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Research Article
ABHI Publications since 2013
International Journal of Pharmacy and Engineering (IJPE)
ISSN 2320-849X

Separation of verapamil enantiomers by high performance liquid chromatography using amylose-based stationary phase

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Abstract

Verapamil is a drug used in the therapy of hypertension, supraventricular arrhythmias and angina pectoris, was separate by high performance liquid chromatography (HPLC) using a semi-preparative column containing amylose tris (3,5-dimethylphenyl) carbamate as chiral stationary phase. The effects of temperature (293–308 K), flow rate (1.0–2.5 mL min^{-1}) and polar modifiers 2-propanol and ethanol ratio (2.5 % - 7.5 %) (by volume) on separation parameters chromatographic were evaluated. The experiments were carried out under normal phase conditions, and the chiral separation exhibit satisfactory values of capacity factors, selectivity and resolution for verapamil enantiomers with a good performance. The preferred operation conditions were 298 K, 1.0 mL min^{-1}, and the 2-propanol and ethanol ratio in the mobile phase 5.0 % (by volume).

Keywords: Verapamil; Chiral; Enantiomer separation; high performance liquid chromatography.

1. Introduction

The pharmaceutical industry has placed its emphasis on gathering enantiomerically enriched compounds before performance evaluations on pharmacokinetic, metabolic, physiological and toxicological evaluation in the search for drugs with great benefits [1,2]. The separation of active chiral compounds when one enantiomer is more efficient causes possible reduction of both dosage and side effects especially on pre-clinical and clinical tests [2,3].

Verapamil (VER), 2-(3,4-dimethoxyphenhyl)-5-[2-(3,4dimethoxyphenyl)ethyl-methyl-amino]-2-propan-2-yl-pentanenitrile, is a calcium channel-blocking drug, and it is widely used in

the therapy of hypertension, supraventricular arrhythmias and angina pectoris [4-9]. VER is a tertiary amine with one asymmetric carbon and exists in two enantiomeric forms (Fig. 1).

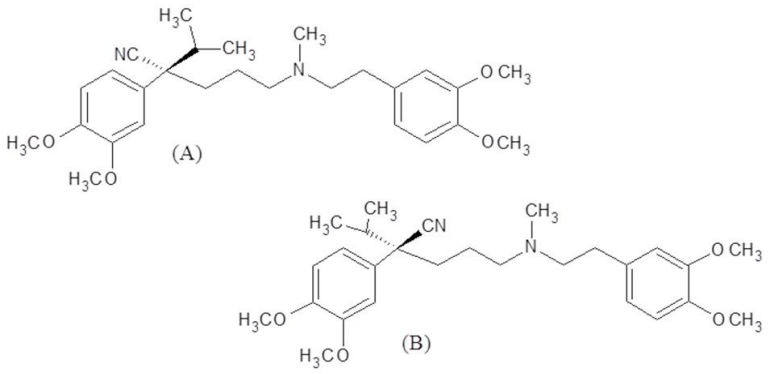

Figure 1. Chemical structures of verapamil enantiomers: (A) *S-(-)*-verapamil, and (B) *R-(+)*-verapamil.

It is usually administered as a racemic mixture of (+)-(*R)*-VER and (-)-(*S*)-VER. However, the enantiomers show different pharmacodynamic and pharmacokinetic properties with (-)-(*S*)-VER being 10-20 times more potent than (+)-(*R*)-VER in slowing cardiac A-V conduction velocity in man, dog, and in the rabbit [5,6,10]. Despite the (+)-(*R*)-VER shown lower antiarrhythmic potency, are reported studies that exhibits its anti-tumor activity acting as an inhibitor of liver cancer [8,11-14].

Chromatographic separation of enantiomers, particularly by high performance liquid chromatography (HPLC), have gained a great reputation in the last years, and have become a useful method for determining optical purity, and for obtaining enantiomers. Several chiral stationary phases (CSPs), including polysaccharide-base CSPs (e.g. cellulose and amylose), have been used for the enantiomeric separation of a wide variety of racemates [2]. It has also been reported in several separation systems that the amylose CSP is a better than cellulose CSP, because of its more helical structure [2,15-17]. Among these, amylose derivatized with chiral selector tris (3,5-dimethylphenyl) carbamate is the most successful for chiral separation [18].

