CHAPTER 4

CHIRAL SEPARATION OF FROVATRIPTAN ENANTIOMERS:
DEVELOPMENT AND VALIDATION OF A CE METHOD USING
CYCLODEXTRIN AS A CHIRAL SELECTOR AND A HPLC METHOD
USING AMYLOSE BASED CHIRAL SELECTOR
4.1 Introduction to Frovatriptan Succinate

Most of the biological interactions and reactions are subject to varying degrees of stereoselectivity, therefore the stereochemical resolution of optically active molecules is an important and essential step in the development of biologically active potential drugs. A number of biological processes e.g., absorption, protein binding, selective tissue uptake of renal or biliary excretion etc. are a few evidences amongst many of the stereoselective metabolic activities that take place in living organisms[1]. About 40% of the pharmaceutical drugs are optically active i.e. contain enantiomers. As enantiomers of racemic drugs may have relatively different pharmacokinetic properties and diverse pharmacological or toxicological effects [2-5], separation of enantiomers has become very important in analytical chemistry, particularly in the pharmaceutical and biological fields. While one enantiomer has the desired therapeutic property, the other enantiomer may simply be inactive, may have the opposite effect or even be toxic. This is one of the most vital reasons, why the regulatory authorities insist more stringent investigation for evaluating the safety and effectiveness of drugs containing chiral centers. Enantiomeric separations have acquired importance in all the stages of drug development and the commercialization process whenever the drug is known to be chiral.

The development of new analytical methods for efficient chiral separations and precise quantifications of the unwanted isomers
using different separation techniques such as 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 [6-11]. As HPLC is a well-developed and rugged technique, this is the first technique of choice across industries and academia. Further, there are several suppliers of commercial chiral stationary phases which can be used for chiral separation of most of the pharmaceutical compounds.

Capillary Electrophoresis as a technique for the separation of enantiomers offers reduced analysis time, high speed, simplicity, high resolution and selectivity, low cost of analysis and small sample volume requirements. With the use of chiral selectors, resolution can be achieved even when there is a typical free energy difference of only 0.03 kJ/mol of interaction between the enantiomers and the chiral selector [12]. The costly columns packed with chiral stationary phases used in HPLC and GC can be avoided and separation can be achieved by the addition of small amounts of chiral selectors to the background electrolyte in CE. But a known limitation of capillary electrophoresis is poor sensitivity as compared to other techniques, because of the very small injection volumes and detector optics owing to online detection [13]. Nevertheless this technique also finds wide applications in micro
and nano analysis owing to the very small sample requirements in areas such as narcotics, drug abuse and forensics.

Frovatriptan which is administered as a pure enantiomer (R)-(+)-3-(Methylamino)-1,2,3,4-tetrahydro-9H-carbazole-6-carboxamide (Fig. 4.1) is a potent 5-hydroxytryptamine receptor agonist. Frovatriptan reverses cerebral vasodilation by activating $5$-$HT_{1B}$, and it prevents neurogenic inflammation by activating $5$-$HT_{1D}$ [14]. Frovatriptan is more potent than other tryptamine derivatives and has a half-life of 26 hours. This means that active ingredient in Frovatriptan formulations remains in the blood for at least 20 hours, longer than any other triptans which are in use. Compared with Sumatriptan and Naratriptan, Frovatriptan has a fourfold higher affinity to $5$-$HT_{1B}$.

Pyroglutamic acid is the starting material for Frovatriptan. The configuration of pyroglutamic acid which is a commercially available reagent used in the synthesis of Frovatriptan, determines the yield of (R)-Frovatriptan. The synthetic scheme of Frovatriptan is presented in Fig. 4.1. (R)-Frovatriptan is predominantly the major product in the presence of L-pyroglutamic acid as evident from the synthetic scheme. Traces of the D-isomer contained as an impurity in L-pyroglutamic acid can result in generation of the process related impurity (L)-Frovatriptan during the synthesis. As (L)-Frovatriptan is the undesired isomer present in the drug substance, this process related impurity needs to be controlled appropriately.
Chapter-4

Fig. 4.1 Synthetic scheme of (R)-Frovatriptan succinate monohydrate

A CE method was developed for the separation of Frovatriptan enantiomers in bulk drug and dosage forms using sulfo-butyl-ether-beta-cyclodextrin as the chiral selector in the BGE system [15]. This method is simple, offers speed and is cost effective.

A simple and efficient chiral HPLC was developed for the separation of enantiomers of Frovatriptan on a commercially available cellulose based chiral stationary phase [16]. The HPLC method is comparatively more sensitive with detection and quantitation limits of 65 and 200 ng/mL which correspond to 0.006 and 0.02% with respect to the analyte concentration.

Both the CE method and the HPLC methods that were developed are capable of separating unwanted ((S)-Frovatriptan)
isomer from (R)-Frovatriptan succinate and also in quantifying the unwanted isomer. These methods were utilized to have a control on the undesired isomer as an in-process check during the synthesis and for quality control analysis of the bulk drug and dosage forms.

### 4.2 Experimental

#### 4.2.1 Instrumentation

An Agilent Technologies 3D Capillary Electrophoresis system having a built-in diode-array detector was used to perform the chiral separations by CE. A bare fused silica capillary having an effective length of 72 cm (length to detector), a total length of 80.5 cm and 50-μm id, with extended light path having a bubble factor of 3 was used. (Agilent Technologies, Waldbronn, Germany). The Agilent ChemStation was used for system control, acquisition of the data and postrun processing.

