column. The mobile phase was prepared by mixing *n*-hexane, ethanol and diethyl amine in the ratio of 70:30:0.5 (v/v/v) and degassed using a vacuum degasser before use. The flow rate was set to 1.0 mL/min, and the column was maintained at ambient temperature. The injection volume was 10 μL and the detector wavelength was tuned at 245 nm.

The column was flushed with a mixture of *n*-hexane and 2-propanol (90:10, v/v) at the end of each day at a flow rate of 0.5 mL/min for 1 h in order to regenerate after using the mobile phase containing DEA.

A preparative HPLC consisting of Shimadzu LC-8A pump, SPD-6AV UV–vis detector, FCV-100B fraction collector and SCL-8A system controller was used for obtaining standards of (*S*)-Frovatriptan and (*R*)-Frovatriptan using a semi-preparative Chiralpak-AD (250 mm × 10 mm) column. A mobile phase consisting of a mixture of *n*-hexane, ethanol and diethyl amine in the ratio of 70:30:0.5 (v/v/v) was pumped at a flow rate of 3.0 mL/min, with the column maintained at ambient temperature. A 1 mL aliquot of a 15 mg/mL solution of the racemic mixture of Frovatriptan was injected repeatedly and the eluent was monitored at 245 nm.

2.3. Sample preparation

2.3.1. Bulk drug

About 10 mg of the bulk drug was weighed in a 10 mL volumetric flask. To this, 2 mL of ethanol was added and sonicated to dissolve the bulk drug. The volume was made up to mark with the mobile phase (*n*-hexane:ethanol:DEA, 70:30:0.5, v/v/v) and the solutions were filtered through 0.2 μm syringe filters.

2.3.2. Tablets

In a mortar five tablets of Frovatriptan (2.5 mg film-coated tablets), which were previously weighed, were pulverized. The powder equivalent to 10 mg of Frovatriptan was weighed and transferred into a 10 mL volumetric flask. To this, 2 mL of ethanol was added and sonicated for 15 min. A volume of 3 mL mobile phase was added and sonicated for 10 min. The volume was finally made up to mark with the mobile phase and the solutions were filtered through 0.2 μm syringe filters.

3. Results and discussion

3.1. Method development

To achieve separation between enantiomers of Frovatriptan, chiral stationary phases (CSPs) containing cellulose and amylose derivatives were evaluated with suitable mobile phase compositions. The chiral discrimination of enantiomers occurs when they bind with the stationary phase forming transient diastereomeric complexes. The most important interactions between the analyte and the CSP are hydrogen bonding, dipole–dipole interactions, and pi–pi interactions, together with the rigid structure (cellulose-based CSP) or helical structure (amylose-based CSP) of the chiral polymer bound to the support [11]. Various combinations of *n*-hexane:2-propanol and *n*-hexane:ethanol were used as the mobile phase in our initial efforts in the normal-phase separation. These trials were made initially in the absence of DEA and then by adding DEA to the mobile phase. Attempts to separate the enantiomers on cellulose carbamate derivertized columns in normal-phase (Chiralcel OD and Chiralcel OD-H) proved futile. Even with the use of cellulose ester derivertized columns (Chiralcel OJ and Chiralcel OB), the enantiomers could not be separated.

The separation was attempted in reversed phase using cellulose and amylose carbamate derivertized columns (Chiralcel OD-RH and Chiralpak AD-RH) with mobile phases consisting

of mixtures of borate buffer (pH 8.5) with acetonitrile or potassium dihydrogen phosphate buffer (pH 7.0) with acetonitrile in various ratios. No separation could be achieved in reversed phase chiral stationary phases. Poor resolution or no resolution would be due to poor affinity of the enantiomers to the CSP or due to the difficulty of the inclusion of analyte into the chiral cavity.

