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Effect of system variables involved in packed column supercritical fluid chromatography of stavudine taken as model analyte using response surface methodology along with study of thermodynamic parameters

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Abstract

A multifactor optimization technique is successfully applied to study the effect of simultaneously varying the system variables on feasibility of stavudine analysis by packed column supercritical fluid chromatography (PC-SFC). The effect of simultaneously varying the pressure, temperature and modifier concentration was studied to optimize the method in order to obtain excellent chromatographic figures of merit. The method is based on isocratic elution using methanol-modified supercritical carbon dioxide as the mobile phase at the flow rate of 3.0 ml/min through a JASCO Finepak SIL-5, ODS [C_{18} (5 μm, 25 cm × 4.6 mm, i.d.)] column support using photodiode array detection. The optimal conditions were determined with the aid of the response surface methodology using 3^3 factorial designs. From the response surface graphs optimum regions were selected to be +1, −1, and +1 for temperature (60 °C), pressure (20 MPa) and percent modifier concentration (17.81%, v/v), respectively. Linearity dynamic range was found to be in the range of 2.0–150.0 μg/ml with significantly high value of correlation coefficient. The method was validated for precision, robustness and recovery to assess the viability of the established method. The chromatographic limit of detection and quantitation were 0.80 and 1.50 μg/ml respectively. The method has been successfully used to analyze commercial dosage form to assess the chromatographic performance of SFC system which was found to be 99.91% ± 1.62. The present work briefs the thermodynamic applications of PC-SFC with an emphasis on the results of stavudine. The foremost of such applications is the determination of solute diffusion coefficient in supercritical mobile phase by Taylor–Aris peak broadening technique.

Keywords: Column liquid chromatography; Supercritical fluid chromatography; Factorial design; Response surface methodology; Stavudine; Thermodynamic parameters; Taylor–Aris peak broadening technique

1. Introduction

The use of response surface methodology on PC-SFC variables interaction study using stavudine as model analyte has been studied. Facility of on-line addition of modifier and controlling of backpressure has made routine SFC analysis of drugs more feasible [1–5]. Temperature, pressure and modifier concentration are the variables that significantly affect the mobile phase density, drug solubility and thermal diffusivities. These variables are expected to exert the interactive effect. The multivariate experiments using suitable statistical designs such as 3^3 factorial designs will elucidate the effect of variables on different chromatographic parameters.

Stavudine (Fig. 1a) is chemically (2′,3′-didehydro-3′-deoxythymidine, d4T), a thymidine nucleoside with in vitro and in vivo inhibitory activity against the reverse transcriptase of human immunodeficiency virus (HIV) [6]. The impurity of stavudine, thymine (Fig. 1b) (5-methyl-2,4 [1H, 3H]-pyrimidinedione) is official in USP [7]. Several methodologies for the individual determination of stavudine in biological fluids have been previously reported in the literature that included radioimmunoassay [8,9], HPLC [10,11] and LC–MS–MS techniques [12–14].

Using an octadecylsilica stationary phase over a temperature range from 303 to 353 K and an average pressure range from 15 to 35 MPa, the thermodynamic and kinetic aspects of retention

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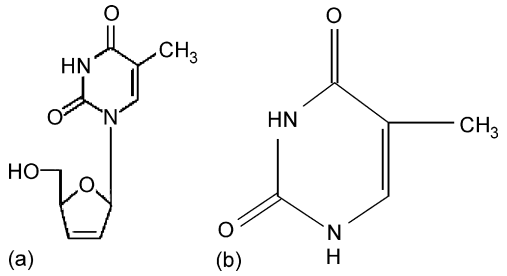

Fig. 1. (a) Structure of stavudine; (b) structure of thymine.

mechanism was examined. Thermodynamic behavior was characterized by the retention factor together with the associated changes in molar enthalpy and molar volume, whereas kinetic behavior was characterized by the rate constants. Determination of diffusion coefficient of analyte by the Taylor–Aris peak broadening technique was carried out. Binary diffusion coefficient of stavudine in methanol-modified supercritical carbon dioxide was determined and the effect of pressure and temperature was studied.

2. Experimental

2.1. Materials and instrumentation

Pharmaceutical grade of stavudine (B. No.: 2113-020801) was kindly supplied as a gift sample by Cipla Ltd., Mumbai, India certified to contain 99.75% (w/w) on dried basis. Thymine reference standard and HPLC grade methanol was purchased from E-Merck Chemicals, India. Carbon dioxide was 99.9% pure (SFE/SFC) obtained from Bombay carbon dioxide, Mumbai, India. The apparatus used was a JASCO SFC 1500-series configured for dynamic mixing with a two-pump system JASCO-PU 2080. System pressure was maintained electronically by backpressure regulator JASCO BP-1580-81. The temperature of the column could be kept at any desired point between 35 and 80 °C using a JASCO CO-2065 series oven. A rheodyne injector, model-7125 (manual loading) with a 20 μl external loop was used. The analyte was chromatographed on a Finepak SIL-5, C_{18} (250 mm × 4.6 mm, 5.0 μm) column, Jasco Corporation, Japan. Detection was done with a photodiode array detector JASCO MD-2010. Cryostat bath (FOURTECH, Mumbai) was used for liquidation of the gas at −10 °C.