The HPLC equipment for the separation studies comprised of Waters 510 HPLC pump, Waters 717 plus auto sampler and Waters 2996 PDA detector (Waters corp., Milford, MA, USA). A Chiralpak AD-H (250 x 4.6) mm column, preceded with a guard column was used for the chiral analysis and validation of the developed HPLC method. Waters Empower software (Build 1154) was used for monitoring the output signals from detector and processing the HPLC chromatograms.
The pure standards of (S)-Frovatriptan and (R)-Frovatriptan were obtained by purification of the racemic mixture on a preparative LC system consisting of Shimadzu LC-8A pump, SPD-6AV UV-Vis detector, FCV-100B fraction collector and SCL-8A system controller. Chiral purification of the racemic mixture was performed on a semi-preparative Chiralpak-AD (250 mm x 10 mm) column.

4.2.2 Chemicals and Reagents

Imidazole (AR grade) was obtained from Merck (India). SB-beta-CD was procured from Advasep® 4 (Cydex Inc, USA). Beta-cyclodextrin (Beta-CD), Heptakis (2,6-di-O-methyl)-beta-cyclodextrin (DM-beta-CD), Heptakis (2,3,6-tri-O-methyl)-beta-cyclodextrin (TM-beta-CD), (2-Hydroxypropyl)-beta-cyclodextrin (HP-beta-CD), 1.0 N and 0.1 N sodium hydroxide (HPCE grade) were procured from Agilent Technologies, (Waldbrohn, Germany). Disodium hydrogen orthophosphate, sodium dihydrogen orthophosphate (AR grade) were procured from S.D. Fine chemicals (Mumbai, India) and Qualigens (Mumbai, India) respectively. Acetonitrile, ethanol and methanol (HPLC grade) were procured from Rankem (New Delhi, India). Water was filtered and deionized with a Milli-Q, Millipore system, (Milford, MA, USA).

n-Hexane (HPLC grade) and Isopropyl alcohol were 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).

Frovatriptan samples and racemic mixture of Frovatriptan were obtained from Bulk-Actives-III of Dr. Reddy’s Laboratories Ltd., (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.

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).

4.2.3 Preparation of solutions

**Preparation of Phosphate Buffer solution for CE**

Appropriate amounts of disodium hydrogen orthophosphate and sodium dihydrogen orthophosphate were dissolved separately in 100 mL of Milli-Q water to obtain each buffer solution having a 50 mM concentration. The 50 mM disodium hydrogen orthophosphate solution was then titrated with 50 mM sodium dihydrogen orthophosphate to a pH of 7.0 using DMS 716 titrino autotitrator.
Preparation of Background electrolyte (BGE) for CE

About 400 mg of SB-beta-CD was dissolved in 10 mL of 50 mM phosphate buffer solution (pH 7.0) for CE by sonication. A 1.0 mL aliquot of acetonitrile was then added to 9.0 mL of this buffer solution and mixed to obtain the BGE (20 mM SB-beta-CD in 50 mM phosphate buffer solution (pH 7.0) : Acetonitrile (9:1 v/v)). The BGE was filtered through 0.2 μm nylon syringe filters.

Preparation of Sample solutions for CE Analysis

Bulk drug

Bulk drug sample solutions were prepared by dissolving 30 mg of the drug in 1.0 mL of acetonitrile by sonication. The volume was then made upto 10 mL with diluent (water: acetonitrile, 90:10 v/v). The sample solution was filtered through 0.2 μm nylon syringe filters.

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 flask, 2 mL of ethanol was added and sonicated for 15 minutes. A 3 mL aliquot of mobile phase was then added and sonicated for 10 minutes. The volume was finally
made up to mark with the mobile phase and the solutions were filtered through 0.2 µm nylon syringe filters.

**Preparation of Sample solutions for HPLC Analysis**

*Bulk drug*

Bulk drug sample solutions were prepared by accurately weighing about 10 mg of the bulk drug weighed in a 10 mL volumetric flask. A 2 mL aliquot of ethanol was added to this flask and sonicated to dissolve the sample. The sample was then diluted up to the marked volume 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.

*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 flask, 2 mL of ethanol was added and sonicated for 15 minutes. A 3 mL aliquot of mobile phase was then added and sonicated for 10 minutes. The volume was finally made up to mark with the mobile phase and the solutions were filtered through 0.2 µm nylon syringe filters.
4.2.4 Electrophoretic conditions and capillary preconditioning

A 30 kV voltage was applied with positive polarity setting (detector towards cathode terminal). The internal standard peak (imidazole) and the enantiomers were monitored at different wavelengths to maximize the sensitivities. A timed wavelength program using a diode array UV detector was adopted and Imidazole was monitored at 205 nm while Frovatriptan enantiomers were monitored at 245 nm. Hydrodynamic injections were made to inject samples by applying pressure at 50 mbar for 3 s followed by injection of a water plug by applying pressure at 50 mbar for 2 s.

Before use, new capillaries (bare fused silica) were flushed with 1.0 N NaOH for 20 min. and then flushed with CE grade water for 10 min. Prior to every use the capillary was conditioned by flushing for 5 min. with water, 2 min. with 0.1 N sodium hydroxide, 2 min. with water and then flushing for 15 min. with the BGE. Between successive runs, the capillary was flushed with the BGE for 2 min. to obtain a high precision in performance.

4.2.5 Chromatographic separation conditions

The HPLC separation was performed on Chiralpak AD-H (250 x 4.6) mm column. The HPLC mobile phase was n-Hexane, ethanol and diethyl amine in the ratio of 70:30:0.5 (v/v/v) which was prepared by mixing 700 mL of n-hexane, 300 mL of ethanol and 5 mL of diethylamine in a 1000 mL measuring jar and then degassed on a
vacuum degasser. A 1.0 mL/min flow rate was set for the mobile phase and the column was maintained at ambient temperature. A 10 µL aliquot of the samples were injected using the autosampler. The detector was tuned at a wavelength of 245 nm to monitor the eluent.

At the end of each day’s analysis, the column was conditioned with a solvent mixture containing n-Hexane and 2-propanol (90:10, v/v) with a 0.5 mL/min flow rate for about 1 hour. This facilitated to clean the column from residues of DEA from the mobile phase and avoid degeneration of the chiral stationary phase.

