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Research Article

Formulation and evaluation of Rifampicin loaded poly-ε-caprolactone nanoparticles using 3^2 factorial design

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ABSTRACT

The objective of this work was to apply the response surface approach in the development of Rifampicin nanoparticle with poly-ε-caprolactone (PCL). Experiments were performed according to a 3^2 factorial design to evaluate the effects of two independent variables, Sonication time (X_1) and PCL amount (X_2) on the percent entrapment efficiency (% EE), mean diameter (MD) of the Rifampicin nanoparticle. The effect of the two independent variables on the response variables was studied by response surface plots and contour plots generated by the Design Expert® software. The compatibility between Rifampicin and the other excipients were confirmed by differential scanning calorimetry (DSC) and Fourier transform infrared (FTIR) studies. The sonication time has an antagonistic effect on % EE and Mean diameter of the nanoparticle but the effect of polymer amount was synergistic one. A nonlinear twisted relationship was obtained for both EE and MD, which indicated an interaction between them at the corresponding factor levels. Kinetic treatment to the dissolution profiles revealed that the drug release follows Highuchi equation which confirms the burst release pattern. The desirability function was used to optimize the response variables, and the observed responses were in agreement with the experimental values. These results indicate that Rifampicin loaded PCL nanoparticles could be effective in sustaining its release for a prolonged period. The PCL NPs could be alternate method for delivery for Rifampicin with prolonged drug release profiles and better therapeutic effect can be achieved for the treatment of tuberculosis. However, further studies are needed to confirm its performance *in vivo*.

Keywords: Factorial design; Nanoparticles; Poly -ε-caprolactone; Rifampicin

INTRODUCTION

Tuberculosis (TB) continues to be a leading cause of death due to infectious disease worldwide, even though adequate treatments are available. Nearly one-third of the world's population is infected with Mycobacterium tuberculosis, the microbe that causes TB in a latent or active form, while more than eight million people develop active TB every year and nearly two million people die annually from TB. Tuberculosis is an infectious disease spread through inhalation of airborne droplets containing M. tuberculosis (Riley RL et al., 1959) and subsequent uptake of the bacterium by alveolar macrophages (Russell DG, 2001). Treatment of tuberculosis is an arduous and lengthy process requiring combination antibiotic therapy administered daily for at least six months (Fox W et al., 1999). During this extended therapy, patients may terminate treatment early due to factors such as unwanted side effects or alleviation of primary symptoms (Thomas C, 2002). Partial treatment of TB can make the disease more difficult to treat since selection of drug-resistant mutants of M. tuberculosis may occur. Thus, an advance in TB therapy that reduces frequency of dosing, body dose and overall treatment time has the potential to be more effective, thereby saving lives. An obvious strategy for reducing the treatment dose of antibiotics for TB treatment involves the steady delivery of antibiotic to infected cellular tissue. Compared to the current approach to therapy, whereby antibiotics are delivered without targeting of infected cells and without sustaining drug levels above therapeutic thresholds, a targeted extended delivery of antibiotics can potentially reduce dosing frequency and overall treatment time simultaneously. A current obstacle to extended release formulation of antibiotics for TB is the relatively large daily antibiotic dose (gram quantities) required for TB treatment. Treatment of tuberculosis is generally successful, except in the case of multiple-drug-resistant strains of Mycobacterium tuberculosis (Nakada Y et al., 1996).

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Rifampicin (RIF) is a first-line drug for use in the therapy of tuberculosis and is included in the list of recommended drug regimens for treatment of latent M. tuberculosis infection in adults (Sharma R et al., 2001). Various polymers have been used in drug delivery research, because they can effectively deliver the drug to a target site and thus increase the therapeutic benefit, while minimizing side effects (Kreuter J, 2001). The best-known class of biodegradable materials for controlled release is the poly lactide-co-glycolides (PLGAs). PLGAs are biocompatible and biodegradable polymers that are hydrolytically degraded into nontoxic oligomers or monomers, lactic acid and glycolic acid (Knight CG and Dingle JT, 1981). Thus far, the application of microsphere technology to the treatment of the initial infection with M. tuberculosis has been studied so that multiple-drug-resistant strains do not develop (Dutt M and Khuller GK, 2001). Modern drug carrier systems play an important role in controlled delivery of a pharmaceutical agent to the target at a therapeutically optimal rate and dose. Among various colloidal drug delivery systems, nanoparticles (NPs) represent a very promising approach to this aim (Maincent P et al., 1992). Being submicron colloidal systems; once in the bloodstream, surface-non modified NPs are rapidly opsonized and massively cleared by the fixed macrophages of mononuclear phagocyte system organs such as liver, lungs, and spleen (Couvreur P et al., 1996).