Several chromatographic techniques have been proposed to separate the verapamil enantiomers by HPLC using a Chiralpak® AD column [4,6,19], a Chiracel® OD-RH column [20], a chiral-AGP column [21], a chiral OD-R column [22], and using a cyclodextrin column [23, 24]. However, studies reported in the literature refer only to separation of the racemic drug in analytical chiral columns whose enantiomers are determined and quantified when present as metabolites in urine [5,26] or blood plasma [4,6,10,19,25]. There is a lack in the literature emphasizing the study of chromatographic separation parameters of verapamil enantiomers in the semi-preparative scale.

Therefore, the aim of this work is to explore the feasibility of enantiomers separation of verapamil by HPLC on the Chiralpak® AD semi-preparative column and evaluate the effects of temperature, flow rate, and polar modifiers ratio (2-propanol and ethanol) on the capacity factor, selectivity, and resolution.

2. Material and Methods

2.1. Materials and equipments

Racemic verapamil was gently donated by EMS® Pharmaceutical Industries (Hortolândia-SP, Brazil). The mobile phase used in this work was a mixture of *n*-hexane, 2-propanol and ethanol HPLC-grade, purchased from TEDIA® (Brazil), and diethylamine (purity 99,5 %) used as additive (Sigma-Aldrich, USA). 1,3,5 tri-*terc*-butylbenzene (TTBB) (Sigma-Aldrich, USA) was used for the determination of the dead time of the chromatographic system.

The semi-preparative stainless steel chiral column (100 mm x 10 mm I.D) packed with amylose tris (3,5-dimethylphenylcarbamate) (Fig. 2) with 20 µm particles was purchased from Chiral Technologies Europe® (France), commercially known as Chiralpak® AD column.

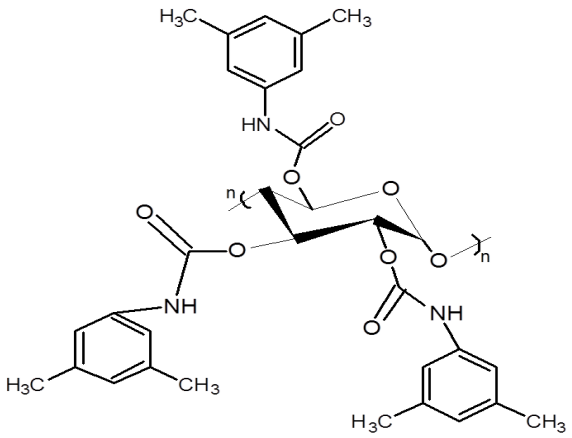

Figure 2. Chemical structure of chiral stationary phase amylose tris (3,5-dimethylphenylcarbamate).

The chromatographic experiments were performed in a HPLC system (Shimadzu®, Japan) equipped with a eluent pump (LC 20 AT model), a UV detector (SPD 20 A model), a system controller (CBM 20 A model), temperature controller, and a digital data acquisition system (LC Solution 1.23 software).

2.2. Experimental procedures

All chromatograms were obtained under isocratic condition using *n*-hexane/ 2-propanol/ ethanol/ diethylamine (90/5/5/0.1 %, by volume) as mobile phase. The retention time was measured and corrected for the dead time contribution of the liquid chromatography using inert compound TTBB (0.5 mg mL^{-1}).. The experiments were performed at different mobile phase flow rates (1.0, 1.5, 2.0 and 2.5 mL min^{-1}) and temperatures (293, 298, 303, and 308 K). Small pulse experiments (20 µL) of dilute solutions (0.5 mg mL^{-1}) were injected into the column after a time interval necessary for the stabilization of the chromatography system. The signal was monitored by the UV detector with a wavelength of 270 nm.

The capacity factors, selectivity and resolution were calculated by Eq. (1), Eq. (2) and Eq. (3), respectively [27,28]

$$k = \frac{(t_r - t_0)}{t_0} \quad (1)$$

$$\alpha = \frac{k_{R(+)}}{k_{S(-)}} \quad (2)$$

$$Rs = 1{,}77 \frac{(t_{r,R(+)} - t_{r,S(-)})}{(w_{b,S(-)} + w_{b,R(+)})} \quad (3)$$

3. Results and Discussion

3.1. Elution profile

Typical elution profile of verapamil enantiomers is shown in Fig. 3. The *S*-(-)-VER was eluted earlier than *R*-(+)-VER. It can be seen that the chiral separation of racemic verapamil on Chiralpak® AD semi-preparative column was satisfactory. Note that the elution peak of TTBB has small base width and relatively short retention time. This behavior is expected since the TTBB, being an inert compound does not undergo stereoselective interaction with the chiral stationary phase and the mass transfer resistances are negligible.