4.2.6 Validation of the methods

The developed CE and HPLC methods for determining the enantiomeric purity of Frovatriptan succinate were validated to demonstrate the applicability suitability of these methods for their intended use. The validation parameters included the assessment of specificity, precision (repeatability, intra-day and inter-day precision), limits of detection and quantitation, linearity and range, accuracy (as percentage recovery) and stability of the drug in analyte solution. The sample solutions were prepared separately as per the methods to validate the CE and the HPLC methods.
4.3 Results and Discussion

4.3.1 CE Method development and optimization

Effect of pH on separation

One of the important parameter which affects the electroosmotic flow (EOF) other than the applied voltage, buffer concentration, capillary temperature and organic modifiers is the pH of the background electrolyte directly. The EOF is directly proportional to the pH of background electrolyte. The pH also controls the ionizing ability of the analyte which depends on its pKa (ionization constant) thereby affecting the migration time and also the peak symmetry. Schmitt and Engelhardt [17] have shown that the migration order of racemic ephedrine was dependent on the pH of buffer. The effect of pH on the symmetry of Frovatriptan enantiomer peaks and the resolution between the enantiomers was evaluated using various buffers at pH of 2.5, 4.6, 7.0 and 9.3. Suitable buffers at each pH were prepared at a constant molar strength (50 mM) and the other separation parameters were kept constant.

Frovatriptan has a pKa value of 10.42 therefore it was expected to be considerably ionized at pH 9.3 and fully ionized under all other experimental conditions namely pH 2.5, 4.6 and 7.0. The background electrolytes at pH 2.5 (phosphate buffer), pH 4.6 (citrate buffer) and pH 7.0 (phosphate buffer) yielded symmetric peaks, however at a higher pH of 9.3 (borate buffer) the enantiomer peaks were found to be asymmetric and tailing. The bad peak shapes at pH 9.3 can be
attributed to partial ionization of Frovatriptan considering its pKa value. The chiral selector SB-beta-CD is highly ionized carrying a negative charge at almost all experimental pH values. The EOF is negligible at a pH of 2.5 and is not significant enough at pH 4.6 to drive this ionized cyclodextrins towards the cathode terminal which is towards the detector side in positive polarity mode. The SB-beta-CD moves counter current and ends up in migrating towards the anode terminal (positive terminal). Therefore the experiments at pH 2.5 and 4.6 using SB-beta-CD were performed in negative polarity mode such that the inclusion complex formed between the enantiomers and the cyclodextrin migrates towards the anode terminal (positive) towards the detector. Resolutions of 1.99 and 2.9 were obtained at pH 2.5 and 4.6 respectively in negative polarity. At a working pH of 7.0 and 9.3 the EOF is strong enough to move the highly ionized and negatively charged SB-beta-CD towards the outlet cathode terminal therefore experiments at pH 7.0 and 9.3 were performed in positive polarity mode. At pH 9.3 a resolution of about 2.65 was obtained between the enantiomers whereas at pH 7.0 the resolution was greater than 3.0.

The migration time of the enantiomers at pH 2.5, 4.6 and 9.3 was high i.e. the peaks migrated after 18 minutes or more. A high resolution (> 3.0) and short migration times (< 14 mins) could be achieved only with the use of phosphate buffer at pH 7.0. It can be inferred that at pH 7.0 there is significant EOF and the analyte molecules are also positively charged. Thus there is a collective force
which aids the cyclodextrin-analyte complex to migrate faster towards the negative outlet terminal. Based on the above findings the buffer solution at pH 7.0 was chosen for further studies.

**Optimization of the chiral selector type and concentration**

A majority of the chiral separations of pharmaceutical molecules achieved by capillary electrophoresis are performed using cyclodextrins as chiral selectors as they form favorable inclusion complexes with the drug molecules. Different cyclodextrins namely native Beta-CD, DM-beta-CD, TM-beta-CD, HP-beta-CD and SB-beta-CD were evaluated for separating the Frovatriptan enantiomers as chiral additives to the BGE.

The enantiomers failed to resolve when native Beta-CD, DM-beta-CD, TM-beta-CD and HP-beta-CD were used as chiral selectors. However with the used of SB-beta-CD, a complete resolution and a baseline separation of the enantiomers could be achieved. Further optimization trials were performed with SB-beta-CD only and the other cyclodextrins were not considered. The beta-CDs are cyclic oligosaccharides consisting of seven glucopyranose units. They form a truncated cone from the carbon skeleton which is hydrophobic in nature, whereas the outer containing the hydroxyl groups is hydrophilic.

Inclusion of bulky hydrophobic groups into the cavity supported by interactions of the hydroxy groups at C2 and C3 at the mouth of the cavity with hydrophilic groups of the analyte is the proposed chiral
recognition mechanism [18]. The charged cyclodextrins such as SB-beta-CD can be used to separate both the charged and neutral analytes. In the ionized state, the charged cyclodextrins migrate with their own electrophoretic mobility therefore, this approach can also be regarded as a kind of electrokinetic chromatography [19] wherein the cyclodextrin phase has a difference mobility when compared to the aqueous phase (BGE) forming a sort of pseudostationary phase.

The chiral selector interacts with the enantiomers preferentially and forms reversible and transient diastereomeric complexes or inclusion complexes. The tendency of the two enantiomers to form these complexes differs due to the differences in the spatial arrangements of the groups attached to the chiral carbon atom. The complex with a higher binding constant, due to its compact size migrates faster than the one with lesser binding constant.

