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Enantioseparation of Tedizolid phosphate by RP-HPLC, using β-Cyclodextrin as a Chiral Mobile Phase Additive

Ajit Anerao*, Vikram Dighe, Satish John, Nitin Pradhan

R&D centre (API), Wanbury Ltd., EL-16, TTC Industrial Estate, Mahape, Navi Mumbai 400710, India.

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ABSTRACT

Objective: Enantiomeric separation and quantification of S-enantiomer of Tedizolid phosphate drug substance.
Material and method: A simple and sensitive reversed phase high performance liquid chromatography (RP-HPLC) method is developed by using β-cyclodextrin (β-CD) as a chiral mobile phase additive. Effect of the pH value of aqueous buffer, concentration of chiral additive, composition of mobile phase, and column temperature on the enantioseparation of Tedizolid phosphate was investigated on the Phenomenex Luna, Phenyl-Hexyl, 250 x 4.6mm, 5µm HPLC column. A satisfactory resolution was achieved at column temperature 20^0C using a mobile phase consisting of a mixture of aqueous buffer of pH 7.0 of disodium hydrogen phosphate with additive β-cyclodextrin, triethylamine and acetonitrile. This analytical method was evaluated by performing method validation as per ICH guideline.
Results: The calibration curve was plotted within the concentration range between 0.30 and 2.25 µg mL^{-1} and the recoveries between 96.9 and 105.3% were obtained, with regression coefficient R^2 is 0.998. The limit of detection (LOD) and limit of quantitation (LOQ) of S- enantiomer of Tedizolid phosphate is 0.10 and 0.30 µg mL^{-1}
Conclusion: The developed method was demonstrated to be accurate, robust and sensitive for the determination of enantiomeric purity of Tedizolid phosphate drug substance.

INTRODUCTION

The chemical name of Tedizolid phosphate is [(5*R*)-(3-{3-Fluoro-4-[6-(2-methyl-2H-tetrazol-5-yl)pyridin-3-yl]phenyl}-2-oxooxazolidin-5-yl]methyl hydrogen phosphate. It has an empirical formula of $C_{17}H_{16}FN_6O_6P$ and a molecular weight of 450.32 g $mole^{-1}$ (FDA, 2014). Tedizolid phosphate is a novel oxazolidinone prodrug antibiotic that is converted *in vivo* by phosphatases to the microbiologically active moiety tedizolid. It has been developed for both oral and intravenous (i. v.) use. Sivextro tablets and lyophilized powder for injection are indicated for the treatment of acute bacterial skin and skin structure infections (ABSSSI) caused by susceptible isolates.

Tedizolid phosphate has one asymmetric center, leading to two possible enantiomers. The absolute configuration at the 5-position of the oxazolidinone ring is the R optical isomer. *In vitro* stability data, showing high stability of the R-isomer, also support the assumption of a low risk for *in vivo* inter-conversion in humans (Elaine and Saba, 2014; EMA, 2015). The US Food and Drug Administration and other regulatory agencies have made it mandatory for the manufacturers to investigate each enantiomer of the chiral drug individually (Rauws and Groen, 1994). According to the International Conference on Harmonization (ICH) guidelines, chiral identity, enantiomeric impurity and chiral assay tests may be needed in drug substance and product specifications (Branch, 2005).

Several research papers have been reported in the literature related to quantification of tedizolid phosphate in human plasma (Santini *et al.*, 2015) and its absorption, distribution, metabolism and excretion (Voon *et al.*, 2014).

* *Corresponding Author*
E-mail: ajit.anerao @ wanbury.com; Mobile +91 7506734840
Telephone +91 2264570555; Fax +91 2264570553

In order to quantitatively determine the enantiomeric composition of tedizolid phosphate, very few analytical methods have been reported. These papers were limited to the chiral purity using capillary electrophoresis (Katarzyna *et al.*, 2016). Because of unavailability of simple, cost effective technique, it is prompted to develop method of analysis which can be performed in any quality control laboratory.

During method development different chiral stationary phases were used along with combination with mixture of polar and non-polar solvents. Normal phase as well as reverse phase chiral chromatography with mobilized and immobilized stationary phases were tried but could not achieve satisfactory resolution. In present research β-cyclodextrin was added in the mobile phase and achieved base to base separation between R and S isomer. Instead of using chiral stationary phases, addition of a chiral additive into the mobile phase form a pair of transient diastereomeric complexes, followed by chromatographic separation on an achiral column. Introducing chiral additives in mobile phase has a number of advantages over using chiral stationary phases (Christodoulou, 2010; Płotka *et al.*, 2011).

