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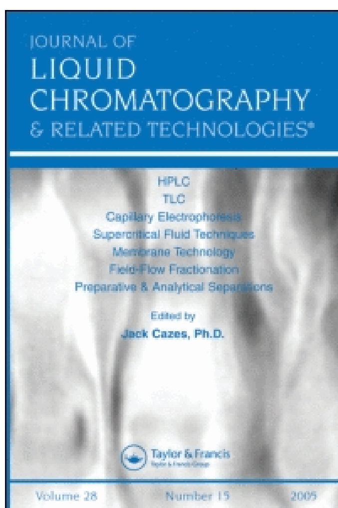

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Abstract: A high-performance liquid chromatographic method for chiral separation of tenofovir enantiomers was developed. The (R) and (S) isomers were separated on intertsil ODS 3V column (150 mm × 4.6 mm i.d., 5 μm) at 25°C. The mobile phase contained the complex of Cu(II) with the optical selector L-phenylalanine (L-PheA). Satisfactory results were achieved with the mobile phase consisting of buffer (3 mM of copper sulfate, 1 mM of L-PheA, and 20 mM of ammonium dihydrogen phosphate, pH adjusted to 4.0) and acetonitrile in the ratio of 95:5. The method was validated for linearity, repeatability, LOD, LOQ, and robustness. The solution stability was studied and found to be stable for the period of 7 days.

Keywords: Column liquid chromatography, Enantiomeric separation, Chiral mobile phase, Tenofovir, L-Phenylalanine, Achiral column

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INTRODUCTION

Chiral discrimination has been an issue in the development and use of pharmaceutical drugs because drug enantiomers may have different pharmacokinetic properties and produce different physiological responses. The administration of highly pure chiral drugs is a major goal of the pharmaceutical industry to protect the client against strains caused by high drug concentration or toxic side effects. Since, there can be quantitative and qualitative differences in pharmacological activity of enantiomers, it is necessary to develop analytical methods for chiral separation to control optical purity and to gain an understanding of the clinical, pharmacological, and pharmacodynamic modes of their actions.

Tenofovir disoproxil fumarate (tenofovir DF1, formerly known as bis(POC)-PMPA) is a bis-ester prodrug of the acyclic nucleoside phosphonate tenofovir. Tenofovir has a strong activity against human immunodeficiency virus infection in humans.[1] However, it is characterized by a permeability limited oral absorption due to its hydrophilic nature.[2] In the prodrug, the two negative charges of tenofovir are masked by isopropyloxycarbonyloxymethyl moieties, which increase the lipophilicity of the compound and, thus, its permeation across membranes. Based on its favorable pharmacokinetic profile compared with other ester prodrugs, tenofovir DF was selected as an orally active form of tenofovir.[3] It was recently approved by the United States Food and Drug Administration (US FDA) for the treatment of human immunodeficiency virus infection with the proprietary name of Viread. The chemical structure of tenofovir and its enantiomer is shown in Figure 1.

Recently, few HPLC analyses were reported in literature[4–8] and all are related to the determination of tenofovir in biological samples using different techniques. However, there was no literature available for enantiomeric separation of tenofovir.

The current study describes the enantiomeric separation of Tenofovir on an achiral HPLC column using L-phenylalanine as the chiral mobile phase additive and using copper sulfate as the complexing agent. The chemical structure of L-phenylalanine is presented in Figure 1.

EXPERIMENTAL

Chemicals and Analytical Standards

Qualified standards of tenofovir and its enantiomer were supplied by the Antiviral Research Laboratory of Matrix Laboratories Limited, Secunderabad, India and were used without any further purification and characterization. HPLC grade acetonitrile was purchased from J.T. Baker, USA. HPLC ammonium dihydrogen phosphate and analytical grade copper sulfate

