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DIRECT CHIRAL SEPARATION AND INVESTIGATION OF CHIRAL DRUGS IN BIOLOGICAL MATRICES WITH PROTEIN PRECIPITATION TECHNIQUES BY RP-HPLC

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ABSTRACT

The current research described about direct chiral separation method on chiral drugs in plasma sample by RP-HPLC method. The combination of Ezetimibe and Tramadol both the drug prescribed in the obesity related rheumatoid arthritis. The separation was done with Chiralpak-ASH analytical column (150 x 4.6 i.d mm, 3 μm) by using acetonitrile: methanol: diethyl amine: formic acid (98.5/1.5/0.1/0.1% v/v/v/v) as mobile phase were delivered at a flow rate of 0.8 ml/min, the detection was measured in PDA detector at the isobestic point of 225 nm. The recovery of the spiked plasma was done with simple protein precipitation techniques. The mean, % recoveries achieved when analyzed plasma samples were, 97, 98% of (+) Ezetimibe and (-)Ezetimibe and 101, 102 % of (+)tramadol and (–)tramadol. The proposed method was validated as per the ICH guidelines and found to be specific, accuracy, precise, linear, selective, etc., The proposed method helpful for both pharmacokinetic and pharmacodynamic study at single stationary and mobile phase.

KEYWORDS: Ezetimibe, Tramadol, Chiralpak-ASH, protein precipitation techniques.

INTRODUCTION

Ezetimibe (EZT) ***(Fig 1.0)*** is an antihyperlipidemic drug selectively forbid the absorption of cholesterol from dietary and biliary genesis by blocking the transport of cholesterol through the gut wall. Ezetimibe is chemically known as (3*R*,4*S*)-1-(4-fluorophenyl)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl) azetidin-2-one. EZT is officially listed in USP NF 32, Indian Pharmacopoeia and British Pharmacopoeia. EZT is a β-lactam derivative having three chiral centers, two are placed on the β-lactam ring and the last one (alcohol) is present in the side chain [Sridevi *et al.*, 2014 and Sree-janardhanan *et al.*, 2012]. The molecule is synthesized as a single enantiomer with the absolute stereochemistry of (2R, 11S, 5S). During the synthetic process development studies of ezetimibe, the opposite OH-enantiomer, which is likely to be detected in the final active pharmaceutical ingredient, is an integral part of the ezetimibe specification. The (*R*)-enantiomer (distomer) is an undesired enantiomer, which can be present as a chiral impurity without having any pharmacological (medicinal uses) and toxicological action.

Tramadol (TRA) ***(Fig 1.0)*** is a centrally acting analgesic (binding μ–opioid receptor) with efficacy and potency ranging between weak opioids and morphine. TRA is chemically known as (±) -trans-2-[(dimethylamino) methyl] - 1 - (3 - methoxyphenyl) Cyclo –hexanol, and it's widely used in the treatment of rheumatoid arthritis and joint pains [Reza *et al.*, 2007]. TRA possesses two chiral centers and exhibit four stereoisomers. *trans*-TRA, a racemate consisting of 1R, 2R-TRA [(+) -TRA], and 1S, 2S-TRA [(−) -TRA], The racemate form is used for therapy. The 1R, 2R-enantiomer exhibits a ten-fold higher analgesic potency than the 1S, 2S-enantiomer.

From the literature, many related analytical methods has been reported for the quantitative determination of EZT enantiomers by HPLC [Kameswararao *et al.*, 2012; Pavani Jagu *et al.*, 2015], Supercritical Fluid Chromatography (SFC) [Kameswararao *et al.*, 2013], LC-MS [Esen Bellur Aticia and Bekir Karli., 2015] in pharmaceutical dosage form and biological matrices. There are various method reports on the direct chiral separation of tramadol based on various chromatographic techniques, *viz.* HPLC [Radimgeryk *et al.*, 2015, Crommen *et al.*, 1997; Pedersen *et al.*, 2003; Blaschke and Overbeck., 1999; Mohammad-Reza *et al.*, 2006], capillary electrophrosis [Saeed Nojavan *et al.*, 2011; Gottfried and Burkhard., 1999; Alireza *et al.*, 2015; Alireza Shafaati *et al.*, 2008; Gottfried Blaschke *et al.*, 2001] and LC/MS/MS [Yunsheng Hsieh., 2005].

