INTERNATIONAL JOURNAL OF RESEARCHES IN BIOSCIENCES, AGRICULTURE AND TECHNOLOGY
© VISHWASHANTI MULTIPURPOSE SOCIETY (Global Peace Multipurpose Society) R. No. MH-659/13(N)
www.vmsindia.org

DEVELOPMENT OF CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF TRAMADOL HYDROCHLORIDE FROM BULK DRUG AND TABLET FORMULATION: PLACKETT-BURMAN DESIGN AND EFFECT OF VARIABLES

Ravindrakumar L. Bakal , Jagdish Manwar, Rajesh Jadhao and Dipak Kumbhar
KYDSCT College of Pharmacy, Sakegaon (Bhusawal) - 425 201, Dist. Jalgaon, MS, India
rlbakal@rediffmail.com

Abstract
Plackett-Burman design was successfully applied for the development of chromatographic method for the determination of tramadol hydrochloride from bulk drug and tablet formulation. The effect of simultaneously varying the flow rate, temperature and concentration of acetonitrile in mobile phase phosphate buffer (KH2PO4, 0.02M, pH 5.8) on the chromatographic responses was studied with the help of response surface methodology (RSM). From RSM optimum regions were selected to be +1, +1 and +1 for flow rate (1 ml/min), temperature (25Â°C) and concentration of acetonitrile in mobile phase phosphate buffer (KH2PO4, 0.02M, pH 5.8) (60%, v/v), respectively. Linearity was observed in the range of 1â€“5 Âµg/ml with r2 value 0.9998. Obtained LOD and LOQ values were found to be 0.024 and 0.052 Âµg/ml, respectively. Developed method was validated as per ICH guidelines and was successfully used for the analysis of tablet formulation.
Keywords: Plackett-Burman design, Response surface methodology, Chromatography, Tramadol hydrochloride

Introduction

Tramadol hydrochloride (Fig. 1), chemically 2-(Dimethylamino) methyl-1-(3â€“methoxy phenyl) cyclo-hexanol is an opoid analgesic used alone or in combination with other agents to treat mild to severe pain [1,2]. Literature survey revealed two UV-spectrophotometric methods for the determination of tramadol hydrochloride in bulk drug and tablet formulation.[3,4] Also, there was report of different method like UV spectroscopy, HPLC, HPTLC methods for the determination of tramadol hydrochloride in combination with other drugs[5,6,7]. There was no report of validated and stability indicating HPLC method for the determination of same drug from bulk drug and pharmaceutical formulation. Therefore, attempts were made to develop validated stability indicating HPLC method for the determination of tramadol hydrochloride from bulk drug and tablet formulation by applying Plackett-Burman design. A Plackett-Burman design is used when we want to screen a large number of factors to identify those that are related to the dependent variable of interest. Response surface methodology (RSM), a surface plotted in three dimensions, provides large information about variables- response relationship. This is a relatively economical method as it allows testing the largest number of factor main effects with the least number of observations with as few runs as possible [8,9]. Here we have selected three experimental variables i.e., flow rate of mobile phase, column temperature and composition of mobile phase, as the possible causes for the change in chromatographic responses [10-14]. These variables are expected to show interactive results. Forced degradation studies involved subjecting the sample to a various stressed conditions to further evaluate the specificity of degradation products.

Material and Methods

Materials and instrumentation Standard drug tramadol hydrochloride was kindly gifted by Abbott Healthcare Pvt. Ltd, Mumbai, India. Acetonitrile (HPLC grade) was procured from E-Merck Chemicals, India. The apparatus used was a The Agilent 1220 Infinity LC system coupled with a gradient mixer and degasser. The temperature of the column could be kept at any desired point between 15 and 50Â°C using oven. A rheodyne injector with loop volume 20 Âµl was used. The analyte was chromatographed on a Nucleosil C-18 (4.6 mm I.D x 250 mm) column. The detection was measured using UV detector. 2.2. Optimization of chromatographic condition 2.2.1. Selection of system variables As flow rate of mobile phase (X1) and column temperature (X2) determines the various responses of HPLC analysis, these two factors were taken as first two experimental variables for optimization of conditions [15-17]. Next to X1 and X2, as the polarity of the mobile phase (i.e., the % of organic solvent content in mobile phase) influence the chromatographic responses, therefore, concentration of acetonitrile (a polar solvent) in mobile phase (X3) was taken as minor variable [18]. 2.2.2. Selection of ranges of variables Range of flow rate (X1) and column temperature (X2) was selected performing