The separations were performed using varying concentrations of SB-beta-CD ranging from 5 mM to 25 mM to study the effect of concentration on resolution. In all these optimization trials, the BGEs were prepared containing 10% acetonitrile as an organic modifier. In the BGE containing 5 mM SB-beta-CD the enantiomer peaks of Frovatriptan were found to be splitting. In these trails, there was an increase in both the enantio-resolution and migration time of the enantiomers, with increasing concentration of SB-beta-CD. However at higher concentrations of the cyclodextrin, an undesirable high current was observed without any significant increase in the
resolution. The high current can result in joule heating of the BGE, a decrease in the buffer viscosity due to heating and eventually a boiling BGE. Therefore the concentration of SB-beta-CD in the BGE system was fixed to 20 mM as it was found to be the most optimum choice considering the increase in resolution, increase in analysis time and an increase in the system current.

An overlay of electropherograms of the enantiomers with difference concentrations of SB-beta-CD are presented in Fig. 4.2. The figs. 4.3a and 4.3b demonstrate graphically the effect of SB-beta-CD concentration on migration times of the enantiomers and the resolution between them.

**Fig. 4.2 Effect of SB-beta-CD concentration on migration time and resolution**
Electrophoretic conditions: Capillary: 72 cm x 50 µm ID, ELP; BGE: 50 mM phosphate: Acetonitrile (90:10 v/v) containing SB-beta-CD; Voltage: + 30 kV; Capillary Cassette temp.: 25 °C.

Fig. 4.3a Effect of SB-beta-CD concentration on resolution

Fig. 4.3b Effect of SB-beta-CD concentration on migration time
Effect of organic modifiers and their concentration on separation

When an organic solvent is added to the background electrolyte it has an impact on several variables such as viscosity of the background electrolyte, the dielectric constant and the zeta potential. In some cases the addition of organic modifiers to the BGE becomes more than necessary as it aids in solubilizing the analytes and also alters the selectivities. Tsuda et al [20] have reported that electro-osmotic velocities obtained with acetonitrile, water and methanol were proportional to the ratios of their dielectric constants to their viscosities.

Different organic modifiers namely acetonitrile, methanol, ethanol and isopropyl alcohol were added to the BGE at 10% (v/v) concentration and their ability to improve the separation was evaluated. The organic modifier acetonitrile was found to produce the most pronounced affect when compared to the other organic modifiers yielding the highest resolution within shortest run times. The overlaid electropherograms for the experimental trials with different organic modifiers are presented in Fig. 4.4. A graphical representation of the resolution and migration time affect arising from different organic modifiers is made in Fig. 4.5a and 4.5b respectively.
Fig. 4.4 Effect of organic modifier on migration time and resolution

Electrophoretic conditions: Capillary: 72 cm x 50 μm ID, ELP; BGE: 50 mM phosphate and organic modifier; (90:10 v/v); 20 mM SB-beta-CD; Voltage: +30 kV; Capillary Cassette temp.: 25 °C.

Fig. 4.5a Effect of organic modifier type on resolution
Wren and Rowe [21-23] have proposed a model to explain the effects of addition of organic solvent on separation. The authors have demonstrated with a mathematical model that the degree of separation depends on the concentration of the chiral selector and that there is an optimum concentration of the chiral selector which yields the maximum separation. Wren and Rowe have presented the role of the organic solvents methanol and acetonitrile on the separation of propranolol enantiomers. The theoretical aspects of the influence of organic modifiers on efficiency and resolution have also been extensively studies by other authors [24-25].

During the complex formation, the hydrophobic part of the analyte molecule enters into the hydrophobic cavity of the cyclodextrin. Therefore when an organic solvent is added to the background electrolyte, this solvent would reduce the affinity of the analyte towards the cyclodextrin cavity, and would in turn increase
the analyte affinity towards the buffer electrolyte. Thus upon addition of an organic solvent to the background electrolyte containing a cyclodextrin, the formation of analyte-cyclodextrin complex will be less favored. Interestingly, this may in turn either increase the separation or decrease the separation depending upon whether the concentration of the cyclodextrin is more than or less than the optimum concentration in the original system. The change in analyte-cyclodextrin binding ability will depend on the polarity of the organic solvent; a non-polar solvent such as acetonitrile is expected to have a more pronounced effect than a polar solvent such as methanol. The decrease in the analyte-cyclodextrin binding ability (decrease in the equilibrium constant) is proportional to the volume of the organic solvent added to the background electrolyte.

The effect of acetonitrile concentration on separation was studied by changing its concentration from 5-20%. Both resolution and migration times decreased with increase in acetonitrile concentration. The decrease in resolution can be explained by the fact that the concentration of cyclodextrin was at the optimum value for an organic free buffer. The decrease in the migration times upon addition of acetonitrile could be due to the increased EOF resulting from a reduced buffer viscosity. The capillary surface area is also modified upon addition of an organic solvent affecting the zeta potential. At 10% (v/v) concentration, acetonitrile provided a fair compromise between resolution and the run time therefore this concentration was
eventually finalized in the BGE system. An overlay of the electropherograms to elucidate the effect of acetonitrile concentration is presented in Fig. 4.6. The graphical representation of the impact of acetonitrile ratio on resolution and migration times is presented in Fig. 4.7a and 4.7b respectively.

**Fig. 4.6 Effect of acetonitrile concentration on migration time and resolution**

*Electrophoretic conditions: Capillary: 72 cm x 50 µm ID, ELP; BGE: 50 mM phosphate: Acetonitrile; 20 mM SB-beta-CD; Voltage: + 30 kV; Capillary Cassette temp.: 25 °C.*
Effect of temperature on separation

The separation was carried out at different temperatures ranging from 15 to 30°C keeping the other separation parameters constant. There was a minor diminishing effect of the increasing capillary temperature on the resolution although not very significant so as to have an adverse impact on the separation. This behavior was expected can be explained based on the fact that lower temperatures
favor the formation of cyclodextrin-analyte complexes which results in a better resolution. The migration times decreased significantly as the experimental temperature was increased due to the decrease in the viscosity of the BGE. As the higher working temperatures may result in joule heating and higher baseline noise, the working temperature was optimized to 25 °C (Fig. 4.8).