The combination therapy of EZT and TRA were prescribed for the remedy of obesity associated rheumatoid arthritis since both are complimentary to each other [Augusto Garcia *et al.*, 2007; Sherine *et al.*, 2013]. In recently reported on simultaneous estimation in pharmaceutical formulation [sathiyasundar *et al.*, 2016], there is no method has been reported on the simultaneous enantiomeric estimation of EZT and TRA in human plasma sample. Hence, there is a need to develop an HPLC method, to find out the chiral purities in spiked plasma sample.

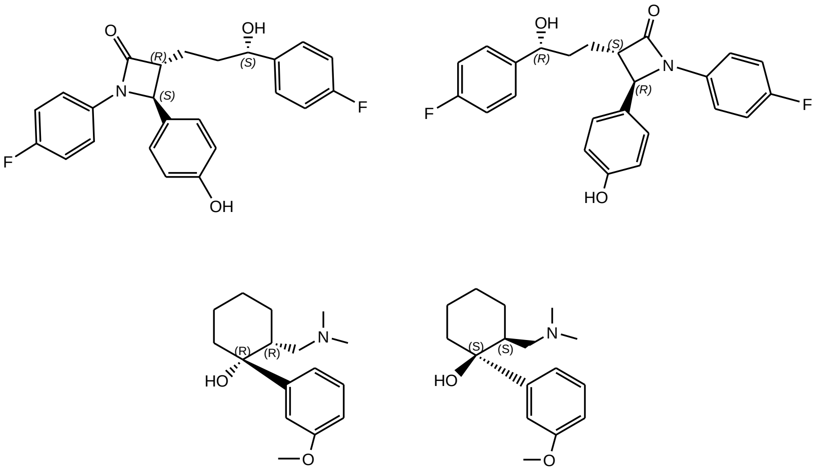

Fig.1. Chemical structure of both enantiomers of Ezetimibe and Tramadol.

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), ethanol, ethyl acetate and diethyl ether 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). The human plasma was gifted by the Raja Muthiah Medical College and Hospital (RMMCH), Annamalai University, Annamalai Nagar, Tamilnadu, India.

HPLC instrumentation

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.

Chromatographic conditions

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. The above stock solution further diluted or spiked with plasma. 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).

Plasma Extraction Technique

There are many methods available for plasma extraction technique among these simple protein precipitation method employed here. In this study we utilized the following organic solvents like cold methanol, cold ethanol, cold ethyl acetate, and cold diethyl ether.

The 1 ml of plasma and equal quantity of cold-Methonal in a glass-stoppered 15 ml centrifuge tube were spiked with the working solutions of EZT and TRA and IS achieve a concentration of 500 µg ml-1 each. The samples were gently shaken for 5 min and centrifuging on a laboratory centrifuge (Remi®, R&C, Remi

Equipment, Mumbai, India) at 5000 RPM for 5min. The supernantant organic layer was transferred to Petri-dish and the contents were evaporated into the air-dry method. The residue was reconstituted in 100 µL of mobile phase and vortex mixed for 60 seconds. Aliquots of 20 µL were injected into the chromatographic system. The same procedure was carried out for blank plasma samples to check the cleanness of the extracts. To assess the efficiency of the extraction procedure, the spiked plasma sample was extracted according to the above procedure, but the addition of IS after extraction. The recoveries of each drug and IS from spiked plasma were determined by comparing the peak area of each analyte after extraction with the respective non-extracted standard solution at the same concentration in both low and high concentrations of each compound was checked. The concentration of the IS were established in 5000 ng/ml. The mean, % recoveries achieved when analyzed plasma samples were, 96, 97% of + Ezetimibe and -Ezetimibe and 101, 102 % of +tramadol and -tramadol with the values are within parenthesis being the % CV of the six replicates. The % CV of the assay results were <15, indicating the precision of the analytical methodology.