preliminary trials using standard stock solution of drug in water (100 Âµg/ml). Flow rate in the range of 0.8-1 ml/min, and temperature in the range of 20â€“25Â°C was selected in combination with acetonitrile as a modifier of mobile phase phosphate buffer (KH2PO4, 0.02M, pH 5.8). As tramadol hydrochloride is a low molecular weight basic drug, use of acidic mobile phase phosphate buffer (KH2PO4, 0.02M, pH 5.8) minimizes the interaction of basic drug with surface silanols on the silica packing because silanols do not ionize at acidic pH [19-21]. By considering the stability and pKa value of drug (pKa 9.8); pH of buffer used was 5.8. This large difference between pKa of drug and pH of buffer keeps the drug unaffected from mobile phase [18]. The concentration range of acetonitrile in phosphate buffer (KH2PO4, 0.02M, pH 5.8) (X3) used was 50-60%, v/v. About 30 min of column equilibration time between each run was maintained. 2.2.3. Experimental Design Chromatographic conditions optimization process to achieve separation of drug with acceptable responses was carried out using Plackett-Burman design 23 trial. According to this design, total 8 trial batches were formed. All the batches were named as SM-1 to SM-8. For investigating the effect, each independent variable was studied at two levels, namely, â€œhighâ€ and â€œlowâ€ . These levels define the upper limit and lower limits of the range covered by each variable. The values of coded levels of independent variables used in the experiment are listed in Table 1. 2.2.4. Response surface methodology The best method for the optimization of experimental conditions is response surface methodology (RSM). This process will not only determine the optimum conditions, but also give the information required to design a process. It is a scientific approach for establishing the optimum conditions. The correlation of three independent variables i.e., flow rate (X1), temperate (X2) and % acetonitrile in phosphate buffer (KH2PO4, 0.02M, pH 5.8) and chromatographic responses i.e. retention time (Y1), peak area (Y2), theoretical plates (Y3) and tailing factor (Y4) was studied. The response surface for each considered response was plotted against two different variables using STATISTICA (Version 8.0.360.0 English, StatSoft Inc., Tulsa, USA) software. The response surface for each considered response was approximated by second order polynomial regression model Eq. (1) [9]. $Y = Î²0 + Î²1X1 + Î²2X2 + Î²3X3$ â€¦â€¦. (1) Where, Y = Chromatographic response; Î²0 = Constant (intercept); Î²1 = Coefficient of X1; Î²2 = Coefficient of X2; Î²3 = Coefficient of X3; X1 = Flow rate of mobile phase, ml/min; X2 = Column temperature, Â°C; X3 = % acetonitrile in phosphate buffer (KH2PO4, 0.02M, pH 5.8), v/v. 2.3. Prediction profiling When the results of an experiment are analyzed, the observed responses on the dependent variables were fitted to a separate prediction equation for each dependent variable (containing different coefficients but the same terms). Once these equations are constructed, predicted values for the dependent variables were computed at combination of levels of the predictor variables. The relationship between observed response values and predicted response values was studied by plotting the linear graph of observed response values against predicted response values calculated from respective regression models of each response separately. 2.4. Analysis of RSM plots and regression models Targeted responses viz. retention time, peak area, theoretical plates, and tailing factor, were studied by one-way ANOVA-based factorial examination. An RSM computation for the current optimization was performed by using software STATISTICA version 8 (Stat-soft, Inc., USA). The obtained data were fitted to the second order regression equation (Eq. 1), and competency of a fitted response was evaluated by ANOVA. By setting the statistical significance to $p<0.05$, produced response surfaces (3D surface plots), and relationship plot between observed and predicted values were critically examined. 2.5. Assay of tablets For assay, an equivalent weight of the tablet (16 mg tramadol hydrochloride per tablet; CantarÂ® mfd. by Abbott Healthcare Pvt. Ltd, Mumbai) content was transferred into a 100 ml volumetric flask containing 30 ml water, shaken for 30 min and sonicated (Metrex Ultra Sonic) for 30 min. Final volume was made up to 100 ml mark with phosphate buffer (KH2PO4, 0.02M, pH 5.8) (2 Âµg/ml). The solution was filtered through Whatman filter paper (0.45Âµ) and was analyzed for drug content. The drug content in sample solution was calculated from the regression equations of standard calibration graph (Fig. 4). 2.6. Purity of peak The peak purity of tramadol hydrochloride was assessed by comparing the spectra at peak start, peak apex and peak end positions at optimum conditions by injecting six replicates of standard solution and sample solution of equal concentration 2 Âµg/ml separately (Fig. 1). 2.7. Validation of method Validation of developed method was carried out as per ICH guidelines [22]. 2.7.1. Accuracy of