2.2. Selection of system variables

Supercritical fluid CO_2 (flow rate 3.0 ml/min) in the pressure range of 20–30 MPa and temperature 40–60 °C was used in combination with methanol as a modifier. Stock solution of standard stavudine was prepared to obtain final concentration of 100 μg/ml. The equilibration time of 15 min between runs was maintained.

2.3. Factorial design

In 3^3 factorial design, levels of factors are independently varied, each factor at three levels (−1, 0, and +1). Responses obtained from the batches of factorial design experiments were subjected to multiple regression analysis using 'UNISTAT version 5.5' software. The response surface for each considered response was approximated by a second order polynomial function Eq. (1) [15,16]:

$$Y = \beta + \beta_1 X_1 + \beta_{11} X_1^2 + \beta_2 X_2 + \beta_{22} X_2^2 + \beta_3 X_3 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{123} X_1 X_2 X_3 \quad (1)$$

where Y is the measured response; β represents intercept; β_1, $\beta_{11}, \beta_2, \ldots$ represents coefficients computed from the responses in the design; X_1 the temperature; X_2 the pressure; X_3 is the flow rate of modifier. Three factors were considered: temperature (X_1), pressure (X_2), and modifier concentration (X_3) at no more than three levels (−1, 0, and +1) (Table 1). A 3^3 factorial design requires 27 experiments.

Table 1
Experimental domain of the factors during response surface study

Factor	Factor ID	Low (−1)	Middle (0)	High (+1)
Temperature (°C)	X_1	40	50	60
Pressure (MPa)	X_2	20	25	30
Modifier (ml/min)	X_3	0.35	0.50	0.65

2.4. Standard solution and calibration graphs

A stock solution of stavudine was prepared in methanol at (500 μg/ml). Standard solutions were prepared by dilution of the stock solution with methanol to give solutions containing stavudine in two concentration ranges of 2.0–50.0 and 50.0–150.0 μg/ml, respectively.

2.5. Method validation

The intra-day and inter-day variation for determination of stavudine was carried out at three different concentration levels 60, 100, and 140 μg/ml using homogeneous, authentic samples (40 mg stavudine per capsule). The parameters for robustness included variation of C_{18} columns from different manufacturers, flow rate of carbon dioxide (%, v/v) of methanol (modifier), column temperature, pressure and methanol of different lots. Two analytical columns, one (Kromasil C-18 column) from Pune, India and the other (Finepak SIL-5, C-18 column) from Japan, were used during the experiment. A signal-to-noise ratio between 3:1 and 10:1 is generally considered acceptable for estimating the detection limit and quantitation limit respectively [17]. For specificity the peak area for stavudine in sample was confirmed by comparing the retention time (t_R) and spectra of the sample with those of standard [18,19]. For assay an equivalent weight of the capsule content was transferred into a 100 ml volumetric flask containing 50 ml methanol, sonicated for 30 min and diluted to 100 ml with methanol. The resulting solution was centrifuged at 3000 rpm for 5 min and supernatant (100 μg/ml) was analyzed for drug content. For detection of the related impurities standard stavudine was prepared to

obtain 100 μg/ml concentration. Similarly standard thymine was prepared at concentration of 100 μg/ml.

2.6. Thermodynamics of retention

Using a temperature range from 303 to 353 K and an average pressure range from 15 to 35 MPa, the thermodynamic and kinetic behaviors are characterized. The van't Hoff equation can be applied to chromatographic retention:

$$\ln k = -\frac{\Delta H_{sm}}{RT} + \frac{\Delta S_{sm}}{R} + \ln \phi \quad (2)$$

ΔH_{sm} and ΔS_{sm} are the enthalpy and entropy change associated with the transfer of solute from one phase to another, R the universal gas constant, ϕ the phase ratio, and T is the absolute temperature [20,21].

2.7. Determination of binary diffusion coefficient of stavudine by Taylor–Aris peak broadening technique

The diffusion coefficient in flowing fluids was first represented in the form of equation by Taylor and Aris:

$$D_{12} = u\frac{[H \pm (H^2 - r^2/3)^{1/2}]}{4} \quad (3)$$

where D_{12} is the binary diffusion coefficient of the solute in supercritical fluid, u the average velocity of mobile phase, H the height equivalent to a theoretical plate and r is the inner radius of the column. The peak-width at half-height was used to calculate diffusion coefficient Taylor–Aris peak broadening technique [22,23].

3. Results and discussion

3.1. Study of response curves

All 27 experiments were run at concentration values of 100 μg/ml. The experimental plan (Table 2) and the corresponding responses are reported in Table 3. Experimental results were computed by unistat software (UNISTAT® version 3.0, MEGALON SA, 1994). The coefficients of the second order polynomial model were estimated by least square regression. The regression model for each considered response was tested through analysis of variance (ANOVA) to determine its significance and validity. The response surface plots were generated after removal of insignificant variables by backward elimination method and adequacy of fitted model was checked by ANOVA [24,25]. The equation model for constant, the regression coefficients and the statistical parameters for each response variable, viz. Y_{PA} (peak area), Y_{PH} (peak height), Y_{TP} (theoretical plates), Y_{CF} (capacity factor) and Y_{AF} (asymmetry factor) are given in Tables 3 and 4.

