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Polysaccharide-based chiral phase under polar organic mode of elution in the determination of the enantiomeric purity of emtricitabine an anti-HIV analogue nucleoside

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Abstract

This work reports the separation of FTC enantiomers using an amylose tris[(*S*)-1-phenylethylcarbamate] coated onto APS-Nucleosil (7 μm particle size, 500 Å pore size, 20% w/w, 15 × 0.46 cm ID) chiral column under polar organic elution mode. Good enantioselectivity ($\alpha = 1.9$) with excellent enantioresolution ($R_S = 3.3$) was achieved by the use of methanol with 0.02% of triethylamine acetate as mobile phase. The method allows the accurate determination of as low as 0.2% of each enantiomer as an impurity. The validated method proved to be reliable and sensitive for the quantification of both enantiomers as impurity in different batches of emtricitabine and β-D-(+)-FTC.

Keywords: Polysaccharide carbamate phase; Enantioseparation; Multimodal elution; Validation; Enatiomeric ratio; FTC

1. Introduction

(−)-Cis-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine [(−)-FTC] is a new synthetic nucleoside undergoing clinical trial phase III for the treatment of human immunodeficiency virus (HIV). β-L-(−)-FTC is also known as emtricitabine, or by the tradename Coviracil and is being developed by Triangle Pharmaceuticals [1,2].

Emtricitabine is a dideoxycytidine nucleoside reverse transcriptase inhibitor, potent and selective against HIV virus types 1 and 2 and hepatitis B virus [3–5]. Emtricitabine (Fig. 1) is approximately 20-fold more potent against HIV in peripheral blood mononuclear cells than its β-D-(+)-enantiomer [3]. Therefore, the development of analytical methods that can enantioseparate and quantify these enantiomers plays a very important role not

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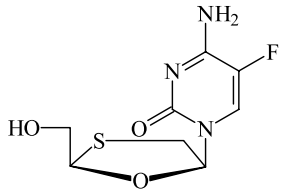

Fig. 1. Structure of emtricitabine.

only at the drug development process but also to assure a reliable quality control of the final manufactured product.

Liquid chromatography using chiral stationary phases has proven to be the most useful among the methods currently used to achieve chiral separation of enantiomeric mixtures. Numerous chiral phases have been investigated and developed during the last decade [6–8]. Polysaccharide-based phases have been identified as very versatile and useful chiral sorbents for the separation of a great variety of different classes of chiral compounds [6,7] and have been efficiently used for the separation of stereoisomers of a number of nucleoside analogues [9–13].

Herein, we report the direct resolution of the enantiomers of FTC by high performance liquid chromatography using an amylose-based chiral stationary phase under polar organic elution mode. The method, developed and validated, is simple and able to quantify, with excellent inter and intra-day precision and accuracy, the enantiomeric purity of the enantiomers when one is an impurity of the other in the percentage range of 0.2–2%.

2. Materials and methods

2.1. Chemicals

Emtricitabine, (+)-FTC, and (±)-FTC were prepared by Microbiologica Química e Farmacêutica LTDA (Rio de Janeiro, Brazil) using procedures adapted from the synthesis of Lamivudine but using 5-F-cytosine instead of cytosine. For Emtricitabine the chiral oxathiolane was made from L-menthylglyoxylate whereas for (+)-FTC, D-menthylglyoxylate was used. (±)-FTC was obtained from the unresolved mixture of oxathiolanes resulting from the reaction of L-menthylglyoxylate with 2-mercaptoethanal [14,15]. Methanol and acetonitrile were HPLC grade and were obtained from Mallinckrodt Baker (St. Louis, Missouri, USA). All the others analytical grade reagents were obtained from Merck (Darmstadt, Germany).

2.2. Instrumentation and HPLC conditions

The HPLC system used consisted of one Shimadzu LC-10ATVP pump, an automatic injector SIL-10ADvp, a SPD-10Avp UV–VIS detector with a SCL 10AVP interface. A photodiode array model SPD-10AVP was also used. Data acquisition was performed using Shimadzu CLASS-VP software.

