chiral HPLC method for separation and quantification of EZT and TRA enantiomers in bulk and their pharmaceutical formulation.

**Experimental**

**Chemicals and reagents**

Working standards of (±)-EZT and (±)-TRA were gifts from Ranbaxy Laboratories Ltd., New Delhi, India and Rilpivirine was gifted from Dr. Reddy’s Laboratory Ltd., Hyderabad, Telungana, India. Acetonitrile (MeCN), Methanol (MeOH) are HPLC grade and diethylamine (DEA), formic acid (FA) other reagents of analytical grade were from SD Fine Chemicals (Mumbai, India). The HPLC-grade water was collected by using Milli-Q water system (Millipore Academic, Bangalore, India).

**HPLC instrumentation and conditions**

The study was performed by using Shimadzu (Japan) chromatography equipped with an LC-20 AD and LC-20 ADvp solvent-delivery module, an SPD-20A PDA detector, rheodyne model 7125 injector valve fitted with a 20µL sample loop. The system was controlled through a system controller (SCL-10A) and a personal computer using a Shimadzu chromatographic software (LC Solution, Release 1.11SP1) installed on it. The mobile phase was degassed using a sonicator (Branson Ultrasonics Corporation, USA). Absorbance spectra were recorded using a UV-Visible spectrophotometer (Model UV-1601PC, Japan) employing quartz cell of 1 cm path length.

Chiral separation of EZT and TRA stereoisomers were carried out on a Chiralpak-ASR chiral column (150 mm × 4.6 mm i.d., 3.0 µm) connected with a guard cartridge (10 mm ×4.0 mm i.d.). The binary mobile phase consisted mixture of MeCN, MeOH (99:1.0 % v/v), and 0.1% formic acid, 0.1% diethylamine mobile phase additives were added. In order to increase the sensitivity for the less concentrated compound and to decrease the background from mobile phase a wavelength of 225 nm were selected for detection. An injection volume of the sample was 20 µL. The HPLC system was used in an air-conditioned laboratory atmosphere (25 ± 2°C).

**Stock and working standard solutions**

Standard stock solutions of (±)-EZT and (±)-TRA (1.0 mg mL−1) were prepared in mobile phase. The prepared stock solution was stored at 4°C protected from the light. The working standard solutions were freshly obtained by diluting the stock standard solutions with mobile phase during the analysis day. Calibration curves reporting peak area ratios of R- and S- EZT, and IS versus drug concentrations were established in the range of 2.0 - 10 µg/ml for R-EZT and S- EZT, 1.0 - 5.0 µg/ml for S-TRA and R-TRA for all the analytes in presence of IS (5.0 µg ml−1).

**Results and Discussion**

**Method development and optimization**

Selection of stationary phase: The selection of an appropriate column is the most important step in method development of chiral separation. The development of direct chiral HPLC methods by employing polysaccharide Chiral Stationary Phase (CSP) in Polar Organic (PO) mode has gained considerable attention [21]. It offers advantages of being fast, efficient and cost-effective in chiral analysis. Therefore, in this study chiral separation was performed using polysaccharide CSPs in PO mode. Preliminary screening studies were carried out to identify the suitable chiral stationary phase for the simultaneous enantiomeric separation of EZT and TRA. In this direction enantiomeric separation of EZT and TRA was
performed on various amylose and cellulose based polysaccharide Chiral Stationary Phases (CSPs) - Lux-Amylose-2 (amylose tris (5-chloro-2-methylphenylcarbamate), Chiralpak-ASH (amylose tris (3,3-dimethylphenyl-carbamate), Chiralpak- ADH (amylose tris (S)-1-phenylethyl-carbamate), Lux-2 (cellulose tris (3-chloro-4-methylphenylcarbamate) and Lux-4 (cellulose tris (4-chloro-3-methylphenylcarbamate) at varying compositions of polar and intermediate polar organic solvents. Among the selected CSPs, only Chiralpak AS-H afforded a partial enantiomeric separation of EZT and TRA in polar organic mode. Hence, Chiralpak AS-H was further explored to achieve quality separation through optimization of the mobile phase composition.