method Accuracy of the method was determined by performing recovery studies using standard addition method [23]. Recovery study was performed by applying the method to preanalysed drug sample to which known amount of standard drug corresponding to 80 and 120% of label claim was added. At each level of the amount six determinations were performed and the results obtained were compared with expected results. 2.7.2. Precision of method Precision of method was determined with respect to both repeatability and reproducibility. An amount of the preanalysed tablet powder equivalent to 100% of the label claim of tramadol hydrochloride was accurately weighed and assayed. System repeatability was determined by six replicate applications and six times measurement of a sample solution at the analytical concentration. The repeatability of sample application and measurement of peak area for active compound were expressed in terms of % RSD (relative standard deviation). Method repeatability was obtained from RSD value by repeating the assay three times in same day for intra-day precision. Inter-day precision was assessed by the assay of three sample sets on different days (inter-day precision). The intra-day and inter-day variation for determination of drug was carried out at three different concentration levels 1, 3 and 4 Âµg/ml. 2.7.3. Linearity and range Linearity of the method was studied by injecting (20 Î¼L) six concentrations of the drug prepared in the water in the range 1-5 Î¼g/ml into the HPLC system. The peak areas were plotted against the corresponding concentrations to obtain the calibration graphs (Fig. 4). 2.7.4. Limit of detection (LOD) and limit of quantitation (LOQ) A signal-to-noise ratio between 3:1 and 10:1 is generally considered acceptable for estimating the limit of detection and limit of quantitation, respectively [22]. LOD and LOQ were experimentally verified by diluting known concentrations of tramadol hydrochloride until the average responses were approximately 3 or 10 times the standard deviation of the responses for six replicate determinations. 2.8. Forced degradation studies In order to determine stability of method (stability-indicating), pure sample of drug was stressed under a variety of conditions to perform forced degradation studies (FDS). A standard stock solution of drug (100 Âµg/ml) was used in FDS to draw an indication of the stability indicating property and specificity of proposed method. In all degradation studies the average peak area of standard drug and degraded sample after application of six replicates were obtained. 2.8.1. Oxidation degradation About 2 ml of hydrogen peroxide (1% v/v) was transferred to 2 ml of standard stock solution of drug, separately and the solution was kept at room temperature. After 30 minutes, resulting solution was diluted to attain concentration 2 ï ¬g/ml, 20ï ¬l of solution was injected and chromatograms were recorded. 2.8.2. Acid degradation About 2 ml of hydrochloric acid (0.01 N) was transferred to 2 ml of standard stock solution of drug, separately and the solution was kept at room temperature. After 30 minutes, resulting solution was diluted to attain concentration 2 ï ¬g/ml, 20ï ¬l of solution was injected and chromatograms were recorded. 2.8.3. Alkali degradation About 2 ml of sodium hydroxide (0.01 N) was transferred to 2 ml of standard stock solution of drug, separately and the solution was kept at room temperature. After 30 minutes, resulting solution was diluted to attain concentration 2 ï ¬g/ml, 20ï ¬l of solution was injected and chromatograms were recorded. 2.8.4. Neutral degradation About 100mg of pure drug was refluxed at 70Â°C in water for 3 hrs. After refluxing, resulting solution was diluted to attain concentration 2 ï ¬g/ml, 20ï ¬l of solution was injected and chromatograms were recorded. 2.8.5. Dry heat (thermal) degradation About 100mg of pure drug was kept in hot air oven at 80Â°C for 6 hrs. After heating, a concentration 2 mcg/ml was prepared using heated drug, 20 microlitre of solution was injected and chromatograms were recorded.

Result and Discussion

3.1. Interpretation of RSM plots and regression models All the batches were run using the concentration 2 Âµg/ml selected from linearity data (Fig. 4). Results obtained from all batches (SM-1 to SM-8) were analyzed by using STATISTICA (v 8.0.360.0 English, StatSoft Inc., Tulsa, USA). The effects and coefficients of regression models were measured by analysis of variance (ANOVA). The RSM plots were generated using same software and the adequacy of fitted model was tested by ANOVA [24-25]. The experimental plan and response are shown in Table 1. From RSM plots following interpretations are concluded.

3.1.1. Retention time The RSM analysis clearly indicated significant effect (p = 0.002431) of all experimental variables on retention time (Y1). To predict the retention time, following regression equation was obtained. Y1 = 54.87000 - 8.48750X1 - 0.10150X2 - 0.62325X3â€¦â€¦.(2) Where, Y1 = Retention time, min; X1 = Flow rate

of mobile phase, ml/min; X2 = Column temperature, Â°C; X3 = % acetonitrile in phosphate buffer (KH2PO4, 0.02M, pH 5.8), v/v. From eq. (2), it is clear that the retention time decreases with increase in experimental variables. How-ever, a high regression coefficient of X1 indicates the flow rate is a most responsible factor affecting retention time. We can increase the flow rate of mobile to decrease retention time, but it may reduce some of peak resolution, and backpressures may be beyond the limits of column. Increase in column temperatures (X2) lower the viscosity of the mobile phase (and thus the pressure) and increase the diffusion coefficient of the analytes and render the analyte to elute earlier [17]. Next to X1, X3 (% of acetonitrile in mobile phase) is another factor that affect the retention time by altering the polarity of mobile phase. Increase in concentration acetonitrile leads to rise in polarity of mobile phase and there rapid equilibrium is established between mobile phase and stationary phase, and retention time is decreased [18,26]. Flow rate of mobile phase (X2) exerts least effect. Response surface plot of retention time (tR) as a function of X1 (flow rate) and X3 (% acetonitrile) is shown in Fig. 2(a). The average retention time of different batches was varied from 7.18 to 15.83 min (Table 1). Statistical data for linear model is given in Table 2. 3.1.2. Peak area The RSM analysis clearly indicated significant effect (p = 0.000435) of all experimental variables on Peak area (Y2). To predict the Peak area, following regression equation was obtained. Y2 = 3487.66 - 1481.73X1 - 7.93X2 - 3.95X3â€¦â€¦.(3) Where, Y2 = peak area; X1 = Flow rate of mobile phase, ml/min; X2 = Column temperature, Â°C; X3 = % acetonitrile in phosphate buffer (KH2PO4, 0.02M, pH 5.8), v/v. From eq. (2), it is clear that the peak area decreases with increase in experimental variables. Relative to temperature and %acetonitrile, flow rate has very high impact on peak area. Variable X2 (temperature) also decrease the peak area insignificantly as compare to flow rate. Concentration of acetonitrile exerts least effect. The decrease in peak area may be due to increased temperature leads to the lowering of density of mobile phase, thereby enhance the mass transfer between phases and hence increase solubility of drug the in the mobile phase. Response surface plot of peak area (pA) as a function of X2 (temperature) and X1 (flow rate) is shown in Fig. 2(b). As shown in Table 1, the average peak area of different batches varied from 1559.77 to 1975.87. Statistical data for linear model is given in Table 2. 3.1.3. Theoretical plates The RSM analysis clearly indicated significant effect (p = 0.000894) of all experimental variables on theoretical plates (Y3). To predict the theoretical plates following regression equation was obtained. Y3 = 10802.12 + 575.39X1 + 6.16X2 â€“ 77.34X3 â€¦â€¦.(4) Where, Y3 = Theoretical plates; X1 = Flow rate of mobile phase, ml/min; X2 = Column temperature, Â°C; X3 = % acetonitrile in phosphate buffer (KH2PO4, 0.02M, pH 5.8), v/v. The theoretical plates analysis revealed positive relationship with two experimental variables namely, flow rate (X1) and temperature (X2). Nevertheless, as evidenced by high positive coefficient for X1, flow rate is the major variable affecting theoretical plates (theor. plates Î± flow rate). This might be due to effect of Van Deemterâ€™s principle which tells that number of theoretical plates (N) is function of height equivalent of a length of column and theoretical plate (H). The relationship between H, N and L is given by equation: N = L / H Where, H = Height equivalent of a theoretical plate, L = Length of column; and N = Theoretical plates. But, H is the function of eddy diffusion (A), longitudinal diffusion (B), resistance to mass to transfer (C) and linear flow velocity of mobile phase (V). The Van Deemterâ€™s equation is given bellow. H = A + B/V + CV Where, A = Eddy diffusion (proportional to particle size dp); B = Longitudinal diffusion (proportional to diffusion coefficients Dm); C = Resistance to mass transfer (proportional to dp2/Dm); V = Linear flow velocity. As the length of column (L), particle size (dp) and ratio of dp2/Dm are constant, numbers of theoretical plates (N) establish proportional relationship with flow rate (i.e. theor. plates Î± flow rate, N Î± V). Thus eq. (5) follows the Van Deemterâ€™s principle [16]. Increase in % of acetonitrile (X3) in mobile phase decreases the theoretical plates. Response surface plot of theoretical plates (tP) as a function of and X3 (% acetonitrile) and X1 (flow rate) is shown in Fig. 2(c). The average theoretical plates of different batches were varied from 6678.85 to 7844.58 (Table 1). Statistical data for linear model is in Table 2. 3.1.4. Tailing factor From RSM, it is clear that there is negative effect of all variables on tailing factor. From the results of tailing factor varying experimental variables, following regression equation was obtained to predict the tailing factor (Y4). Y4 = 2.532500 - 0.100000X1 - 0.013000X2 - 0.020000X3 â€¦â€¦.(5) Where, Y4 = Tailing factor; X1 = Flow rate of mobile phase, ml/min; X2 = Column temperature, Â°C; X3 = %

