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Research paper

# Dissolution test for site-specific release isoniazid pellets in USP apparatus 3 (reciprocating cylinder): Optimization using response surface methodology

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**Abstract**

The present work aims to predict drug release from novel site-specific release isoniazid pellets, in USP dissolution test apparatus 3, using the response surface methodology (RSM). Site-specific release isoniazid pellets were prepared by extrusion-spheronization followed by aqueous coating of Acryl-EZE®. RSM was employed for designing of the experiment, generation of mathematical models and optimization study. A $3^2$ full factorial design was used to study the effect of two factors (at three levels), namely volume of dissolution medium (150, 200, 250 ml) and reciprocation rate (5, 15, 25 dips per min). Amount of drug released in 0.1 N hydrochloric acid at 2 h and in pH 6.8 phosphate buffer at 45 min were selected as responses. Results revealed that both, the volume of medium and reciprocation rate, are significant factors affecting isoniazid release. A second order polynomial equation fitted to the data was used to predict the responses in the optimal region. The optimized conditions resulted in dissolution data that were close to the predicted values. The proposed mathematical model is found to be robust and accurate for optimization of dissolution test conditions for site-specific release isoniazid pellets.


*Keywords:* Isoniazid; Site-specific release; USP apparatus 3; Response surface methodology; Optimization; Full factorial design

## 1. Introduction

Isoniazid, an isonicotinic acid hydrazide, a first-line antitubercular agent, is an integral part of intensive as well as continuation phase of six months treatment schedule against tuberculosis [1]. Isoniazid has an aqueous solubility of approximately 125 mg $ml^{-1}$ [2]. In order to minimize its interaction with rifampicin in acidic environment of stomach, Shishoo et al. [3] emphasized the need to develop a site-specific release formulation of isoniazid. Isoniazid is less permeated through the stomach and is mainly absorbed through the intestine because it occurs in the protonated form at acidic pH ($pK_a = 2$) [4]. Therefore, it can be considered as a good candidate for the development of a site-specific release formulation. Enteric coating is a popular and a widely accepted technique for achieving the site-specific drug release in the intestine. Considering the popularity and the robustness of the multiparticulate system (e.g., pellets, granules, etc.) as a means of tailoring the release profile of a drug [5], this approach has been adopted in the formulation of isoniazid pellets. Pellets offer various advantages over single unit dosage form including minimal risk of dose dumping, flexibility of blending units with different release patterns, as well as short and reproducible gastric residence time [6].

Dissolution test has proved to be an essential *in vitro* test to characterize the performance of an oral drug delivery system [7]. The significance of a dissolution test is that, for a drug to be absorbed from gastrointestinal tract and to be available to the systemic circulation, it must be previously solubilized [8]. Therefore, dissolution test is used not

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only for the quality control of a finished product to assess batch-to-batch consistency of drug release from solid dosage forms, but is also essential in the development of a formulation for screening and proper assessment of different formulations. In fact, the creative use of dissolution technique can speed up the formulation development, particularly in the case of modified-release products, enabling prompt identification of potential problems in drug release rate. Essentially, dissolution test makes it possible to assess the dissolution properties of the drug itself and thereby to select the most appropriate excipients and appropriate proportions among them for obtaining the desired drug release behavior. Among the several dissolution methods specified in United States Pharmacopoeia (USP), apparatus 1 (basket) has been extensively employed to evaluate the dissolution of site-specific release formulations. However, USP apparatus 3 (reciprocating cylinder) provides sound hydrodynamic conditions for the evaluation of pellets. In contrast to the movement of media in USP apparatus 1, the dosage form moves freely through the dissolution medium in case of reciprocating cylinder. USP apparatus 3 is considered as the first line apparatus in product development of controlled release products and especially the pellets, because of its usefulness and convenience in exposing products to mechanical as well as variety of physicochemical conditions which eventually influence the release of a product in the gastrointestinal tract. USP apparatus 3 has a relatively short history and was incorporated into USP in 1991 as apparatus 3 [9]. There exist only a few reports in the literature on the use of USP apparatus 3 for testing drug release rate and for comparing it to those obtained from other methods. Most of these reports, however, focus on extended release dosage forms [10–13].