Choice of mobile phase solvents & effect of additives: The mobile phase consisting of ACN/MeOH (99/1 % v/v) showed partial enantioresolution of the analytes with poor peak shape. Hence, it was decided to introduce acidic and basic organic modifiers like Acetic Acid (AA), formic acid (FA) diethylamine (DEA) and triethylamine (TEA) for further improvement on the enantiomeric resolution were incorporated into the mobile phase. The effects of organic mobile phase additives on the racemates separation were investigated. When 0.1% DEA was added to the mobile phase, TRA enantiomers were well separated whereas, EZT enantiomers remained with partial separation. When 0.1% FA was added into the mobile phase, EZT enantiomers were separated but TRA enantiomers were partially resolved. Hence, a mixture of DEA (0.1% v/v) and FA (0.1% v/v) was incorporated into the mobile phase.

The mobile phase composition containing a mixture of MeCN/MeOH/DEA/FA viz, 99/1/0.1/0.1 % v/v/v/v respectively, resulted in a good enantioresolution of EZT and TRA. A reasonable runtime was obtained with 1.0 mL min⁻¹ flow rate. The elution order of R-EZT, S-EZT, S-TRA and R-TRA enantiomers was found to be 2.12, 2.40, 4.01, and 4.50 min. respectively. The corresponding chromatogram was shown in Figure 2b. The method development summary and system suitability was given in Table 1.

Choice of internal standard: To select a suitable Internal Standard (IS) for the analysis, various drug substances were examined. Among the tested compounds, Rilpivirine (IS) met all the typical requirements of a compound to be used as an IS, i.e. it was stable during the analysis, well resolved, and its elution time was shorter than that of last eluting analyte peak.

Method validation

The proposed liquid chromatographic method was validated by following ICH guidelines. Validation parameters like selectivity, specificity, linearity, limit of detection and quantification, accuracy, precision, stability and robustness were addressed.

Specificity: The specificity of the method was evaluated by assessing the chromatograms of most commonly used excipients (starch, lactose monohydrate, methyl cellulose, titanium dioxide and magnesium stearate) with that of the standard drugs. There were no excipient peaks co-eluted with the analytes, indicating that the optimized assay method is selective and specific in relation to the excipients used in this study. All placebo chromatograms showed no interference peaks Figure 2a.

Table 1: Method development summary and system suitability results.

<table>
<thead>
<tr>
<th>#</th>
<th>Chromatographic conditions</th>
<th>Remarks</th>
<th>System suitability</th>
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</thead>
<tbody>
<tr>
<td>1.</td>
<td>SP: Amylose-2 (250 mm x 4.6 mm, 5µ)</td>
<td>No enantiomeric separation was observed for both EZT and TRA.</td>
<td>k₁</td>
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<td></td>
<td>MP: ACN:MeOH:DEA:FA (99/1/0.1% v/v/v/v)</td>
<td></td>
<td>-</td>
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<tr>
<td></td>
<td>Flow rate: 1.0 ml/min</td>
<td></td>
<td></td>
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<tr>
<td>2</td>
<td>SP: Lux-2 (250 mm x 4.6 mm, 5µ)</td>
<td>EZN enantiomeric peaks were not resolved, and TRA enantiomers were partially separated.</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>MP: MeCN:MeOH:DEA:FA (99/1/0.1% v/v/v/v)</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Flow rate: 1.0 ml/min</td>
<td></td>
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<tr>
<td>3</td>
<td>SP: Lux-4 (250 mm x 4.6 mm, 5µ)</td>
<td>No enantiomeric separation of EZT but peak broadening was observed for TRA.</td>
<td>-</td>
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<tr>
<td></td>
<td>MP: MeCN:MeOH:DEA:FA (99/1/0.1% v/v/v/v)</td>
<td></td>
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<tr>
<td></td>
<td>Flow rate: 1.0 ml/min</td>
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<td></td>
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<tr>
<td>4</td>
<td>SP: Chiralpak-ADH (150 mm x 4.6 mm, 5µ)</td>
<td>EZN was Partially separated</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>MP: MeCN:MeOH:DEA:FA (99/1/0.1% v/v/v/v)</td>
<td>Good separation was observed in TRA.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Flow rate: 1.0 ml/min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>SP: Chiralpak-ASH (150 mm x 4.6 mm, 5µ)</td>
<td>Excellent enantio separations were observed for EZT and TRA.</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>MP: MeCN:MeOH:DEA:FA (99/1/0.1% v/v/v/v)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Flow rate: 1.0 ml/min</td>
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</table>