First, the proposed method is less expensive because achiral column is used which is less expensive as compared to chiral columns. In general, chiral columns are of high price, less stability, low capacity and have a relatively short lifetime. Secondly, this method offers greater flexibility. Diverse achiral columns and chiral additives can be used. Moreover, the approach based on chiral mobile phases can work in both a normal phase and a reversed phase operation mode.

Cyclodextrins separate the enantiomers utilizing the phenomenon of host guest complexation. The affinity of the analyte for cyclodextrin is due to the hydrophobic interaction between the analyte and the cyclodextrin cavity and the hydrogen bonding between the analyte and the functional groups on the cyclodextrin ring.

Cyclodextrin and its derivatives (Han, 1997; Bressolle *et al.*, 1996) have been recognized as the most prevalent chiral mobile phase additives due to the nature of being nontoxic, non-flammable, non-volatile, and stable over a wide range of pH and negligible absorption in the UV ranges broadly used in chromatographic detection.

They, therefore, have been successfully applied in the enantiomeric separation of a variety of racemates reported in a review article (Bressolle *et al.*, 1996), and recently, of mandelic acid derivatives (Tong *et al.*, 2014), pantoprazole (Guan *et al.*, 2008), cathinone and amphetamine derivatives (Taschwer *et al.*, 2014), phenyllactic acid (Xu *et al.*, 2013), 2-arylpropionic acid (Ye *et al.*, 2010), sertraline (Reyes-Reyes *et al.*, 2014), beta-carboline alkaloids (Le´On *et al.*, 2008), and amlodipine (Xie *et al.*, 2014). The proposed analytical method is validated as per International conference on harmonization guidelines ICH Q2-R1 (ICH, 2005) and USFDA (FDA, 2009).

Structure of R-Tedizolid phosphate, S-Tedizolid phosphate and β-cyclodextrin are given in figure I to III.

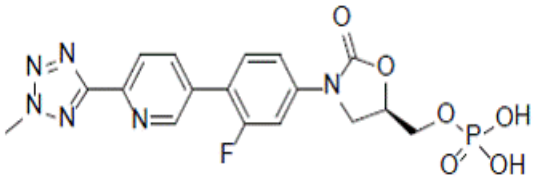

Fig. I: R-Tedizolid phosphate.

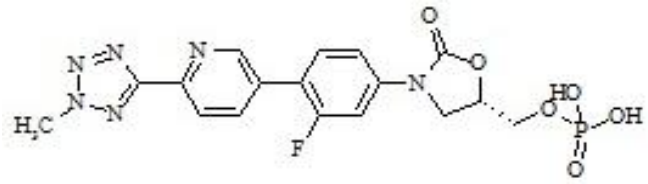

Fig. II: S-Tedizolid phosphate.

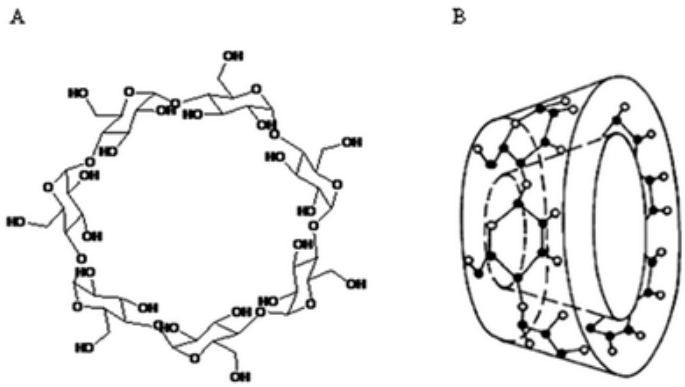

Fig. III: β-cyclodextrin.

MATERIALS AND METHODS

Chemical and reagents

β-cyclodextrin CAS No.7585-39-9 was purchased from Spectrochem, India. R-Tedizolid phosphate and S-enantiomer is synthesized and characterized at Wanbary R&D center. All HPLC grade chemicals required for mobile phase and sample preparation, acetonitrile, triethylamine, disodium hydrogen phosphate and o-phosphoric acid were purchased from Merck-India. HPLC grade N,N-dimethylsulfoxide from Rankem and Siemens Labostar water purification system was used for HPLC grade water to prepare the mobile phase and diluents. Mobile phase was filtered through 0.45 µm nylon filter before use.