Response surface methodology (RSM) is a widely practiced approach in the development and optimization of drug delivery systems [14,15]. Based on the principle of design of experiments, the methodology encompasses use of various types of experimental designs, generation of polynomial equations and mapping of the response over the experimental domain to optimize formulation as well as processing conditions. The advantage of such methodology is in providing a rationale for simultaneous evaluation of several variables. The technique requires minimum experimentation and time, thus proving to be far more efficient and cost effective than conventional methods of product development. For implementation of RSM, factorial designs (FDs; full or fractional) are the most popular statistical designs. Full factorial design (FFD) involves the study of the effect of all factors at various levels and is considered as an efficient approach to estimate the influence of individual variables (main effect) and their interactions. Until date, application of RSM has not been reported in the development and optimization of the dissolution test method for USP apparatus 3. Most of the publications however focus on the optimization of dissolution test conditions for USP apparatus 1 and 2 [14,16].

The current study illustrates the evaluation of *in vitro* release characteristics of site-specific release isoniazid pellets, under defined hydrodynamic conditions in USP apparatus 3. Computer-aided optimization techniques using $3^2$ FFD were employed to investigate the effect of two factors viz., volume of the dissolution medium and reciprocation rate, on release of isoniazid from the site-specific release pellets. A FD for 2 factors at 3 levels each ($3^2$) is considered identical to a two-factor composite design and has an added advantage of determining a quadratic response surface [14,15,17].

## 2. Materials and methods

### 2.1. Materials

Isoniazid I. P. was kindly supplied by Cadila Pharmaceuticals Ltd., Ahmedabad, India. Microcrystalline cellulose (Avicel® PH 101, Signet Chemical Corporation, Mumbai, India), Polyvinylpyrrolidone (PVP K-90, Kollidon® 90, BASF, Germany) and Acryl-EZE® (Colorcon Asia Pvt. Ltd., Mumbai, India) were used as excipients and were obtained from the indicated sources. All other ingredients and reagents were of analytical grade and were used as received.

### 2.2. Preparation of site-specific release isoniazid pellets

#### 2.2.1. Preparation of isoniazid loaded pellets

Powder components of the formulation (Isoniazid – 55% w/w, Avicel® PH 101 – 42% w/w and Kollidon® 90 – 3% w/w; Batch size – 500 g) were mixed in a small scale planetary mixer (Kalweka, Karnavati Eng. Ltd., India) for 10 min. Purified water (40% w/w of total solids) was added to get a wet mass. Extrudates were obtained by feeding the wet mass in gravity fed cylinder extruder (R. R. Enterprises, India). Extrudates were spheronized in a spheronizer (R. R. Enterprises, India) to obtain spherical pellets. The pellets were dried in fluid-bed dryer (Nero-Aeromatic, Switzerland) at 50 °C for 20 min. Fraction of pellets, 16/25#, was subjected to coating process.

#### 2.2.2. Enteric coating of isoniazid pellets for site-specific release of isoniazid

Isoniazid pellets were coated with 10% w/w aqueous suspension of Acryl-EZE® using fluid-bed coater (Nero-Aeromatic, Switzerland) to achieve 35% weight gain. The process conditions were 'pre-warming of the cores at 40 °C for 10 min; spray nozzle diameter, 1 mm; atomizing air pressure, 1 bar; air flow rate, 80 $m^3\ h^{-1}$; inlet air temperature, 40 °C; product temperature 32–35 °C; spray rate, 1.5 ml $min^{-1}$; post drying at 40 °C for 10 min.

