Thermodynamic Evaluation of Immobilized Cellulose Tris(3,5-Dichlorophenylcarbamate) as a Stationary Phase for Liquid Chromatographic Separation of Darunavir Enantiomers

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Liquid chromatographic separation of darunavir (DRV) enantiomers was studied on Chiralpak IC column containing immobilized cellulose tris(3,5-dichlorophenylcarbamate) using a variety of mobile phase solvents at different temperatures. The separations were accomplished under normal phase conditions using different compositions of n-hexane, organic modifier (2-propanol, ethanol or 1-propanol) and diethyl amine (0.1%) as mobile phase solvents. The effect of volume and nature of organic modifier and column temperature on retention, separation and resolution were studied. Van’t Hoff plots (ln $k'$ vs $1/T$) were drawn from the chromatographic retention data to calculate to apparent thermodynamic parameters and explain the interactions between the DRV enantiomers and cellulose tris(3,5-dichlorophenylcarbamate) immobilized on silica.

Introduction

The importance of chirality in various fields of science such as pharmaceutical, biomedical, agrochemical and environmental is well known. Enantiomers, the non-super imposeable mirror images, are difficult to separate as they possess similar physicochemical properties but differ in biological activity. The stringent regulations proposed by regulatory authorities on marketing of chiral drugs have led to a great demand for developing analytical and preparative methods for enantioseparations (1). Among several techniques, chromatography on polysaccharide chiral stationary phases (CSPs) is most widely used for chiral separations. Recently, M/s Daicel Technologies, Japan has introduced three commercial immobilized polysaccharide-based CSPs, namely Chiralpak IA, Chiralpak IB and Chiralpak IC containing amyllose tris(3,5-dimethylphenylcarbamate), cellulose tris(3,5-dimethylphenylcarbamate) and cellulose tris(3,5-dichlorophenylcarbamate), respectively, immobilized on silica (Figure 1) (2–5). Previously, the same polysaccharide CSPs were coated on silica support but their use with mobile phase solvents such as tetrahydrofuran (THF), chloroform (CHCl$_3$), dichloromethane (DCM), ethyl acetate (EtOAc), pyridine, acetone and certain ethers was limited. Hence, organic reactions conducted in such solvents could not be monitored by coated polysaccharide CSPs. The immobilized characteristic nature of the CSP broadens not only the choice and range of mobile phase solvents but also sample diluents and the possibility of mobile phase modifiers from 0 to 100%. A wide range of mobile phase solvents such as acetonitrile/ethanol or 2-propanol and methanol/ethanol or 2-propanol could be used with these columns (5). Evaluation of such immobilized chiral columns (6–9) at different temperatures is of great importance to know their separation efficiency and strength of interactions between the enantiomers and CSP to understand the mechanism of chiral separations.

Darunavir [(-)-(3S,3a,6aR)-hexahydrofuro[2,3-b]furan-3-yl-(2S,3R)-4-(4-amino-N-isobutylphenylsulfonamido)-3-hydroxy-1-phenylbutan-2-ylcarbamate] (DRV) (I) (Figure 2) is a new HIV-1 protease inhibitor effective against viral strains that are not sensitive to other antiretrovirals (10). During its synthesis, its enantiomer (3S,3aR,6aS)-hexahydrofuro[2,3-b]furan-3-yl((2R,3S)-4-(4-amino-N-isobutylphenylsulfonamido)-3-hydroxy-1-phenylbutan-2-ylcarbamate) (Figure 2) could be formed most likely as an impurity at significant levels. Therefore, their separation is important not only to assure the therapeutic efficacy but also safety of DRV-containing pharmaceutical preparations. Several LC–LC–MS and LC–MS–MS methods have been reported for separation of (-) DRV from other antiretroviral drugs in human plasma. A few LC–MS methods for the simultaneous determination of DRV along with other antiretroviral agents (11), on dried plasma spots (12) and in plasma of HIV-infected patients (13,14), were reported. LC–MS–MS (15, 16) was used for quantification of DRV and other antiretroviral agents in human plasma. It was also used for quantification of DRV in presence of protease inhibitors (atazanavir, lopinavir and ritonavir) and non-nucleoside reverse transcriptase inhibitors (efavirenz and nevirapine) on dried blood spots (17). Martin et al. (18) proposed a column switching LC–MS–MS method for determination of the plasma concentration of DRV along with other 10 antiretroviral agents (10) with UV and UPLC with MS–MS detection (19) were reported for quantification of DRV in human plasma. Recently, the authors have studied the separation of (-) DRV not only from (+) enantiomer but also from four of its stereoisomers on two different columns, namely Chiralpak AD-H and IA columns containing (i) coated and (ii) immobilized with amylose tris(3,5-dimethylphenylcarbamate) stationary phase, respectively (20, 21). However, its separation on a commercially available new Chiralpak IC column containing cellulose tris(3,5-dichlorophenylcarbamate) immobilized on silica was not reported so far in the literature.