Choice of internal standard

Different chemically inert compounds were tested on IS. Among these rilpivirine was found suitable as it had similar wavelength and does not interfere with main peak (analyte peak). The solution stability was conducted with 48 hrs.

RESULTS AND DISCUSSION

Method development and Optimization

The choice of an appropriate column is the primary goal in method development of chiral separation. Preliminary screening studies were carried out to identify the suitable chiral stationary phase for the simultaneous enantiomeric separation of EZT and TRA. In this direct enantiomeric separation of EZT and TRA was performed on amylose (amylose tris (5-chloro-2-methylphenylcarbamate), (amylose tris (3,5-dimethylphenyl-carbamate), (amylose tris (S)-1-phenylethyl-carbamate) and cellulose based polysaccharide chiral stationary phases (CSPs) such as (cellulose tris (3-chloro-4- methylphenylcarbamate) and (cellulose tris (4-chloro-3- methylphenylcarbamate) at different proportions of polar and intermediate polar organic solvents. Among the selected CSPs, only Chiralpak AS-H afforded a partial enantiomeric separation of EZT and TRA. Hence, Chiralpak AS-H was further explored to achieve a quality separation through optimization of the mobile phase composition.

To start with, enantiomeric separation of EZT and TRA was tried in the polar organic mode. In polar organic mode screening a mobile phase consisting of 100% MeOH, EtOH, ACN and their binary combinations were tested. During the preliminary investigatory runs, the mobile phase consisting of ACN/MeOH (98/2.0 % v/v) showed partial enantioresolution of the analytes with poor peak shape. Hence, further optimization was required. In order to improve the resolution between the enantiomers and peak shape, different organic modifiers like acetic acid (AA), formic acid (FA) diethylamine (DEA) and triethylamine (TEA) were incorporated in the mobile phase. The effects of organic additives on the racemates separation were investigated. When 0.1% DEA was added to the mobile phase, TRA enantiomers are well separated but EZT enantiomers remained with partial separation. Opposite behaviour was observed when 0.1% FA added to mobile phase that separates EZT enantiomers but TRA enantiomers still remained with partial separation ***(Fig 2)***.

Considering the above observation, a mixture of DEA and FA (0.1% each) was incorporated into the mobile phase. The mobile phase composition with both the mobile phase additive, MeCN/MeOH/DEA/FA (98.5/1.5/0.1/0.1 % v/v/v/v), resulted in a good enantioresolution between EZT and TRA. The above developed method done with API, then further studies like flow rate, temperature has been modified with plasma sample. A reasonable runtime was obtained with 0.8 mL min^{-1} and the flow rate was adjusted to reduce the overall runtime. The elution order of R-EZT, S-EZT, S-TRA and R-TRA enantiomers was found to be 3.12, 3.32, 6.01, and 6.87 min respectively, the order of elution were check with single enantiomeric form of respective enantiomers (spiked human plasma sample).

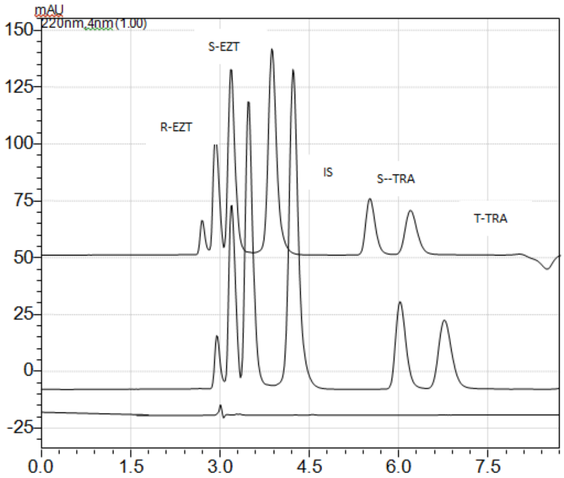

Fig. 2.0 Chiral separation Chromatograms of R-EZT, S-EZT, IS (Rilpivirine) and S-TRA, R-TRA represented here.