### 2.3. Dissolution methodology

Dissolution studies were carried out in USP dissolution apparatus 3 (Hanson Research B-3 release rate tester;

Hanson Research Corporation, Chatsworth, CA). For carrying out release rate study, USP method B, for delayed release formulations, was followed [18]. Test was carried out in 0.1 N hydrochloric acid (HCl) for 2 h followed by pH 6.8 phosphate buffer USP for 45 min. Dissolution medium, pH 6.8 phosphate buffer were prepared by combining appropriate amounts of HCl and tri-basic sodium phosphate. Table 1 summarizes the general conditions followed in this study. During the study, the reciprocating cylinder containing pellets moved between the rows successively and switched from one medium to another.

All the dissolution samples were filtered through 0.22 μm Millipore® (Polyvinylidene difluoride, PVDF) filter and analyzed immediately after the completion of dissolution test by UV-Visible spectrophotometer (Shimadzu UV-2450, UV-vis scanning spectrophotometer, Japan). Isoniazid released in 0.1 N HCl was estimated as per method specified in USP [19] and isoniazid released in pH 6.8 phosphate buffer was measured at $\lambda_{max}$ 263 nm by a validated spectrophotometric method [20]. The analytical method was found to be specific, linear in the concentration range of 5–30 μg/ml, precise (%CV: 1.05–3.16) and accurate (98.5–102.0%). For each dissolution run, a mean of six determinations was recorded.

### 2.4. Experimental design

A $3^2$ FFD was used for the dissolution testing optimization procedure. Volume of dissolution medium ($X_1$, ml) and reciprocation rate ($X_2$, dips per minute, dpm) were the two factors (independent variables) studied. The levels for $X_1$ and $X_2$ were chosen in accordance with the preliminary data and were representative of the entire range of operating conditions of the USP apparatus 3. The responses (dependent variables) studied were amount of isoniazid released in 0.1 N HCl at 2 h ($Y_1$, %) and amount of isoniazid released in pH 6.8 phosphate buffer at 45 min ($Y_2$, %). Table 2 summarizes independent and dependent variables along with their levels. Experimental dissolution testing runs are listed in Table 3.

Table 1
The test conditions followed in the dissolution testing of site-specific release isoniazid pellets in USP apparatus 3

| Parameter | Dissolution test conditions |
|---|---|
| Dissolution medium | 0.1 N HCl and pH 6.8 phosphate buffer USP |
| Temperature (°C) | 37.0 ± 0.5 |
| Volume (ml) | 150/200/250 (as per the statistical design) |
| Reciprocation speed (dpm) | 5/15/25 (as per the statistical design) |
| Sample holder | Reciprocating cylinder (#40) |
| Volume of the sample withdrawn (ml) | 5.00 |
| Test duration | 2 h in 0.1 N HCl followed by 45 min in pH 6.8 phosphate buffer |

Table 2
Factors (independent variables), factor levels and responses (dependent variables) used in $3^2$ full factorial experimental design

| Factors | Factor levels used | | | Responses |
|---|---|---|---|---|
| | −1 | 0 | 1 | |
| $X_1$ = Volume of dissolution medium (ml) | 150 | 200 | 250 | $Y_1$ = amount of isoniazid released in 0.1 N HCl at 2 h (%) and |
| $X_2$ = Reciprocation rate (dpm) | 5 | 15 | 25 | $Y_2$ = amount of amount released in pH 6.8 phosphate buffer at 45 min (%) |

Table 3
Dissolution test runs carried out on site-specific release isoniazid pellets as per $3^2$ full factorial experimental design

| Dissolution test run | Factor $X_1$ (Volume of dissolution medium, ml) | Factor $X_2$ (Reciprocation rate, dpm) |
|---|---|---|
| 1 | 150 | 5 |
| 2 | 200 | 5 |
| 3 | 250 | 5 |
| 4 | 150 | 15 |
| 5 | 200 | 15 |
| 6 | 250 | 15 |
| 7 | 150 | 25 |
| 8 | 200 | 25 |
| 9 | 250 | 25 |

