

Direct Chiral HPLC Method for the
Simultaneous Separation of Ezetimibe
and Tramadol Enantiomers Using a
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CC-BY 4.0Keywords Chiral HPLC; Ezetimibe;
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Abstract

In the present study, a new and simple direct chiral HPLC method was developed and validated for the simultaneous enantiomeric separation of ezetimibe and tramadol. The enantiomeric separation was carried on Chiralpak-ASH analytical column (150 x 4.6 i.d mm, 3 μm) by using acetonitrile: methanol: diethyl amine: formic acid (99/1.0/0.1/0.1% v/v/v/v) as mobile phase. Solvent mixtures were delivered at a flow rate of 1.0 ml/min and peaks were detected at 225 nm. The retention time of R-EZT, S- EZT and S-TRA, R-TRA was found to be 2.12, 2.40 and 4.01, 4.50 min respectively. The calibration curve were plotted in the range of 2.0-10 μg/ml for R-EZT, S- EZT and 1.0-5.0 μg/ml for S-TRA, R-TRA respectively. The proposed method was validated as per the ICH guidelines and found to be specific, linear, selective, and precise. The obtained results indicated that the proposed method can be utilized for the simultaneous enantiomeric purity determination of ezetimibe and tramadol in active pharmaceutical ingredient and their pharmaceutical formulation.

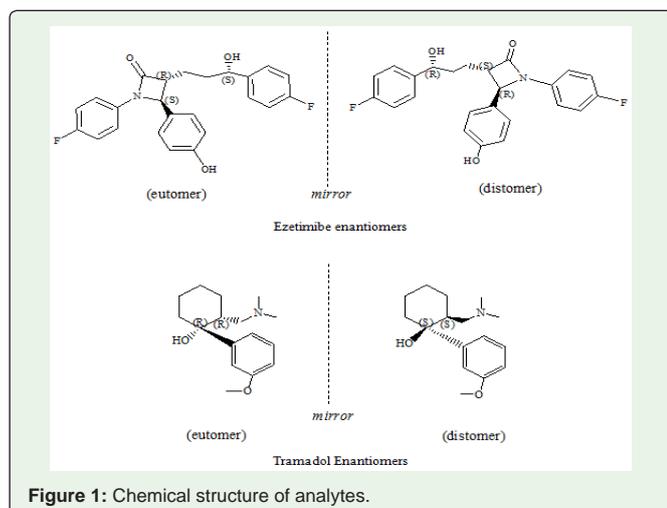
Introduction

Chiral separation has become a very important topic in analytical chemistry driven to a great extent by recent developments in methodology for both the analytical and preparative resolution of racemic drug mixtures in order to eliminate the unwanted isomer from the preparation. Chiral HPLC method has proven to be one of the best methods for chiral separation, and quantification of enantiomers of chiral drugs.

Ezetimibe (EZT) (Figure 1) is chemically known as (3R,4S) -1-(4-fluorophenyl) -3-[(3S) -three-(4-fluorophenyl) -3-hydroxypropyl] -4-(4-hydroxyphenyl) azetidin-2-one is an antihyperlipidemic agent. EZT is selectively prevents the absorption of cholesterol from dietary and biliary sources by blocking the transport of cholesterol through the intestinal wall, thereby it reduces the overall delivery of cholesterol to the liver and subsequent reduction of serum LDL-C [1,2]. EZT is a β-lactam derivative having three stereogenic centers. Two of the chiral centre are located in the β-lactam ring and the last one (alcohol) is present in the side chain. EZT is manufactured as a single enantiomer with absolute stereochemistry of (2R, 11S, and 5S) as eutomer. EZT products may contain traces of (2R, 11S, and 5R) distomer, residual chiral impurity was obtained from EZT synthesis [3].

Tramadol (TRA) (Figure 2) is chemically known as (±)-Trans-2-[(dimethylamino) methyl]-1 - (3 -methoxyphenyl) cyclohexanol, is widely used for the treatment of rheumatoid arthritis [4]. TRA possesses two chiral centers and exhibit four stereoisomers, trans-T, a racemate consisting of 1R, 2R-T[(+) -T], and 1S, 2S-T [(-) -T], is a centrally acting analgesic with efficacy and potency ranging between weak opioids and morphine. The (+)-form of trans-tramadol (eutomer) is approximately ten-fold more active than the (-)-form (distomer). Hence, it is necessary to identify and determine both the drugs EZT and TRA enantiomers for safety and efficacy of drug therapy. The monograph of EZT and TRA is officially listed in Indian Pharmacopoeia, British Pharmacopoeia and United States Pharmacopoeia. The combined dose of EZT and TRA prescribed commonly for the treatment of obesity related rheumatoid arthritis since both are exhibit complimentary activity to each other [5,6].