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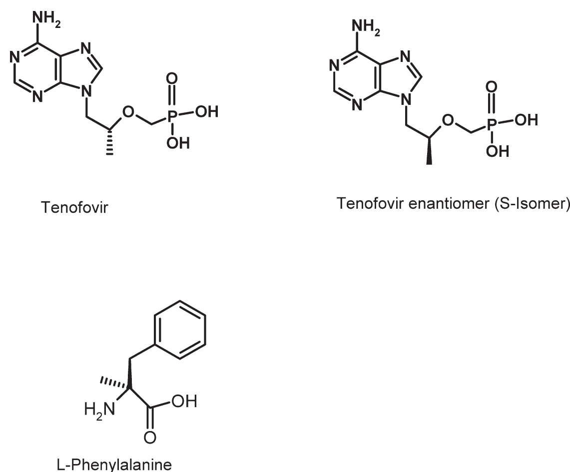

Figure 1. Chemical structures of L-phenylalanine, tenofovir and its enantiomer.

(anhydrous) were procured from Merck, Germany. L-Phenylalanine was purchased from Fluka (Buches, Switzerland). Analytical reagent grade ortho phosphoric acid was purchased from Ranbaxy Fine Chemicals, India.

Apparatus

All analyses were performed with a Waters HPLC system consisting of 2695 separation module, 2487 dual wavelength UV detector, auto sampler, column heater, degasser, and sample cooler. The same instrument was used in development and validation studies. Waters Empower software was used for data acquiring and calculation of system suitability parameters.

Buffer Preparation

The buffer was prepared by dissolving appropriate quantities of copper sulfate, L-phenylalanine, and ammonium dihydrogen phosphate to obtain the concentrations of 3 mM of copper sulfate, 1 mM of phenylalanine, and 20 mM of ammonium dihydrogen phosphate. The pH was adjusted to 4.0 with ortho phosphoric acid, filtered through a 0.45 μm porosity membrane filter.

Preparation of Mobile Phase

The mobile phase was prepared by mixing the buffer solution and acetonitrile in the ratio of 95:5, respectively, degassed and filtered through a 0.45 μm membrane filter. Mobile phase was used as the diluent in all solution preparations and subsequent dilutions.

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Preparation of Stock Solutions

Samples of tenofovir and its enantiomer were prepared in the diluent to obtain a concentration of 1.0 mg/mL.

Chromatographic Conditions

The optimized chromatographic conditions were achieved by using the mobile phase consisting of buffer and acetonitrile in the ratio of 95:5, respectively. The analytical column used for analysis of the sample was an Intertsil ODS-3V with the dimensions of 150 mm × 4.6 mm, 5 μm particle size, purchased from M/s. GL Sciences, USA. The flow rate was set at 0.6 mL/min and the peak responses were measured at 260 nm. The column temperature was maintained at 25°C. A typical chromatogram obtained with the optimized chromatographic conditions, showing enantiomeric separation of tenofovir is presented in Figure 2.

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Method Validation

Limit of Detection and Limit of Quantification of Enantiomer Impurity

The limit of detection (LOD), defined as lowest concentration of analyte that can be clearly detected above the baseline signal, is estimated as three times the signal to noise ratio. LOD and LOQ were measured by injecting a series of diluted solutions of the enantiomer.

The LOD of the enantiomer content was estimated by a series of dilutions of stock solution at the concentration of 0.02 μg/mL solution. This solution was prepared by diluting 5 mL of stock solution (1 mg/mL), initially, to 50 mL (dilution 1), followed by 2 mL to 100 mL (dilution 2), and finally, 1 mL of dilution 2 solution to 100 mL, to give a concentration equivalent to 0.02 μg/mL.

The LOQ of the enantiomer was measured by determining the signal to noise ratio of the solution. The solution was prepared by diluting 3.5 mL of dilution 2 solution to 100 mL, to give a concentration of 0.07 μg/mL.

Analytical Method Precision

The precision of the developed chiral method was evaluated by analyzing six test solutions of the enantiomer prepared at the LOQ level. Peak response corresponding to each test solution was measured and the relative standard deviation was calculated.