### 2.5. Statistical analysis of the data and validation of the model

Various RSM computations for the current study were performed employing Design-Expert® software (Version 7.1.2, Stat-Ease Inc., Minneapolis, MN). Polynomial models including interaction and quadratic terms were generated for all the response variables using multiple linear regression analysis. Statistical validity of the polynomials was established on the basis of ANOVA and the 3D response graphs were constructed using Design-Expert® software. To validate the chosen experimental design and polynomial equations, optimum test condition was selected. The tests corresponding to this optimum dissolution condition and three additional random dissolution test conditions were carried out in the experimental matrix to determine the validity of the model generated. Subsequently, the resultant experimental data of the response properties were quantitatively compared with those of the predicted values. Also, the linear regression plots between observed and predicted values of the response properties were drawn using MS-Excel.

## 3. Results and discussion

In developing a novel drug delivery system, particularly, in the case of site-specific release product, dissolution test is a helpful *in vitro* tool for the assessment and adjustment of the drug release profile from a candidate formulation, enabling easy and fast evaluation of the effects of formulation changes. However, this test is sensitive to many

parameters such as temperature, agitation, dissolution medium, pH of the medium, volume of dissolution medium and shape of the vessel [21,22]. The precision of dissolution test is essential for the reliability of the results. Earlier experiments in our laboratory using USP dissolution apparatus 1 indicated a non discriminatory dissolution test (data not shown). Therefore, USP dissolution apparatus 3 was chosen for the current study. A multivariate optimization strategy was carried out with the aim of finding the optimum conditions for the testing of drug dissolution behavior from the site-specific release isoniazid pellets.

### 3.1. Experiments of $3^2$ FFD

Response data for all the 9 experimental runs of $3^2$ FFD, performed in accordance with Table 3, are presented in Table 4. In 0.1 N HCl only 11.40% to 15.90% of isoniazid was released in 2 h and pellets were found to be completely intact at the end of 2 h. Acid resistance test is a significant index of drug dissolution performance of an enteric coated formulation. Polymers used for formulating enteric coated formulation should be able to withstand the lower pH values of stomach and be able to disintegrate in the range of pH 6–7. At pH 6.8, 67.90% to 80.60% of isoniazid was released at the end of 45 min. This indicates that Acryl-EZE® effectively controls the release of isoniazid (a borderline BCS class-I and class-III drug, [23]) from site-specific release isoniazid pellets.

### 3.2. Mathematical modeling

Mathematical relationship was generated between the factors (dependent variables) and responses (independent variables) using the statistical package Design-Expert® for determining the levels of factors, which yield optimum dissolution responses. A second order polynomial regression equation that fitted to the data is as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1^2 + \beta_4 X_2^2 + \beta_5 X_1 X_2, \quad (1)$$

where $\beta_0$ is the intercept representing the arithmetic averages of all the quantitative outcomes of 9 runs; $\beta_1$ to $\beta_5$ are the coefficients computed from the observed experimental values of $Y$; and $X_1$ and $X_2$ are the coded levels of factors. The terms $X_1X_2$ and $X_i^2$ ($i = 1$ and 2) represent the interaction and quadratic terms, respectively. The equations of the responses are given below:

$$Y_1 = 11.52 - 0.58X_1 + 0.01X_2 + 0.58X_1X_2 + 2.82{X_1}^2 + 0.47{X_1}^2 \quad (2)$$

$$Y_2 = 70.72 + 1.88X_1 + 3.38X_2 + 4.9X_1X_2 + 0.88{X_2}^2 - 1.32{X_2}^2 \quad (3)$$

The equation represents the quantitative effect of factors ($X_1$ and $X_2$) upon the responses ($Y_1$ and $Y_2$). 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. Positive sign in front of the terms indicates synergistic effect while negative sign indicates antagonistic effect of the factors.