The response surfaces were drawn, evaluating the effects of the two factors at a time and setting the other at fixed value. As shown in Fig. 2a–e, the analysis produces three-dimensional graphs by plotting the response model against two of the factors, while the third is held constant. From the response curves following interpretations have been reported:

Table 2
List of experiments in the factorial design (3^3) for method optimization

Run order	X_1	X_2	X_3
1	−1	−1	−1
2	−1	−1	0
3	−1	−1	+1
4	−1	0	−1
5	−1	0	0
6	−1	0	+1
7	−1	+1	−1
8	−1	+1	0
9	−1	+1	+1
10	0	−1	−1
11	0	−1	0
12	0	−1	+1
13	0	0	−1
14	0	0	0
15	0	0	+1
16	0	+1	−1
17	0	+1	0
18	0	+1	+1
19	+1	−1	−1
20	+1	−1	0
21	+1	−1	+1
22	+1	0	−1
23	+1	0	0
24	+1	0	+1
25	+1	+1	−1
26	+1	+1	0
27	+1	+1	+1

Table 3
List of responses from the factorial design ($n = 3$)

Run order	Y_{PA} (mAU s)	Y_{PH} (mAU)	Y_N	Y_{CF}	Y_{AF}
1	911893	72068	13094	3.6	1.09
2	844310	106108	13334	2.0	1.13
3	821122	139209	13611	1.3	1.24
4	924591	79491	13106	3.2	1.07
5	900212	117347	13301	1.9	1.08
6	867720	147688	13257	1.2	1.10
7	1035367	96322	13172	3.0	1.10
8	940079	130390	13262	1.7	1.26
9	908333	164059	13570	1.2	1.19
10	876270	69412	13330	3.8	1.11
11	797360	98900	13467	2.1	1.23
12	780431	130563	13451	1.3	1.22
13	829420	73610	13325	3.4	1.16
14	853979	110649	13400	1.9	1.18
15	829213	145841	13603	1.2	1.15
16	895978	81529	13051	3.1	1.09
17	878843	124108	13393	1.7	1.20
18	890743	160987	13495	1.2	1.22
19	841634	63670	13508	4.1	1.17
20	811874	97361	13573	2.3	1.08
21	773009	130113	13656	1.4	1.13
22	878733	70113	13042	3.6	1.13
23	894336	107043	12914	1.7	1.10
24	818553	141189	13450	1.4	1.15
25	916032	81758	13026	3.1	1.09
26	887970	118755	13131	1.8	1.06
27	860038	156049	13451	1.1	1.08

Y_{PA}, peak area; Y_{PH}, peak height; Y_N, height equivalent to theoretical plates; Y_{CF}, capacity or retention factor; AF, asymmetric factor.