The objective of this work was to entrap Rifampicin within PCL nanoparticles by O/W emulsion solvent evaporation with ultrasonification method and to optimize the encapsulation formulation. To achieve this goal, this study was designed to assess the influence of formulation variables on the characteristics of nanoparticles such as encapsulation efficiency, rifampicin loading, particle size, zeta potential, and morphology. The formulation variables were solvent in the oil phase, concentration of PCL, ultrasonification time and concentration of Surfactant (PVA).

MATERIALS AND METHODS

Materials

Rifampicin was kindly supplied by Macleod Pharmaceuticals, Mumbai as a gift sample. Poly-ε-caprolactone (PCL M_W 65000 g/mol) was purchased from Sigma Aldrich. Polyvinyl alcohol (PVA M_w 22000 g/mol), dichloromethane (DCM), acetonitrile (ACN), phosphate buffer and ascorbic acid were of analytical grade and purchased from Qualichens, New Delhi.

Method of preparation of PCL nanoparticles

In this procedure, specific amount of PCL was dissolved in 10 ml of solvent i.e. Methylene chloride containing 30 mg of rifampicin. A specific concentration of PVA solution was prepared in PBS solution. The PCL solution was added to 40 ml of the PVA solution. The total mixture in 250 ml centrifuge bottle was then placed in an ice bath and emulsified using a Vibra-cell Probe Sonicator (VC 5040, Sonics and Maternals, USA) a specific time to obtain an oil-in-water emulsion. Another 40 ml of the PVA solution was then added to the emulsion. The final emulsion was stirred for 12 hour at 300 rpm on a magnetic stir plate to allow the evaporation of methylene chloride to allow the formation of the nanoparticles. The suspension was then centrifuged at 10,000 g for 20 min. The pellet was resuspended in distilled water and centrifuged two more times at 1200 g for 20 min each. Then washing steps were followed to remove un-encapsulated PVA and Rifampicin. The nanoparticles were collected and frozen at -80^{o}C for at least 1.5 hour and subsequently freeze dried. The freeze dried nanoparticles were stored at 4^{o}C.

Physical characterization

Measurement of particle size, polydispersity index and zeta potential

Particle size distribution of rifampicin loaded PCL nanoparticles was determined by laser scanning technique using Malvern instrument after appropriate dilution with distilled water. Approximately 5 mg of dried particles were re-suspended into 1.0 ml of distilled water and the resulting solution was briefly vortexed and treated in a bath sonication for 3 min. This suspension was analyzed at an obscuration of 10-20% on a Malvern Mastersizer. The mean particle size, polydispersity index and zeta potential were calculated for each formulation maintained at 25^{o}C and polydispersity index will measure the size distribution of nanoparticles population.

Scanning electron microscopy (SEM)

The SEM analysis of prepared PCL nanoparticles was performed for morphological studies. The formulations are poured in to circular aluminum stubs using double adhesive tape, and coated with gold in HUS-5GB vacuum evaporator , and observed in Hitachi S-3000N SEM at an acceleration voltage of 10 KV and a magnification of 5000 X.

Differential scanning calorimetry (DSC)

DSC analysis was performed in order to investigate the melting and recrystallization behavior of crystalline materials like PCL nanoparticles. The samples were sealed in aluminum pans and measurements were recorded using DSC instrument. The samples were heated from 25 to 200^{o}C at a heating rate of 10^{o}C /min under nitrogen atmosphere.

Total drug content

Encapsulation efficiency was determined by an extraction method. Dried nanoparticles (10 mg) were dissolved in 5 ml of methylene chloride and 5 ml of distilled water. The mixture was vigorously vortexed for 1 min and sonicated 5 min in order to extract the rifampicin into the organic solution. Then, methylene chloride was evaporated and replaced with methanol. The 2 ml of organic solution was filtered and the Rifampicin

content of the solution was analyzed. The amount of isoniazid was determined using UV spectrophotometer at 332 nm. The placebo formulation prepared similarly to drug loaded PCL nanoparticles is used as blank. The total drug content was calculated.