The experiments were done for a flow rate of 1.0 mL min^{-1}, 298 K, and the ratio of 2-propanol and ethanol in the mobile phase 5 % (by volume). The relative standard deviations (RSD) (n = 3) of capacity factors of *S*-(-)-VER and *R*-(+)-VER, selectivity, and resolution were 0.81 %, 1.02 %, 0.19 %, and 0.41 %, respectively.

3.2. Effect of temperature and flow rate on capacity factor

The capacity factors of *S*-(-)-VER and *R*-(+)-VER are plotted against temperature as shown in Figs. 4 and 5, respectively, with ratio of 2-propanol and ethanol in the mobile phase 5 % (by volume). It is observed that the capacity factors decreases with increasing temperature at constant flow rate, since the increases in temperature reduces the viscosity of the mobile phase and provides greater mobility of the chiral molecules through the pores of the stationary phase, which consequently increasing the interaction rate (adsorption and desorption) verapamil enantiomers with adsorbent. This effect reduces the residence time of molecules within the chromatographic column and therefore their retention times. It may also note that the capacity factors remain constant when increasing the flow rate for each temperature evaluated.

In this work were obtained capacity factors values greater than 2.0 and less than 5.0. According to [27], chromatographic separations are considered satisfactory when capacity factors values are between 1.0 to 10.0.

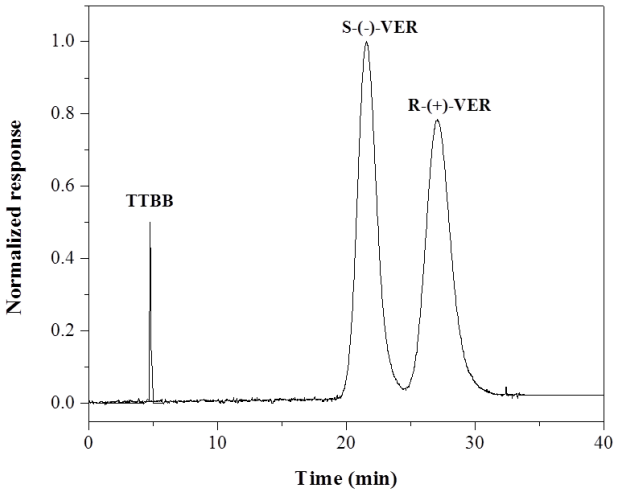

Figure 3. Chromatogram of racemic verapamil and 1,3,5-tri-*terc*-butylbenzene. Chromatographic conditions: racemate concentration, 0.5 g L^{-1}; mobile phase, *n*-hexane/ 2-propanol/ ethanol/ diethylamine (90/5/5/0.1 %, by volume); stationary phase, Chiralpak® AD column (100 x 10 mm, 20 µm); detection, 270 nm; injection volume, 20 µL.

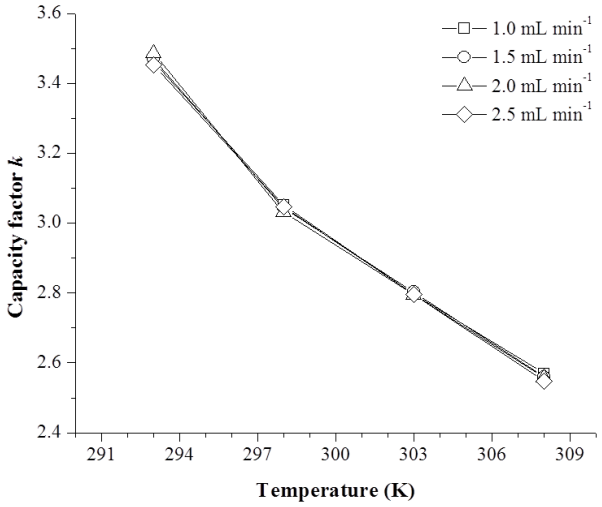

Figure 4. Effect of temperature and flow rate on capacity factor of *S-(-)*-VER enantiomer. Chromatographic conditions: racemate concentration, 0.5 g L^{-1}; mobile phase, *n*-hexane/ 2-propanol/ ethanol/ diethylamine (90/5/5/0.1 %, by volume); stationary phase, Chiralpak® AD column (100 x 10 mm, 20 µm); detection, 270 nm; injection volume, 20 µL.

