Original Research Paper

Acta Chromatographica 27(2015)4, 583–595
DOI: 10.1556/AChrom.27.2015.4.1

Enantiomeric Separation and Quantitation of Tenofovir Disoproxil Fumarate Using Amylose-Based Chiral Stationary Phases by High-Performance Liquid Chromatography

R. HEYDARI[1,*] AND M. SHAMSIPUR[2,]**

[1]Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, P.O. Box 68149-89468, Khorramabad, Iran
[2]Department of Chemistry, Razi University, Kermanshah, Iran
*E-mail: rouhollahheydari@yahoo.com, **E-mail: mshamsipur@yahoo.com

Summary. A rapid high-performance liquid chromatography (HPLC) method for chiral purity determination of tenofovir disoproxil fumarate in raw material and pharmaceutical formulations was developed. The (*S*)-enantiomer appears to be as an impurity and pharmacologically inactive. The effects of various stationary phases, mobile phase composition, and column temperature on enantiomeric separation of tenofovir disoproxil were investigated and optimized. Chromatography resolution of tenofovir disoproxil enantiomers was performed on NUCLEOCEL ALPHA-RP S column (250 × 4.6 mm i.d., 5 μm). The elution was achieved by using 95:5% (*v*/*v*) methanol–acetonitrile, containing 0.1% triethylamine at a flow rate of 0.8 mL min^{-1}. The ultraviolet (UV) detector was set at 260 nm. Calibration curves were linear in the range of 1–100 μg mL^{-1} and 0.2–20 μg mL^{-1} for (*R*)-tenofovir disoproxil and (*S*)-enantiomer, respectively. Limits of detection and quantitation for (*S*)-enantiomer were 0.06 and 0.2 μg mL^{-1}. The run time of analysis was less than 7.0 min. The proposed method was used successfully for separation and quantification of tenofovir disoproxil enantiomers in raw material and pharmaceutical formulations.

Key Words: tenofovir disoproxil fumarate, NUCLEOCEL ALPHA-RP S column, enantiomeric separation, high-performance liquid chromatography

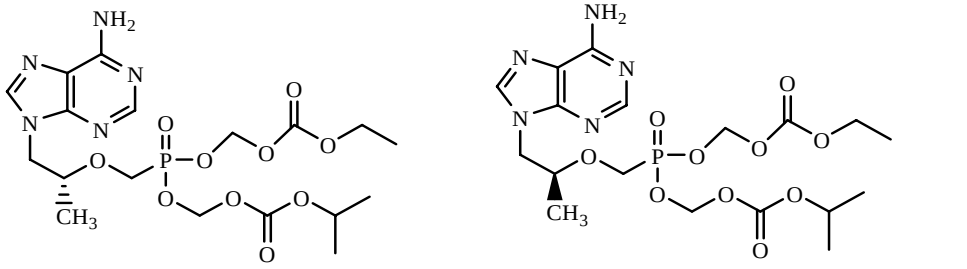
Introduction

Chiral separations are important, especially in the pharmaceutical industry, as enantiomers often possess different health benefits [1]. The analysis and preparation of a pure enantiomer usually involve its separation from the antipode. Among all the chiral separation techniques, high-performance liquid chromatography (HPLC) has proven to be the most convenient, reproducible, selective, sensitive, and widely applicable method. A chiral selector was used as chiral stationary phase (CSP) in most HPLC methods. These CSPs are prepared by using either a polymeric structure or a small ligand as

chiral selector. The polymeric CSPs were classified in two categories: synthetic and naturally chiral polymers [2–5]. Cellulose and amylose are the most abundant natural polymers used in chiral separations.

Amylose-based columns were used as a suitable CSP for many racemates [6, 7]. The chiral recognition ability of CSP depends on two factors: the size of cavities and chirality of the side chain. For example, the (*S*)-1-phenylethylcarbamate of amylose shows a higher resolving ability than the (*R*) derivative.