Entrapment efficiency

The prepared PCL nanoparticles dispersion was centrifuged at 15000 rpm for 30 min at 0°C using REMI cooling centrifuge. Then the supernatant is analyzed for the free drug content. The concentration of RIF in the supernatant was determined by UV–Visible spectrophotometry at 332 nm. Entrapment efficiency was using the below Equation.

$$\text{Entrapment efficiency (\%)} = (RFM_{initial} - RFM_{supernatant})/RFM_{initial} \times 100$$

Drug loading

Measured quantity of freeze-dried nanoparticles were dissolved in methanol by sonication for 5 min and assayed for drug content on a UV spectrophotometer UV1650PC, Schimadzu Corporation US at λ_{max} of 332 nm. Percent drug loading (% DL) was calculated using the following equation:

$$DL\ (\%) = W_{DL}/W_{NP} \times 100$$

Where W_{DL} is the weight of drug in RIF nanoparticles and W_{NP} is the weight of RIF nanoparticles.

***In vitro* drug release**

Drug release studies were performed by the dialysis method (Li Y et al., 2008). Nanoparticles (equivalent to 10 mg drug) were loaded into a pre-treated dialysis bag (Sigma, molecular weight cut-off 12–14 kDa) and introduced into the basket of USP apparatus-I. Phosphate-buffered saline (900 ml) containing 1%*w*/*v* ascorbic acid at a pH of 7.2, was used as the dissolution medium (Muttil P et al., 2007). Aliquots (5 ml) were withdrawn at specific time intervals and analyzed for RIF by UV spectroscopy at λ_{max} 332 nm. Percent drug release *vs*. time profiles were plotted. Negligible leakage of the

Table 1: Factors (independent variables), factor levels and responses (dependent variables) used in 3-level factorial experimental design

Factors	Type of Factor	Factors Level			Response
		-1	0	1	
X_1 = Sonication time (min)	Numeric	1	3	5	Y_1 = Entrapment efficiency (%)
X_2 = Amount of PCL (mg)	Numeric	300	400	500	Y_2 = Mean diameter (nm)

Table 2: Results for entrapment efficiency and mean diameter of prepared Rifampicin nanoparticles with 3-level factorial experimental design

Std.	Run	X_1 (Min)	X_2 (mg)	Y_1 (%)	Y_2 (nm)
1	1	1	300	36.61	287.42
5	2	3	400	35.95	321.23
8	3	3	500	52.41	327.49
2	4	3	300	31.98	296.33
9	5	5	500	41.39	215.08
4	6	1	400	43.19	375.16
3	7	5	300	25.81	245.2
6	8	5	400	27.33	276.41
7	9	1	500	62.31	387.27

Table 3: Fit summary of model for the measured responses Y1 (%Entrapment efficiency) and Y2 (Mean diameter, nm)

Model	Y_1		Y_2	
	f-value	P value	f-value	P value
Linear vs. Mean	28.93	0.0008	22.94	0.0008
$2F_1$ vs. Linear	1.63	0.2599	4.18	0.0962
Quadratic vs. $2F_1$	7944.14	0.0001	32.92	0.0091

Table 4: Model summary statistics of responses to select suitable model to fit data

Source	Linear			$2F_1$			Quadratic		
	Adj R^2	Pred R^2	PRESS	Adj R^2	Pred R^2	PRESS	Adj R^2	Pred R^2	PRESS
Y_1	0.8747	0.7811	244.4933	0.8863	0.7622	265.5759	1.0000	0.9998	0.1813
Y_2	0.8458	0.6975	44377.95	0.8992	0.6894	45568.00	0.9927	0.9696	4458.44

*Adj. = adjusted; Pred. = predicted

particles from the dialysis tube was confirmed by testing the blank RIF Np, which showed no absorbance in the release medium.

In vitro drug-release data were fitted to kinetic models such as zero order, first order, Higuchi equation and Korsmeyer–Peppas equation. The regression analysis of *Q vs*. *t* (zero order), log *Q vs*. *t* (first order), *Q vs*. square root of *t* (Higuchi), log%*Q vs*. log%*t* (Korsmeyer–Peppas), where *Q* is the amount of drug released at time *t*, was performed (Thakkar VT et al., 2009).