Instrumentation

Shimadzu HPLC system LC-2010 CHT with UV detector and LC solutions software or its equivalent was used. The analysis was carried out on Phenomenex Luna Phenyl-Hexyl, 250 x 4.6mm, 5µm Part No.00G-4257-E0 with column temperature is 20^0C. Separation was achieved with gradient elution of mobile phase-A and mobile phase-B with timed programme T_{min}/A:B: T_0/100:00; T_{50}/100:00; T_{55}/00:100; T_{65}/00:100; T_{66}/100:00; T_{75}/100:00. The flow rate was 1.0 mL/min and sample injection volume was 5 µL. Detector wavelength is 300 nm.

Preparation of solutions

Diluent

A mixture of equal volume of acetonitrile and water was prepared.

Buffer preparation

Buffer was prepared by weighing and mixing 5.70 g of β-Cyclodextrin and 5.60 g of Di-sodium hydrogen phosphate in 1000 ml of water. It was sonicated to dissolve and then added 8.0 mL of Triethylamine. Adjusted the pH to 7.0 with Ortho-phosphoric acid and filtered through 0.45µ filter.

Mobile Phase A

A mixture of 1000 mL of buffer and 150 mL of acetonitrile was prepared, mixed and sonicated to remove air bubbles.

Mobile Phase B

A mixture of 500 mL of water and 500 mL of acetonitrile was prepared, mixed and sonicated to remove air bubbles.

Blank preparation

Transferred 5.0 mL of N,N-dimethylsulphoxide into 50 mL volumetric flask and diluted up to the mark with diluent.

Standard Stock solution

Accurately weighed and transferred 15.0 mg of S-enantiomer of Tedizolid phosphate reference standard into 100 mL volumetric flask. 10 mL of N,N-dimethylsulphoxide was added and diluted up to the mark with diluent.

Standard solution-A

5.0 mL of standard stock solution was transferred to 50 mL volumetric flask, 5.0 mL of N,N-dimethylsulphoxide was added and diluted up to the mark with diluent.

Standard solution-B

5.0 mL of standard solution-A was transferred to 50 mL volumetric flask, added 5.0 mL N,N-dimethylsulphoxide and diluted up to mark with diluent.

System Suitability solution

Weighed and transferred accurately about 50.0 mg of (R)-Tedizolid phosphate reference standard in to 50 mL volumetric flask. First dissolved in 5 mL of N,N-dimethylsulphoxide. Added 5.0 mL of standard solution-A and diluted up to the mark with diluent.

Test solution

Weighed and transferred accurately about 50.0 mg of sample in to 50 mL volumetric flask, dissolved in 5 mL of N,N-dimethylsulphoxide and diluted up to the mark with diluent.

Procedure

Using auto sampler injector all solutions of equal injection volume (5µL) was injected, blank preparation followed by system suitability solution, standard solution-B six replicates and test solution. In system suitability solution R-Tedizolid phosphate peak is found eluting at retention time of about 37 minutes and the relative retention time of S-enantiomer is about 0.93. Resolution between both peaks was more than 1.5 and relative standard deviation (RSD) of peak area of six replicate injection of standard solution-B for S-enantiomer peak was found less than 5.0 %. S-enantiomer content in Tedizolid phosphate drug substance was calculated by external standard method.

RESULTS AND DISCUSSION

Analytical method development

Key parameters that affected the enantioseparation included the type and concentration of the chiral selector, whether an organic solvent was added or not, type of organic solvent, regarding to its protic or aprotic properties, pH of the mobile phase, stationary phase and column temperature. These parameters were optimized to achieve the highest enantioseparation in the Tedizolid phosphate enantiomers.

Stationary phase selection

Considering the structural similiarity of oxazolidine of linezolid, first trial was carried out as per the method of USP linezolid In-Process Revision (USP, 2014). Chiralpak AD-H, 250 mm X 4.6mm, 5µm column consist of mobile phase Hexane : absolute alcohol : Trifluroacetic acid (650:350:1). In this method both enantiomers are resolved but recovery of S-enantiomer peak is not achieved because both peaks are asymmetrical. Different Mobile phase composition of solvents like n-Hexane, ethanol, n-Heptane, isopropyl alcohol, acetonitrile and TFA (trifluoroacetic acid) trials were taken but recovery is not achieved. Solubility of Tedizolid phosphate is major constrain while trying with chiralpak ADH column. API is soluble only in N,N-dimethylsulfoxide which is not advisable to use with ADH column because it spoils the stationary phase. Normal phase chromatography with another chiral stationary phases were tried such as Chiralcel OD-H and Lux Amylose-2 with different solvents and modifiers including n-Hexane, isopropyl alcohol, ethanol , trifluroacetic acid to achieve the separation and recovery of S-isomer but no satisfactory results were found.