Method Validation

The last step of the present study was to check method's validation for specificity, linearity, accuracy, intra/inter-day precision, robustness and ruggedness. The optimized HPLC method was specific in relation to the blank plasma used in this study and there is no peak interference**.** An linearity was established at five levels over the concentration ranges of 2.0 – 10.0 µg/ml for R-EZT, S- EZT and 1.0-5.0 µg/ml for S-TRA, R-TRA with R^2 of more than 0.998 for all the analytes and 5.0 µg/ml of IS was fixed. The slope and intercept of the calibration curve were 1.106 and - 0.178 for R-EZT, 1.521 and 0.129 for S- EZT, 1.001 and - 0.047 for S-TRA, 1.009 and - 0.039 for R-TRA respectively. Since the correlation coefficient are good indicators of linearity performance of an analytical procedure and additionally a one way ANOVA was performed. For all the analytes, the calculated F- Value (F_{Calc}) was found to be below than the theoretical F-Value (F_{Crit}) at 5.0 % significance level, indicating that there was no significance difference between replicate determinations for each concentration level.

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, assessed by spike recovery, in which the % recovery of both enantiomers it's at each level (n = 3) and mean % recovery (n = 9) were found to be 96, 97,101,102 % 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, ± 5 %. 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_{Crit}=2.306), at 5.0 % significance level, the null hypothesis was accepted. These results indicate that the method is accurate and therefore the absence of interference from blank plasma used in this study. These results indicate that the method is accurate and therefore the absence of interference from placebo excipients used in this study. The intra and inter-day precision (n = 6) was confirmed since, the % CV were well within the target criterion of ≤ 8 and ≤ 10 respectively. The variations in MeCN concentration (98.5 % ± 0.5), the flow rate (0.8 ± 0.05) and the formic acid (0.1 ± 0.02 %) did not alter the sample values of both enantiomers more than 5 % and therefore it would be concluded that the method conditions are robust.

Application of the method

This proposed RP-HPLC method was applicable to know the information related to the pharmacokinetic and pharmacodynamic study of Ezetimibe and Tramadol enantiomers in biological real samples.

REFERANCE

1 Alirezashafaatia, Majid, Sayyed. Chiral separation of basic and zwitterionic drugs by highly sulfated cyclodextrins using short-end injection capillary electrophoresis, Iranian J Pharmaceu Rese., 2008; 7: 275-281.

2 Augusto G, Segami I, Claudia S, Henry N, Eduardo A, Elmer G. Obesity is independently associated with impaired quality of life in patients with rheumatoid arthritis, Clin Rheumatol, 2007; 26: 1831-1835.

3 Blaschke and Overbeck. Direct determination of tramadol glucuronides in human urine by high-performance liquid chromatography with fluorescence detection, J. Chromatogr. B., 1999; 732: 185-192.

4 Crommen J, Ceccato A, Chiap P, Hubert. Automated determination of tramadol enantiomers in human plasma using solid-phase extraction in combination with chiral liquid chromatography. J. Chromatogra. B., 1997; 698: 161-170.

5 Esen Bellur, Bekir Karlıga. Identification, synthesis and characterization of process related impurities of benidipine hydrochloride, stress-testing/ stability studies and HPLC/UPLC method validations, J. Pharmace. Analysis, 2015; 5: 256-268.

6 Gottfried and Burkhard. Achiral and chiral determination of tramadol and its metabolites in urine by capillary electrophoresis, Electrophoresis., 1999; 20: 555-563.