Statistical analysis

Statistical analysis was performed using EASE Design - Expert-8 for its factorial design. 3^2 factorial designs were selected for optimization study of above mention formulation. 3-D (response surface) and 2-D (contour plot) shows the effect of polymer and ultrasonication time in response variable. Polynomial models including interaction and quadratic terms were generated for all the response variables using multiple linear regression analysis. Equations were derived and coefficients of interactions were calculated to determine the effect of each variable on the formulation characteristics. Statistical validity of the model was established on the basis of Analysis of variance (ANOVA) and the 3D response graphs were constructed using Design-Expert software.

RESULT AND DISCUSSION

Optimization data analysis

Response data for all the 9 experimental runs of 3-level

Table 5: Influence of concentration of polymer (PCL) in the organic phase (PCL + methylene chloride) on nanoparticles mean size taking PVA 1% w/v and sonication time 3 minutes

Mass of PCL (mg)	DCM (ml)	Mean size of NP (nm)	Encapsulation efficiency (%)
100	10	421±9	21.63
200	10	447±5	26.54
300	10	453±7	28.38
500	10	468±4	39.72
800	10	675±9	29.54

Table 6: Influence of concentration of polymer (PCL) in the organic phase (PCL + Methylene chloride + Acetonitrile) on nanoparticles mean size taking PVA 1% w/v and sonication time 3 minutes

Mass of PCL (mg)	DCM:CAN (10 ml)	Mean size of NP (nm)	Encapsulation efficiency (%)
100	1:1	215±6	12.24
200	1:1	241±9	16.38
300	1:1	267±4	24.56
500	1:1	275±8	29.47
800	1:1	319±12	30.19

Table 7: Batch compositions used for constituting nanoparticles and properties of rifampicin loaded nanoparticles

Formula	Sonication time	PCL Amount	Encapsulation	Loading	Mean size	Polydispersity	Zeta potential
NPR 1	1	300	36.61	3.03±0.39	287.42	0.23±0.07	- 19.63±0.17
NPR 2	1	400	43.19	3.48±0.17	375.16	0.17±0.06	-16.48±0.41
NPR 3	1	500	62.31	3.89±0.73	387.27	0.29±0.09	-15.29±0.26
NPR 4	3	300	31.98	1.43±0.16	296.33	0.85±0.22	-21.3±0.22
NPR 5	3	400	35.95	1.96±0.38	321.23	0.67±0.21	-17.24±.39
NPR 6	3	500	52.41	2.46±0.31	327.49	0.55±0.09	-16.67±0.26
NPR 7	5	300	25.81	1.29±0.15	245.2	0.89±0.14	-28.23±0.81
NPR 8	5	400	27.33	1.87±0.18	276.41	0.47±0.24	-24.95±0.22
NPR 9	5	500	41.39	1.94±0.21	215.08	0.64±0.27	-14.52±0.64

Table 8: Observed and predicted value for the optimized formula

Predicted	Y_1	Y_2
	41.957	225.59
Observed	41.023	229.57
Predicted Error	2.22608861	1.76426

Predicted error (%) = (observed value − predicted value)/predicted value × 100

factorial design, performed in accordance with Table 1, are presented in Table 2.

Mathematical modeling

Mathematical relationship was generated between the factors (independent variables) and responses (dependent variables) using the statistical package Design-Expert. First step in mathematical modeling was fitting the experimental data to appropriate model. A suitable model was selected by software on the basis of different parameter obtained from regression analysis such as p-value, adjusted R^2, predicted R^2 and Predicted Residual Sum of Square (PRESS) value (Tables 3 and 4). Table 2 lists the values of various response parameters of the prepared batches. ANOVA was applied for estimating the significance of model, at 5% significance level. If more than one model was significant ($p < 0.05$) for the response, the adjusted R^2 and PRESS value of the model were compared to select the best mathematical model for that response. Focus on maximizing the value of adjusted R^2 and predicted R^2. Low PRESS value indicated adequate fitting of model (Huang YB et al., 2004).

General quadratic equation for two independent variables is as follow:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1 X_2 + \beta_4 X_1^2 + \beta_5 X_2^2 \ldots$$

Where: β_0 is the intercept representing the arithmetic averages of all the quantitative outcomes of 9 runs. β_1 to β_5 are all coefficients calculated from the observed experimental values of Y. X_1 and X_2 are the coded levels of factors. The terms X_1X_2 and X^2 represent the interaction and quadratic terms, respectively. Coefficients with one factor represent the effect of that particular factor while the coefficients with more than one factor and those with second order terms represent the interaction between those factors and the quadratic nature of the phenomena, respectively. Synergistic effect and antagonistic effect of factor were indicated by positive sign and negative sign in front of that factor term, respectively.

