OPTIMIZATION OF PIROXICAM MONOETHANOLAMINE LOADED GOLD NANO PARTICLES USING 2³ FULL FACTORIAL DESIGN APPROACH.

Jessy Shaji* and Seema Darade

Dept. of Pharmaceutics, Prin. K. M. Kundnani College of Pharmacy, Cuffe Parade, Mumbai 400005, India.

ABSTRACT

The objective of the present study was to investigate the combined effect of three independent variables in the fabrication of Piroxicam monoethanolamine (PRX-MEA) loaded gold nanoparticles (PM-AuNPs) using a 2³ full factorial design. PM-AuNPs were prepared by green chemistry approach using methanolic green tea (Camellia sinesis) extract with factors viz. gold salt concentration X₁ (1.57 to 3.93 mg), PRX-MEA concentration X₂ (2.86 to 8.12 mg) and reaction time X₃ (60 to 180 min.) and were evaluated for their particle size Y₁ (nm) and % loading efficiency Y₂ (% LE). The mean particle size of PM-AuNPs batches ranged between 43 to 158.3 nm. Preliminary trials of PM-AuNPs revealed that on increasing the gold salt concentration the particle size was also increased. The % LE was found to be between 81.94 % to 97.78%. Process variables such as gold salt and PRX-MEA concentration had positive impact on percent drug loading efficiency. The effects of all the tested independent variables were significant (P-values<0.05). Using design check point analysis the optimum formulation comprising of 1.96 mg of gold salt, 6.58mg of PRX-MEA and stirring time of 180 min was selected. This formulation demonstrated particle size of 51.02 ± 45 nm and LE of 88.05 ± 0.36 %. PM-AuNPs with small particle size and excellent loading efficiency were successfully produced using this method. Characterization of optimum formulation by evaluating the experimental data showed no significant difference between predicted and observed values.
KEYWORDS: $2^3$ full factorial design, loading efficiency (%LE), Gold nanoparticles, Optimization, Piroxicam Monoethanolamine (PRX-MEA).

INTRODUCTION
Gold nanoparticles has gained much attention and emerged as an attractive carrier for delivery in the field of nanotechnology. Gold nanoparticles (AuNPs) are hybrid materials featuring an inorganic gold core possessing negative charge surrounded by organic monolayer which depicts the reactivity and solubility of the nanoparticles. AuNPs have numerous applications in biomedical sciences including drug delivery, tissue/organ imaging, photo thermal therapy and identification of pathogens in clinical specimens. AuNPs have the advantage of ease of synthesis, ready functionalization through thiol (sulfur moiety) and amine linkages, enhanced permeability and retention (EPR effect), high stability and modulation of drug release at remote place which defines the versatility of AuNPs as drug delivery carriers.$^{[1-3]}$

AuNPs can be easily synthesized by most common method of chemical synthesis like citrate reduction [as described by Turkevich and Frens] and Out Brust synthesis which are quite expensive with byproducts toxic to the environment.$^{[4]}$ The most safe and economical alternative method for the synthesis of AuNPs is by green chemistry using various plant antioxidants.$^{[5]}$ Green tea polyphenols have been reported to act as both reducing and stabilizing agents for AuNPs synthesis by conjugating some of its components on the surfaces of gold. The method for preparation is a simple stirring of gold salt and green tea extract at optimum temperature and speed followed by addition of drug solution and appropriate stabilizing agents to prevent particle agglomeration if any.$^{[6-7]}$

Piroxicam (PRX) is a potent anionic non-steroidal anti-inflammatory drug (NSAID’s) which can be ionized as a zwitterion with two pKa values, $pK_a1=1.86$ and $pK_a2=5.46$. It possesses a large intermolecular multipole moment due to its multiple oppositely charged groups. Hence it has a low polar solubility as well as low lipophilicity. Although Piroxicam belongs to the BCS class II drugs, a bioavailability study revealed that it takes more than 2h to reach maximum concentration. Bioavailability of such drug was remarkably increased by preparing the alkaline ethanolamine salts.$^{[8]}$ The undesirable side effects of Piroxicam like GI adverse effects, stomach ulcers have been expressed after oral administration. Hence PRX-MEA loaded gold nanoparticles (PM-AuNPs) were formulated with the ability to evade GI toxicities of piroxicam and to provide relatively consistent drug levels for prolonged periods.
with rapid penetration.\cite{9} Rodrigue et al. studied the incorporation of Piroxicam in water soluble gold nanoparticles based on imidazolium gemini amphiphiles which supports the above hypothesis.\cite{10}

The Design of the Experiments (DOE technique) consisting of statistical equations were utilized for the pharmaceutical product development.\cite{11-12} This technique would provide an efficient method to collect the necessary information that effectively explores the relationship between the independent variable and responses from the experimental data output. Optimization process also acquires information to understand main effects and the interaction between the factors which can results in breakthrough improvement in the design model.\cite{13} The primary goal of this study was to investigate the utility of $2^3$ factorial design and optimization process in the development of improved PM-AuNPs formulation using the empirical model equations.\cite{14-15}

**MATERIALS AND METHODS**

**Materials**

Commercial grade Piroxicam was a kind gift by Ramdev chemicals, Mumbai. Gold (III) Chloride trihydrate (HAuCl$_4$.3H$_2$O) was purchased from Himedia Laboratories, Mumbai. Tween 80 was obtained as a gift sample from Croda, India. Monoethanolamine, Diethanolamine, Triethanolamine were purchased from S.D. Fine chemicals, Mumbai. All the other chemicals used were of Analytical grade.