acetonitrile in phosphate buffer (KH2PO4, 0.02M, pH 5.8), v/v. Eq. (5) indicate that the effect of all variables have very low effect on the tailing factor and is found to be statistical significant (p = 0.009525). The equation indicates the tailing factor is decrease with increase in value of all variables. How-ever, a higher negative coefficient for X1 indicates the flow rate is a major factor affecting tailing factor. Usually, tailing factor is increases with flow rate. This causes increase in column back pressure due to rise in flow rate that causes the peak to becomes more non-Gaussian. But in this case, tailing is decreasing. This may be because of dominant effect of basic nature of drug as basic compounds do not causes higher tailing on silica [18]. Second reason for this low tailing is may be effect of steric hindrance of the access to silanols [27,28]. Once they are freely accessible, no peak distortions are encountered. Third reason for this low tailing may be due to the restricted dipole interactions between drug molecule and stationary phase resulting in decrease in tailing factor [26]. Next to X1, X3 (% of acetonitrile in mobile phase) is second most important factor that affect tailing factor. Increase in concentration acetonitrile increases polarity of mobile phase thereby causing rapid equilibrium between stationary phase and mobile phase. This causes drug to elute faster with decreasing affinity towards stationary phase without causing peak distortion. Response surface plot of tailing factor (tF) as a function of X1 (flow rate) and X3 (% acetonitrile) is shown in Fig. 2(d). The average tailing factors of different batches were varied from 1.02 to 1.46 (Table 1). Statistical data for linear model is given in Table 2. 3.2. Relationship between Observed versus predicted values The regression line for each response expresses the best prediction of the dependent variables i.e. retention time, peak area, theoretical plates and tailing factor given the independent variables. However, nature was perfectly predictable, and was substantial variation of the observed points around the fitted regression line (Fig. 3). 3.3. Optimized set of chromatographic conditions From the RSM study, it is clear that the selected variable X1 (flow rate), X2 (column temperature), and X3 (% acetonitrile in phosphate buffer (KH2PO4), 0.02M, pH 5.8) are important for the regression model and their interactive effect has been observed on chromatographic responses. From RSM optimum regions were selected to be +1, +1 and +1 for flow rate (1 ml/min), temperature (25Â°C) and concentration of acetonitrile in mobile phase phosphate buffer (KH2PO4, 0.02M, pH 5.8) (60%, v/v), respectively (Batch SM-3). This optimized set of conditions was further used for construction of calibration graph and method validation studies. 3.4. Validation of method 3.4.1. Accuracy of method Accuracy was determined by performing recovery studies at two levels i.e. 80 and 120% by using standard addition method. Good recoveries (101.33â€“99.40%) at each concentration level with a very low % RSD (0.97â€“0.11%) indicated the accuracy of method (Table 3). 3.4.2. Precision of method The average intra-day and inter-day precision were found to be 0.91 and 0.78, respectively (Table 3). 3.4.3. Linearity and range Linearity and range study was performed by determining the drug concentration in series of standard working solutions of concentrations 1-2 Âµg/ml in triplicate (Fig. 4). The RSD of slope was less than 2 (Table 3). 3.4.4. LOD and LOQ LOD and LOQ of method were confirmed by diluting the drug solution of known concentrations 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 taken as LOD and LOQ, respectively. The LOD and LOQ values were found to be 0.024 Âµg/ml and 0.052 Âµg/ml, respectively (Table 3). 3.4.5. Purity of peak In peak purity study of tramadol hydrochloride, good correlation (r = 0.9991) was observed between standard and sample spectra. The average retention time for candesartan cilexetil was found to be 7.41 (SD Â± 0.057) for six replicates (Fig. 4). 3.4.6. Assay of tablets and recovery study The percent drug content of tramadol hydrochloride in tablets was found to be 99.78 (%RSD Â± 0.54). The mean recovery from the tablet formulation was found to be 100.42 % (%RSD Â±0.68) (Table 4). 3.4.7. Forced degradation studies Forced degradation studies clearly indicate that the drug tramadol hydrochloride is susceptible to oxidation, acid, alkali and heat (thermal). The highest amount of drug degradation found in acid (92.85%) followed by oxidation study (43.61%). Little degradation was observed in alkali (9.18%) and in thermal degradation (4.81%) no degradation was observed.