Analysis of variance (ANOVA) was applied for estimating the significance of the model, at 5% significance level. A model is considered significant if the $p$-value (significance probability value) is less than 0.05. From the $p$-values presented in Table 5, it can be stated that for both the responses the linear contribution of the model was not significant. However, for response $Y_1$, quadratic contribution of the response was significant, whereas, for response $Y_2$, the cross product contribution was significant.

In Table 6, factor effects of $3^2$ FFD model and associated $p$-values for the responses $Y_1$ and $Y_2$, are presented. A factor is considered to influence the response if the effects significantly differ from zero and the $p$-value is less than 0.05.

Data in Table 6 show that the response $Y_1$ was significantly affected by the synergistic effect of quadratic term

Table 4
Results of dissolution studies carried out on site-specific release isoniazid pellets as per $3^2$ full factorial experimental design: response $Y_1$ (amount of isoniazid released in 0.1 N HCl at 2 h, %) and response $Y_2$ (amount of isoniazid released in pH 6.8 phosphate buffer at 45 min, %)

| Dissolution test run | Response $Y_1$ (%)[a] | Response $Y_2$ (%)[a] |
|---|---|---|
| 1 | 15.9 ± 0.7 | 68.9 ± 3.6 |
| 2 | 15.3 ± 1.5 | 70.6 ± 3.6 |
| 3 | 14.5 ± 1.2 | 67.9 ± 4.2 |
| 4 | 11.4 ± 0.9 | 69.8 ± 1.7 |
| 5 | 11.6 ± 0.7 | 68.1 ± 4.5 |
| 6 | 12.5 ± 1.2 | 72.5 ± 3.1 |
| 7 | 14.0 ± 0.5 | 62.0 ± 4.5 |
| 8 | 13.3 ± 0.8 | 76.1 ± 2.1 |
| 9 | 14.9 ± 0.5 | 80.6 ± 2.3 |

[a] Mean of 6 ± SD.

Table 5
Summary of analysis of variance (ANOVA) for the measured response $Y_1$ (amount of drug released in 0.1 N HCl at 2 h) and response $Y_2$ (amount of drug released in pH 6.8 phosphate buffer at 45 min)

| Source of variation | $Y_1$ | | $Y_2$ | |
|---|---|---|---|---|
| | $F$ | $p$-value | $F$ | $p$-value |
| Linear contribution | 0.34 | 0.7273 | 2.00 | 0.2157 |
| Quadratic contribution | 10.36 | 0.0413 | 3.40 | 0.1714 |
| Cross-product contribution (2FI) | 0.33 | 0.8067 | 8.00 | 0.0235 |

Table 6
A summary of each factor effect and its $p$-values for, response $Y_1$ (amount of isoniazid released in 0.1 N HCl at 2 h) and for response $Y_2$ (amount of isoniazid released in pH 6.8 phosphate buffer at 45 min)

| Factor | $Y_1$ | | $Y_2$ | |
|---|---|---|---|---|
| | Factor effect | $p$-value | Factor effect | $p$-value |
| $X_1$ | −0.580 | 0.1035 | +1.880 | 0.1584 |
| $X_2$ | +0.100 | 0.7179 | +3.380 | **0.0309** |
| $X_1X_2$ | +0.580 | 0.1592 | +4.900 | **0.0169** |
| ${X_1}^2$ | +2.820 | **0.0075** | +0.880 | 0.7342 |
| ${X_2}^2$ | +0.432 | 0.3632 | −1.320 | 0.6174 |

Significant effects of factors on individual responses are shown in bold type.

of volume of dissolution medium ($X_1^2$) ($p$-value, 0.0075). Significant factors affecting the response $Y_2$ were reciprocation rate ($X_2$) with $p$-value, 0.0309 and interaction effects (cross-product terms) with $p$-value, 0.0169. Both the above-mentioned factors show the synergistic effect and increase the release of isoniazid from site-specific release isoniazid pellets.