The enantiomers could be separated only on amylose carbamate derivertized CSP (Chiralpak AD and Chiralpak AD-H) with mobile phase comprising either mixtures of *n*-hexane, 2-propanol and DEA or *n*-hexane, ethanol and DEA. As expected owing to the smaller particle size of the stationary phase the Chiralpak AD-H column yielded a better separation. In the case of CSPs with carbamate derivatives, binding of solute to the CSPs is achieved through the interactions between the solutes and the polar carbamate groups on CSPs [12]. Solutes can bind to the carbamate groups on the CSPs forming transient diastereomers through hydrogen bonding using the C=O and NH groups and also through dipole–dipole interaction using the C=O moiety. Frovatriptan has NH and H_2N–C=O (amide) functional groups and these could well be contributing to the interactions with the carbamate groups on CSP, resulting in separation. The aromatic ring on the solute could provide additional stabilizing effect to the solute–CSP complex as reported by Wainer et al. [13].

The use of ethanol in mobile phase provided better selectivity and resolution than 2-propanol (Fig. 2a and b). A comparison of the system suitability results obtained using ethanol and 2-propanol clearly indicate ethanol is the solvent of choice (Table 1). The addition of DEA up to 0.5% (by volume) to the mobile phase resulted in improved peak shapes, better resolution and shorter run times.

The effect of ethanol concentration, DEA concentration, temperature and flow rate on resolution (R_S), retention time (t_R) and selectivity (α) were examined and the most optimum conditions were found to be a mobile phase consisting of *n*-hexane:ethanol:diethyl amine (70:30:0.5, v/v/v) at flow rate of 1.0 mL/min with the column maintained at ambient temperature.

3.2. Limit of detection and limit of quantitation

The limits of detection and quantitation of Frovatriptan enantiomers were estimated by obtaining the detector signal for the peaks and by performing serial dilution of a solution of known concentration. The limits of detection and quantitation were found to be 65 ng/mL and 200 ng/mL, respectively with the peak signal to noise ratios of about 2–3 at LOD level and 9–12 at LOQ level [14]. These results suggest that the proposed LC method is sufficiently sensitive for the determination of Frovatriptan enantiomers.

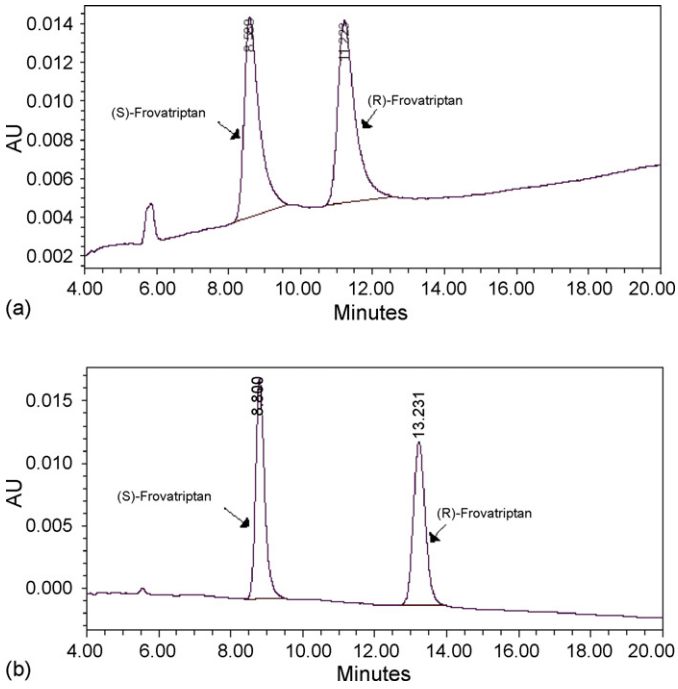

Fig. 2. (a) Effect of 2-propanol on separation of Frovatriptan enantiomers; mobile phase *n*-hexane:2-propanol:DEA (75:25:0.5, v/v/v). (b) Effect of ethanol on separation of Frovatriptan enantiomers; mobile phase *n*-hexane:ethanol:DEA (70:30:0.5, v/v/v).

3.3. Precision (repeatability and reproducibility)

The repeatability of the method was evaluated by calculating the RSD in area of the unwanted isomer in spiked samples for six replicate injections and the reproducibility was expressed in terms of RSD in area obtained for analyses performed on three consecutive days, six times each day. The precision studies for (*S*)-Frovatriptan were performed at the limit of quantification (LOQ) and at 1% of analyte concentration. The results were precise for estimation of the unwanted isomer (Table 2).