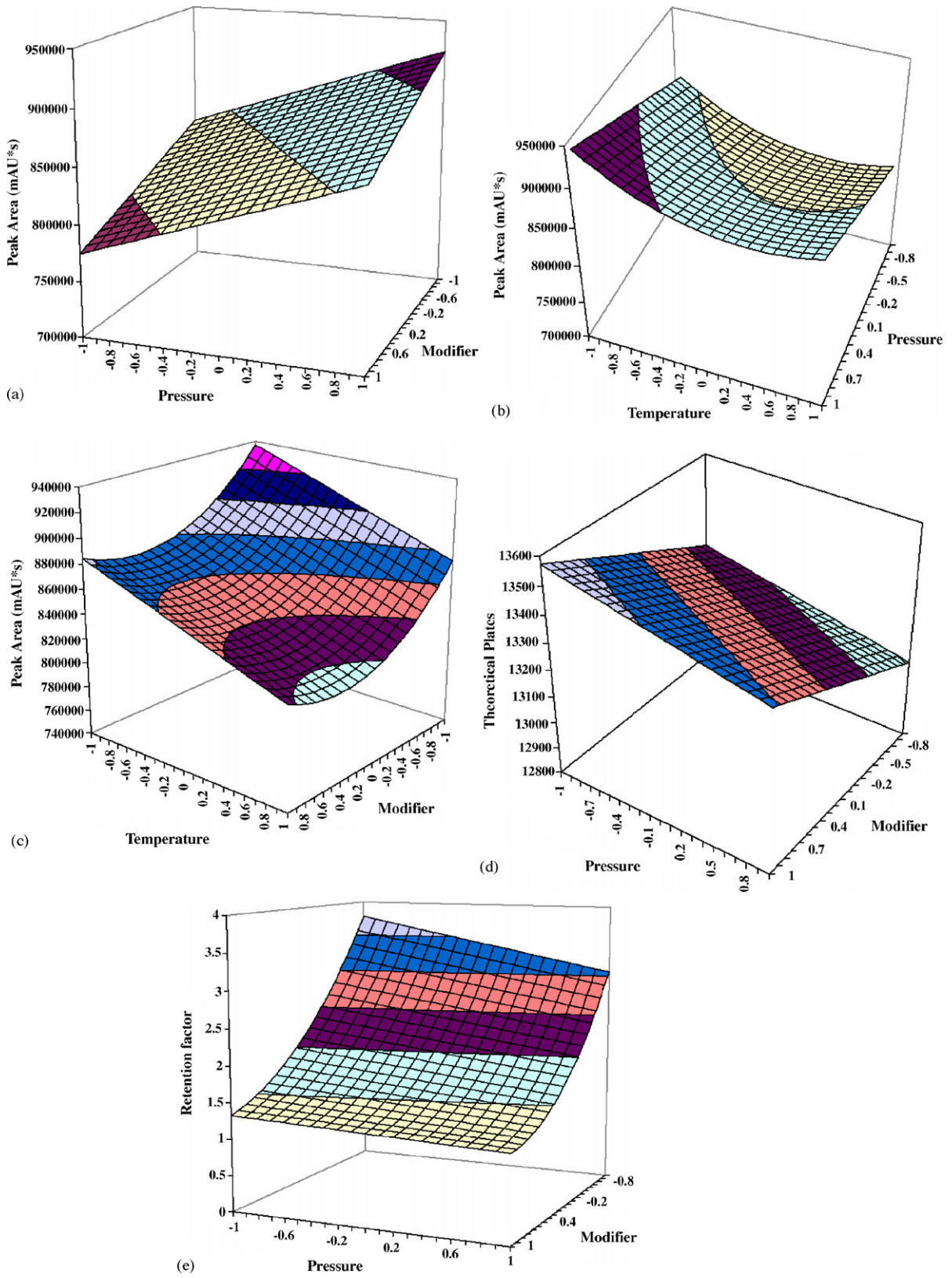

Fig. 2. (a) Plot of the response surface showing variation of the response Y_{PA} (peak area) as a function of X_2 (pressure) and X_3 (% modifier), fixed factor X_1 (temperature). (b) Plot of the response surface showing variation of the response Y_{PA} (peak area) as a function of X_1 (temperature) and X_2 (pressure), fixed factor X_3 (% modifier). (c). Plot of the response surface showing variation of the response Y_{PA} (peak area) as a function of X_1 (temperature) and X_3 (% modifier), fixed factor X_2 (pressure). (d) Plot of the response surface for Y_{TP} (theoretical plate). Variation of the response as a function of X_2 (pressure) and X_3 (% modifier), fixed factor X_1 (temperature). (e) Plot of the response surface for Y_{CF} (capacity or retention factor). Variation of the response as a function of X_2 (pressure) and X_3 (% modifier), fixed factor X_1 (temperature).

Table 4
Summary of regression results for the measured responses

Coefficients	Parameters			
	Y_{PA}	Y_{PH}	Y_{TP}	Y_{CF}
β_0	848027	110330	3332.7	1.90
β_1	−26192	−481283	81.7	0.08
β_2	41971	11475	–	−0.21
β_3	31153	34874	160.6	−1.08
β_{12}	–	–	−91.2	−0.06
β_{23}	–	2229	–	0.14
β_{13}	–	–	–	−0.06
β_{11}	31741	–	–	–
β_{22}	–	1968	–	–
β_{33}	–	–	–	0.44
β_{123}	–	–	–	–
r^2	0.845	0.996	0.614	0.994
p	2×10^{-4}	3×10^{-4}	1×10^{-4}	0.0

With the increase in pressure the density of the methanol-modified supercritical CO_2 increases resulting in decrease in mean collision frequency (diffusitivity). The λ_{max} of the analyte has not changed with the increase in pressure throughout the experimentation. This has been confirmed with the help of PDA detector. The changes in absorbance value have been noticed with pressure due to the varying degree of interaction with the mobile phase that also contributes for changes observed in peak area while the λ_{max} remained constant at 263 nm. As the modifier concentration is increased, the polarity of mobile phase increases resulting in rapid elution of solute from the column. The reduction in peak area due to increase in temperature results from the lower density of the fluid and hence lower solubility of the analyte in the mobile phase (Fig. 2a–c).

It has been observed that the interactive effect of pressure and modifier concentration has significant effect on chromatographic figures of merit. Increase in pressure results decrease in theoretical plates and retention factor while theoretical plates increases with increasing modifier concentration. With increase in pressure the peak shape attains more non-Gaussian nature due to restricted dipole interactions between analyte and stationary phase resulting in decrease in theoretical plates. With increase in modifier concentration theoretical plate increases and retention factor decreases. This is due to increasing polarity of mobile phase resulting in establishment of rapid equilibrium of analyte between stationary phase and mobile phase (Fig. 2d and e).

From the above study it is clear that the selected factors X_1 (temperature), X_2 (pressure), and X_3 (concentration of modifier) are significant for the regression model and their interactive effect has been observed on chromatographic parameters. From the above response surface graphs optimum regions were selected to be +1, −1, and +1 for temperature (60 °C), pressure (20 MPa) and modifier concentration (17.80%, v/v), respectively and further utilized for construction of calibration curves and validation studies.

3.2. Calibration curves

Linearity was evaluated by determining standard working solutions containing 2.0–50.0 μg/ml (low range: $r^2 = 0.997$, slope = 0.94 ± 0.64, intercept = 10.38 ± 1.94) and 50.0–150.0 μg/ml (high range: $r^2 = 0.999$, slope = 1.28 ± 0.48, intercept = 18.41 ± 1.27), respectively of standard in triplicate. The standard deviation of intercept value was less than 2. No significant difference was observed in the slopes of standard curves (ANOVA; $p < 0.05$).