The present manuscript describes separation of DRV enantiomers on Chiralpak IC column containing cellulose tris(3,5-dichlorophenylcarbamate) immobilized on silica using a range of mobile phase solvents composed of different organic modifiers in different quantities. The chromatographic retention, separation and resolution were investigated by varying the volume...
and type of organic modifier at different column temperatures. The retention data were used to derive apparent thermodynamic parameters, namely entropy, enthalpy and free energy to explain the important aspects of enantiomeric interactions with cellulose tris(3,5-dichlorophenylcarbamate) immobilized on silica.

Experimental

Chemicals
All the solvents used were of HPLC grade (E. Merck, Mumbai, India). Analytical reagent grade diethyl amine (DEA) (Spectrochem, Mumbai, India) was used. The enantiomers of DRV obtained from a local pharmaceutical manufacturing unit were used.

Chromatography
The HPLC system consisting of two LC-20 AD pumps, photodiode array detector, an SIL-20 AC auto sampler, a DGU-20 A5 degasser and CBM-20A controller (Shimadzu, Kyoto, Japan) was used. The chromatographic and the integrated data were recorded using HP-Vectra (Hewlett Packard, Waldbronn, Germany) computer system using LC-solution data acquiring software (Shimadzu). Chiralpak IC (25 cm × 4.6 mm ID, 5 μm) (Daicel, Tokyo, Japan) was used for chromatographic separations. Mobile phase solvents were filtered through a 0.45-mm membrane filter paper and degassed using an ultrasonic bath. The analytes were dissolved in 2-propanol at ~0.1 mg mL⁻¹ and filtered through a 0.45-μm membrane filter paper prior to injection. The flow rate was 1.2 mL min⁻¹. The injection volume was 5 μL. The UV detection wavelength was set at 266 nm. Retention factors (k') were calculated using the formula (tᵣ - t₀)/t₀, where tᵣ is the retention time of a particular enantiomer of DRV and t₀ is the retention time of unretained peak. The void volume (v₀) of the column was determined by injecting 1,3,5-tri-tert-butylbenzene and determining the retention time (tᵣ) of the unretained peak. Separation factors (α) for pair of enantiomers of DRV were calculated from k₂/k₁, where k₂ and k₁ are the retention factors for the second and first eluted enantiomers, respectively. Origin software was used to draw the graphs and linear Van’t Hoff plots.

Results and Discussion
Preliminary experiments using a range of solvents such as THF, DCM, EtOAc and CHCl₃ as mobile phase organic modifiers were carried out to separate the (+) and (−) enantiomers of DRV. But in all the cases, the separation was not successful. However, when solvents such as ethanol, 2-propanol and 1-propanol were tried good separations were achieved. The performance of Chiralpak IC was compared with Chiralpak IA and AD-H both containing (i) immobilized and (ii) coated amylose tris(3,5-dimethylphenylcarbamate) as a chiral selector. Figure 3 compares the retention parameters (k', α, Rₛ) of (−) and (+) DRV on the three columns using (i) ethanol and (ii) 2-propanol as organic modifiers. The variations in separation of DRV enantiomers on Chiralpak IA, IC and AD-H columns could be seen from Figure 3. Similar retention, enantioseparation and resolutions were observed on Chiralpak IA and IC columns using ethanol as an organic modifier. However, large separations and resolutions were obtained on Chiralpak AD-H when compared with other two columns. Interestingly, when 2-propanol was used as an organic modifier, both Chiralpak IA and AD-H columns showed similar retention, enantioseparation and resolutions while large separations and resolutions were obtained on Chiralpak IC. The retention was found to increase from ethanol to 2-propanol on Chiralpak IA and IC columns while a decrease
on Chiralpak AD-H. This could be due to the incorporation of different size/shape of alcohol modifiers into the chiral cavities of cellulose tris(3,5-dichlorophenylcarbamate) which might alter the steric environment influencing the solute retention ($k'$) as reported elsewhere (22).