**Fig. 4.8 Effect of capillary cassette temperature on migration time and resolution**

_Electrophoretic conditions: Capillary: 72 cm x 50 µm ID, ELP; BGE: 50 mM phosphate: Acetonitrile (90:10 v/v); 20 mM SB-beta-CD; Voltage: +30 kV;_
4.3.2 HPLC Method development and Optimization

The chiral stationary phases (CSPs) play the most important role in bringing about chiral separation by HPLC. A large variety of compounds such as native or derivatized amino acids, derivatized polysaccharides, cyclodextrins, proteins, chiral crown ethers, macrocyclic antibiotics have been successfully used as chiral stationary phases [26]. The polysaccharide based CSPs namely cellulose and amylase derivatives have become very popular due to their ease of use, reproducibility in achieving the separation and a wide range of applications [27-31] and are commercially available.

HPLC columns containing different chiral stationary phases (CSPs) of cellulose and amyllose derivatives were evaluated to achieve enantioresolution between Frovatriptan enantiomers using various mobile phase systems. As discussed earlier, the chiral discrimination is a result of the enantiomers binding with the stationary phase to form transient diastereomeric complexes which differ in their binding constants. 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 (amyllose-based CSP) of the chiral polymer bound to the support [32]. In the initial efforts, different mobile phase systems comprising of n-Hexane and isopropyl alcohol, n-Hexane and ethanol in different proportions were used in the normal phase chromatographic separation. These trials were made initially in the
absence of an organic modifier such as DEA but the peaks were found to be tailing due to the secondary retention. The later trials were made by including DEA as an organic modifier in the mobile phase. The Frovatriptan enantiomers could not be resolved on cellulose carbamate derivertized columns (Chiralcel OD and Chiralcel OD-H) in normal phase mode and these attempts proved futile. No separation of the enantiomers could be achieved when separation was attempted in columns containing cellulose ester derivertized stationary phases (Chiralcel OJ and Chiralcel OB) too.

As a shift from normal phase to reversed phase can provide an alternate selectivity due to a significant difference in the polarities of the stationary phase and the mobile phase, the enantiomeric resolution was evaluated in reversed phase columns containing cellulose and amylose carbamate derivertized stationary phases (Chiralcel OD-RH and Chiralpak AD-RH). The mobile phases in reversed phase mode used were mixtures of borate buffer (pH 8.5) with acetonitrile or potassium dihydrogen phosphate buffer (pH 7.0) with acetonitrile in different proportions. The enantiomers could not be resolved in reversed phase chiral stationary phases. When the enantiomers have poor affinity or no affinity to the CPS or when the inclusion of enantiomers into the chiral cavity is difficult either insufficient and unacceptable or totally no resolution between the enantiomers is achieved.
The Frovatriptan enantiomers could be successfully resolved only on an amylose carbamate derivertized CSP (Chiralpak AD and Chiralpak AD-H) using mixtures of n-Hexane, 2-propanol and DEA or n-Hexane, ethanol and DEA as the mobile phase. The separation on the Chiralpak AD-H column was better when compared to the separation on Chiralpak AD column. This is an expected phenomenon and can be attributed to the smaller particle size of the stationary phase (5µm) as against 10µm in the latter case. This is best explained by the Van deempter theory of separation and shall not be discussed here.

The binding of solute to the carbamate derivatives CSPs is achieved through the interactions between the solutes and the polar carbamate groups present on the CSPs [33]. The enantiomeric solutes can bind to the carbamate groups on the CSPs through hydrogen bonding using the C=O and NH groups and also through dipole-dipole interaction using the C=O moiety. Frovatriptan has a secondary amine (NH) and an amide (H₂N-C=O) functional groups. The resultant separation can be explained based on the fact that these functional groups present in the analyte molecules could well be contributing to the interactions with the carbamate groups on CSP. An additional stabilizing effect on the solute-CSP complex is also obtained by the presence of an aromatic ring on the solute as reported by Wainer et al. [34].
Selectivity and resolution were better with the mobile phase containing ethanol when compared to the mobile phase containing isopropyl alcohol which is presented in Fig.4.9a and 4.9b. Higher resolution (R), higher selectivity (α), higher theoretical plates (N) and more symmetric peaks (T) were obtained with ethanol as compared to isopropyl alcohol. The system suitability results obtained using ethanol and isopropanol are presented in Table-4.1 and these clearly indicate that ethanol is the solvent of choice. When DEA was added to the mobile phase upto 0.5% (by volume), improved peak shapes, better resolution and shorter analysis times could be achieved. As the analyte molecules are basic in nature the addition of DEA helps in keeping the analyte in an un-ionized state and also in avoiding the secondary retentions arising from the presence of basic moieties on the analyte.

The optimization of the mobile phase and the flow rate was performed by evaluating the effects of ethanol concentration, DEA concentration, column temperature and flow rate on resolution (Rs), retention time (tR) and selectivity (α). 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 a flow rate of 1.0 mL/min with the column maintained at ambient temperature.
**Fig. 4.9a: Effect of 2-propanol on separation of Frovatriptan enantiomers**

Mobile phase n-Hexane:2-propanol:DEA (75:25: 0.5 v/v/v)

**Fig. 4.9b: Effect of Ethanol on separation of Frovatriptan enantiomers**

Mobile phase n-Hexane:Ethanol:DEA (70:30: 0.5 v/v/v)
Table 4.1  HPLC System suitability for Frovatriptan enantiomers