3.3. Validation of the method

3.3.1. Precision and robustness of the method

The average intra-day and inter-day precision were found to be 1.18 and 1.28, respectively. Results, presented in Table 5 indicate that the selected factors remained unaffected by small variations of these parameters. Insignificant differences in peak areas and less variability in retention time were observed.

3.3.2. LOD and LOQ

LOD and LOQ were experimentally verified by diluting known concentrations of stavudine until the average responses were approximately 3 or 10 times the standard deviation of the responses of the blank for six replicate determinations. The signal/noise ratios 3:1 and 10:1 were considered as LOD and LOQ, respectively. The LOD and LOQ were found to be 0.80 and 1.50 μg/ml, respectively.

3.3.3. Peak purity and recovery studies

The peak purity of stavudine was assessed by comparing the spectra at peak start, peak apex and peak end positions, i.e., r(S, M) = 0.9995 and r(M, E) = 0.9992. Good correlation (r = 0.9998) was also obtained between standard and sample spectra. The average retention time ± standard deviation for stavudine was found to be 2.20 ± 0.05 for six replicates. The mean recovery from the marketed formulation was found to be 99.43 ± 0.84.

3.4. Analysis of the marketed formulation

The peak at t_R 2.39 min for stavudine was observed in the chromatogram of the drug samples extracted from capsules. The drug content was found to be 99.91% ± 1.62 (%R.S.D. of 0.02) for stavudine. Statistical evaluation was performed using Student's t-test and the F-ratio at 95% confidence level.

3.5. Detection of related impurity

It was found that a small peak (thymine impurity) was well resolved from the parent peak (stavudine) at 20 MPa, 60 °C and 0.65 ml/min of modifier concentration [7]. It was observed that on increasing the polarity of mobile phase from 0.35 to 0.65 ml/min and temperature from 40 to 60 °C the resolution of the impurity from the parent drug was improved at 20 MPa (Fig. 3). With the help of photodiode array detector, the λ_{max} of the impurity was found to be 259 nm and its retention time was 1.96 min. The peak area of the impurity was significant, i.e., the

Table 5
Robustness evaluation[a] of the SFC method ($n = 6$)

Chromatographic changes		Stavudine			
Factor[b]	Level	t_R[c]	k[d]	AF[e]	Area (mAU s) (%R.S.D.)
Flow rate of CO_2 (ml/min)					
2.90	−1	2.39	1.39	1.05	773122
3.00	0	2.37	1.37	1.03	773009
3.10	1	2.35	1.35	1.01	772985
Mean ± S.D. ($n = 6$)		2.37 ± 0.02	1.37 ± 0.02	1.03 ± 0.02	773038 (0.009)
% age of methanol in the mobile phase (v/v)					
17.36	−1	2.38	1.40	1.04	773298
17.81	0	2.37	1.37	1.03	773009
18.25	1	2.33	1.33	1.02	772765
Mean ± S.D. ($n = 6$)		2.36 ± 0.03	1.37 ± 0.04	1.03 ± 0.01	773024 (0.03)
Temperature (K)					
331	−1	2.38	1.38	1.04	773319
333	0	2.37	1.37	1.03	773009
335	1	2.35	1.35	1.02	772851
Mean ± S.D. ($n = 6$)		2.37 ± 0.02	1.37 ± 0.02	1.03 ± 0.01	773059 (0.03)
Columns from different manufacturers					
Kromasil		2.36	1.36	1.05	772981
Finepak		2.37	1.37	1.03	773009
Mean ± S.D. ($n = 6$)		2.37 ± 0.01	1.37 ± 0.01	1.04 ± 0.01	772995 (0.002)
Solvents of different lots					
First lot		2.37	1.37	1.03	773009
Second lot		2.36	1.36	1.02	773225
Mean ± S.D. ($n = 6$)		2.37 ± 0.01	1.37 ± 0.01	1.03 ± 0.01	773117 (0.019)

[a] Average of three concentrations 20, 50, 80 μg/ml for stavudine.
[b] Four factors were slightly changed at three levels (1, 0, −1); each time a factor was changed from level (0) the other factors remained at level (0).
[c] Retention time.
[d] Capacity factor.
[e] Asymmetry factor.

average percent area of the impurity with respect to the parent peak was found to be 1.62% ± 0.08 (for six replicate injections).

3.6. Thermodynamic behavior

3.6.1. Retention factors

It is apparent that the retention factor for stavudine increases with increase in temperature and decreases with increase in pressure (Table 6). This can be explained by an increase in temperature leads to lower density of the mobile phase, which in turn leads to greater retention. Likewise an increase in pressure leads to increase in density and decrease in retention. It has been observed that at 30 MPa the retention factor decreases with increase in temperature which can be attributed to compression of the stationary phase resulting in lesser interaction of the alkyl chains with the planar solutes like stavudine.