In the literature, various analytical methods have been reported for the quantitative determination of EZT enantiomers including HPLC [7,8], Supercritical Fluid Chromatography (SFC) [9] and LC-MS [3] in pharmaceutical formulation and biological matrices. Similarly, several analytical methods were reported for direct chiral separation of tramadol. They include methods based on HPLC [10-14], capillary electrophoresis [15-19] and LC/MS/MS [20] in various sample matrices. To the best of our knowledge, no method has been reported for the simultaneous enantiomeric separation of EZT and TRA. Hence, the objective of the present study was to develop a new simultaneous direct



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 (\pm)-EZT and (\pm)-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 20 μ L 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 \times 4.6 mm i.d., 3.0 μ m) connected with a guard cartridge (10 mm \times 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 μ L. The HPLC system was used in an air-conditioned laboratory atmosphere (25 \pm 2 $^{\circ}$ C).

Stock and working standard solutions

Standard stock solutions of (\pm)-EZT and (\pm)-TRA (1.0 mg mL⁻¹

) were prepared in mobile phase. The prepared stock solution was stored at 4 $^{\circ}$ 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 μ g/ml for R-EZT and S- EZT, 1.0 - 5.0 μ g/ml for S-TRA and R-TRA for all the analytes in presence of IS (5.0 μ g mL⁻¹).

Formulation sample preparation

Twenty tablets were weighed and finely powdered thoroughly and the amount of pharmaceutical products powder equivalent to 10 mg of (\pm)-EZT and (\pm)-TRA was accurately weighed and transferred in a 50 ml volumetric flask; suitable quantity of IS (5.0 mg) was added followed by 25 ml of mobile phase. This mixture was subjected to sonication for 10 min for complete extraction of drugs and the solution was made up to the mark with mobile phase to obtain a final concentration of (\pm)-EZT as 10 μ g/ml, IS and (\pm)-TRA as 5.0 μ g/ml, respectively. The solution was centrifuged at 4000 rpm for 10 min; the clear supernatant was collected and filtered through a 0.2 μ m syringe filter (Gelman Science, India) and 20 μ L of this solution was injected for HPLC analysis.

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

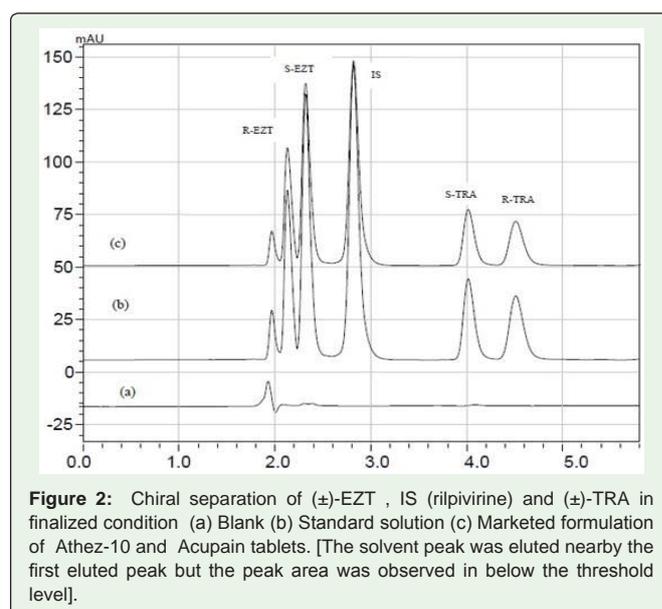


Table 1: Method development summary and system suitability results.