<table>
<thead>
<tr>
<th>Mobile phase</th>
<th>Compound</th>
<th>$t_R$</th>
<th>$N$</th>
<th>$R_s$</th>
<th>$\alpha$</th>
<th>$T$</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Hexane: 2-propanol:DEA (75:25:0.5 v/v/v)</td>
<td>(S)-Frovatriptan</td>
<td>8.59</td>
<td>2170</td>
<td>--</td>
<td>--</td>
<td>1.66</td>
</tr>
<tr>
<td></td>
<td>(R)-Frovatriptan</td>
<td>11.22</td>
<td>2864</td>
<td>3.20</td>
<td>1.35</td>
<td>1.63</td>
</tr>
<tr>
<td>n-Hexane: Ethanol:DEA (70:30:0.5 v/v/v)</td>
<td>(S)-Frovatriptan</td>
<td>8.80</td>
<td>6312</td>
<td>--</td>
<td>--</td>
<td>1.32</td>
</tr>
<tr>
<td></td>
<td>(R)-Frovatriptan</td>
<td>13.23</td>
<td>7687</td>
<td>8.28</td>
<td>1.57</td>
<td>1.14</td>
</tr>
</tbody>
</table>

$t_R$: Retention time (min), $N$: Theoretical plates, $R_s$: Resolution, $\alpha$: Selectivity and $T$: Tailing factor.

Chromatographic conditions: Column: Chiralpak AD-H (250 x 4.6) mm, 5 µ with guard column, Flow: 1.0 mL/min and UV: 245 nm.

4.3.3 Validation of the Methods

The optimized CE method and the HPLC method for determining the chiral purity and quantification of was validated for quantification of S-Frovatriptan were validated in accordance with the ICH guidelines for analytical method validation [35]. The CE method was validated using imidazole while as the internal standard while no internal standard was used in the HPLC method. The CE method validation included the demonstration of specificity, precision in migration time and peak area, linearity, accuracy and stability of drug in solution. The HPLC method validation involved the specificity, precision, linearity and range, accuracy and stability in analytical solution.

Specificity

Specificity of a method is its ability to detect the component of interest without any interference. As per the ICH Q2B guideline, Specificity is the ability to assess unequivocally the analyte in the
presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Specificity is often demonstrated as the spectral purity of the peaks of interest which may be the drug peak or its related impurities. The specificity of the method is also evaluated by the physical inspection of the chromatogram/electropherogram to ensure that there is no co-elution of other components with the targeted component. In both the CE method and the HPLC method, a complete resolution of the Frovatriptan enantiomers was achieved. Further, there was no other component of significant response found to co-elute with the enantiomers. The photodiode array detection method was applied to evaluate the peak purity and homogeneity of the peaks. A typical electropherogram of racemic Frovatriptan in the presence of internal standard is shown in Fig. 4.10 which shows a baseline separated enantiomers and a complete resolution between the other minor unknown components, the Frovatriptan enantiomers and the internal standard. A representative HPLC chromatogram of Frovatriptan sample (R)-Frovatriptan spiked with (S)-Frovatriptan is presented in Fig. 4.11. A complete resolution and no interference of other components can also be observed in the HPLC method.
Fig. 4.10 Electropherogram of Frovatriptan enantiomers spiked with the internal standard imidazole.

Fig. 4.11: A typical HPLC chromatogram of (R)-Frovatriptan spiked with (S)-Frovatriptan
**Precision**

*Precision of the CE method*

The precision of the CE method was determined as the repeatability, intraday and inter-day precision (intermediate precision) of migration times and corrected peak area ratios. In capillary electrophoresis, the peak area is dependent on the electrophoretic mobility of that component. The analytes which migrate slower result in spending more time in the detector cell and thus give a higher response. Therefore the corrected areas which are obtained by dividing the peak area by the migration time of that peak is used which remains constant and corrects the differences in responses arising from differential electrophoretic mobilities. The corrected peak area ratio is obtained as the ratio of the corrected peak area of analyte peak and the internal standard.

To evaluate the repeatability of the CE method, six replicate injections of a standard solution spiked with the unwanted isomer at Limit of Quantitation (LOQ) level and at 1.0% level were performed. For these six injections, the relative standard deviation (RSD) for migration time, correct peak area and corrected peak area ratios were calculated (Table-4.2). It can be observed that the precision in corrected peak area ratios which is calculated with the internal standard is better than the precision of corrected peak areas itself. Since a not so good precision of the hydrodynamic injection system of the CE is a known phenomenon, the use of internal standard is
necessary in order to compensate the poor precision observed with the hydrodynamic injection, and hence to achieve good method precision [36].

**Table-4.2 CE Precision data for (S)-Frovatriptan**

<table>
<thead>
<tr>
<th></th>
<th>LOQ level</th>
<th>1.0% level</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Repeatability</strong>*</td>
<td>% RSD</td>
<td>% RSD</td>
</tr>
<tr>
<td>Migration time</td>
<td>0.68</td>
<td>0.25</td>
</tr>
<tr>
<td>Corrected peak area</td>
<td>8.33</td>
<td>1.74</td>
</tr>
<tr>
<td>Corrected peak area ratio</td>
<td>3.53</td>
<td>1.50</td>
</tr>
<tr>
<td><strong>Intra-day precision</strong>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Migration time</td>
<td>0.83</td>
<td>0.88</td>
</tr>
<tr>
<td>Corrected peak area</td>
<td>5.86</td>
<td>5.21</td>
</tr>
<tr>
<td>Corrected peak area ratio</td>
<td>3.42</td>
<td>2.13</td>
</tr>
<tr>
<td><strong>Inter-day precision</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Migration time</td>
<td>1.35</td>
<td>1.61</td>
</tr>
<tr>
<td>Corrected peak area</td>
<td>4.70</td>
<td>3.33</td>
</tr>
<tr>
<td>Corrected peak area ratio</td>
<td>1.86</td>
<td>1.22</td>
</tr>
</tbody>
</table>

* n = 6 determinations

The intermediate precision of the CE method was evaluated by performing 6 determinations on each day of analysis for three different days. The results of intermediate precision (Table-1) also suggest that the precision in corrected peak area ratios was as good as the precision obtained in repeatability and intraday precision experiments. The RSD was less than 1.61 % in migration time and
3.53% in corrected peak area ratios considering the repeatability, intraday precision and the intermediate precision.