Chiral columns (150 × 4.6 mm I.D.) were prepared as described in the literature [16,17] and consisted of cellulose and amylose tris(3,5-dimethylphenylcarbamate), amylose tris(3,5-dimethoxyphenylcarbamate) and amylose tris[(*S*)-1-phenylethylcarbamate] and coated (20% w/w) onto APS-Nucleosil (7 μm particle size and 500 Å pore size).

The preferred chiral column was the amylose tris[(*S*)-1-phenylethylcarbamate]. Elution was carried out isocratically at a flow rate of 0.5 ml min^{-1} using acetonitrile:methanol (95:05 v/v) with triethylamine (TEA) and glacial acetic acid (AcOH), at 0.02% (v/v) of each, as a triethylamine acetate buffer (TEAA) with detection at $\lambda = 280$ nm.

2.3. Preparation of stock solutions and calibration standards

Stock solutions of two different concentrations were prepared using the selected mobile phase as the solvent, one at 1 mg ml^{-1} and other of 5 μg ml^{-1} of each enantiomer.

Using the appropriate stock solution, standards solutions were prepared at concentrations range of 0.2–2.0 μg ml^{-1} of emtricitabine at 100 μg ml^{-1} solution of β-D-(+)-FTC. Standards solutions of 0.2–2.0 μg ml^{-1} of β-D-(+)-FTC at 100 μg ml^{-1} solution of emtricitabine were also prepared. The

standards solutions were prepared in triplicate. Calibration curves were constructed by plotting the peak area against the concentration of standard solutions of each enantiomer. The data were subjected to linear regression analysis and showed good linearity with a correlation coefficient of 0.9990 for emtricitabine and 0.9995 for β-D-(+)-FTC.

2.4. Inter day and intra-precision and accuracy

The inter day and intra-precision were determinate use three quality controls. The quality controls standard solutions were prepared at 0.5, 1.1 and 1.8 μg ml^{-1} of one enantiomer in a 100 μg ml^{-1} solution of the other. The peak–area ratios of the quality controls were used for evaluating the inter-day and intra-day variability. Five samples of each control were prepared and analyzed in 3 different days.

The accuracy of the method was evaluated by back-calculation; it was also tested using blinded unknowns, at two different concentrations, which were prepared by a different analyst.

2.5. Stability studies

Using the same three quality controls solutions the stability of the enantiomers samples was evaluated over a period of 48 h with analysis at 0, 24 and 48 h. No evidence of degradation of the analytes was observed in the chromatograms during all this period.

2.6. Selectivity

A photodiode array detector was used to identify the separated enantiomers and to check peak purity in the bulk samples examined.

The elution order was determined by injecting the solutions of the racemic mixture, and then each enantiomer separately.

2.7. Limits of detection and quantification

The limit of detection (LOD) was calculated taking a signal-to-noise ratio of 3 as criteria and it was measured by preparing samples in serial dilutions of β-D-(+)-FTC in a 100 μg ml^{-1} solution of emtricitabine. The acceptance criteria for the lowest limit of quantification (LOQ) were that for three prepared samples the CV and accuracy were under 10% variability. For calculating LOD and LOQ for emtricitabine in samples of β-D-(+)-FTC, serial dilutions of emtricitabine sample solutions in a 100 μg ml^{-1} solution of β-D-(+)-FTC were prepared.

3. Results and discussion

The development of new methodologies for chiral discrimination has been of great concern. Among the most useful and versatile chiral analytical columns described in the literature in recent years are the coated carbohydrate carbamate columns, which were originally developed by Okamoto et al. These chiral columns have been used extensively both in analytical mode and in preparative scale with success [18,19].