**Effect of organic modifier**

Three different organic modifiers, namely ethanol, 2-propanol and 1-propanol with n-hexane and 0.1% DEA were tried to study their effect on separation of enantiomers of DRV. Figure 4 shows the chromatograms demonstrating the effect of percent of organic modifier on retention, separation and resolution of the enantiomers of DRV at 25°C. It could be seen from Figure 4 that as the percentage of 2-propanol increases (vertical comparison), retention factors were decreased, enantioseparations did not change much and resolutions were decreased. Similar trends were observed in case of ethanol and 1-propanol too. This could be understood in terms of hydrogen-bonding interactions between the enantiomers and CSP. These interactions may decrease with increased alcohol content in the mobile phase leading to faster elution. Table I gives the retention data of enantiomers of DRV at different column temperatures. It could be seen from Table I, better enantioseparations ($\alpha = 2.27$) and resolutions ($R_s = 7.96$) were obtained using 35% of 2-propanol both at 20 and 40°C, respectively. It could be seen from Figure 4 that the increase in retention of DRV enantiomers (horizontal comparison) from ethanol, 1-propanol to 2-propanol was in the order of their polarity indices. The enantioseparation and resolutions were also found to increase from ethanol to 1-propanol to 2-propanol. Overall, better enantioseparations and resolutions were obtained using 2-propanol as an organic modifier.

**Effect of column temperature**

The effect of column temperature was evaluated using different compositions of mobile phases between 20 and 40°C with an interval of 5°C. Figure 5 shows the effect of temperature on retention ($t_R$), separation ($\alpha$) and resolution ($R_s$) of enantiomers of DRV on Chiralpak IC column using ethanol, 2-propanol and 1-propanol as organic modifiers, respectively. As shown in Figure 5, retention times, enantioseparations decreased and resolutions increased from 20 to 40°C in all cases. Overall, better resolutions were obtained at 40°C in all organic modifier systems.

**Thermodynamic study**

In a chromatographic enantioseparation, the relation between chromatographic retention data and temperature of the chiral
where \( k' \) is retention factor of the particular enantiomer and \( \alpha \) is separation factor for a given enantiomeric pair. \( \Delta H^0 \) and \( \Delta S^0 \) represent the differences in the enthalpy and entropy, respectively, when one enantiomer transfers from mobile phase to the stationary phase. \( R \) is universal gas constant. \( \Phi \) is phase ratio. \( \Delta S^* \) is used to substitute for the expression \( \Delta S^0 / R + \ln \Phi \). The \( \Delta S^* \) values were used mainly for comparison, but not for determination of the \( \Delta S^0 \) values as the phase ratio was unknown. The \( \Delta \Delta H^0 \) and \( \Delta \Delta S^0 \) represent the differences of \( \Delta H^0 \) and \( \Delta S^0 \) for a given pair of enantiomers, respectively. If the plots of \( \ln k' \) or \( \ln \alpha \) against \( 1/T \) are linear in a temperature range, the corresponding thermodynamic parameters \( \Delta H^0 \) and \( \Delta S^* \), which are temperature independent, can be deduced from the slope \( (\Delta H^0 = -\Delta \) slope \() \) and the intercept \( (\Delta S^* = \) intercept \( \times R) \) of the straight lines. The linear character also suggests that the conformation of the CSP does not change substantially within the range of experimental temperatures. The thermodynamic parameters derived by this method are apparent, not intrinsic (9) and comparison of these thermodynamic parameters \( \Delta H^0 \), \( \Delta S^* \), \( \Delta \Delta H^0 \) and \( \Delta \Delta S^* \) may give some understanding of chiral recognition mechanism. In this study, 2-propanol and 1-propanol systems gave linear Van’t Hoff plots indicating that the conformation of the CSP did not change in the studied temperature range, whereas the use of ethanol, unusually did not give linear Van’t Hoff plots (27) indicating that a change in conformation of the CSP in the studied temperature range.