3.4. Linearity and range

The linearity of the method was studied over a concentration covering a range of 200 ng/mL (LOQ) to 6150 ng/mL (1.2% of analyte concentration). The standard drug was spiked with (*S*)-Frovatriptan at concentrations of 200, 2050, 3070,

Table 1
System suitability report for Frovatriptan enantiomer separation

Mobile phase	Compound	t_R	*N*	R_s	α	*T*
n-Hexane:2-propanol:DEA (75:25:0.5, v/v/v)	(*S*)-Frovatriptan	8.59	2170	–	–	1.66
	(*R*)-Frovatriptan	11.22	2864	3.20	1.35	1.63
n-Hexane:ethanol:DEA (70:30:0.5, v/v/v)	(*S*)-Frovatriptan	8.80	6312	–	–	1.32
	(*R*)-Frovatriptan	13.23	7687	8.28	1.57	1.14

t_R: Retention time (min), *N*: theoretical plates, R_s: resolution, α: selectivity and T: tailing factor.
Chromatographic conditions: column: Chiralpak AD-H (250 × 4.6) mm, 5 μm with guard column, flow: 1.0 mL/min and UV: 245 nm.

Table 2
Precision for (*S*)-Frovatriptan

	% RSD
Repeatability at LOQ level ($n=6$)	
Retention time	0.01
Peak area	3.69
Repeatability at 1% level[a]	
Retention time	0.01
Peak area	0.75
Intra-day precision at 1% concentration[a]	
Retention time	0.02
Peak area	0.54
Inter-day precision at 1% level[a]	
Retention time	0.52
Peak area	1.36

[a] $n=6$ determinations.

Table 3
Accuracy data for (S)-Frovatriptan

Amount spiked (μg/mL)	Amount recovered[a] (μg/mL)	Percentage recovery
0.20	0.21 ± 0.08	103.0
3.17	3.13 ± 0.04	98.9
4.04	4.03 ± 0.09	99.8
4.86	4.88 ± 0.04	100.4

[a] $n=3$ determinations.

4100, 5120 and 6150 ng/mL and injected in triplicate. The data were subjected to linear regression analysis. The calibration graph showed good linearity, with a regression coefficient of 0.9998. The straight-line equation for (*S*)-Frovatriptan was $y = 80318x + 1886.1$.

3.5. Accuracy

The accuracy of the method was evaluated by spiking the standard drug with known amounts of the unwanted isomer ((*S*)-isomer) at LOQ, 0.8, 1.0 and 1.2% of analyte concentration in triplicate. The recoveries were calculated from the slope and the intercept obtained for the calibration curve of (*S*)-Frovatriptan standard. The recoveries ranged from 98.9 to 103.3% (Table 3). A typical chromatogram of spiked standard is shown in Fig. 3.

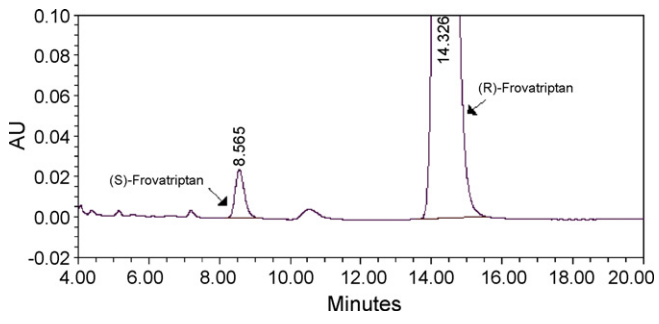

Fig. 3. A typical chromatogram of (*R*)-Frovatriptan spiked with 1% (w/w) (*S*)-Frovatriptan.