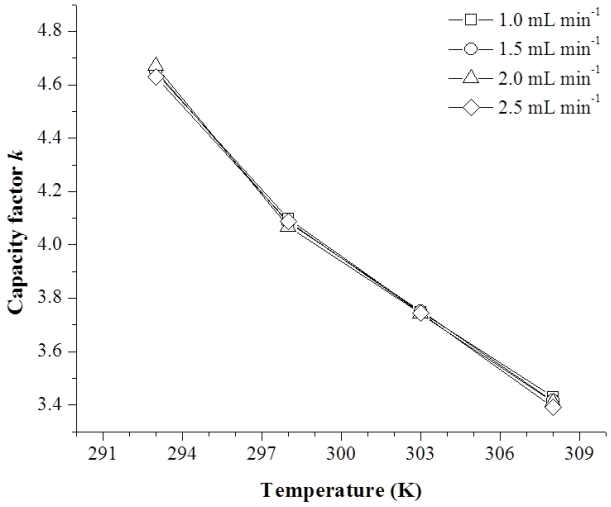

Figure 5. Effect of temperature and flow rate on capacity factor of *R-(+)*-VER enantiomer. Chromatographic conditions: racemate concentration, 0.5 g L^{-1}; mobile phase, *n*-hexane/ 2-propanol/ ethanol/ diethylamine (90/5/5/0.1 %, by volume); stationary phase, Chiralpak® AD column (100 x 10 mm, 20 µm); detection, 270 nm; injection volume, 20 µL.

3.3. Effects of temperature and flow rate on selectivity and resolution

The system presented good performance separation. As shown in Fig. 6 selectivity (α) shows little variation with increasing temperature and remained practically constant with increasing mobile phase flow rate. However, α values were higher than 1.33. According to [29] in most separation with polysaccharide phases, complete separation of enantiomers is attained if selectivity is larger than 1.2.

In most cases, selectivity and resolution of the enantiomers decreases with an increase in temperature. However, α doesn't adequately describe the separation of enantiomers, since it doesn't include information about peak width [30,31]. Resolution (*Rs*) is given by the relationship between the difference of retention time (t_r) of each enantiomer and the bandwidth (w_b) of each elution peak, according to Equation (3).

In Fig. 7, the resolution of verapamil is plotted against the temperature with ratio of 2-propanol and ethanol in the mobile phase 5 % (by volume). It can be seen that the resolution is influenced by the column temperature. When the temperature increases, there is less interaction between enantiomers and chiral stationary phase, i.e. there is smaller binding energy between molecules and chiral adsorbent [30]. Therefore, the low interaction provides quick elution of the enantiomers and hence reducing the retention time and the bandwidth of the chromatographic peaks. However, in this work, the reduction of the bandwidth showed a greater effect on the resolution, contributing to the increase of the chromatographic parameters values as indicated by Eq. (3).

It was also found that the flow rate of mobile phase affects the resolution. An increase in the flow rate causes a decrease in the resolution due to a decrease in the difference between the retention time and reduced in bandwidth of each elution peak. However, in this case, the resolution

was mainly affected by the reduction in retention time which were more pronounced during the experiments.

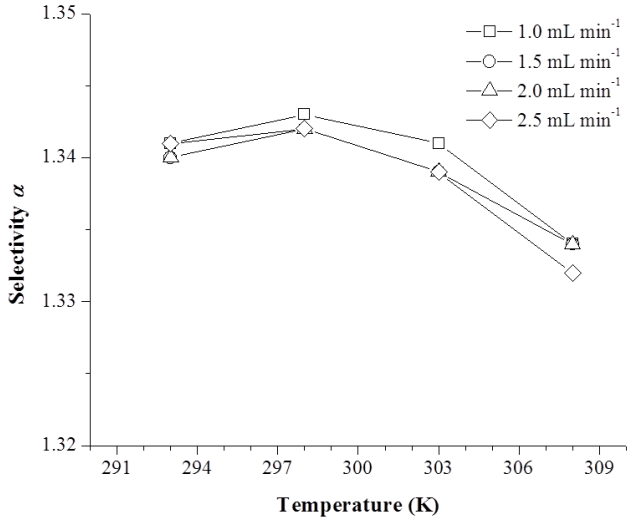

Figure 6. Effect of temperature and flow rate on selectivity of verapamil enantiomers. Chromatographic conditions: racemate concentration, 0.5 g L^{-1}; mobile phase, *n*-hexane/ 2-propanol/ ethanol/ diethylamine (90/5/5/0.1 %, by volume); stationary phase, Chiralpak® AD column (100 x 10 mm, 20 µm); detection, 270 nm; injection volume, 20 µL.