Effect of formulation variables on entrapment efficiency (EE)

From the p-values presented in Table 3, linear model and quadratic model was found to be significant for EE. Quadratic model was selected on the basis of maximum value of adj. R^2 and low PRESS value indicating adequate fitting of model (Table 4). Quadratic model was significant with model f-value of 7944.14 (p-value < 0.0008). The quadratic equation generated by software is as follows:

$$EE = +35.94 - 7.93 * X_1 + 10.28 * X_2 - 2.53 * X_1 * X_2 - 0.67 * X_1^2 + 6.26 * X_2^2$$

Equation reveals that both factors (X1 and X2) affect EE of nanoparticles significantly. Equations also indicated that the effect of the change in sonication time having antagonistic effect on the EE where as the PCL amount has synergistic effect on the EE of the Nanoparticles. The combined effect of factors X_1 and X_2 can further be elucidated with the help of response surface plots (Figure 1), which demonstrated that Y_1 varies in a non linear fashion with the two independent factors. However, the steeper ascent in the response surface with PCL amount (X_2) – instead of sonication time (X_1) – is clearly discernible from response surface plots, indicating that the effect of PCL amount is comparatively more pronounced than that of sonication time. From this discussion, one can conclude that the EE may be changed by appropriate selection of the levels of X_1 and X_2. Figure 1B shows a linear relationship between the observed response values and the predicted values indicating the correctness of the model.

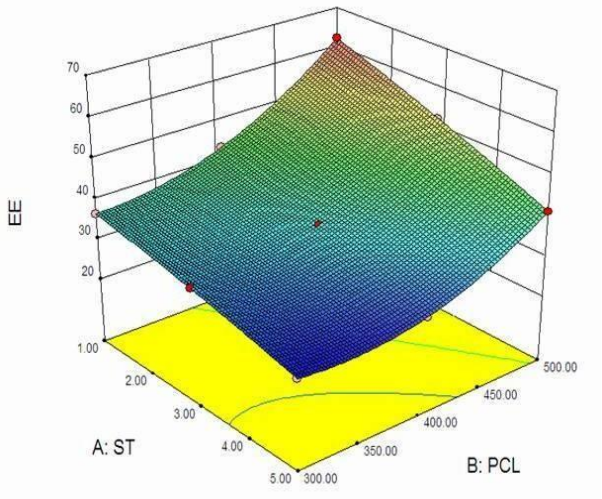

Figure 1: Showing the interaction of X1 and X2 on the Entrapment efficiency

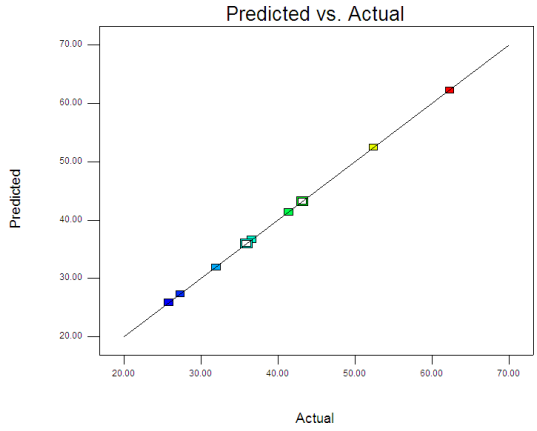

Figure 2: The predicted and original value for EE Effect of formulation variables on mean diameter of Nanoparticles

From the p-values presented in Table 3, linear model and quadratic model was found to be significant for MD. Quadratic model was selected on the basis of maximum value of adj. R^2 and low PRESS value indicating adequate fitting of model (Table 4). Quadratic model was significant with model f-value of 32.92 (p-

value < 0.0008). The quadratic equation generated by software is as follows:

$$MD = +335.77 - 52.19 * X_1 + 16.82 * X_2 - 32.49 * X_1 * X_2 - 17.26 * X_1 2 - 31.14 * X_2 2$$

The above equation shows that the increase in sonication time decreases the particle size whereas increase in polymer concentration leads to increase in mean diameter of the Rifampicin nanoparticles.