**Precision of the HPLC method**

The repeatability of the HPLC method was evaluated by as a measure of the RSD in area of the unwanted enantiomer in the spiked sample for six replicate injections. The reproducibility of the HPLC method was assessed as the RSD in area obtained for analyses performed on three different days, with six determinations on each day. The precision studies for (S)-Frovatriptan were performed at the limit of Quantification (LOQ) and at 1.0% of analyte concentration. The HPLC precision results for the retention time and the peak areas presented in Table-4.3 endorse that the method is indeed precise and reproducible for estimating the enantiomeric purity of Frovatriptan.

**Table-4.3 HPLC Precision for (S)-Frovatriptan**

<table>
<thead>
<tr>
<th>Validation parameter</th>
<th>Percentage RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Retention time</td>
</tr>
<tr>
<td>Repeatability at LOQ level (n=6)</td>
<td>0.11</td>
</tr>
<tr>
<td>Repeatability at 1.0% level*</td>
<td>0.20</td>
</tr>
<tr>
<td>Intra-day precision at 1.0% concentration*</td>
<td>0.05</td>
</tr>
<tr>
<td>Inter-day precision at 1.0% level*</td>
<td>0.52</td>
</tr>
</tbody>
</table>

* n = 6 determinations
Linearity

Linearity of the CE method

The linearity of the CE method was evaluated as the proportional change in the detector response to the varying sample concentrations. The (S)-Frovatriptan was spiked to the standard drug at five different concentrations ranging from LOQ (3.0 µg/mL) to 2.0% (60 µg/mL) of the nominal analyte concentration. Triplicate analyses were performed for each sample that was spiked with the unwanted isomer and the internal standard. The corrected peak area ratios were plotted against the corresponding concentrations and the regression coefficients calculated using the least squares method. The linear equation for (S)-Frovatriptan was $y=0.0326x + 0.081$ with a coefficient of regression ($R^2$) of 0.9986.

Linearity of the HPLC method

The linearity of the HPLC method was studied in the concentration range of LOQ (200 ng/mL) to 1.2% (6150 ng/mL) of the analyte concentration. The standard drug was spiked with (S)-Frovatriptan at concentrations of 200, 2050, 3070, 4100, 5120 and 6150 ng/mL and triplicate determinations were made. The HPLC linearity data were subjected to linear regression analysis. The HPLC calibration graph showed an excellent linearity of the response with respect to the analyte concentration. A regression coefficient of 0.9998
was obtained and the straight-line equation for (S)-Frovatriptan was
\[ y = 80318x + 1886.1. \]

**Limits of detection (LOD) and quantification (LOQ)**

*Sensitivity of the CE method*

Limit of detection of a compound is defined as the lowest concentration that can be detected. The LOD demonstrates the sensitivity of the method in detection of the compound. A signal-to-noise ratio of approximately 2-3 is considered acceptable as the detectable limits. The LOD of each of the enantiomers in the CE method was found to be 1.0 \( \mu g/mL \) which corresponds to 0.03% with respect to the analyte concentration. The limit of quantification is the lowest concentration of a compound that can be quantified using the method with reasonably acceptable precision and accuracy. A concentration with a typical signal-to-noise ratio of 9-12 is regarded as the LOQ level. The LOQ level for each of the Frovatriptan enantiomers in the CE method was found to be 3.0 \( \mu g/mL \) which corresponds to 0.09% with respect to the sample concentration. Thus the CE method was found to be sensitive enough to detect and quantify the unwanted isomer.

*Sensitivity of the HPLC method*

The limits of detection and quantitation of Frovatriptan enantiomers by the HPLC method were determined by obtaining the
detector response for the enantiomer peaks. Serial dilutions of a racemic solution of known concentration were performed and injected to obtain the signal response. The limits of detection and quantitation of the HPLC method were found to be 65 ng/mL and 200 ng/mL respectively yielding signal-to-noise ratios of about 2-3 at LOD level and 9-12 at LOQ level respectively for the enantiomer peaks. The LOD of the HPLC method with respect to the analyte concentration is 0.0065% and the LOQ of this method respect to the analyte concentration is 0.02%. These results suggest that the developed LC method is sufficiently sensitive and infact more sensitive than the CE method for the determination of Frovatriptan enantiomers. The higher sensitivity of HPLC method when compared to the CE method is an expected phenomenon owing to the differences in the detector optics, the optical pathlengths and the amount of samples injected in both the techniques.

**Accuracy**

**Accuracy of the CE method**

Accuracy of the CE method was demonstrated by performing recovery experiments. The accuracy samples were prepared in triplicate by independently spiking (S)-Frovatriptan to the standard drug at levels of LOQ, 0.8%, 1.0% and 1.2% with respect to the sample concentration. The percentage recoveries for the unwanted isomer were calculated from the slope and intercept obtained from the
standard curve. The percentage recoveries of the unwanted isomer ranged from 98.75 to 105.38% as shown in Table-4.4

**Table-4.4 Accuracy of the CE method for (S)-Frovatriptan**

<table>
<thead>
<tr>
<th>Amount spiked (µg/mL)</th>
<th>Amount recovered* (µg/mL)</th>
<th>Percentage recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.02</td>
<td>5.29 ± 0.10</td>
<td>105.38</td>
</tr>
<tr>
<td>24.03</td>
<td>24.32 ± 1.46</td>
<td>101.21</td>
</tr>
<tr>
<td>30.04</td>
<td>30.84 ± 3.03</td>
<td>102.66</td>
</tr>
<tr>
<td>36.05</td>
<td>35.60 ± 0.73</td>
<td>98.75</td>
</tr>
</tbody>
</table>

* n=3 determinations

**Accuracy of the HPLC method**

The accuracy of the method was evaluated by spiking the standard drug with known amounts of the unwanted isomer ((S)-Frovatriptan) at levels of LOQ, 0.8, 1.0 and 1.2% of the analyte concentration. The spiking at each level was done independently in triplicate. The recoveries were calculated from the slope and intercept obtained for the calibration curve of (S)-Frovatriptan standard. The recoveries in the HPLC method ranged from 98.9 to 103.3% which are presented in Table-4.5.