Table 6
Retention factors (k) for stavudine ($n = 3$)

Temperature (K)	k at 25 MPa	k at 30 MPa	Pressure (MPa)	k at 313 K
303	–	1.26	–	–
308	–	1.23	–	–
313	1.19	1.18	15	1.37
318	1.20	1.17	20	1.27
323	1.29	1.15	25	1.19
328	1.43	–	30	1.18
333	1.50	–	35	1.06

3.6.2. Molar enthalpy

The change in molar enthalpy at constant pressure (25 MPa) is found to be 1.56 kcal/mol ± 0.18. The graph for solute is linear ($r^2 = 0.993$) and the slope is negative. A negative slope is indicative of a positive change in molar enthalpy and suggests that the transfer from the mobile to stationary phase is not enthalpically favorable but it is entropically driven. The entropy value is found to be 5.11 kcal/°. This indicates the spontaneity of the process. At constant pressure of 30 MPa and in temperature range of 30–50 °C the graph for solute is linear ($r^2 = 0.9997$) and the slope is positive. A positive slope is indicative of a negative change in molar enthalpy and is found to be −0.435 kcal/mol ± 0.09. This suggests that the transfer from the mobile to stationary phase is enthalpically favorable at 30 MPa.

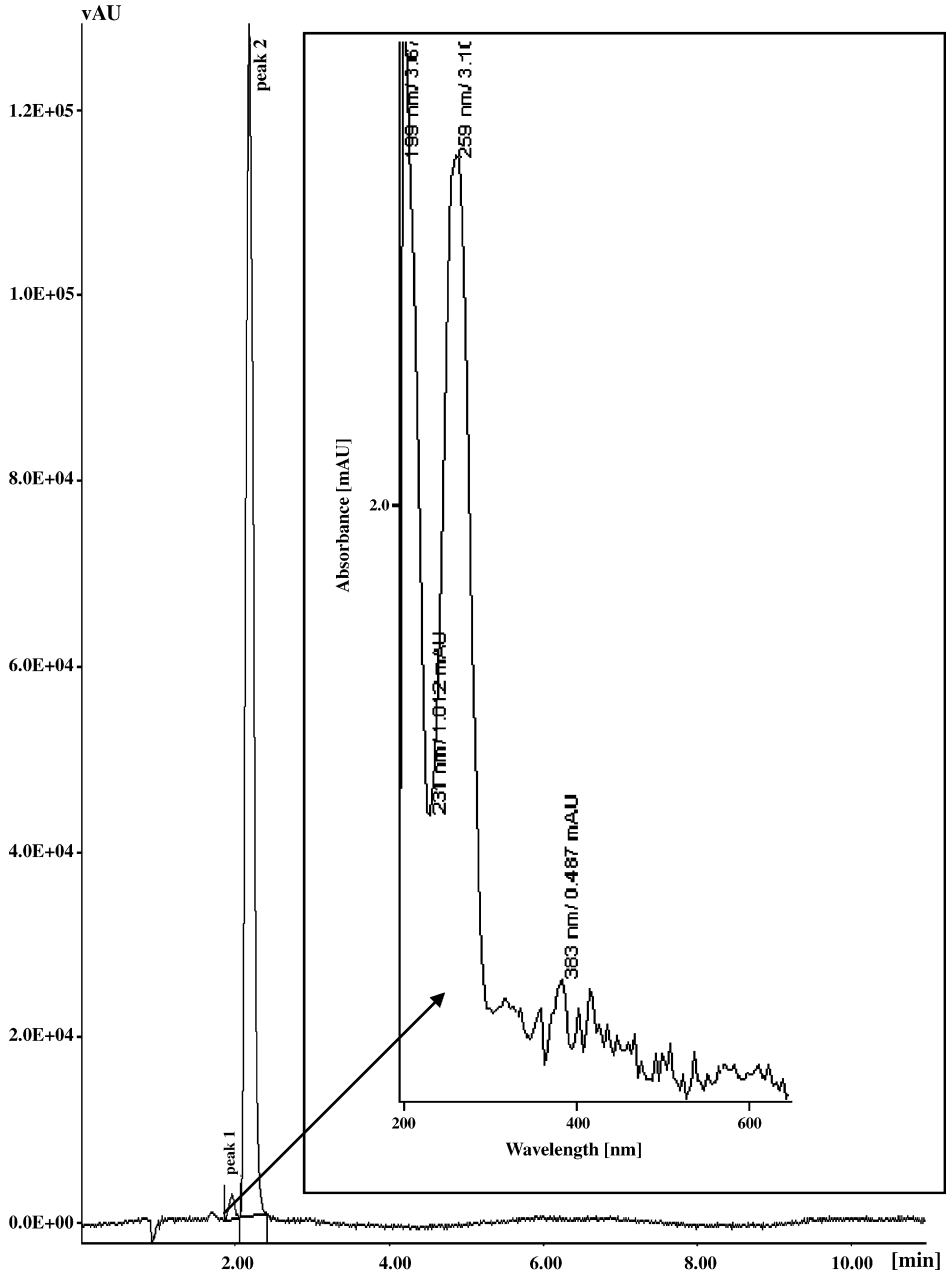

Fig. 3. Chromatogram showing impurity (peak 1: $t_R = 1.96$ min) and parent drug (peak 2: $t_R = 2.37$ min, $\lambda_{max} = 263$ nm) along with the UV spectrum of impurity ($\lambda_{max} = 259$ nm) at optimized conditions obtained from the response surface graphs +1, −1, and +1 for temperature (60 °C), pressure (20 MPa) and percent modifier concentration (17.81%, v/v), respectively.