### 3.3. Response surface analysis

The 3-dimensional response surface plots were drawn to estimate the effect of independent variables on each response. Figs. 1 and 2 show the effect of two hydrodynamic conditions in the dissolution test on the release of isoniazid in 0.1 N HCl and release of isoniazid in pH 6.8,

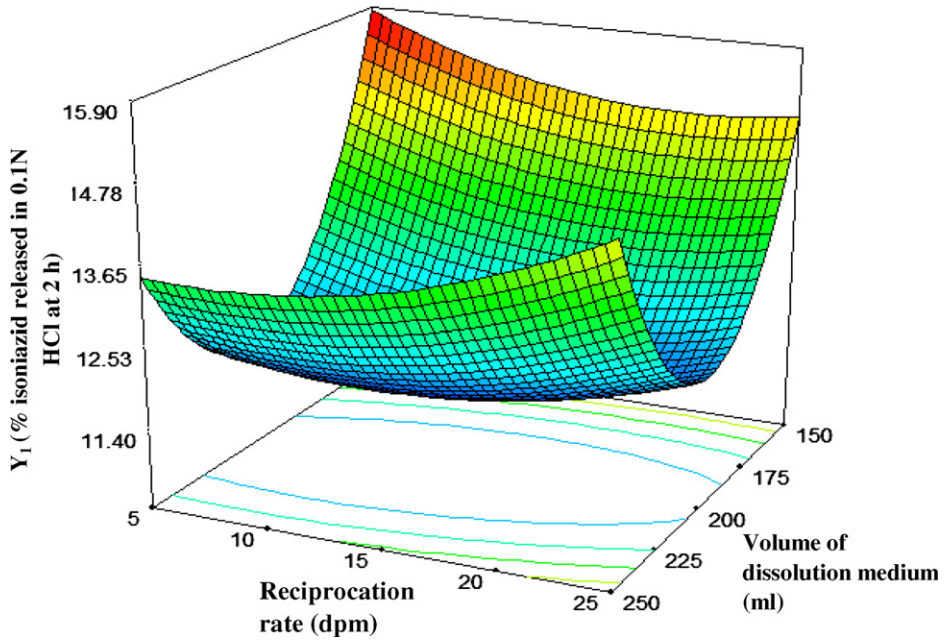


Fig. 1. Response surface plot showing the influence of volume of dissolution medium and reciprocation rate on response $Y_1$ (amount of isoniazid released in 0.1 N HCl at 2 h, %).