#	Chromatographic conditions	Remarks	System suitability			
			k_1	$Rs_{1,2}$	$Rs_{4,5}$	tR_5
1.	^a SP: Amylose-2 (250 mm x 4.6 mm, 5 μ) *MP: MeCN:MeOH:DEA:FA (99/1.0/0.1/0.1 % v/v/v/v) Flow rate: 1.0 ml/min	No enantiomeric separation was observed for both EZT and TRA.	-	-	-	-
2	SP: Lux-2 (250 mm x 4.6 mm, 5 μ) MP: MeCN:MeOH:DEA:FA (99/1.0/0.1/0.1 % v/v/v/v) Flow rate: 1.0 ml/min	EZT enantiomeric peaks were not resolved, and TRA enantiomers were partially separated.	-	-	1.2	7.9 min
3	SP: Lux-4 (250 mm x 4.6 mm, 5 μ) MP: MeCN:MeOH:DEA:FA (99/1.0/0.1/0.1 % v/v/v/v) Flow rate: 1.0 ml/min	No enantiomeric separation of EZT but peak broadening was observed for TRA.	-	-	1.4	8.3 min
4	SP: Chiralpak-ADH (150mm x 4.6mm, 5 μ) MP: MeCN:MeOH:DEA:FA (99/1.0/0.1/0.1 % v/v/v/v) Flow rate: 1.0 ml/min	EZT was Partially separated Good separation was observed in TRA.	0.9	1.1	2.8	5.9 min
5	SP: Chiralpak-ASH (150 mm x 4.6 mm, 5 μ) MP: MeCN:MeOH:DEA:FA (99/1.0/0.1/0.1 % v/v/v/v) Flow rate: 1.0 ml/min	Excellent enantio separations were observed for EZT and TRA.	1.4	1.98	2.8	4.9 min

^aSP: Stationary Phase, *MP: Mobile Phase.

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 (3,5-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 enantiomeric peaks are 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.

Linearity : The linearity of the method was established at five levels over the concentration ranges of 2-10 $\mu\text{g}/\text{mL}$ for EZP and 1.0-5.0 $\mu\text{g}/\text{mL}$ TRA approximately from 20 to 200% of nominal range of analyte. The concentration of IS was fixed at 5.0 $\mu\text{g}/\text{mL}$. Peak areas (y) of EZP and TRA were plotted versus their respective concentrations (x) and linear regression analysis performed on the resultant calibration curves (n=6). The slope and intercept of the calibration curve were 1.216 and - 0.190 for R-EZT, 1.671 and 0.140 for S- EZT, 1.080 and - 0.059 for S-TRA, 1.079 and - 0.050 for R-TRA respectively. The correlation coefficients (R^2) were found to be more than 0.995.

Limits of Detection (LOD) and Limits of Quantitation (LOQ): In accordance with ICH recommendations, the approach based on the standard deviation of the response and the slope of the calibration plots was used to determine detection and quantification limits. LOD and LOQ values were estimated as [(standard deviation of repeatability)/(slope of the regression equation)] by multiplying with 3.3 and 10 respectively. Using the above equations, the LOD and LOQ were estimated at 0.51 and 1.56 ng/ml for R-EZT, 0.27 and 0.84 ng/mL for S- EZT, 0.18 and 0.54 ng/ml for S-TRA, and 0.15 and 0.46 ng/mL for R-TRA respectively.

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) \times 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 found to be 99.68, 99.72, 99.68 and 99.72% for R-EZT, S-EZT, S-TRA and R-TRA respectively. The recoveries of enantiomers at each level were found well within the acceptable criteria of bias, \pm 2.0 %. The mean % recovery (n = 9) for each enantiomer was also tested for significance by using Student t-test. Since the t_{Calc} is less than the theoretical t value ($t_{\text{crit}} = 2.306$), 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 analyte (2.0, 6.0, 10.0 $\mu\text{g}/\text{mL}$ for EZP and 1.0, 3.0 and 5.0 $\mu\text{g}/\text{mL}$ for TRA with 5 $\mu\text{g}/\text{mL}$ 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 \leq 3%. The intra and inter-day precision (n = 6.0) was confirmed since, the % CV were well within the target criterion of \leq 2.0 and \leq 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 % \pm 0.5), the flow rate (1.0 \pm 0.05) and the formic acid (0.1 \pm 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_{Calc} 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 $t_{\text{crit}} = 2.571$ 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 (\pm)-EZT and (\pm)-TRA in bulk drugs and pharmaceutical formulations. The optimized chromatographic condition enabled baseline resolution of the both (\pm)-EZT and (\pm)-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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