Thousands of different chiral compounds have been efficiently enantioresolved by polysaccharide-based columns [18–20] and a number of different mobile phases have been described for achieving enantioselectivity. The selection of an appropriate polysaccharide chiral column and mobile phase for a given separation is normally a difficult task. Thus, our first effort was to resolve a racemic mixture of FTC under different elution conditions using four different polysaccharide chiral phases.

The amylose tris[(*S*)-1-phenylethylcarbamate] chiral phase showed the highest enantioselectivity for the racemic mixture of FTC under the chromatographic conditions used (Table 1). Under the same elution conditions the phases cellulose tris(3,5-dimethylphenylcarbamate) and amylose tris(3,5-dimethoxyphenylcarbamate) were unable to enantioresolve (±)-FTC; amylose tris(3,5dimethylphenylcarbamate) showed median selectivity poor resolution with hexane–ethanol (60:40 v/v) as the mobile phase.

Good enantioselectivity ($\alpha = 1.43$) and resolution ($R_S = 1.46$) were obtained with the amylose tris[(*S*)-1-phenylethylcarbamate] chiral phase using hexane–ethanol (60:40 v/v) as mobile phase at a flow rate of 1 ml min^{-1} (Table 1). However,

Table 1
Influence of the mobile phase on the enantioselectivity of the phase amylose tris[(*S*)-1-phenylethylcarbamate] coated onto APS-Nucleosil (7 μm particle size, 500 Å pore size, 20% w/w, 15 × 0.46 cm ID)

Mobile phase	K_1	α	R_S
Hex/EtOH 60:40	1.92	1.43	1.46
CH_3CN/MeOH 90:10	0.54	1.49	1.61
CH_3CN/MeOH 85:15	0.22	1.77	1.24
CH_3CN/EtOH 90:10	1.10	1.50	1.30
CH_3CN/EtOH 85:15	0.32	1.97	1.26
MeOH 100%	0.13	1.77	0.86
EtOH 100%	1.60	1.34	1.58
CH_3CN/H_2O 50:50	0.60	1.00	–
$MeOH/H_2O$ 30:70	0.51	1.00	–
$EtOH/H_2O$ 30:70	0.30	1.93	1.46

*Flow rate 1.0 ml min^{-1}.

we tried to direct our experiments aiming the development of a method that could be easily used not only for measuring the enantiomeric ratio of FTC enantiomers at the quality control level but also at the synthetic process stage, by the use of reversed-phase or polar organic elution mode.

The mobile phase in chiral chromatography plays a crucial role in the interaction process [18–21]. It affects not only the retention factor but also the enantioselectivity or enantioresolution [11,12]. Example of this was the lack of enantioresolution observed under reversed-phase mode of elution when acetonitrile or methanol was used as mobile phase. However, good enantioselectivity was achieved by the use of ethanol as the modifier but the resolution obtained was not good enough for measuring β-D-(+)-FTC with precision and accuracy in presence of emtricitabine in a large enantiomeric excess.

Schinazi and collaborators have reported the separation of (±)-FTC enantiomers using a Chiralpak® AS column with 2-propanol as the mobile phase [4,5]. Due to the high backpressures normally associated with the use of 100% 2-propanol, the use of other mobile phases compositions was investigated (Table 1). The optimization of the elution strength and flow rate of acetonitrile–methanol mobile phases increased the enantioselectivity and resolution; however an accurate determination of the enantiomeric ratio could not be made due to poor band symmetry.

TEA has been used efficiently as a silanol suppressor [22,23] and in this case the use of 0.01% of TEA increased the R_S from 1.90 to 2.37. The ionic behavior of the solute is an important factor in controlling enantioresolution [6]. The use of an organic base with an organic acid for controlling the degree of ionization of basic solutes is well established [23–25]. Thus, the further addition of 0.01% AcOH to the mobile phase to form TEAA improved the enantioresolution to 2.79, and by increasing the concentration of TEAA to 0.02% a selectivity of 1.9 with excellent resolution ($R_S = 3.3$) and good band profile was obtained for the FTC enantiomers. Under these elution conditions emtricitabine was the first enantiomer to elute. This order of elution was observed in all conditions stated in Table 1. Fig. 2 shows a typical chromatogram obtained during the method development. This condition was then selected as the methodology for measuring the enantiomeric ratios of FTC enantiomers and it was then validated.