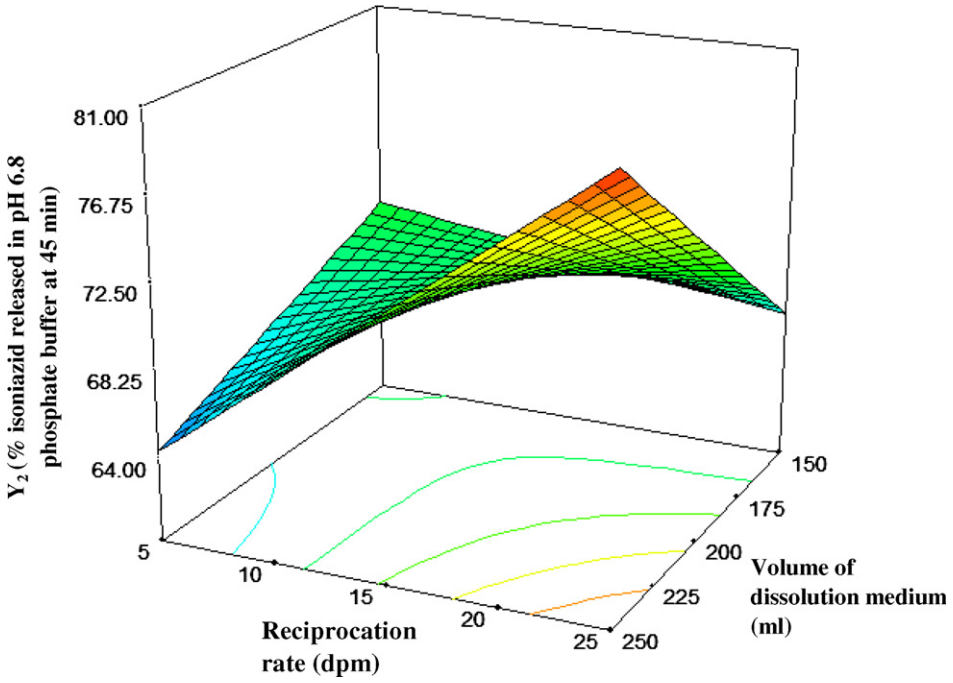


Fig. 2. Response surface plot showing the influence of volume of dissolution medium and reciprocation rate on response $Y_2$ (amount of isoniazid released in pH 6.8 phosphate buffer at 45 min, %).

respectively. The Fig. 1 shows 'a region of minima' lying between the intermediate to higher levels of both the factors. However, the effect of volume of dissolution medium seems to be more pronounced as compared with that of speed. This receives confirmation from the mathematical model generated for response (Eq. 2).

Fig. 2 depicts a nonlinear twisted relationship for $Y_2$ at intermediate and high levels of both the factors. This can be attributed to the potential occurrence of interaction between the two independent variables at the corresponding factor levels, construing that each independent variable is tending to modify the effect of another towards the release of isoniazid in pH 6.8. However, the effect of speed seems to be more pronounced as compared with that of volume of dissolution medium. This is in agreement with Eq. (3) as well as Fig. 2.

With the help of polynomial equation, the process was optimized for both the responses. The final optimal experimental parameters were calculated by satisfying the requirements for each response in the set. Thus, to obtain site-specific release of isoniazid, it is desirable to minimize $Y_1$, and maximize $Y_2$. In this study optimization was performed with constraints for $Y_1$ ($\leqslant$15%) and $Y_2$ ($\geqslant$ 80%). The optimal calculated parameters were

- Volume of dissolution medium, $X_1 = 225$ ml
- Reciprocation rate, $X_2 = 25$ dpm

The test carried out with the above-mentioned dissolution test conditions showed $Y_{1\text{Experimental}}$ as 12.00% ($Y_{1\text{Predicted}}$, 12.31%; percentage prediction error, −2.58) and $Y_{2\text{Experimental}}$ as 82.63% ($Y_{2\text{predicted}}$, 80.6%; percentage prediction error, 2.46) as shown in Table 7. Low values of prediction percentage error indicate that the predicted and observed values are in good agreement.

### 3.4. Validation of response surface model

In order to assess the reliability of the developed mathematical model, dissolution tests corresponding to the above-mentioned optimum dissolution conditions and three additional random dissolution tests with conditions covering the entire range of experimental domain were performed. For each of these test runs, responses were estimated by use of the generated mathematical model and by the experimental procedures. Table 7 lists the dissolution test conditions of the optimum and the random check points, their experimental and predicted values for both the response variables. Fig. 3A and B shows linear correlation plots between the observed and predicted response variables. The graphs demonstrate high values of correlation coefficient, $r^2$ (>0.9) indicating excellent goodness of fit. Therefore, it can be concluded that, model functions $Y_1$