**Table-4.5 Accuracy of HPLC method for (S)-Frovatriptan**

<table>
<thead>
<tr>
<th>Amount spiked (µg/mL)</th>
<th>Amount recovered* (µg/mL)</th>
<th>Percentage recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.20</td>
<td>0.21 ± 0.08</td>
<td>103.0</td>
</tr>
<tr>
<td>3.17</td>
<td>3.13 ± 0.04</td>
<td>98.9</td>
</tr>
<tr>
<td>4.04</td>
<td>4.03 ± 0.09</td>
<td>99.8</td>
</tr>
<tr>
<td>4.86</td>
<td>4.88 ± 0.04</td>
<td>100.4</td>
</tr>
</tbody>
</table>

* n=3 determinations
Stability of the drug in analyte solution

**Stability in CE method**

To evaluate the stability of the sample in solution prepared for CE analysis, samples were injected immediately after preparation and analyzed for the content of unwanted isomer. The sample solution was stored at benchtop at room temperature for 24 hours and then re-analyzed. No significant difference in either the peak area ratios or in the enantiomeric composition was observed which confirms the integrity of the drug in the analyte solution. The results concluded that there was no degradation or inter conversion of the enantiomers for at least 24 hours.

**Stability in HPLC sample solution**

Standard solutions of (S)-Frovatriptan and (R)-Frovatriptan were prepared at the analyte concentration using the diluent. After preparation, each standard solution was analyzed immediately. The chromatograms of the pure enantiomers are presented in Fig. 4.12 and Fig. 4.13. Each of the standard solutions were then divided into two parts. While one part was stored at 2-8 °C in a refrigerator, the other part of the same solution was stored at bench top in tightly capped volumetric flasks. The solutions stored in the refrigerator and at bench top were analyzed again after 24 hours. There was no change in the enantiomeric purity and no new peaks were observed. Further, the peak area of each enantiomer after 24 hours did not show any
significant difference when compared to the area of initial samples. It can be concluded that both the enantiomers were stable in the diluent for at least 24 hours when stored either at 2-8 °C or at room temperature.

**Fig. 4.12:** A typical chromatogram of (S)-Frovatriptan standard
(Chem purity: 99.81%; Chiral purity: 99.97%)

**Fig. 4.13:** A typical chromatogram of (R)-Frovatriptan standard
(Chemical purity: 99.90%; chiral purity: 100.00%)
4.3.4 Determination of Enantiomeric purity of Frovatriptan Bulk Samples and Formulations (Tablets)

Various batches of bulk drug and formulations were analyzed using the validated CE and HPLC methods. Each sample was analyzed in triplicate and the content of (S)-Frovatriptan was determined. The results by both techniques were found to be reproducible and a good agreement between the CE and HPLC results for the enantiomeric content was obtained as presented in Table-4.5. The results demonstrate the complementarity of the CE and HPLC methods for determining enantiomeric purity of Frovatriptan.

A typical HPLC chromatogram of a real time sample (bulk drug) analysis is shown in Fig-4.14.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Batch</th>
<th>% (S)-Frovatriptan*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>By HPLC</strong></td>
</tr>
<tr>
<td>Bulk Drug</td>
<td>A</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>32.42 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>0.12 ± 0.00</td>
</tr>
<tr>
<td>Frova® 2.5 mg</td>
<td>I</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>tablets</td>
<td>II</td>
<td>0.08 ± 0.02</td>
</tr>
</tbody>
</table>

*n= 3 determinations

Table-4.6 Chiral analysis of Frovatriptan bulk drug and tablets
4.4 Conclusion

Two analytical methods involving different separation techniques were developed for determining the enantiomeric purity of Frovatriptan succinate, a drug used for the treatment of migraine. While the separation between the enantiomers was achieved using SB-beta-CD as the chiral selector in Capillary Zone Electrophoresis method, the enantio-resolution in the HPLC method was obtained on commercially available cellulose based chiral stationary phase.

The CZE method was found to be simple, cost effective and efficient for estimating the enantiomeric purity. The effect of various electrophoretic parameters on the enantio-resolution were studied. The pH of the background electrolyte, the type of cyclodextrin and its concentration in the BGE, the type and concentration of organic modifiers and the capillary temperature were found to have an impact
on the separation efficiencies. The best electrophoretic conditions were chosen from the method optimization trials to finalize the method.

The separation of Frovatriptan enantiomers in the HPLC method was achieved on an amylose carbamate derivertized (Chiralpak AD-H) column. Different commercially available chiral stationary phases in normal phase and reverse phase were evaluated for their ability to enantio-separate the isomers. The effects of mobile phase modifiers (ethanol and isopropyl alcohol) and the mobile phase additive (diethylamine) on separation were studied to select the best separation conditions.

Both methods were validated as per the ICH guidelines to establish their suitability of use. The CE and HPLC methods were found to be specific, linear, accurate, precise and reproducible. The methods were utilized to estimate the content of (S)-isomer in the bulk drug samples of Frovatriptan and also in Frovatriptan tablets.
4.5 References


16. Muzaffar Khan, Balaji Viswanathan, D. Sreenivas Rao, Rajasekhar Reddy, Chiral separation of Frovatriptan isomers by


31. Dingenen J., Polysaccharide phases in enantioseparations, in *A Practical Approach to Chiral Separations by Liquid


35. ICH, (Q2B): Validation of analytical procedures-methodology, November 1996.