The response of the UV detector at 280 nm was linear from 0.2 to 2.00 μg ml^{-1} for each enantiomer. The regression equations found were of $y = 60\,953x + 1324$ with a correlation coefficient of 0.9995 for the β-D-(+)-FTC and $y = 59\,590x + 1768$ with a correlation coefficient of 0.9990 for the emtricitabine.

The stability of the stock solutions of both enantiomers was assessed by periodic analysis of solutions maintained at ambient temperature for 0, 24 and 48 h. All solutions were stable during the period studied.

The intra and inter-day precision and accuracy of the method were determined by analyzing five replicates of three quality controls on 3 non-consecutive days. Precision are expressed as coefficients of variation (CV%) and the accuracy was evaluated by back-calculation and expressed as the percent deviation between amount found and amount added at the three concentrations examined. The results are shown on Table 2 and led to satisfactory intra- and inter-assay precision, with coefficient of variation $\leq 2\%$ for both enantio-

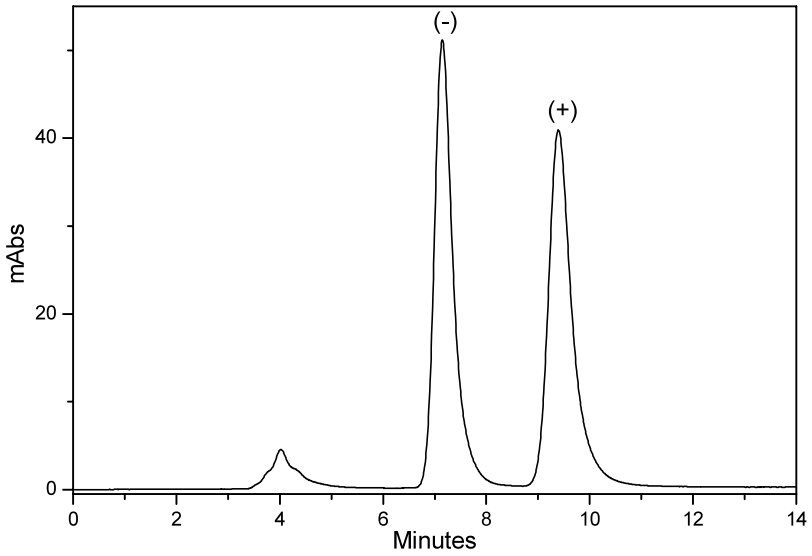

Fig. 2. Chromatogram of β-(±)-FTC on the column amylose tris[(*S*)-1-phenylethylcarbamate] using acetonitrile–methanol (95:05 v/v) with 0.02% of TEAA, as mobile phase with a flow rate of 1 ml min^{-1} and detection at $\lambda = 280$ nm.

mers. The accuracy varied from 96 to 102% for emtricitabine and from 100 to 107% for β-D-(+)-FTC.

The limit of detection and quantification were determined as 0.06 and 0.20% for emtricitabine and 0.07 and 0.20% for β-D-(+)-FTC.

Two blind samples containing unknown concentrations to the analyst produced accuracies in the range of 98.1–100% at the concentration levels of 0.45 and 1.45 μg ml^{-1} with CV% at the range of 0.7–1.9 for the duplicate analysis of both enantiomers.