Table 1: Study of experimental variables by factorial design

Batch	Coded variables			Natural condition			t_R		p_A		t_P		t_F	
	X_1	X_2	X_3	X_1	X_2	X_3	Exp.	Pred.	Exp.	Pred.	Exp.	Pred.	Exp.	Pred.
SM-1	+1	+1	-1	1	25	50	11.73	11.73	1570.91	1596.06	7726.90	7767.19	1.31	1.30
SM-2	+1	-1	-1	1	20	50	12.23	12.24	1680.12	1654.97	7844.58	7804.29	1.46	1.47
SM-3	+1	+1	+1	1	25	60	7.41	7.39	1559.77	1534.62	6798.70	6758.41	1.02	1.03
SM-4	-1	-1	-1	0.8	20	50	15.83	15.83	1885.01	1910.16	7345.67	7385.96	1.29	1.28
SM-5	-1	+1	+1	0.8	25	60	7.18	7.19	1846.98	1872.13	6906.30	6946.59	1.20	1.19
SM-6	-1	-1	+1	0.8	20	60	7.71	7.70	1917.65	1892.50	6888.15	6847.86	1.13	1.14
SM-7	-1	+1	-1	0.8	25	50	15.35	15.37	1975.87	1950.72	7448.60	7408.31	1.26	1.27
SM-8	+1	-1	+1	1	20	60	7.92	7.90	1629.33	1654.48	6678.85	6719.14	1.17	1.16
					Minimum		7.18	7.18	1559.77	1534.62	6678.85	6719.14	1.02	1.03
					Maximum		15.83	15.83	1975.87	1950.72	7844.58	7804.29	1.46	1.47
					Mean		10.84	10.84	1760.13	1755.10	7216.12	7216.12	1.23	1.23

*** Where, X_1 = Flow rate, ml/min; X_2 = Column temperature, °C; X_3 = % Acetonitrile in mobile phase phosphate buffer (KH_2PO_4 0.02M, pH 5.8), v/v; t_R = Retention time, min; p_A = Peak area; t_P = Theoretical plates; t_F = Tailing factor; Exp. = Experimental result; Pred. = Predicted result.**

Table 2: Statistical data for linear model of responses (n = 8)

Response		B Coefficient	Std.Err.	t-value	p-value
Retention time	Mean/Interact.*	54.87000	8.057912	6.80946	0.002431
	X_1	-8.48750	4.781491	-1.77507	0.150546
	X_2	-0.10150	0.191260	-0.53069	0.623729
	X_3	-0.62325	0.095630	-6.51732	0.002862
Peak Area	Mean/Interact.*	3487.66	326.4981	10.68201	0.000435
	X_1*	-1481.73	193.7410	-7.64797	0.001570
	X_2	-7.93	7.7496	-1.02314	0.364079
	X_3	-3.95	3.8748	-1.02056	0.365163
Theoretical plates	Mean/Interact.*	10802.12	1218.533	8.86485	0.000894
	X_1	575.39	723.067	0.79576	0.470725
	X_2	6.16	28.923	0.21307	0.841692
	X_3	-77.34	14.461	-5.34831	0.005892
Tailing Factor	Mean/Interact.*	2.532500	0.542408	4.66900	0.009525
	X_1	0.100000	0.321860	0.31069	0.771550
	X_2	-0.013000	0.012874	-1.00976	0.369733
	X_3*	-0.020000	0.006437	-3.10694	0.035978

* $P<0.05$ (significant for a 95% confidence level); X_1 = Flow rate, ml/min; X_2 = Column temperature, °C; X_3 = Conc. of acetonitrile in mobile phase phosphate buffer (KH_2PO_4 0.02M, pH 5.8), v/v.

Table 3: Results of linearity and precision study (n=6).

Linearity study			Precision study				
Parameter	**Result**	**%RSD**	**Conc.**	**Intra-day**	**%R.S.D**	**Inter-day**	**%RSD**
Range (µg/ml)	1-5		1 µg/ml	99.95	1.65	99.51	1.49
r^2	0.9998	0.74	3 µg/ml	98.11	0.58	99.15	0.34
Slope	916.95	0.56	4 µg/ml	100.24	0.50	100.17	0.51
LOD (µg/ml)	0.12	-	Mean	99.43	0.91	99.61	0.78
LOQ (µg/ml)	0.33	-		-	-	-	-

Table 4: Results of assay of tablet formulation and recovery study (n=6).