7 Gottfried Blaschke, Utabirgitta, Marcoliver, Haraldschulz, Peterandresen. Direct chiral assay of tramadol and detection of the phase II metabolite O-demethyl tramadol glucuronide in human urine using capillary electrophoresis with laser-induced native fluorescence detection. J.Chromatogr. B., 2001; 765: 3-13.

8 Kameswara Rao, Venkatanarayana Gudala, Madhusudhan Gutta, Srinivasulu Polisetty and Sai Venkata Srinivas Koduri. Development and validation of chiral hplc method for identification and quantification of (R)-enantiomer in ezetimibe, American J. Anal Chem., 2012; 3: 478-483.

9 Kameswararao, Venugopal, Madhusudhan, Srinivasulu, Saivenkata. Isolation and characterization of R-Enantiomer in Ezetimibe, American J. Anal Chem., 2013; 4: 488-495.

10 Mohammad-Reza, Yalda, Faezeh. Development and validation of a rapid HPLC method for simultaneous determination of tramadol, and its two main metabolites in human plasma, J. Chromatogr. B., 2006; 830: 207-211.

11 Pavani Jagu, VijayKumar, BM Raoa, Someswara Rao. A noval enantioselective reverse phase HPLC method for the determination of ezetimibe and its S-enantiomers, World. J. Pharma. Research, 2015; 4: 1605-1619.

12 Pedersen, Brosen, Nielsen. Enantioselective HPLC method for quantitative determination of Tramadol and O-Desmethyltramadol in plasma and urine: Application to clinical studies, Chromatogr, 2003; 57: 279-285.

13 Radim Geryk, Kveta Kalikova, Jiri Vozka, Eva Tesarova. Immobilized Polysaccharide Based Stationary Phases for Enantioseparation in Normal Versus Reversed Phase HPLC, Chromatographia, 2015.

14 Reza Mehvar, Katherine, Ridhi, Okponanabofa. Stereospecific high performance liquid chromatographic analysis of tramadol and its O-demethylated (M1) and N,O-demethylated (M5) metabolites in human plasma, J. Chromatogr. B., 2007; 852: 152-159.

15 Saeed Nojavan, Ali Mohammadi, Mohammadreza, Ali Reza. Stability evaluation of tramadol enantiomers using a chiral stability-indicating capillaryelectrophoresis method and its application to pharmaceutical analysis. J.Sep. Sci., 2011; 34: 1613-1620.

16 Sathiyasundar R, Kanthiah S and Kannappan V. Direct Chiral HPLC Method for the Simultaneous Separation of Ezetimibe and Tramadol Enantiomers Using a Polysaccharide Stationary Phase. Ann Chromatogr Sep Tech., 2016; 2(3): 1025.

17 Sherine E, Cynthia S, Eric L, John M. Contribution of obesity to the rise in incidence of rheumatoid arthritis, Arthritis Care Res., 2013; 65: 71-77.

18 Sree Janardhanan, R Manavalan, K Valliappan. Chemometric technique for the optimization of chromatographic system: Simultaneous HPLC determination of Rosuvastatin, Telmisartan, Ezetimibe and Atorvastatin used in combined cardiovascular therapy, Arabian Journal of Chemistry, 2012.

19 Sridevi R, Badal K, Sameer G. Stability-Indicating HPLC method for the simultaneous determination of valsartan and ezetimibe in pharmaceuticals, Trop. J Pharmace Res., 2014; 13: 809-817.

20 Tabani H, Mahyari M, Sahragard A, Fakhari AR, Shaabani A. Evaluation of sulfated maltodextrin as a novel anionic chiral selector for the enantioseparation of basic chiral drugs by capillary electrophoresis, Electrophoresis, 2015; 36: 305-311.

21 Yunsheng H, Jiwen C, Walter A. Chiral liquid chromatography-tandem mass spectrometric methods for stereoisomeric pharmaceutical determinations. J.Chromatographia. B., 2005; 820: 1-8.