Typical chromatograms of the analysis of emtricitabine and of β-D-(+)-FTC obtained during the validation study are shown in Fig. 3. The validated methods were suitable for the quantitative analysis of enantiomeric composition of a variety emtricitabine batches and also of β-D-(+)-FTC (Table 3). The selectivity of the present method was evaluated by analyzing the different batches of the emtricitabine and of β-D-(+)-FTC using the photodiode-array detector. No co-elution of interfering peaks with the investigated enantiomers was noticed. The method developed is precise and accurate to measure β-D-(+)-FTC in the range of 0.2–2.0% as an impurity. The method is also suitable for the analysis of the enantiomeric purity of β-D-(+)-FTC if desired.

Table 2
Inter and intra-day precision and accuracy data for the enantiomers of FTC

Enantiomers	Concentration (μg ml^{-1})	1st day		2nd day		3rd day	
		CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
Emtricitabine	0.5	1.03	102	1.87	100	1.88	102
	1.1	1.51	100	1.54	100	0.86	101
	1.8	0.62	96	0.47	96	0.65	96
β-D-(+)-FTC	0.5	1.39	102	1.44	100	0.39	101
	1.1	0.88	107	1.79	106	1.52	103
	1.8	2.06	102	0.56	102	0.87	102

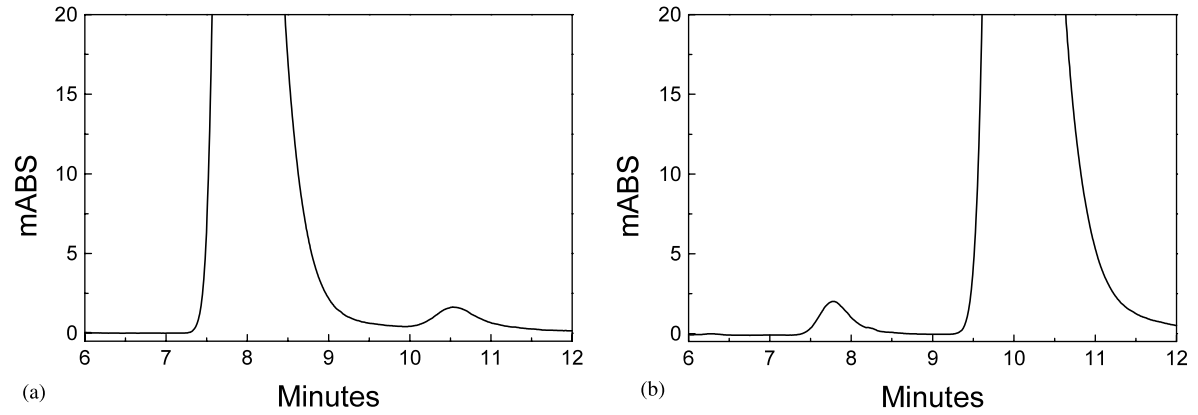

Fig. 3. (a) Chromatogram of emtricitabine spiked with 1% of β-D-(+)-FTC; and (b) chromatogram of β-D-(+)-FTC spiked with 1% of emtricitabine.

Table 3
Analysis of the batches of β-D-(+)-FTC and emtricitabine

Sample	β-D-(+)-FTC (μg ml^{-1})	Emtricitabine (μg ml^{-1})
Emtricitabine batch 1	Not detected	–
Emtricitabine batch 2	Not detected	–
β-D-(+)-FTC batch 1	–	Not detected
β-D-(+)-FTC batch 2	–	0.8

4. Conclusion

The methods presented here showed an excellent enantioselectivity for FTC enantiomers. The good linearity, precision, accuracy, sensitivity and selectivity obtained under polar organic elution mode allow it to accurately determine down to 0.2% of each enantiomer as an impurity. This limit meets the requirement of the pharmaceutical industry, so it can be used efficiently for assessing enantiomeric purity in bulk products.