Further trials planned with immobilized chiral stationary phases as Chiralpak IA, ID-3 column were carried out with different composition of solvents and modifiers like ethanol, methyl tert. butyl ether, dichloromethane, trifluroacetic acid, diethylamine but resolution was not satisfactory.

Further trials planned on reverse phase chiral stationary phases like Chiralcel OD-RH, Chiralpak AGP column tried with different buffers such as ammonium carbonate, disodium hydrogen phosphate, potassium dihydrogen phosphate at pH 2.2, 5.0, 8.8 with solvent composition like acetonitrile, methanol but no

resolution found. Crown-pack CR (+) column tried with buffer as perchloric acid pH 1.0, 1.5 and 2.0 but no resolution observed.

Chiradex HR column trials taken using different buffers like ammonium formate, sodium dihydrogen phosphate, ammonium dihydrogen phosphate, potassium dihydrogen phosphate, triethylamine at pH 3.0, 5.0 and 7.0. By using buffer 25 mM disodium hydrogen phosphate at pH 5.0 : acetonitrile (70:30) with flow rate 0.8 mL/min resolution found 1.4. Recovery was found 98.0% to 102.0% but both enantiomers are eluting very late and run time was 180 min. Different trials taken to shorten the run time but at shorter run time resolution was reduced and recovery was not achieved.

Out of these many trials both enantiomers were found resolved only on Chiradex column which contain β-cyclodextrin. So the next trial was carried out using β-cyclodextrin additive in a buffer and acetonitrile as organic solvent with C-18 column but this gives no resolution.

Finally desired resolution is obtained with Phenomenex Luna Phenyl-Hexyl column along with combination of additive β-Cyclodextrin, disodium hydrogen phosphate and acetonitrile. Resolution between R and S-enantiomer is 1.7 and achieved base to base separation between both enantiomers. Run time was reduced to 75.0 minutes.

Effect of composition of mobile phase

The mobile phase composition was found to influence the retention time and resolution of tedizolid phosphate enantiomers. Further investigation was conducted by varying the volumetric ratio of aqueous buffer and acetonitrile under the following conditions: pH of 7.0, β-CD concentration of 5 mM and disodium hydrogen phosphate concentration of 40 mM. The variation of organic modifiers' content has substantial impact on enantioseparation of Tedizolid phosphate. Upon increasing the proportion of acetonitrile, both retention time and resolution decreased accordingly. In order to obtain satisfactory resolution and retention time, the volume ratio of mobile phase A 1000 : 150 was selected.

Effect of the Concentration of β-cyclodextrin

The effect of β-CD concentration on enantioseparation was investigated under the pH of 7.0. A range of β-CD concentrations from 1mM to 10mM were tested. The concentration of β-CD has a significant impact on the resolution. Resolution is getting reduced after reducing concentration to 1mM. By increasing concentration both enantiomers are base to base resolved. Resolution was found sufficient about 1.7 at 5mM as well as 10 mM concentration of β-CD. The β-CD concentration of 5 mM was chosen as a compromise between a good resolution and a relatively short retention time.

Effect of pH on resolution

Chiral recognition is based on the formation of a stable inclusion complex and hydrogen bonding interaction with the guest enantiomer and pH plays an important role in maintaining the stability of the complex. The influence of mobile phase pH in the range of 3.0 to 7.0 with 5 mM chiral additive and 40 mM disodium hydrogen phosphate was investigated. As shown in figure-IV with decrease in pH of the mobile phase, resolution decreased.

At pH 3.0 and 5.0 both enantiomers are not separate, at 6.5 pH resolution is 1.3 and at 7.0 pH resolution is achieved 1.7 and peaks are base to base separate. Hence pH 7.0 was found to be the optimum pH for the analysis. Along with the pH, triethylamine and concentration of discodium hydrogen phosphate was studied. It was observed that the band broadened without triethylamine, resulting in lower resolution. One unknown impurity was eluting very closely with S-enantiomer peak which is got separated by addition of disodium hydrogen phosphate.