Tenofovir disoproxil fumarate is a fumaric acid salt of bis-isopropoxycarbonyloxymethyl ester derivative of tenofovir. The structures of (*R*)-tenofovir disoproxil and tenofovir disoproxil enantiomer ((*S*)-enantiomer) are shown in *Fig. 1*. The chemical name of (*R*)-tenofovir disoproxil fumarate is [[(1*R*)-2(6-amino-9H-purin-9-yl)-1-methylethoxy]methyl]phosphonate, bis-(isopropyloxycarbonyloxymethyl ester), fumarate (1:1). Tenofovir, a nucleotide reverse transcriptase inhibitor, is an antiviral agent that plays a function in current anti-human immunodeficiency virus (HIV) therapy [8]. A prodrug of tenofovir, tenofovir disoproxil fumarate (tenofovir DF), is used in combination with other antiretroviral drugs for the treatment of patients above 18 years of age infected with the HIV who failed or are intolerant to nucleoside analog therapy or are not controlled by their current antiretroviral regimen [8]. The (*S*)-isomer appears to be pharmacologically inactive. In addition, the presence of an impurity can significantly affect the therapeutic properties of the drug. Hence, it is important to develop separation methods for chiral purity control of the active pharmaceutical ingredient (API) and finished products. Limit of (*S*)-enantiomer in raw material using a suitable chiral chromatographic method should not be more than 1.0%.



(*R*)-Tenofovir disoproxil Tenofovir disoproxil enantiomer ((*S*)-enantiomer)

Fig. 1. Structures of (*R*)-tenofovir disoproxil and tenofovir disoproxil enantiomer ((*S*)-enantiomer)

Recently, several methods for the simultaneous determination of tenofovir and other antiretroviral nucleosides or tenofovir alone in biological samples using different detection techniques have been reported in the literature. These include HPLC methods with UV detection [9–11], spectrofluorimetric detection [12, 13], and tandem mass spectrometry (MS/MS) detection [14–17]. There is also a report on the enantiomeric separation of tenofovir on an achiral HPLC column by using L-phenylalanine as chiral mobile phase additive and copper sulfate as complexing agent [18]. However, most of the reported methods require time-consuming sample preparation methods or expensive equipments such as liquid chromatography–mass spectrometry (LC–MS) that are not economically feasible for routine analysis. Therefore, the development of rapid, simple, and inexpensive separation methods for chiral purity control of the API and finished products is necessary.

Rapid analysis techniques will reduce cost and save time. The purpose of this study was to develop and validate a new, rapid, and simple method for enantiomeric separation and quantitation of (*R*)-tenofovir disoproxil and its enantiomer in raw material and pharmaceutical preparations by using chiral HPLC.

Experimental

Chemicals

(*R*)-tenofovir disoproxil fumarate, its enantiomer ((*S*)-isomer), and pharmaceutical raw material were obtained from Hetero (Hyderabad, India). Potassium hydrogen phosphate dibasic, sodium hydroxide, phosphoric acid, methanol, acetonitrile, and triethylamine were purchased from Merck (Darmstadt, Germany). TENOBIOVIR tablets (containing 300 mg tenofovir disoproxil fumarate) were obtained from Bakhtar Bioshimi Pharmaceutical Company (Kermanshah, Iran). VIREAD tablets (containing 300 mg tenofovir disoproxil fumarate) were obtained from Gilead Sciences (USA).

Apparatus and Conditions

A high-pressure Shimadzu HPLC system (Model LC-10AD vp, Japan) with a UV–vis detector (SPD-10A vp) was used. The NUCLEOCEL ALPHA-RP S column (250 × 4.6 mm, 5 µm), consisting of amylose tris(3,5-dimethylphenyl carbamate) as stationary phase, was purchased from Macherey-Nagel (Germany). The CHIRALPAK AD-H column (250 × 4.6 mm, 5 µm) was pur-