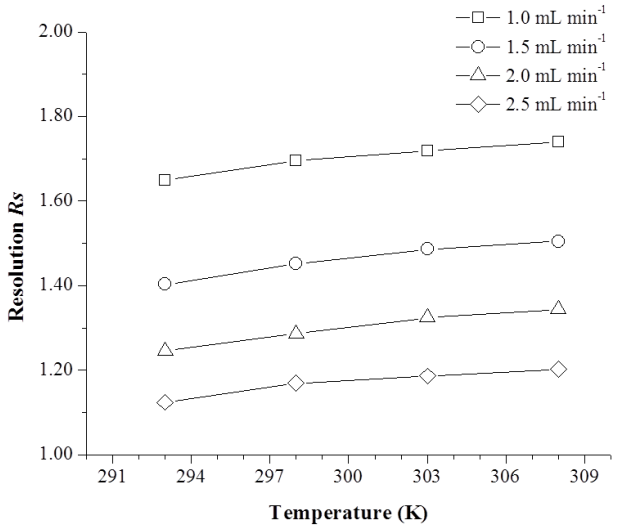

Figure 7. Effect of temperature and flow rate on resolution of verapamil enantiomers. Chromatographic conditions: racemate concentration, 0.5 g L^{-1}; mobile phase, *n*-hexane/ 2-propanol/ ethanol/ diethylamine (90/5/5/0.1 %, by volume); stationary phase, Chiralpak® AD column (100 x 10 mm, 20 µm); detection, 270 nm; injection volume, 20 µL.

3.4. *Effect of 2-propanol and ethanol ratio in the mobile phase*

Experiments were carried out under preferred conditions of flow rate 1.0 mL min^{-1}, and temperature 298 K. The results are shown in Table 1.

It may be observed that when the *n*-hexane and diethylamine ratios are fixed in 90 % (by volume) and 0.1 % (by volume), respectively, and the polar modifiers ratio are varied simultaneously, the chromatographic parameters values ($k_{S(-)\text{-VER}}$, $k_{R(+)\text{-VER}}$, α, and *Rs*) increases with the addition of 2-propanol and ethanol in the mobile phase. The best values are obtained for the alcohols ratios equal to 5.0 % (by volume). From this ratio, chromatographic parameters values decrease again with the addition of 2-propanol and removal of ethanol. Therefore, as a result, the preferred polar modifiers ratio in the mobile phase was 5.0 % (by volume) in the range examined.

Proportion alcoholic *(%, by volume)*		***Chromatographic parameters***			
2-propanol	Ethanol	$k_{S(-)\text{-VER}}$	$k_{R(+)\text{-VER}}$	α	*Rs*
0.0	10.0	2.58	2.95	1.15	0.84
2.5	7.5	2.95	3.64	1.24	1.33
5.0	5.0	2.98	3.98	1.34	1.68
7.5	2.5	2.68	3.55	1.32	1.54
10.0	0.0	2.46	3.23	1.31	1.39

Table 1. Effects of 2-propanol and ethanol ratio (%, by volume) on capacity factors (*k*), selectivity (*α*), and resolution (*Rs*)

4. Conclusions

This paper reports the separation of verapamil enantiomers was performed in a semi-preparative chromatographic column using stationary phase amylose tris (3,5-dimethylphenyl) carbamate. The best chromatographic parameters values were obtained for the mobile phase containing polar modifiers (2- propanol and ethanol) in the ratio 5 % (by volume). The chromatographic parameters (capacity factors, selectivity, and resolution) showed satisfactory separation of the verapamil enantiomers when evaluated at different temperatures and flow rates of the mobile phase.

It is worth noting that the present work is a contribution to understand temperature and flow rate effects in the chromatographic separation of the verapamil enantiomers by HPLC using Chiralpak® AD column in semi-preparative scale.

Nomenclature

k	capacity factor
t_r	retention time
t_0	dead time
α	selectivity
Rs	resolution

w_b bandwidth

Acknowledgements

The authors gratefully acknowledge the financial support from São Paulo Research Foundation (FAPESP - Proc. 2007/02872-4), and CAPES Foundation (Proc. Pro-Eng 613/2008).

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