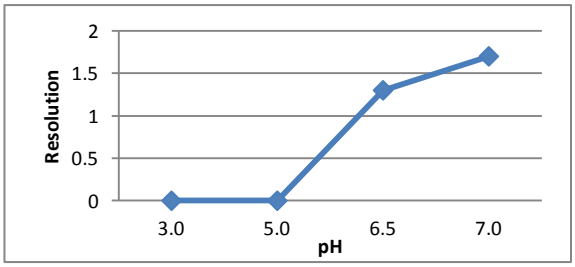

Fig. IV: Effect of pH on resolution of R and S enantiomers.

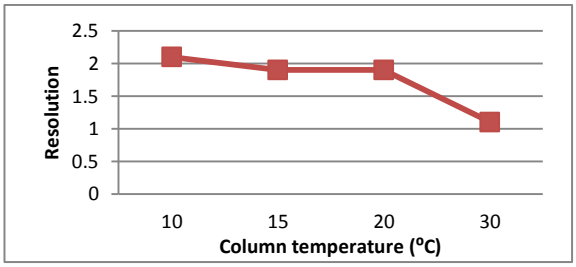

Fig. V: Effect of column temperature on resolution of R and S enantiomers.

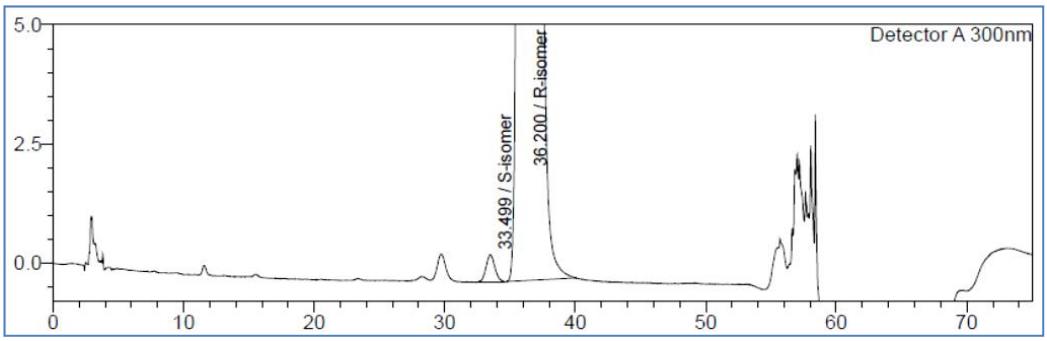

Fig. VI: System suitability solution chromatogram where R-Tedizolid phosphate is eluting at RT 36.2 minutes and S-enantiomer at relative retention time 0.93.

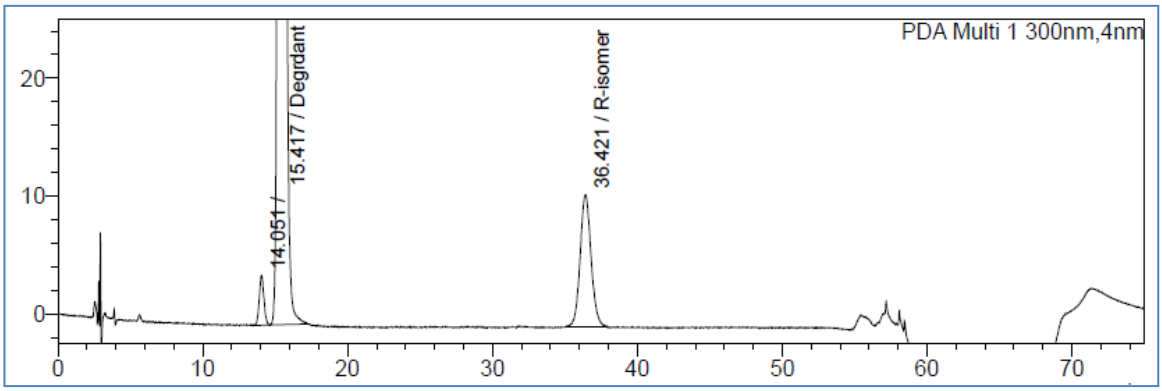

Fig. VII: Chromatogram of Tedizolid phosphate exposed to 5N sodium hydroxide where major degradant is eluting at RT 15.4 minutes.