Assay of tablet		**Recovery study**			**Degradation study**	
Parameter	**Result**	**% Level**	**%Recovery**	**RSD**	**Condition**	**%Degraded**
Label claim* (mg/tablet)	2	80	100.36	0.52	Oxidation	43.61
					Acid	92.85
% Estimated	99.78	120	100.30	0.85	Base	9.18
% RSD	0.11				Thermal	4.81
					Photo	0.00

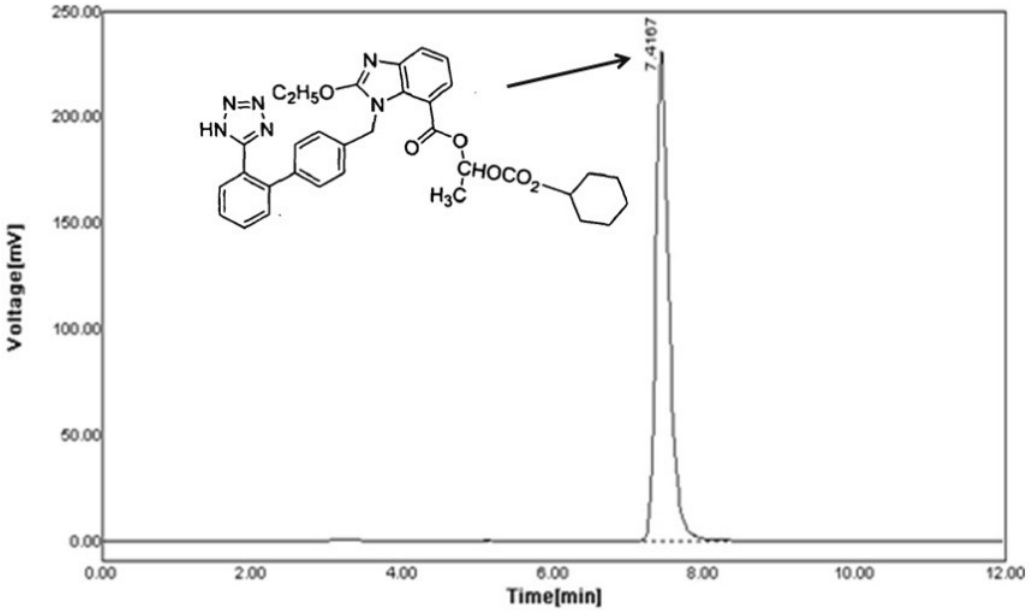

Figure 1: HPLC Chromatogram of tramadol hydrochloride.