chased from Daicel Chemical Industries (Japan). The chiral-AGP column (150 × 4.0 mm, 5 μm), consisting of immobilized α_1-acid glycoprotein as stationary phase, was purchased from ChromTech (Stockholm, Sweden). The NUCLEODEX β-OH (200 × 4.0 mm, 5 μm), consisting of β-cyclodextrin as stationary phase, was purchased from Macherey-Nagel (Germany). Methanol–acetonitrile (95:5% (*v*/*v*)) containing 0.1% (*v*/*v*) triethylamine was used as mobile phase. The temperature of the column was maintained at 20 °C by a column oven (CTO-10AS vp). The mobile phase flow rate was kept constant at 0.8 mL min^{-1}. The analytes were detected at 260 nm. The injection volume was 5 μL.

Preparation of Stock and Working Standard Solutions

The stock solutions of (*R*)-tenofovir disoproxil and tenofovir disoproxil enantiomer ((*S*)-enantiomer) were prepared by dissolving 10 mg of each compound in 10 mL of mobile phase, separately. The corresponding working standard solutions were prepared by dilution of individual stock solutions with mobile phase. From these solutions, serial dilutions were made to obtain different concentrations of 1.0, 2.0, 5.0, 10.0, 20.0, 40.0, 60.0, 80.0, and 100 μg mL^{-1}, for (*R*)-tenofovir disoproxil, and 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, and 20.0 μg mL^{-1}, for (*S*)-enantiomer.

Preparation of Sample Solutions

The raw material solution was prepared by transferring of 40 mg of tenofovir DF raw material to a 100-mL volumetric flask with added 100 mL of mobile phase, to obtain a sample solution containing 0.4 mg mL^{-1} of drug. The tablet solutions were prepared by dissolving an amount of tablet equivalent to 40 mg of tenofovir DF into a 100-mL volumetric flask containing mobile phase. Sample solutions were stirred for 10 min. Before injection to HPLC system, sample solutions passed through a disposable 0.45 μm PTFE membrane.

Results and Discussion

In order to separate two enantiomers of tenofovir disoproxil, various CSPs with different mobile phases were examined. Four columns including NUCLEOCEL ALPHA-RP S, CHIRALPAK AD-H, NUCLEODEX β-OH, and AGP were used in this study. NUCLEOCEL ALPHA-RP S and CHIRALPAK AD-H contain amylose tris(3,5-dimethylphenylcarbamate) as CSP, whereas NUCLEODEX β-OH and AGP columns contain β-cyclodextrin and

α_1-acid glycoprotein as stationary phase, respectively. These columns (NUCLEODEX β-OH and AGP) were operated with phosphate buffer containing various percents of acetonitrile (5–15%, *v*/*v*) as mobile phase. Under the above conditions no resolution was observed with two columns.

Therefore, NUCLEOCEL ALPHA-RP S and CHIRALPAK AD-H columns were selected for further investigations.

Comparison of NUCLEOCEL ALPHA-RP S and CHIRALPAK AD-H Columns

Fig. 2 shows the chromatograms acquired using two different columns with similar stationary phases (amylose tris(3,5-dimethylphenylcarbamate)) under the same conditions. As observed, enantiomeric separation occurred with two columns. However, the run time with the NUCLEOCEL ALPHA-RP S column was shorter than that of the CHIRALPAK AD-H column. Other important column performance parameters are shown in *Table I*. The