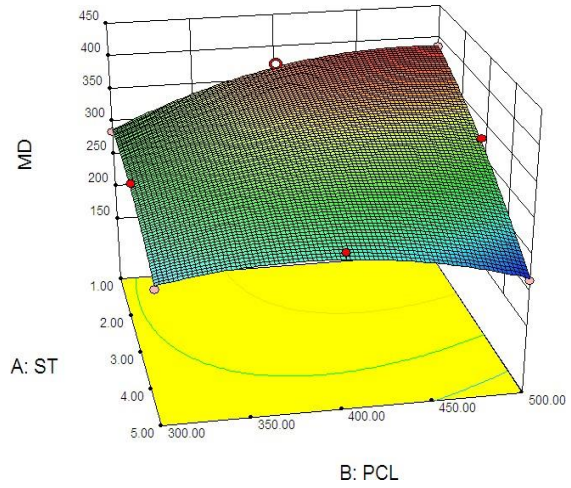

Figure 3: Showing the interaction of X1 and X2 on the entrapment efficiency

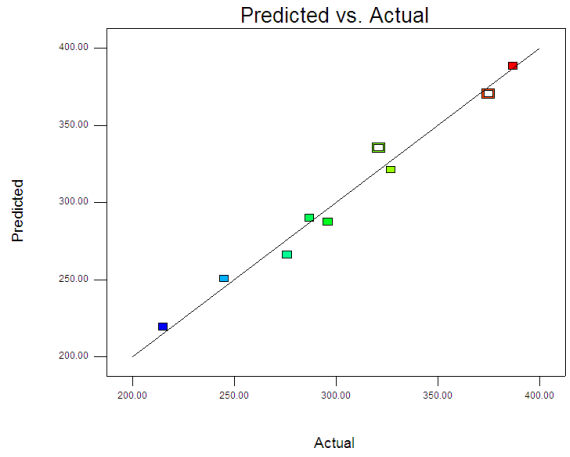

Figure 4: predicted and original value for EE

The solvent composition can be an input factor in controlling the solvent removal rate and the final size of the polymeric nanoparticles. In this study, the particles mean size of the nanoparticles prepared by taking the mixture of equal amount of DCM and ACN as the solvent in the oil phase was smaller than that prepared by DCM only (Table 5-6). The solvent in the oil phase dissolves into the aqueous phase and then, evaporates in the O/W emulsion solvent evaporation method. The amount and rate of solvent transfer from the oil phase into the aqueous phase depends on the solubility of the organic phase. The water-miscible solvent rapidly diffused out of the polymer solution. As the water solubility of ACN was greater than DCM and therefore the rate of precipitation was higher than the rate with DCM. Due to the high water solubility of ACN, it had higher diffusion rate before hardening which was the major reason for the smaller particle mean size prepared by mixture of DCM and ACN. In this study, encapsulation efficiency was increased when DCM was used as the solvent in the oil phase. By increasing the particle mean size, the RIF NP diffusion into the aqueous solution decreased because there was a longer distance to RIF travel. Consequently, higher encapsulation efficiency was associated with an increase in the particle mean size.

The effect of polymer concentration on properties of nanoparticles

Polymer concentration is also a key factor. In this study, two PCL concentrations, 1 to 8 g/100 ml, were taken in the preliminary test. The concentrations showed the best result in preliminary test is 3 to 5 g/100 ml. The encapsulation efficiency was increased by increasing PCL concentration from 3 to 5 g/100 ml (Table 5-6). By increasing the polymer concentration in the organic phase, the viscosity of the solution was increased. Increasing viscosity can decrease the RIF diffusion into the aqueous phase and thus increase the RIF incorporation into the nanoparticles (Ito F et al., 2007). Consequently, the encapsulation efficiency of nanoparticles was increased by increasing the polymer concentration. Conversely, RIF loading (%) was decreased by increasing PCL concentration. Increasing viscosity increased the total mass of PCL in the nanoparticles. This decreased the ratio of RIF to the total mass of nanoparticles, thus decreasing RIF loading (%).

The effect of ultrasonification time on properties of nanoparticles

Ultrasonification time is another input factor. In this study, particle mean size was decreased by increasing ultrasonification time from 1 to 5 min (Table 2). The increased time of ultrasonification led to the formation of smaller nanoparticles. It was also observed that encapsulation efficiency was decreased by increasing ultrasonification time from 1 to 5 min (Table 2). Increasing the ultrasonification time resulted in a reduction in the encapsulation efficiency due to the decreasing particle mean size (Song X et al., 2008).