Table I gives the retention and enantioseparation data of DRV on Chiralpak IC column using ethanol, 1-propanol and 2-propanol as organic modifiers at different column temperatures. In case of 1-propanol, the \( k' \) values decreased as the column temperature was increased at all volumes of the organic modifier. Figure 6 shows that the Van’t Hoff plots of (i) \( \ln k' \) vs \( 1/T \) and \( \ln k'_2 \) vs \( 1/T \) were highly linear in the temperature range of 20–40°C indicating that the conformation of the CSP did not change in the studied temperature range. The corresponding thermodynamic parameters \( \Delta H^0 \) and \( \Delta S^* \) were calculated from these plots and given in Table II. Because of the fluctuation of \( \alpha \) values under these conditions, the \( \Delta \Delta H^0 \) and \( \Delta \Delta S^* \) were determined directly from the differences of \( \Delta H^0_2 \) and \( \Delta H^0_1 \), \( \Delta S^* \) and \( \Delta S^* \). It could been seen from Table II, as the percentage of 1-propanol increases, the absolute \( \Delta H^0_1 \) values of DRV decrease from \(-6.640 \) to \(-6.409 \) \( \text{kJ mol}^{-1} \) gradually. It indicates the decrease of heat of adsorption from \( 30 \) to \( 35 \% \) while an increase from \(-6.409 \) to \(-7.510 \) \( \text{kJ mol}^{-1} \) indicates the increase of heat of adsorption from \( 35 \) to \( 45 \% \). These results indicate that the interactions between \(-\) DRV and the CSP decrease from \( 30 \) to \( 35 \% \) and then increase from \( 35 \) to \( 45 \% \) of the organic modifier. However, the increase in absolute \( \Delta H^0_1 \) values of DRV (\(-10.517 \) to \(-11.211 \) \( \text{kJ mol}^{-1} \)) indicates an increase of heat of adsorption from \( 30 \) to \( 45 \% \). There results show that the interactions between \(+\) DRV and the CSP increase from \( 30 \) to \( 45 \% \) of the organic modifier. However, the

\[
\ln k' = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} + \ln \Phi, \quad \ln k'_2 = -\frac{\Delta H^0_2}{RT} + \Delta S^* \quad (1)
\]

\[
\ln \alpha = -\frac{\Delta \Delta H^0}{RT} + \frac{\Delta \Delta S^0}{R} \quad (2)
\]
Figure 5. Effect of temperature on retention factor ($k'$), separation ($\alpha$) and resolution ($R_s$) of enantiomers of DRV on Chiralpak IC column by using (A) n-hexane : ethanol : DEA (65 : 35 : 0.1, v/v/v), (B) n-hexane : 1-propanol : DEA (70 : 30 : 0.1, v/v/v) and (C) n-hexane : 2-propanol : DEA (60 : 40 : 0.1, v/v/v) as mobile phases.

Figure 6. Van't Hoff plots for (−) and (+) DRV using different volume (%) of (A) 1-propanol and (B) 2-propanol as mobile phase organic modifiers.
\[ \Delta H^0 \] and \[ \Delta S^* \] values in all cases were negative, suggesting that the enantioseparation was enthalpy driven.

In case of 2-propanol, the \( k' \) values decreased as the column temperature was increased. Figure 6 shows that the Van’t Hoff plots of \( \ln k'_1 \) vs \( 1/T \) and \( \ln k'_2 \) vs \( 1/T \) were highly linear in the temperature range of 20–40°C indicating that the conformation of the CSP did not change in the studied temperature range. The corresponding thermodynamic parameters \( \Delta H^0 \) and \( \Delta S^* \) were calculated from the plots of \( \ln k'_1 \) vs \( 1/T \) and \( \ln k'_2 \) vs \( 1/T \) and given in Table II. It could be seen from Table II, as the percentage of 2-propanol increases, the absolute \( \Delta H^0 \) values of DRV enantiomers increase (−11.746 to −14.080 and −17.123 to −19.019 kJ mol\(^{-1}\)) indicating the increase of heat of adsorption from 40 to 55% reflecting stronger interactions between DRV enantiomers and cellulose tris(3,5-dichlorophenylcarbamate). The absolute \( \Delta H^0 \) and \( \Delta S^* \) values in all cases were negative and suggest that the enantioseparation was enthalpy driven.

**Conclusions**

The effect of nature and content of organic modifier at different column temperatures on the retention and enantioseparation of DRV on Chiralpak IC column containing cellulose tris(3,5-dichlorophenylcarbamate) were studied. The apparent thermodynamic parameters derived from the Van’t Hoff plots (\( \ln k' \) vs \( 1/T \)) were used to explain the chiral recognition mechanism of the new immobilized cellulose tris(3,5-dichlorophenylcarbamate) on silica. The plots of \( \ln k \) against \( 1/T \) were highly linear, and the negative values of all the thermodynamic parameters in the temperature range of 20–40°C in 1-propanol and 2-propanol systems suggest that the conformation of the CSP did not change in the studied temperature range. The optimized conditions may be of use in development of LC–MS–MS methods for pharmacokinetics and therapeutic drug monitoring of (−) and (+) DRV enantiomers in biological matrices.

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<th>Table II</th>
<th>Effect of Volume [%] of 1-Propanol and 2-Propanol in Mobile Phase on Thermodynamic Parameters of (−) DRV (1) and (+) DRV (2)</th>
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