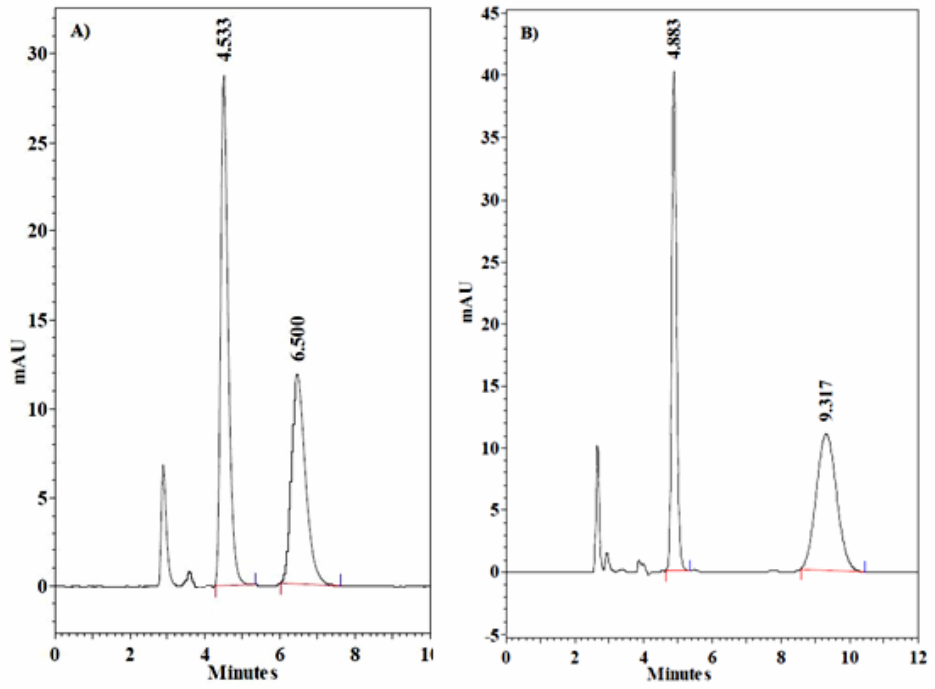


Fig. 2. Typical chromatograms of (*R*)-tenofovir disoproxil and its enantiomer by two columns: NUCLEOCEL ALPHA-RP S column (A) and CHIRALPAK AD-H column (B). Conditions: flow rate, 0.8 mL min^{-1}; column temperature, 20 °C; mobile phase, methanol–acetonitrile (95:5 (*v*/*v*))

tailing factors (*T*) were not very different for the two columns *(Table I)*. Therefore, the NUCLEOCEL ALPHA-RP S column was used for further investigations. However, due to high-resolution enantiomer separation using the CHIRALPAK AD-H column, this column can be used in preparative HPLC analyses.

Table I. System suitability parameters. Conditions: flow rate, 0.8 mL min^{-1}; column temperature, 20 °C; ratio of methanol–acetonitrile in mobile phase, 95:5 (*v*/*v*)

Column	Compound	RT	Rs	*N*	*T*
NUCLEOCEL ALPHA-RP S	(*R*)-tenofovir disoproxil	4.533	–	2649	1.24
	(*S*)-enantiomer	6.500	1.79	1399	1.08
CHIRALPAK AD-H	(*R*)-tenofovir disoproxil	4.883	–	6128	1.22
	(*S*)-enantiomer	9.317	4.22	1111	1.17

RT: retention time in minute; Rs: the resolution factor is calculated between each peak and its nearest preceding neighbor; *N*: number of theoretical plate; *T*: the tailing factor is calculated at 5% of peak height according to USP method.

The Effect of Acetonitrile Content on Enantioselectivity

The effect of acetonitrile content of mobile phase on enantioseparation in the range of 0.0–40.0% (*v*/*v*) was studied. As observed from *Fig. 3*, with the addition of 5.0% acetonitrile, enantioselectivity (α) increases from 1.75 to