3.6.3. Molar volume

The change in molar volume is found to be 0.012 ml/mol ± 0.09. The graph for solute is linear ($r^2 = 0.996$) and the slope is negative. A negative slope is indicative of a positive change in molar volume and suggests that the solute occupies less volume in the mobile phase than in the stationary phase.

3.6.4. Kinetic behavior

3.6.4.1. Rate constants. From Tables 7 and 8 it is clear that the rate constants for stavudine increase with increasing temperature [26,27]. This behavior is a consequence of the increased diffusion coefficients and the enhanced fluidity of the stationary

Table 7
Rate constants for stavudine as a function of temperature ($n = 3$)

Temperature (K)	k_{ms} (s^{-1})	k_{sm} (s^{-1})
313	2.25	2.52
318	2.39	2.86
323	2.57	3.31
328	2.85	4.07
333	2.99	4.49

k_{ms}, rate constant from mobile to stationary phase calculated at 25 MPa; k_{sm}, rate constant from stationary to mobile phase calculated at 25 MPa.

Table 8
Rate constants for stavudine as a function of temperature ($n = 3$)

Temperature (K)	k_{ms} (s^{-1})	k_{sm} (s^{-1})
303	2.52	3.18
308	2.47	3.04
313	2.40	2.88
318	2.35	2.76
333	2.30	2.65

k_{ms}, rate constant from mobile to stationary phase calculated at 30 MPa; k_{sm}, rate constant from stationary to mobile phase calculated at 30 MPa.

phase. As more kinetic energy is imparted, the alkyl chains become more liable and can more readily undergo rotation of the carbon–carbon bonds from the trans to the gauche conformation. This increased liability enables the stavudine to diffuse in and out of the stationary phase more freely. As shown in Table 9, the rate constants for stavudine decrease with increasing pressure. This behavior is a consequence of the compression of the alkyl chains, which impedes the solute diffusion in and out of the stationary phase.

3.6.5. Activation enthalpy

The activation enthalpy for ΔH_m^* and ΔH_s^* at constant pressures (25 and 30 MPa), is found to be 1.57, 10.71 and −0.440, −0.872 kcal/mol, respectively. As the activation enthalpy is compared with molar enthalpy, a trend emerges: $\Delta H_s^* > \Delta H_m^* > \Delta H_{sm}$. Thus, the enthalpic barrier for the transition is significantly greater than the thermodynamic change in molar enthalpy at 25 MPa but reverse trend was observed at 30 MPa.

3.6.6. Activation volume

The activation volume for ΔV_m^* and ΔV_s^* is found to be 0.013 and 0.025 ml/mol, respectively. Similar to the enthalpic trends, the activation volumes and the change in molar volume demonstrate the trend of $\Delta V_s^* > \Delta V_m^* > \Delta V_{sm}$. Hence, the volumetric barrier for the transition is large, even though the overall change in molar volume is relatively small.

3.7. Taylor–Aris peak broadening technique for determination of binary diffusion coefficient of stavudine

The diffusion coefficient was determined at 30, 35, 40, 45, 50, 55, and 60 °C and in the pressure range of 15, 20, 25, 30, and 35 MPa. Asymmetry factors used for the diffusivity results reported were less than 1.1. The effect of temperature

Table 9
Rate constants for stavudine as a function of pressure ($n = 3$)

Pressure (MPa)	k_{ms} (s^{-1})	k_{sm} (s^{-1})
15	2.73	3.74
20	2.54	3.22
25	2.38	2.84
30	2.24	2.51
35	2.13	2.26

k_{ms}, rate constant from mobile to stationary phase calculated at 313 K; k_{sm}, rate constant from stationary to mobile phase calculated at 313 K.

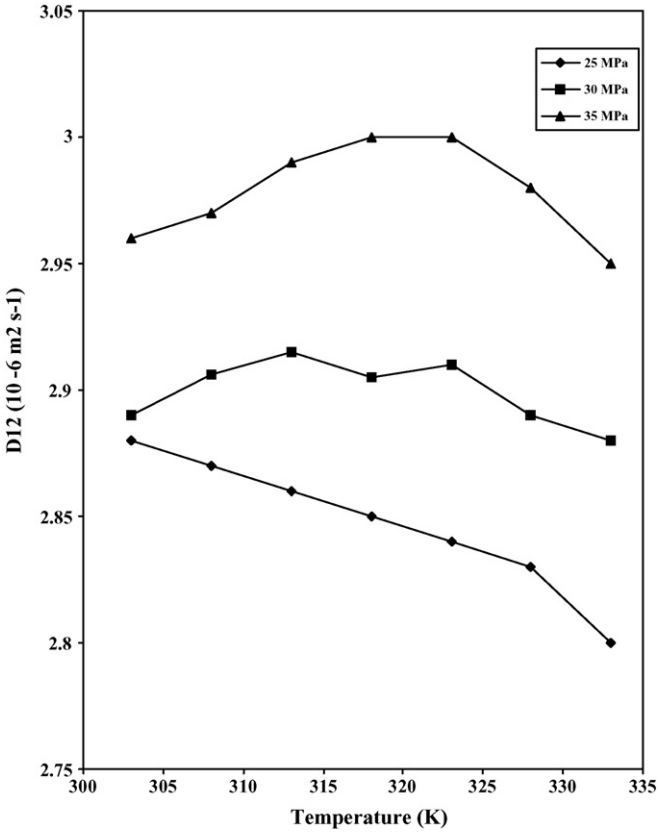

Fig. 4. Effect of temperature on the diffusion coefficient (D_{12}) of stavudine in supercritical fluid carbon dioxide at 25, 30, and 35 MPa.