Linearity of Enantiomer Impurity

The detector's linear response was evaluated by preparing six calibration solutions of the enantiomer at concentrations of the LOQ to 120% of the

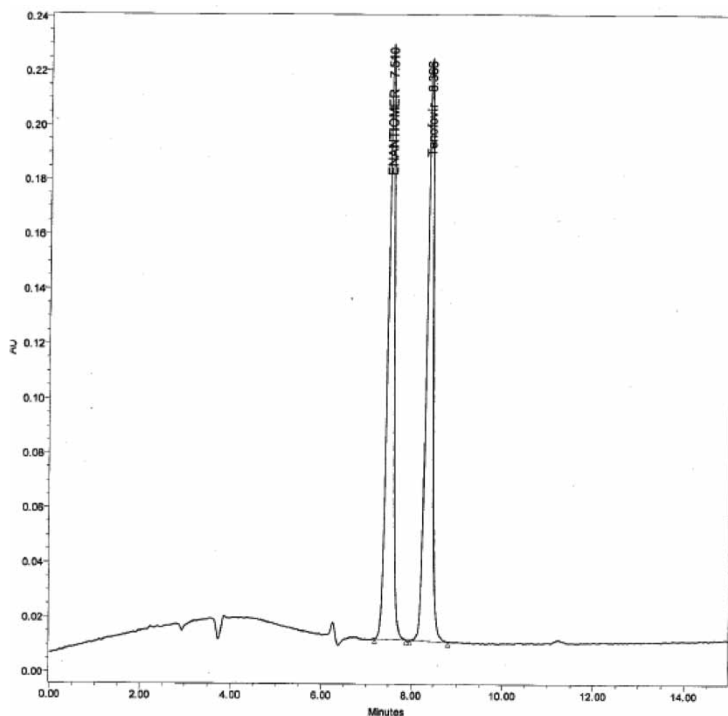

Figure 2. Typical chromatogram of enantiomeric separation of tenofovir.

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specification limit of the enantiomer, prepared from the stock solutions in the diluent.

The detector linear response was evaluated over the concentration range of 0.02 μg/mL to 6.0 μg/mL using six different concentrations prepared in mobile phase using stock solutions.

The calibration curve was constructed by plotting peak area versus concentrations of enantiomer and the regression equation was calculated.

Method Reproducibility

In order to determine the repeatability of the method, replicate injections ($n = 6$) of the mixture are made having a concentration of 1.0 mg/mL solution, containing 0.1% of enantiomer. Of the enantiomer stock solution, 1 mL was initially diluted to 100 mL, from which 2.5 mL was further diluted to 50 mL in a volumetric flask containing 25 mL of tenofovir stock solution. The intermediate precision was also evaluated over three days by performing six successive injections on each day by preparing the solutions similar to the above.

Robustness of the Method

Robustness of the method was studied with the solution of 0.1% enantiomer in tenofovir by deliberate changes in mobile phase flow, variation in column temperature, variation in pH of buffer, and content of acetonitrile.

The effect of the flow rate was studied by deliberate changes in the standard flow rate of the mobile phase by ± 0.2 units (0.4 mL/min to 0.8 mL/min).

The effect of temperature was studied by altering the column temperature by $\pm 5^{\circ}C$ keeping the other operational conditions unaltered.

The effect of buffer pH was studied by altering the pH values by ± 0.5 units, while other components were kept unaltered.

The effect of change in acetonitrile content in mobile phase was studied by altering the composition in mobile phase by ± 1 units, while other components of mobile phase were kept unchanged.

Solution Stability and Mobile Phase Stability

The stability of tenofovir and its enantiomer were studied by retaining the sample containing 0.1% of the enantiomer in tenofovir in a tightly closed volumetric flask at room temperature in the laboratory for 7 days. The content of the enantiomer was checked after every 8 h interval during the study period, and the peak responses were compared.

Mobile phase stability was evaluated by a fresh preparation of enantiomer solution at a 0.1% level with respect to tenofovir working concentration at 8 h intervals for a period of 7 days.