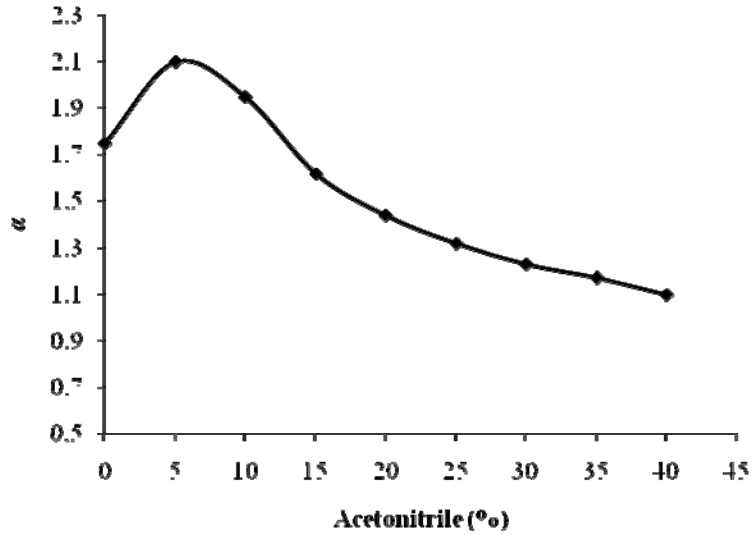


Fig. 3. Effect of acetonitrile percent in methanol–acetonitrile mobile phase on enantioselectivity. Conditions: flow rate, 0.8 mL min^{-1}; column temperature, 20 °C

2.1. Further addition of acetonitrile content to mobile phase decreased the enantioselectivity. Therefore, a 95:5% (*v*/*v*) mixture of methanol–acetonitrile was selected as mobile phase.

The Effect of Column Temperature on Enantioselectivity

Temperature has a major impact on retention, enantioselectivity, resolution, and column efficiency in chiral separations. Consequently, the variation of the column operation temperature has been frequently exploited as an optimization parameter in gas and liquid chromatographic separations of enantiomers [19–22].

Generally, chiral selectivity increases exponentially with decreasing column temperature. In this work, the effect of column temperature on enantioselectivity was investigated over the range of 5.0–50.0 °C. As seen, the enantioselectivity decreased by increasing the column temperature gradually *(Fig. 4)*. However, in the range of 5.0–30.0 °C, the variations of chiral selectivity were small. Therefore, a column temperature of 20.0 °C was selected for further studies.

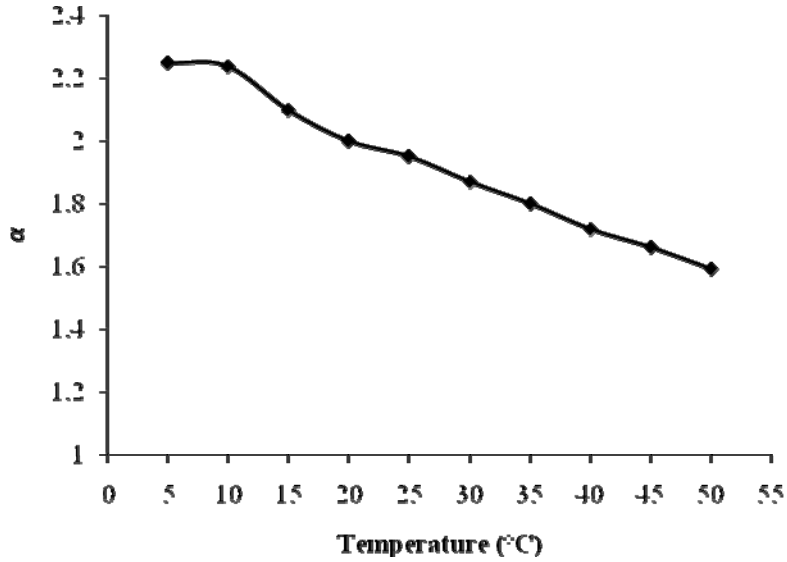


Fig. 4. Effect of column temperature on enantioselectivity. Conditions: flow rate, 0.8 mL min^{-1}; mobile phase, methanol–acetonitrile (95:5 (*v*/*v*))

Method Development and Validation

Method validation is the process of demonstrating that analytical procedures are suitable for their intended use. Calibration curves were obtained by analyses of working standard solutions of the individual enantiomers at various concentrations. Linearity ranges were evaluated based on the correlations between the peak areas and the concentrations of the enantiomers. The statistical parameters of the resulting linear calibration curves for the two enantiomers of tenofovir disoproxil are summarized in *Table II*.