and pressure on the diffusion coefficient of stavudine is shown in Figs. 4 and 5. At 25 MPa, there is significant decrease in the D_{12} value with increase in the temperature. At 30 and 35 MPa initially the D_{12} increases up to 315 K and then decreases with increase in temperature and increases with pressure.

3.8. PC-SFC versus LC

The established SFC method has following advantages over the existing LC methods [10–14]. No preparation of high concentration of buffer solutions is required. The use of buffers can damage the stationary phase and therefore high column washing time is mandatory. Huge consumption of organic solvents like acetonitrile in LC methods generates more disposable waste, which makes them less economical. The reported LC methods for stavudine required the use of gradient elution, pH adjustments up to 4.0 and high column saturation time. These adjustments make these methods more tedious. In PC-SFC the necessity of the preparation of a mobile phase is obviated, as retention factor can be easily and widely tuned by the proper choice of modifier. While the SFC and reported LC methods yielded similar accuracy and precision, the number of samples per hour handled by the PC-SFC method is more since the speed of PC-SFC method (less than 5 min) was found to be better than gradient LC methods. PC-SFC offers dramatically high resolution that improves throughput, pure product recovery and replaces toxic and flammable solvents used in HPLC [28,29].

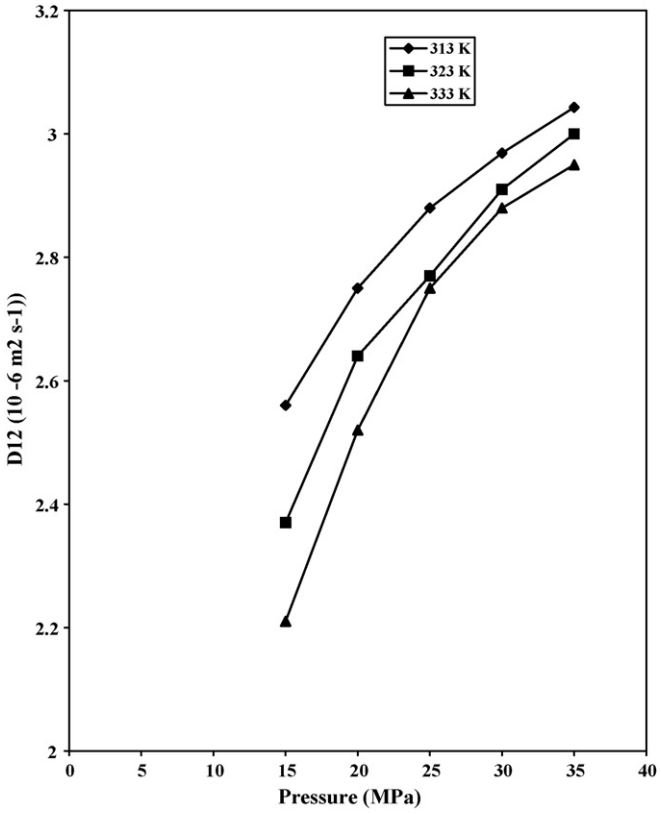

Fig. 5. Effect of pressure on the diffusion coefficient (D_{12}) of stavudine in supercritical fluid carbon dioxide at 313, 323, and 333 K.

The major interest is that higher flow rates can be used in PC-SFC to take advantage of the high diffusivity of supercritical fluids reducing analysis time without compromising efficiency. Thus column equilibration occurs within a few minutes speeding the optimization of chromatographic parameters.

4. Conclusion

The optimization study to control the variables was successfully performed employing response surface methodology from the data obtained from 3^3 factorial designs using stavudine as model drug. From the response surface graphs optimum regions were selected to be +1, −1, and +1 for temperature (60 °C), pressure (20 MPa) and percent modifier concentration (17.80%, v/v), respectively maintaining constant flow rate of supercritical CO_2 at 3.0 ml/min. Potential thymine impurity can be reliably found and quantified at 1.62% (w/w) of the bulk drug. Statistical tests indicate that the proposed SFC method reduces the duration of analysis and appear to be suitable for routine determination of drugs in pharmaceutical formulation in quality control laboratories, where economy and time are essential. The kinetic data suggests that the barrier for transition between mobile and stationary phase is small because the changes in molar enthalpy and molar volume are relatively less. The low values for activation enthalpy and activation volume suggest that the transition is more likely to occur in a single step. A new finding of the study is that at 30 MPa, in the temperature range of 30–50 °C, the process is enthalpically favorable where as at 25 MPa the process is entropically driven. This study also demonstrates that the operating pressure and temperature are important parameters affecting the binary diffusion coefficient of stavudine in supercritical carbon dioxide.

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