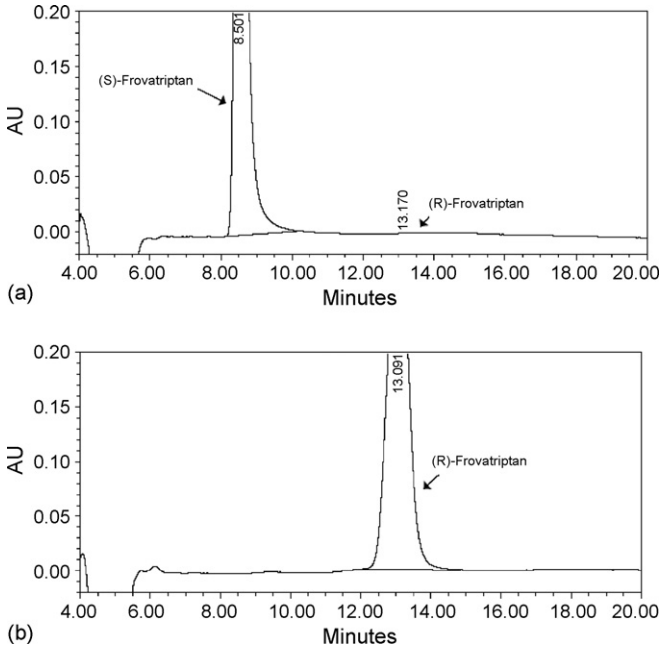

Fig. 4. (a) A typical chromatogram of (*S*)-Frovatriptan standard (chemical purity: 99.81%; chiral purity: 99.97%). (b) A typical chromatogram of (R)-Frovatriptan standard (chemical purity: 99.90%; chiral purity: 100%).

3.6. Stability in solution

Standard solutions of (*S*)-Frovatriptan and (*R*)-Frovatriptan were prepared in the mobile phase at analyte concentration. Each standard solution was analyzed immediately after preparation (Fig. 4a and b) and divided into two parts. One part was stored at 2–8 °C in a refrigerator and the other at bench top in tightly capped volumetric flasks. The stored solutions of each isomer were reanalyzed after 24 h. No change in either the chemical or enantiomeric purity was observed. The area obtained for each isomer after 24 h did not show any significant change compared with the area of initial analysis. This indicates that both isomers were stable in the mobile phase for at least 24 h when stored either at 2–8 °C or at bench top.

4. Chiral analysis of bulk drug and formulations

Various batches of bulk drug and formulations were analyzed in triplicate and (*S*)-Frovatriptan was quantified. The results

Table 4
Chiral analysis of Frovatriptan bulk drug and formulation batches

Sample	Batch	% (*S*)-Frovatriptan[a]	
		By HPLC	By CE
Bulk drug	A	0.14 ± 0.02	0.13 ± 0.03
	B	0.10 ± 0.01	0.10 ± 0.02
	C	32.42 ± 0.04	32.45 ± 0.05
	D	0.07 ± 0.01	0.08 ± 0.01
	E	0.12 ± 0.00	0.12 ± 0.02
Formulation	I	0.11 ± 0.01	0.10 ± 0.02
	II	0.08 ± 0.02	0.08 ± 0.02

[a] $n=3$ determinations.

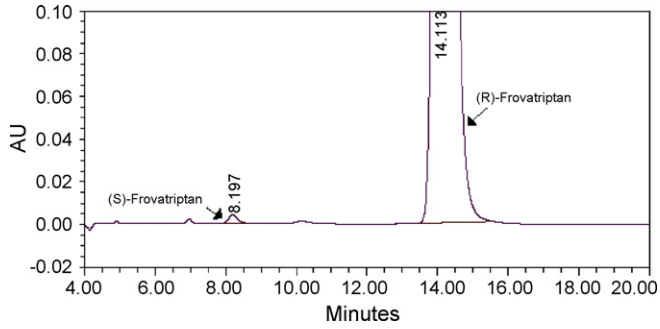

Fig. 5. A typical chromatogram of bulk drug containing 0.07% of unwanted isomer.

show consistency between determinations with a low standard deviation as shown in Table 4. The results of analysis for various batches of bulk drug and formulation were compared with the results obtained by our previously developed chiral CE method and were found to be in very good agreement. A typical chromatogram of the bulk drug is shown in Fig. 5.

5. Conclusion

A chiral HPLC method for the separation of Frovatriptan enantiomers was developed and validated. The chiral separation was achieved in amylose carbamate derivertized column (Chiralpak AD-H). This method is simple, accurate and has provided good linearity, precision and reproducibility. The results of analysis obtained with this HPLC method and a validated CE method are comparable. The practical applicability of this method was tested by analyzing various batches of the bulk drug and formulations of Frovatriptan.

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