The effect of amount of PVA on properties of nanoparticles

Lowering the amount of PVA in the aqueous medium led to an improvement in encapsulation efficacy, but this improvement was not so significant (Feng SS et al., 2001). Because RIF is slightly water soluble, higher surfactant concentration causes the drug to penetrate more to the continuous phase. When no PVA was used in the external phase, no NP was formed. At 0.25% (w/v) of PVA, larger particles were produced. The use of higher amount of PVA (0.5%, 1% and 1.5%) did not affect the particle size of NPs ($P<0.05$).

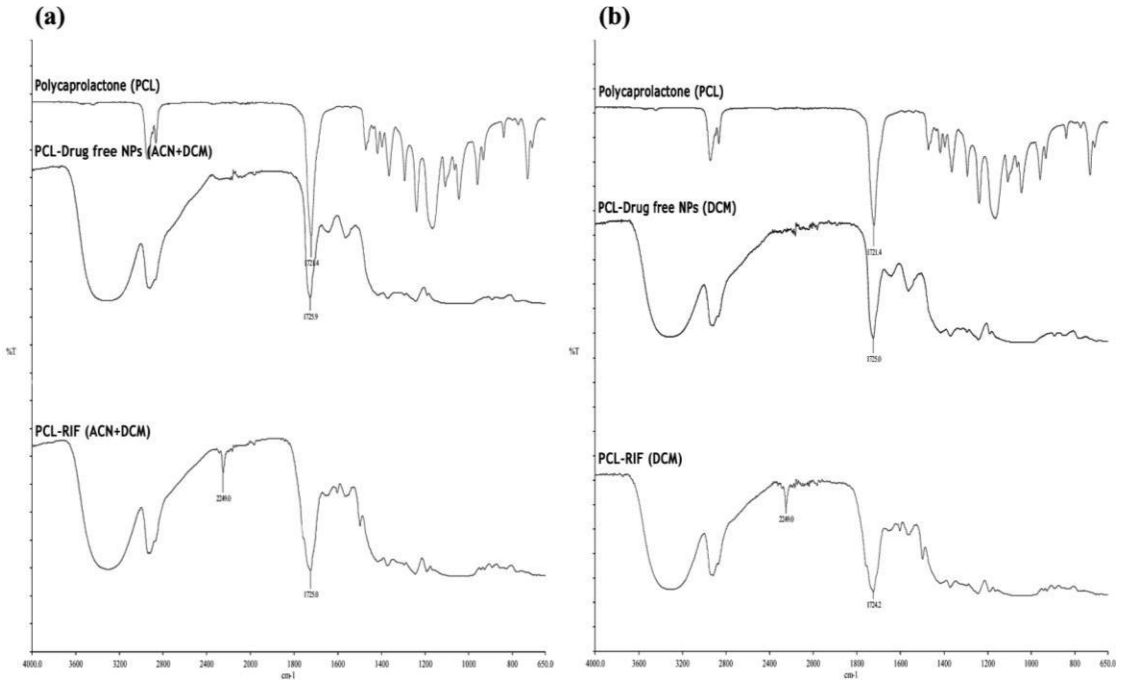

Figure 5: DSC thermograms of rifampicin nanoparticles with (a) PCL in DCM and (b) DCM +ACN

Optimization

A numerical optimization technique by the desirability approach was used to generate the optimum settings for the formulation. The process was optimized for the dependent (response) variables Y_1 and Y_2 and the optimized formula was arrived at by keeping the EE maximum. The mean diameter was targeted to minimize. The optimized formulations revolved around the NPR 9 having PCL amount 500 mg and sonication time were 4.9 minutes. To gainsay the reliability of the response surface model, a new optimized formulation was prepared according to the predicted model and evaluated for the responses. The results in Table 8 illustrate a good relationship between the experimental and predicted values, which confirms the practicability and validity of the model. The predicted error for all the response variables was below 6% indicating that the RSM optimization technique was appropriate for optimizing the rifampicin nanoparticles.

CONCLUSION

The study suggests that the nanoparticles of rifampicin can be designed using PCL to provide extended release. The nanoparticles showed a promising release profile for a longer period which is intended. The rifampicin containing nanoparticles can provide an alternative to the conventional dosage form for the treatment of tuberculosis which need further *in-vivo* test.

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