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References

[1] K. Das, X. Xiong, H. Yang, C.E. Westland, C.S. Gibbs, S.G. Sarafianos, E. Arnold, J. Virol. 75 (2001) 4771–4779.
[2] A. Sykes, C. Wakeford, F. Rousseau, A. Rigney, E. Mondou, 9th Conference on Retroviruses and Opportunistic Infections, Session 89, February 24–28, 2002, Seattle, WA, USA.
[3] C.V. Abobo, L. Ni, R.F. Schinazi, J. Pharm. Sci. 63 (1994) 96–99.
[4] R.F. Schinazi, A. Mcmillan, D. Cannon, R. Mathis, R.M. Lloyd, A. Peck, J.P. Sommadossi, M. St. Clair, J. Wilson, P.A. Furman, G. Painter, W.B. Choi, D.C. Liotta, Antimicrob. Agents Chemother. 36 (1992) 2423–2431.
[5] R.F. Schinazi, F.D. Boudinot, S.S. Ibrahim, C. Manning, H.M. Mclure, D.C. Liotta, Antimicrob. Agents Chemother. 36 (1992) 2432–2438.
[6] K. Tachibana, A. Ohnishi, J. Chromatogr. A 906 (2001) 127–154.
[7] N.M. Maier, P. Franco, W. Lindner, J. Chromatogr. A 906 (2001) 3–33.
[8] A. Berthod, T.L. Xiao, Y. Liu, W.S. Jenks, D.W. Armstrong, J. Chromatogr. A 955 (2002) 53–69.
[9] N. Mesplet, Y. Saito, P. Morin, L. Agrofoglio, J. Chromatogr. A 983 (2003) 115–124.
[10] E. Lipka-Belloli, C. Len, G. Mackenzi, G. Ronco, J.P. Bonte, C. Vaccher, J. Chromatogr. A 943 (2002) 91–100.
[11] A. Magor, S. Abu-Lafi, S. Levin, J. Chromatogr. A 866 (2000) 183–194.
[12] S. Levin, M. Sterin, A. Magora, A. Popescu, J. Chromatogr. A 752 (1996) 131–146.

[13] M.P. Di Marco, C.A. Evans, D.M. Dixit, W.L. Brown, M.A. Siddiqui, H.L.A. Tse, H. Jin, N. Nguyen-Ba, T.S. Mansour, J. Chromatogr. A 645 (1992) 107–114.
[14] M.D. Goodyear, P.O. Dwyer, M.L. Hill, A.J. Whitehead, R. Hornby, P. Hallett, (Glaxo Group Ltd., UK). PCT Int. Appl. (1995), 24 pp, WO 9529174, CA 124:146759.
[15] H. Jin, M.A. Siddiqui, C.A. Evans, H.L.A. Tse, T.S. Mansour, M.D. Goodyear, P. Ravenscroft, C.D. Beels, J. Org. chem. 60 (1995) 2621–2623.
[16] S.A. Matlin, M. Elizabeth Tiritan, Q.B. Cass, D.R. Boyd, Chirality 8 (1996) 147–152.
[17] S.A. Matlin, M.E. Tiritan, A.J. Crawford, Q.B. Cass, D.R. Boyd, Chirality 6 (1994) 135–140.
[18] E.R. Francotte, J. Chromatogr. A 906 (2001) 379–397.
[19] E. Yashima, J. Chromatogr. A 906 (2001) 105–125.
[20] Q.B. Cass, A.L.G. Degani, N.M. Cassiano, J. Pedrazolli, Jr., J. Chromatogr. B 766 (2002) 156–160.
[21] P. Piras, C. Roussel, J. Pierrot-Sanders, J. Chromatogr. A 906 (2001) 443–458.
[22] Q.B. Cass, M.E. Tiritan, S.A. Calafatti, S.A. Matlin, J. Liq. Chromatogr. Rel. Technol. 22 (1999) 3091–3099.
[23] M.W. Matchett, S.K. Branch, T.M. Jefferies, Chirality 8 (1996) 126–130.
[24] Y. Tang, Chirality 8 (1996) 136–142.
[25] G. Török, A. Péter, D.W. Armstrong, D. Tourwé, G. Tóth, J. Sápi, Chirality 13 (2001) 648–656.