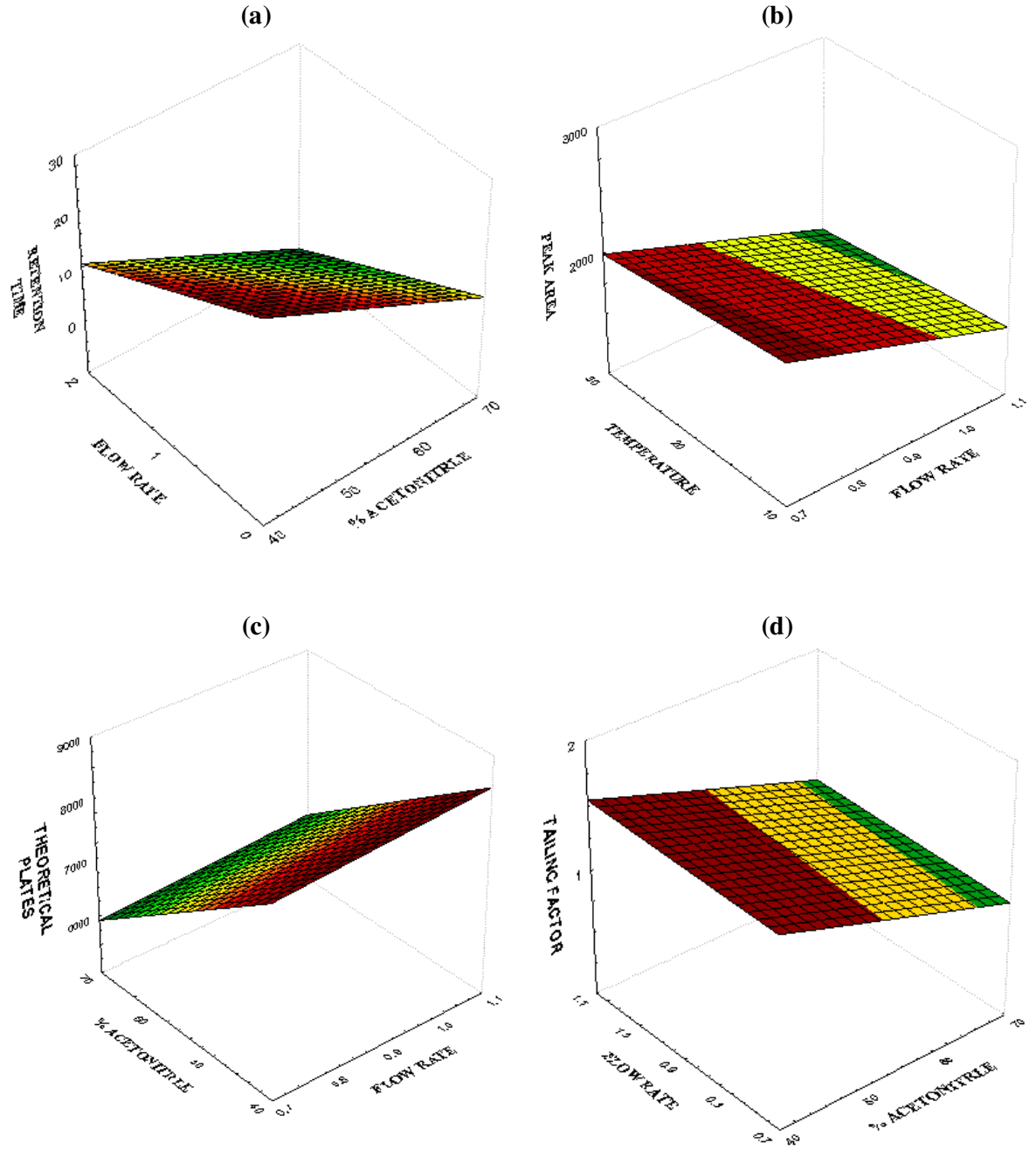

Figure 2: (a) Response surface plot of retention time *(t_R)* as a function of X_1 (flow rate) and X_3 (% acetonitrile). (b) Response surface plot of peak area *(p_A)* as a function of X_2 (temperature) and X_1 (flow rate). (c) Response surface plot of theoretical plates *(t_P)* as a function of X_3 (% acetonitrile) and X_1 (flow rate). (d) Response surface plot of tailing factor *(t_F)* as a function of X_1 (flow rate) and X_3 (% acetonitrile).

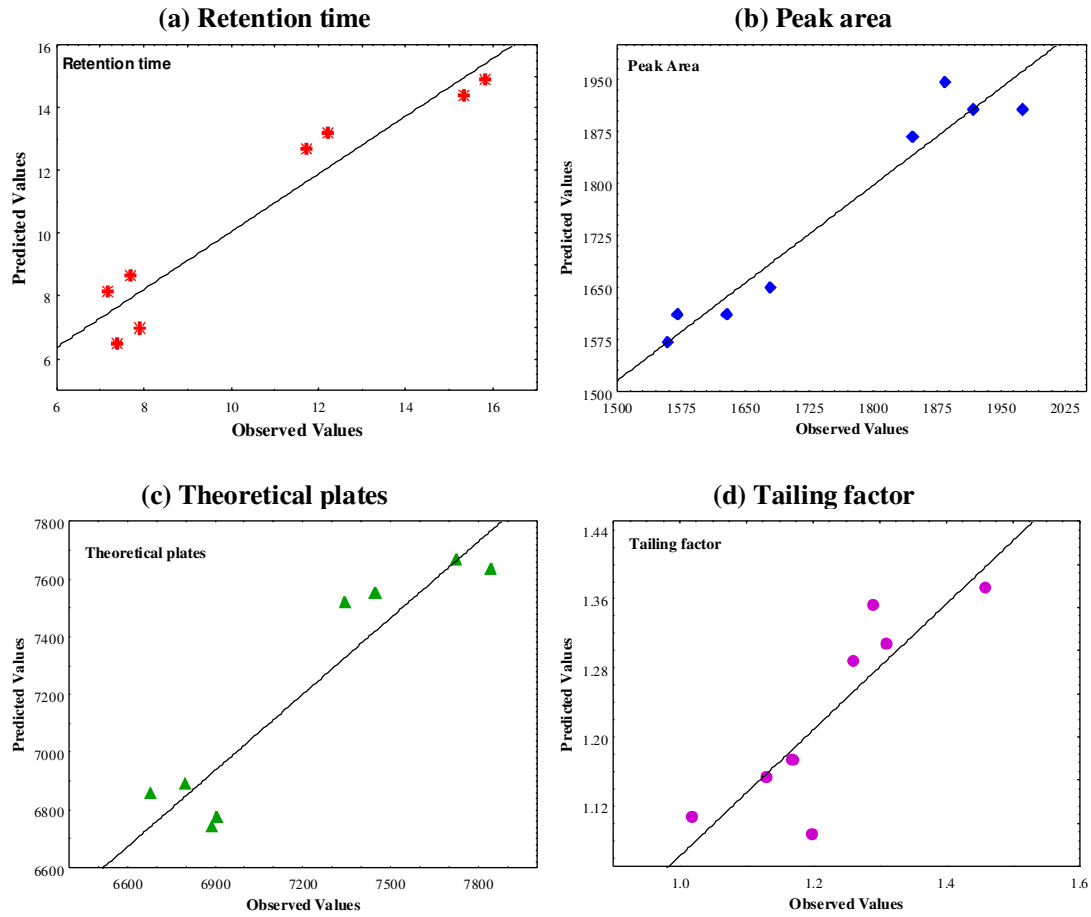

Figure 3: Relationship between observed response versus predicted response values of (a) retention time, (b) peak area, (c) theoretical plates, and (d) tailing factor.