Table II. Linear analytical response statistical summary for compounds using peak area

Compound	Calibration range (μg mL^{-1})	R^2	Intercept	Slope	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
(*R*)-tenofovir disoproxil	1–200	0.9996	44758.0	36106.0	–	–
(*S*)-enantiomer	0.2–20	0.9984	3720.2	35205.0	0.06	0.2

In order to investigate the repeatability of the proposed method, known concentrations of tenofovir disoproxil and its enantiomer (typically 5.0 μg mL^{-1}) were injected to the HPLC system, under the optimized experimental conditions, six times. The mean values of relative standard deviation (RSD) were 0.58% for tenofovir disoproxil and 0.42% for (*S*)-enantiomer.

Within and between-day precisions were calculated by analyzing three standard solutions with different concentration levels (5, 20, and 100 μg mL^{-1} for (*R*)-tenofovir disoproxil and 1, 5, and 20 μg mL^{-1} for (*S*)-enantiomer) for each analyte. Results are shown in *Table III*.

The accuracy of the method was evaluated using spiked samples containing (*R*)-tenofovir disoproxil (400 μg mL^{-1}) with the (*S*)-enantiomer on different concentration levels (0.05% and 0.1% of main peak, 0.2 and 0.4 μg mL^{-1}, respectively) *(Fig. 5)*. The recoveries of (*S*)-enantiomer were found to be between 99.2 and 105.0%, and the average RSDs were below 2.0%.

The LOD and LOQ for the (*S*)-enantiomer were calculated experimentally from serial dilutions. Results indicated the values of LOD and LOQ are 0.06 and 0.2 μg mL^{-1}, respectively. The LOD and LOQ for (*R*)-tenofovir disoproxil were not reported because this compound exists as a major component in real samples.

The proposed method was applied for determination of (*S*)-enantiomer in a raw material sample from Hetero Company (Hyderabad, India),

Table III. Precision of within and between run analysis (n = 9; three sets for 3 days)

Compound	Concentration (μg mL^{-1})	RSD (%)[a]
Within-day (n = 3)		
(*R*)-tenofovir disoproxil	5.0	0.51
	20.0	1.14
	100.0	0.80
(*S*)-enantiomer	1.0	0.66
	5.0	0.10
	20.0	0.40
Between-day (n = 9)		
(*R*)-tenofovir disoproxil	5.0	1.04
	20.0	1.37
	100.0	1.22
(*S*)-enantiomer	1.0	1.62
	5.0	1.56
	20.0	1.16

[a]Relative standard deviation.

Table IV. Results of determined amounts of tenofovir DF and its enantiomer in real samples

Real sample		Results (assay %) ± SD
Raw material	Tenofovir DF	99.00 ± 0.37
	(*S*)-enantiomer	Not detected
VIREAD tablet	Tenofovir DF	98.22 ± 0.59
	(*S*)-enantiomer	Not detected
TENOBIOVIR tablet	Tenofovir DF	98.98 ± 0.41
	(*S*)-enantiomer	Not detected