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Quantification of Impurity in Bulk Sample

The bulk sample of tenofovir provided by the Process Research Laboratory of Matrix Laboratories showed the enantiomer content was below the detection limit. Standard addition and recovery experiments were conducted to determine the accuracy of the analytical method for quantification of the enantiomer content in bulk samples.

The study was carried out in triplicate at 0.1, 0.25, and 0.5% of the tenofovir target analyte concentration. The recovery was calculated from the slope and intercept of the calibration curve obtained through a linearity study. A typical chromatogram of bulk sample analysis was presented in Figure 3.

RESULTS AND DISCUSSION

Development and Optimization of the Method

Copper (II) complexes of L-amino acids (L-AA) and their derivatives have been used as chiral mobile phase additives (CMPA) for resolution of the enantiomers

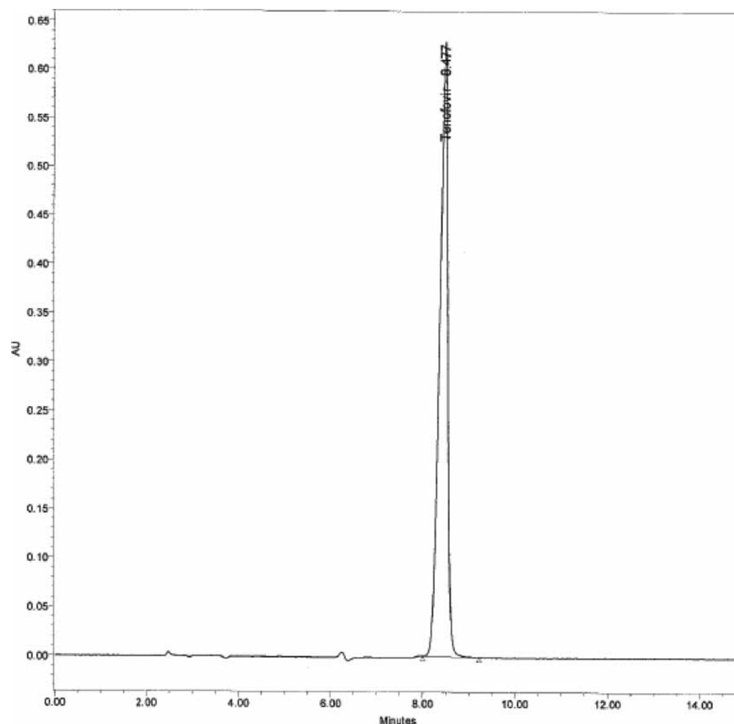

Figure 3. Typical chromatogram of tenofovir bulk sample.

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of free or derivatized amino acids by reverse-phase HPLC.[9–12] In chiral ligand-exchange HPLC, the factor most responsible for chiral discrimination seems to be the affinity of the diastereomeric ternary ligand complexes for the stationary phase.[13–14] It has been observed that the concentration of Cu(II) and the ratio of L-phenylalanine (L-PheA) in the mobile phase has affected the separation of enantiomers. Initially, the experiments were carried out with a 1:1 ratio of Cu(II)/L-PheA and the separation was not achieved. The concentration was increased to 3:1 where good separation was achieved and further levels are not investigated due to formation of precipitation.

Enantioselectivity was found to be pH dependent of the buffer solution. The optimum selectivity was achieved at the pH of 4.0. The higher pH studies were not performed due to the formation of precipitate at pH of 5.0.

The buffer was finally modified by maintaining the Cu(II)/L-PheA ratio at 3:1, with 20 mM of ammonium dihydrogen phosphate, and the pH was adjusted to 4.0 with ortho phosphoric acid. The mobile phase was optimized at buffer and acetonitrile ratio at 95:5, using a reverse-phase column of C_{18} stationary packing of 5 μm particle size. Typical retention times of tenofovir and its enantiomer were found at 8.37 and 7.51 minutes, respectively. The system suitability parameters are presented in Table 1.

Table 1. System suitability parameters

Compound (n = 3)	RT	R	N	T
Enantiomer	7.51	—	3933	0.92
Tenofovir	8.37	3.22	16118	0.95

RT: Retention time in minutes; R: Resolution; N: No. of theoretical plates; T: Tailing factor.