*SP: Stationary Phase, *MP: Mobile Phase.
Accuracy: The accuracy of the method was determined by analyzing Quality Control (QC) standards prepared at three levels of 80, 100 and 120% of the expected assay value or label claim of the analytes in the commercial formulation. QC samples were prepared as three replicates at each concentration level by spiking the standard drugs with the placebo excipients, which were left overnight to allow matrix-analyte interactions to occur. The recovery of the analytes at each level (n = 3) and mean % recovery (n = 9) were determined and % accuracy was expressed as [(calculated amount/predicted amount) × 100]. Accuracy, assessed by spike recovery, in which the % recovery of both enantiomers it is at each level (n = 3) and mean % recovery (n = 9) were determined and % accuracy was accepted. The recoveries of enantiomers at each level were found well within the acceptable criteria of bias, ± 2.0 %. The mean recovery (n = 9) for each enantiomer was also tested for significance by using Student t-test. Since the t calculated (t calc) is less than the t critical (t crit) at 5% significance level, the null hypothesis (the recovery is unity or 100%) was accepted.

Precision: The precision was established by injecting three different concentrations of each enantiomer (2.0, 6.0) µg/mL for EZP and 1.0, 3.0, and 5.0 µg/mL for TRA with 5 µg/mL - 1 of IS each in six replicates, for intraday precision (repeatability) and on three consecutive days for the intermediate precision (reproducibility). Precision was expressed by the %RSD of the analyte peak area. Results for all studied compounds met the proposed requirement %RSD ≤ 3%. The intra and inter-day precision (n = 6.0) was confirmed since, the % CV were well within the target criterion of ≤ 2.0 and ≤ 3.0 respectively.

Robustness: The robustness of the proposed method was assessed to provide an indication of its reliability during normal usage with respect to small, but deliberate variations in experimental parameters such as variations in MeCN concentration (99% ± 0.5%), the flow rate (1.0 ± 0.05) and the formic acid (0.1 ± 0.02 %) did not alter the assay values of both enantiomers more than 1.0 % and therefore it would be concluded that the method conditions are robust.

Application of the method

The proposed HPLC method was applied to the quantitative estimation of commercially available tablet dosage forms of EZT (Athez 10) and TRA (Acupain). Assay results obtained for Athez tablets were found to be 5.1 mg of R-EZT and 4.48 mg of S- EZT respectively. When analyzing the Acupain tablets, the obtained results were, 99.78 (24.8 mg) of S-TRA and 99.66 (24.7 mg) of R-TRA respectively. Good agreement was found between the assay results and the label claim of the product.

The mean recoveries for each enantiomer were also tested for significance to realize whether the recovery means are different from the label claim of the tablet by Student t-test. The values of t crit for R-EZT (1.001), S-EZT (0.769) and S-TRA (0.872), R-TRA (0.798) were obtained to be less than the t crit = 2.571 at 5.0 % significance levels, suggested that there was no significant difference between the mean recoveries of the enantiomers and the label claim of the analyzed product. The respective chromatogram was shown in Fig. 2c.

Conclusion

An efficient direct chiral liquid chromatographic method was developed and validated for the simultaneous estimation of the (±)-EZT and (±)-TRA in bulk drugs and pharmaceutical formulations. The optimized chromatographic condition enabled baseline resolution of the both (±)-EZT and (±)-TRA enantiomers in a reasonable analysis time. The analytical results obtained lead to the conclusion that the developed method performs well with regard to both precision and accuracy, and allows to detect chiral impurities. Therefore, it could be successfully adopted for the routine analysis of ezetimibe and tramadol enantiomers (R-EZT, S-EZT and S-TRA, R-TRA) in bulk drugs and pharmaceutical formulations. The rapid and sensitive chiral separation performance of the developed method can also be utilized for determining the enantiomeric excess of single enantiomeric products.

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References