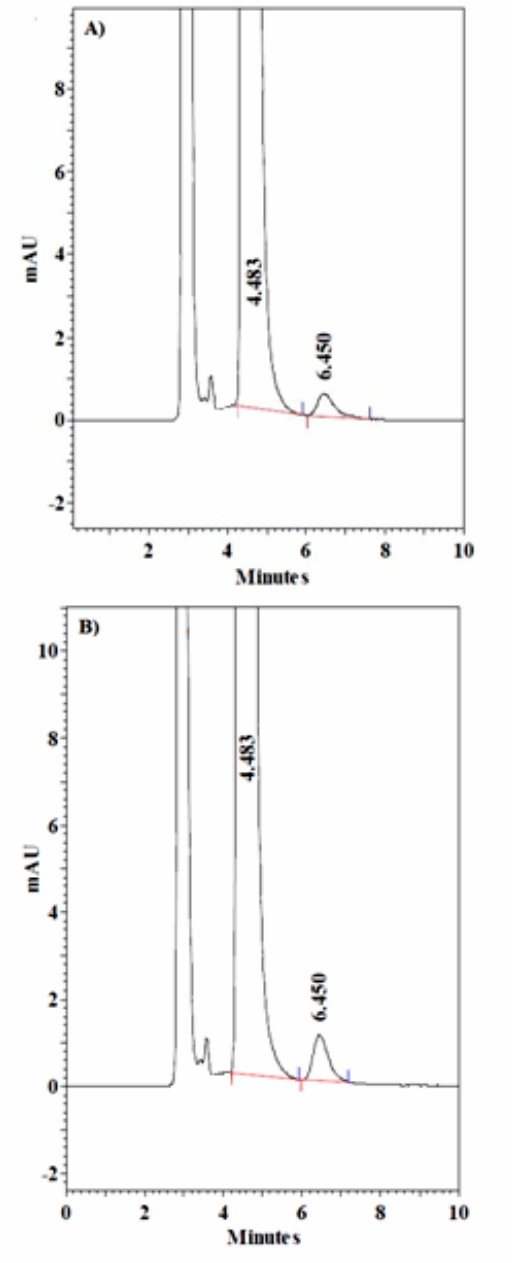


Fig. 5. Chromatograms of spiked sample with various percentages of (*S*)-enantiomer: 0.05% (A) and 0.1% (B). Conditions: flow rate, 0.8 mL min^{-1}; column temperature, 20.0 °C; mobile phase, methanol–acetonitrile (95:5 (*v*/*v*))

TENOBIOVIR tablets (Bakhtar Bioshimi, Iran), and VIREAD tablets (Gilead Sciences, USA). Results are shown in *Table IV*. *Fig. 6* shows the HPLC chromatograms of the raw material *(Fig. 6A)* and spiked raw material with (*S*)-enantiomer *(Fig. 6B)*.

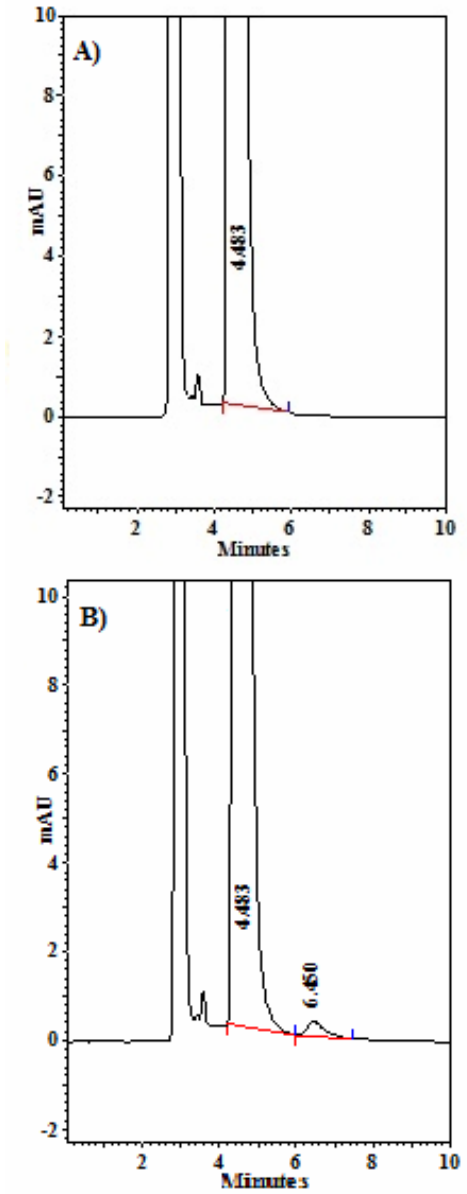


Fig. 6. Chromatograms of raw material (A) and raw material spiked with (*S*)-enantiomer (0.2 μg mL^{-1}, 0.05% of main peak) (B). Conditions: flow rate, 0.8 mL min^{-1}; column temperature, 20.0 °C; mobile phase, methanol–acetonitrile (95:5 (*v*/*v*))

Conclusion

A rapid, simple, and direct chiral separation method for enantiomeric separation and quantitation of (*R*)-tenofovir disoproxil and its (*S*)-enantiomer has been developed using the amylose tris(3,5-dimetylphenylcarbamate) stationary phase (NUCLEOCEL ALPHA-RP S). The results were shown under applied chromatographic conditions, resolution of these two enantiomers was complete. The proposed method was validated for linearity, repeatability, accuracy, LOD, and LOQ. The present method can be used in quality control laboratories for separation and quantification of tenofovir DF enantiomers in raw material and pharmaceutical formulations.