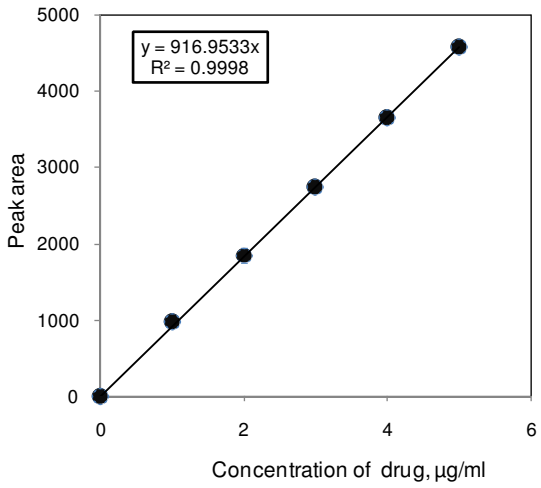

Figure 4: Calibration graph of tramadol hydrochloride.

Conclusion

Plackett-Burman design was successfully employed for the chromatographic separation of tramadol hydrochloride from bulk drug and tablet formulation. From RSM, optimum set of conditions for chromatographic separation of drug was found to be flow rate 1 ml/min, temperature 25Â°C, and % acetonitrile in phosphate buffer (KH2PO4, 0.02M, pH 5.8) 60% v/v. The studies indicate that flow rate and temperature are the two most important variables responsible for change in chromatographic responses. Results of forced degradation study indicate develop method is stability indicating. The method was validated as per ICH guidelines and results were found statistically significant. As the method separates the drug from its degradation products, it can be employed as a stability indicating for quantitative analysis for determination of tramadol hydrochloride in bulk drug and tablet formulation, without any interference from the excipients and in the presence of its acidic, alkaline, and oxidative degradation products.

Reference

1. Lee C.R., McTavish, D., Sorkin, E.M. Drugs1993;46:313. doi:10.2165/00003495-199346020-00008

2. Grond, S., Sablotzki, A., Clinical Pharmacokinetics 2004;43:879. doi:10.2165/00003088-200443130-00004.

3. Gogulamudi, L., Sujana, K., 2012. Int. J. Pharm. Pharm. Sci.. 4. 275.

4. Setty, K.N., Ramachar, K.,. Chakravarthy, I. E., Prabhavathi, K., 2012. Chem. Sci. Trans. 1. 317. DOI:10.7598/cst2012.199.

5. Gharge, D., Dhabale, P., 2010. Int. J. PharmTech Res. 2. 1119.

6. Shukla R., Shivkumar R., Kumar, N.S, 2011. Bulletin of Pharmaceutical Research. 1. 62.

7. Chandra, P., Rathore, A., Lohidasan, S., Mahadik, K.R., 2012. Sci Pharm. 80. 337.

8. Weiyong, L., Henrik, T., Rasmussen., 2003. J. Chromatogr. A. 1016. 165.

9. Manwar, J., Mahadik, K., Paradkar, A., 2013. Ferment. Technol. 1. 109. http://dx.doi.org/10.4172/2167-7972.1000109.

10. Heyden, Y.V., Hartman C., Massart, D.L., Michel, L., Kiechle, P., Erni, F., 1995. Analytica Chimica Acta. 316. 15.

11. Manwar, J.V., Sonawane, B.V., Patil, S.V., Takle, S.P., 2011. Der Chemica Sinica. 2. 152.

12. Manwar, J.V., Mahadik, K.R., Paradkar, A.R., Takle, S.P., Sathiyanarayanan, L., Patil, S.V., 2012. Der Pharmacia Sinica. 3. 41.

13. Nora H. Al-Shaalan. 2010. J. Saudi Chem. Soc. 14.15. doi:10.1016/j.jscs.2009.12.004.

14. Rao, K.L.M., Krishnaiah, c., Sudheer Babu K., K., Reddy, p., 2014. J. Saudi Chem. Soc. 2014. 593.

15. Neue, U.D., 1997. HPLC Columns: Theory, Technology, and Practice, Wiley-VCH, New York.

16. Van Deemter J.J., Zuiderweg, F.J., Klinkenberg, A., 1956. Chem. Eng. Sc. 5. 271.

17. David, V.M., 2000. J. Chromatogr., A. 902. 311. doi:10.1016/S0021-9673(00)00924-9.

18. Ahuja, S., Dong, M.W., 2005. Handbook of Pharmaceutical Analysis by HPLC. Elsevier Academic Press.

19. Snyder, L.R., Kirkland, J.J., Glajch, J.L., Practical HPLC Method Development, 1997. 2, Wiley-Interscience, New York.

20. Meyer, V. R., Practical HPLC. 1998. Wiley, New York.

21. David Harvey, Modern Analytical Chemistry, McGraw-Hill Higher Education, USA.

22. ICH, Q2A, International Conference on Harmonization. 1994. October, Geneva.

23. Bakal R.L., Manwar J.V., Sahare A.Y., Bhajipale N.S., Manikrao A.M., 2008. Indian J.Pharm. Educ. Res. 42.23.

24. Carlson, R., 1992. Design and Optimization in Organic Synthesis. Elsevier, Amsterdam.

25. Lewis, G.A., Mathieu, D., Phan-Tan-Luu, R., 1999. Pharmaceutical Experimental Design, Marcel Dekker, New York.

26. Kirkland, J.J., Snyder, L.R., 1997. Practical HPLC method development. Wiley inter science publication, New York.

27. Neue, U.D., Murphy, B.P., 1979. unpublished results.

28. Meyer, E., Engelhardt, H., Fresenius Z. 1989. Anal. Chem. 333.734.