Results from Validation of the Method

The relative standard deviation (RSD) in the repeatability study was found to be less than 0.3% for retention time and peak responses of both enantiomers. The RSD values in the intermediate precision study were found in the same order of magnitude as those obtained in the repeatability test.

The LOD and LOQ concentrations of the enantiomer were measured as 0.02 μg/mL and 0.07 μg/mL, respectively, with the signal to noise ratio of about 3 and 10, respectively.

The RSD value of the method precision at the LOQ level of the enantiomer was found to be less than 2%.

Good linearity was observed for the enantiomer over the concentration range of LOQ to 120% of the specification limit with respect to analyte concentration (0.07 μg to 6 μg/mL, with the linear regression equation $y = 4143.2x + 85$ (correlation coefficient R = 0.99992). The RSD of slope and intercept was evaluated over the same concentration range for three consecutive days and was found to be less than 0.3% and less than 2.5%, respectively. Summary data of the validation results are presented in Table 2.

The standard addition and recovery experiments were carried out for the enantiomer in the tenofovir bulk sample in triplicate at concentration levels of 0.1%, 0.25%, and 0.5% of analyte concentration (analyte concentration was set at 1 mg/mL). The recoveries were found to be in the range of 98.67% to 101.0% with the RSD values in the range of 0.99% to 3.07%.

The robustness of the method is the ability of the method to remain unaffected by small changes in parameters such as flow rate, mobile phase composition, and column temperature. To evaluate the robustness of the method, the experimental conditions were deliberately modified and the resolution between tenofovir and its enantiomer was evaluated. The chromatographic resolution experiments for enantiomers were estimated to evaluate the method robustness under the altered conditions. The resolution in the flow rate variation experiments, column temperature variations, variation in buffer pH, and acetonitrile content variations was found in the range of 3.06 to 3.27, 3.23 to 3.47, 2.77 to 3.41, and 3.09 to 3.27, respectively. This indicates that the resolution between tenofovir and its enantiomer found in all robustness experiments was not less than 3.0, and demonstrates that the method was sufficiently robust. The summary of the robustness data is presented in Table 3.

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Table 2. Validation results of analytical method

Validation parameter	Result
Linearity of enantiomer	
Calibration range	0.07 μg – 6.0 μg
Calibration points	6
Correlation coefficient	0.99992
Slope (%RSD)	0.22
Intercept (%RSD)	2.01
LOD & LOQ data	
Limit of detection	0.02 μg
Limit of quantitation	0.07 μg
Precession at LOQ (%RSD)	1.97%
Repeatability data (%RSD)	
Retention time (enantiomer)	0.22
Retention time (tenofovir)	0.26
Peak area (enantiomer)	0.39
Peak area (tenofovir)	0.44
Intermediate precision data (n = 18, %RSD)	
Retention time (enantiomer)	0.21
Retention time (tenofovir)	0.27
Peak area (enantiomer)	0.41
Peak area (tenofovir)	0.43

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Table 3. Robustness data

Parameter altered	Resolution
Flow rate	
0.4 mL/min	3.27
0.6 mL/min	3.22
0.8 mL/min	3.06
Column temperature	
20°C	3.23
25°C	3.22
30°C	3.47
Buffer pH	
3.5	2.77
4.0	3.22
4.5	3.41
Acetonitrile (in percent)	
3	3.27
5	3.22
7	3.09

The RSD of enantiomeric content evaluated through solution stability and mobile phase stability was found to be $<0.7\%$ in both cases. Thus, it is evident that the mobile phase and/or the prepared solutions are stable for at least 7 days.

CONCLUSIONS

A simple isocratic chiral method was developed for enantiomeric separation of tenofovir using L-phenylalanine as the chiral mobile phase additive and was fully validated. Satisfactory results were obtained in all validation experiments and can be conveniently used in quality laboratories for quantification of enantiomeric contents.

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