Effect of the column temperature

The effect of column temperature on the resolution of Tedizolid phospahte enantiomers was studied in a range of 10^0C to 30^0C. As shown in figure-V increase in column temperature, the resolution decreased, at 30^0C the resolution was 1.1. Resolution increases after reducing column temperature, where at 20^0C, 15^0C and 10^0C, the resolution is 1.9, 1.9 and 2.1 respectively. By reducing temperature, resolution is increased but during long analysis sequence instrument stop due to leak detection because of water condensation in column compartment. To achieve a fair balance between higher resolutions and avoid water condensation, 20^0C was chosen as the operating temperature.

Analytical Method Validation

The proposed method was validated through the examination of specificity, solution stability, limit of detection (LOD), limit of quantitation (LOQ), linearity, accuracy, ruggedness and robustness study as per ICH guideline.

Specificity and Force degradation study

Specificity is defined as the ability of the method to determine accurately and specifically the analyte of interest in the presence of other components in a sample matrix that may be expected to be present in the sample matrix under the stated conditions. Specificity of the method was evidenced by comparing blank, R-Tedizolid phosphate, S-Tedizolid phosphate and all specified impurities separate injections as well as spiking S-enantiomer into R-Tedizolid phosphate test solution. There are no interfering peaks at the retention times of R-Tedizolid phosphate eluting at 36.2 minutes and S-enantiomer at relative retention time 0.93. Referring to figure VI of system suitability solution chromatogram The peak purity of both enantiomers is checked with photo diode array detector (PDA). It is found that peak purity index of R-Tedizolid phosphate and S-enantiomer is 1.00 and 0.99 respectively which proves that both enantiomer peaks are pure without any interference. Force degradation is performed to ensure interference of any degradant at the retention time of S-enantiomer peak. Tedizolid phosphate sample was exposed to different stress conditions and then tested using the proposed method of analysis.

The API batch was heated at 105^0C for 24 hours and then the test solution was prepared and injected. Similarly sample was treated with base 5 N sodium hydroxide, with 5N hydrochloric acid exposed to ultra-violet light for 24 hours and 3% hydrogen peroxide solution 24 hours. After exposure samples were prepared separately and tested using the proposed chiral purity method with photo diode array (PDA) detector. It was observed that Tedizolid phosphate is stable when exposed to heat, UV light, acid and oxidation. In base degradation one major impurity is formed about 92% eluting at retention time 15.4 minutes. The base degradation chromatogram is given in figure VII. On basis of forced degradation study it can be concluded that no degradant is eluting at the retention time of R and S-Tedizolid phosphate. Peak purity of both enantiomer peaks is ensured by PDA detector found passing in all degraded samples.

Solution stability

Drug stability in Active Pharmaceutical Ingredient is a function of storage conditions and chemical properties of the drug and its impurities. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. Stability data are required to show that the concentration and purity of analyte in the sample at the time of analysis corresponds to the concentration and purity of analyte at the time of sampling. The solution stability till twenty three hours of Tedizolid phosphate API had been checked by injecting system suitability solution. Solution was prepared fresh before injection and immediately injected and same solution was injected after twenty three hours. Tedizolid phosphate and S-enantiomer is found stable in solution form till twenty three hours.

Limit of detection

The limit of detection (LOD) is the lowest concentration of analyte in a sample that can be detected but not necessary quantified. The obtained LOD value of S-enantiomer of Tedizolid phosphate is tabulated in Table-I.

$$LOD = 3.3 \times \sigma / S$$

Where, σ = the standard deviation of the response and S= slope of the calibration curve

Limit of quantitation

The limit of quantitation is the lowest concentration or amount of analyte that can be determined quantitatively within an acceptable level of repeatability precision and trueness.

Limit of quantitation (LOQ) = $10.0 \times \sigma / S$

Where, σ = the standard deviation of the response and S= slope of the calibration curve

Precision at LOQ is confirmed by six replicate analyses of S-enantiomer at LOQ level. LOD, LOQ and RSD at LOQ level is tabulated in Table I.

Table I: LOD, LOQ and precision.