Acknowledgments

The support of this work by Iran National Science Foundation (INSF) and Bakhtar Bioshimi Pharmaceutical Company, Kermanshah, Iran, is gratefully acknowledged. The authors thank Mr. Bahman Barazesh for his excellent collaboration.

References

[1] V.K. Kakumanu, V. Arora, and A.K. Bansal, Eur. J. Pharm. Biopharm., **64**, 255 (2006)
[2] Y. Okamoto, S. Honda, I. Okamoto, H. Yuki, S. Murata, R. Noyori, and H. Takaya, J. Am. Chem. Soc., **103**, 6971 (1981)
[3] K.R. Lindner and A. Mannschreck, J. Chromatogr. A, **193**, 308 (1980)
[4] J. Hermansson, J. Chromatogr. A, **269**, 71 (1983)
[5] Y. Okamoto, M. Kawashima, K. Yakamoto, and K. Hatada, Chem. Lett., **13**, 739 (1984)
[6] Y. Okamoto, Y. Kaida, H. Hayashida, and K. Hatada, Chem. Lett., **19**, 909 (1990)
[7] Y. Kaida and Y. Okamoto, J. Chromatogr. A, **641**, 267 (1993)
[8] E. De Clercq, Biochim. Biophys. Acta, **1587**, 258 (2002)
[9] S. Sentenac, C. Fernandez, A. Thuillier, P. Lechat, and G. Aymard, J. Chromatogr. B, **793**, 317 (2003)
[10] N.L. Rezk, R.D. Crutchley, and A.D. Kashuba, J. Chromatogr. B, **822**, 201 (2005)
[11] V. Bezy, P. Morin, P. Couerbe, G. Leleu, and L. Agrofilio, J. Chromatogr. B, **821**, 132 (2005)
[12] V. Jullien, J-M. Tréluyer, G. Pons, and E. Rey, J. Chromatogr. B, **785**, 377 (2003)
[13] R.W. Sparidans, K.M.L. Crommentuyn, J.H.M. Schellens, and J.H. Beijnen, J. Chromatogr. B, **791**, 227 (2003)
[14] T. Delahunty, L. Bushman, and C.V. Fletcher, J. Chromatogr. B, **830**, 6 (2006)
[15] T. Delahunty, L. Bushman, B. Robbins, and C.V. Fletcher, J. Chromatogr. B, **877**, 1907 (2009)

[16] N.A. Gomes, V.V. Vaidya, A. Pudage, S.S. Joshi, and S.A. Parekh, J. Pharm. Biomed. Anal., **48**, 918 (2008)
[17] R.S. Jansena, H. Rosing, W. Kromdijk, R.T. Heine, J.H.M. Schellens, and J.H. Beijnen, J. Chromatogr. B, **878**, 621 (2010)
[18] U. Seshachalam, B. Rajababu, B. Haribabu, and K.B. Chandrasekhar, J. Liq. Chromatogr. Relat. Technol., **31**, 410 (2008)
[19] E. Kusters, V. Loux, E. Schmidt, and P. Floersheim, J. Chromatogr. A, **666**, 421 (1994)
[20] K. Cabrena and D. Lubda, J. Chromatogr. A, **666**, 433 (1994)
[21] K.M. Kirkland and D.A. McCombs, J. Chromatogr. A, **666**, 211 (1994)
[22] V. Schurig, J. Chromatogr. A, **906**, 275 (2001)