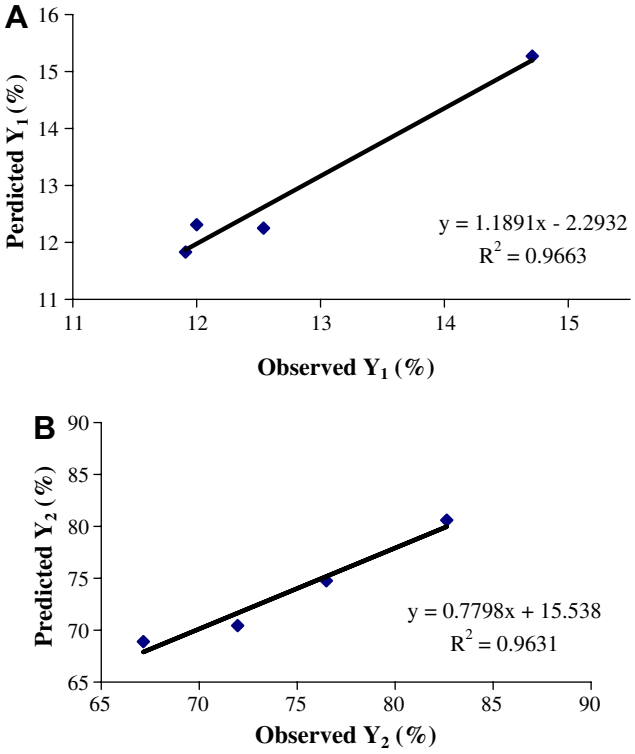


Fig. 3. Linear correlation plots (A and B) between observed and predicted values for response $Y_1$ (amount of isoniazid released in 0.1 N HCl at 2 h,%) and response $Y_2$ (amount of isoniazid released in pH 6.8 phosphate buffer at 45 min, %).

Table 7
The experimental and predicted values for response $Y_1$ (amount of isoniazid released in 0.1 N HCl at 2 h, %) and for response $Y_2$ (amount of isoniazid released in pH 6.8 phosphate buffer at 45 min, %) along with percentage prediction error observed for the optimum dissolution test condition (A) and random dissolution test conditions (B, C, and D)

| Dissolution test | Test conditions[a] $X_1$ (ml)/$X_2$ (dpm) | Response | Experimental value | Predicted value | Percent prediction error[b] |
|---|---|---|---|---|---|
| A | 225/25 | $Y_1$ | 12.00 | 12.31 | −2.58 |
| | | $Y_2$ | 82.63 | 80.6 | 2.46 |
| B | 200/10 | $Y_1$ | 11.91 | 11.83 | 0.67 |
| | | $Y_2$ | 67.15 | 68.9 | −2.60 |
| C | 225/20 | $Y_1$ | 12.54 | 12.25 | 2.31 |
| | | $Y_2$ | 76.49 | 74.76 | 2.26 |
| D | 150/10 | $Y_1$ | 14.71 | 15.27 | −3.80 |
| | | $Y_{21}$ | 71.96 | 70.44 | 2.11 |

[a] $X_1$, volume of the dissolution medium (ml) and $X_2$, reciprocation rate (dpm).
[b] Percent error was calculated using the formula (Experimental value − Predicted value)/Experimental value × 100.

and $Y_2$ well interpreted, the variable data of isoniazid release in 0.1 N HCl at 2 h and isoniazid release in pH 6.8 phosphate buffer at 45 min. Thus, the lower magnitude of error (−3.8 to 2.31 for $Y_1$ and −2.6 to 2.46 for $Y_2$) as well as significant values of $r^2$ (>0.9) in the current study indicate the robustness of the mathematical model and high prognostic ability of RSM.

## 4. Conclusion

A statistical model has been established to predict the release properties of the isoniazid from the site-specific pellets, by simultaneously studying the effect of various hydrodynamic factors in USP dissolution test apparatus 3, using RSM. The $3^2$ FFD strategy was found to point out the significant factors affecting drug release from the site-specific release isoniazid pellets, in the considered experimental domain. A set of optimum conditions for dissolution test, with respect to the release of the isoniazid, were found to be 225 ml of dissolution medium with 25 dpm reciprocation rate. High degree of prognosis obtained for $3^2$ full factorial design corroborates that RSM is an efficient tool in optimization experiments.

This approach could be applied for other dissolution procedures as well as for other solid dosage forms. Examination of dissolution data discussed in this work will help research scientist in collection of scientifically sound data and its interpretation.

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