Name of the compound	LOD in % (wrt test concentration)	LOQ in % (wrt test concentration)	LOQ precision (% RSD of six replicate)
S-Tedizolid phosphate	0.01%	0.03%	2.3%

Linearity

The ability of the method to obtain test results proportional to the concentration of the analyte within a given range. It was evaluated by linear regression analysis and calculated by the least square regression method. Under the experimental conditions, the S-enantiomer peak area vs. concentration plot for the proposed method was found to be linear over the range of LOQ concentration, 25%, 50%, 80%, 100%, 120% and 150% of the specified limit (0.30 µg mL^{-1} to 2.25 µg mL^{-1}) with a regression coefficient 0.998. The regression coefficient (r^2) is > 0.99 is generally considered as evidence of acceptable fit of the data to the regression line.

Accuracy

Accuracy can be defined as the closeness of agreement between a test result and the accepted reference value. Accuracy of the method was determined by recovery study. Analytical method may be considered validated in terms of accuracy if the mean value is within ± 20% of the actual value. During recovery study, Tedizolid phosphate API batch was analyzed and then S-enantiomer of known concentration is spiked in the API at LOQ level, 50%, 100% and 150% with respect to the limit of S-enantiomer.

Table II: Recovery study.

Name of the compound	LOQ level	50% level	100% level	150% level
S-Tedizolid phosphate	96.9%	100.9%	104.3%	105.3.0%

As per ICH guideline Q3A-R2 the limit of known impurity should be 0.15% if daily maximum dose is less than 2.0 g (ICH, 2006). The dose of Tedizolid phosphate is 200 mg/day with oral and parenteral route of administration (Drugs.com, 2014). So the limit of S-enantiomer as known impurity is decided 0.15%. Recovery of S-enantiomer was found in the range of 80.0% to 120.0%, which was well within the acceptance criteria. Results are tabulated in Table-II.

Ruggedness study

The (intra-laboratory tested) behavior of an analytical process when small changes in environment and/or operating condition are made. The ruggedness of the method was evaluated by estimating % RSD of standard solution tested by two different analysts using different HPLC instrument and columns on different days. % RSD of area of S-enantiomer peak on two different days is found 1.8% and 1.0% with resolution between R and S-enantiomers in system suitability solution is found 1.78 and 2.02 respectively. Six preparations of S-enantiomer spiked in R-Tedizolid phosphate was prepared by two different analysts and analyzed on two different days. The % RSD of S-enantiomer of total twelve preparations is found 3.99% which proves that the method is rugged and delivers accurate and consistent results.

Robustness

Robustness is a measure of the capacity of the analytical procedure to remain unaffected by small but deliberate variations in method–performance parameters, which provides an indication of its reliability during normal usage. Robustness of the method was determined by analyzing the system suitability solution and batch analysis with deliberate change in the parameters like (a) flow rate of mobile phase ± 0.1 ml/min (b) mobile phase solvent ratio variation ±10%, (c) mobile phase pH ± 0.1 and (d) column temperature ± 5°C. Results are discussed in table III.

Table III: Robustness study.

Change in the chromatographic parameter	Resolution
Flow rate	
0.9 mL/min	1.7
1.1 mL/min	1.5
Mobile phase solvent variation	
+10% Acetonitrile	1.02
-10% Acetonitrile	1.7
Mobile phase pH	
pH 6.9	1.6
pH 7.1	1.5
Column temperature	
Temperature 15°C	1.6
Temperature 25°C	1.5

Critical parameter in the method is resolution that is studied during robustness evaluation. It had been observed that flow rate, pH of the mobile phase and column temperature not affecting much in day to day variation of chromatographic parameters. But the concentration of acetonitrile in the mobile phase-A has significant impact on enantioseparation.

CONCLUSION

This paper describes a new and reliable method for Tedizolid phosphate enantioseparation by HPLC, using β-CD as a

chiral mobile phase additive. β-cyclodextrin was used for the first time as a chiral mobile phase additive for determining the enantiomeric composition of racemic Tedizolid phosphate, rather than involving costly chiral stationary phases. The RP-HPLC chiral analytical method satisfies all validation parameters like system suitability, precision, specificity, accuracy, linearity of detector response, ruggedness and robustness. It indicates that the method is stable and suitable for the quantification of S-enantiomer of R-Tedizolid phospahte. Hence, the simple cost effective validated method can be used for routine analysis in quality control laboratories in the pharmaceutical industry.

Future plan is decided to identify the degradant impurity which is observed about 92% during force degradation study. This obvious degradant is required to synthesize or enrich for structure elucidation purpose. After quanlification of impurity standard it can be quantified in Tedizolid phosphate API either using chiral or achiral purity method.

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Conflict of Interests: There are no conflicts of interest.

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