**Methods**

**Preparation of PRX-MEA loaded gold nanoparticles (PM-AuNPs)**

PM-AUNPs were synthesized using green chemistry approach. In a typical synthesis, 30μL of methanolic Camellia sinesis extract (1.85g in 100ml methanol) was added to the solution of Gold (III) chloride trihydrate at room temperature under constant stirring at 800 rpm on a magnetic stirrer (1 MLH, Remi, India). Appearance of light red color indicated the redox change due to the presence of polyphenolic compounds present in CS extract Further the accurate concentration of PRX-MEA solution and stabilizer tween 80 (2.5 %v/v) was added to the gold chloride solution.\cite{6,10}\ The reaction was stirred for definite time period and kept overnight in dark for complete reduction of gold ions and attachment of drug to the surface of the gold nanoparticles were indicated by dark purple color.
Experimental design
A $2^3$ full factorial design using Design Expert version 9. 6. 0. 2 (Stat-Ease Inc., and Minneapolis, MN) with three central points and 2 replicates was employed for modeling and analysis of problem. The design predicted the optimized critical formulation variables based on their effect on the response of an interest.$^{[16]}$ The independent variables or factors were concentration of gold salt ($X_1$), concentration of drug ($X_2$) and reaction time ($X_3$). The values were chosen based on our initial experimental trials. The concentration of Tween 80 was fixed at 2.5% W/V. The dependent variables or responses were mean particle size ($Y_1$) and loading efficiency (% LE) ($Y_2$).$^{[17-18]}$

The following is the general formula for the model selected:
\[ Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{23} X_2 X_3 + \beta_{123} X_1 X_2 X_3 + E \quad \ldots \ldots \quad (1) \]

Where $\beta_0$ is the intercept term, $\beta_1$-$\beta_{123}$ are the coefficients of the respective variables and their interaction terms, and $E$ is an error term.

The estimation of main effects of controllable variable ($X_1$, $X_2$ and $X_3$) and their possible interactions ($X_1 X_2$, $X_2 X_3$, $X_1 X_3$ and $X_1 X_2 X_3$) were elaborated using the half-normal plot and Pareto chart. The data were analyzed by means of analysis of variance and P-values less than 0.05 were considered to be statistically significant. Additionally, three dimensional response surface plots were constructed for each dependent response to study the effects of both formulation variables simultaneously along with the behavior of the system.$^{[19]}$

The experimental design was diagnosed using the Normal probability plot of the studentized residuals to check for normality of residuals, studentized residuals versus predicted values to check for constant error and Box-Cox plot for power transformations. The validity of the developed factorial equations was verified using design check point analysis by comparing the predicted and observed experimental response values for selected optimized batch.$^{[20]}$

Particle size
Particle size of the PM-AuNPs was determined by using Nanosight NS 500 (Malvern Instruments Ltd, UK) with computer controlled motorized stage and charge-couple device (CCD) that allows visualization and tracking of laser illuminated particles undergoing Brownian motion in suspension. 0.2 μl PM-AuNPs colloidal dispersion was diluted to 2 ml using ultrapure water and filtered using 0.22 μm nylon membrane filters to remove the contamination of dust and other particles. The samples were introduced directly into the
chamber using a syringe. Video images were analyzed by NTA analytical software version 3.2. Measurements were carried out with red laser (638 nm) at 25 °C.\[^{21}\]

**Drug loading efficiency (% LE)**

PRX-MEA loading onto the AuNPs was expressed as the percentage of PRX-MEA in the produced nanoparticle with respect to the initial amount of PRX-MEA that was used for synthesizing the nanoparticles. The colloidal dispersion of PM-AuNPs was vortexed (Remi, India) for 2 minutes and centrifuged at 15,000 rpm (Eltek, Mumbai) for 20 minutes. The supernant after filtration through (mesh size 4 whatmann filter) was analyzed by UV-Vis spectrophotometry (EVOLUTION 6000) at 354 nm to estimate unassociated PRX-MEA salt.\[^{21}\]

The % LE was determined by the following equation:

\[
\text{% LE} = \frac{\text{Amount of drug added during preparation} - \text{Amount of drug in the supernatant}}{\text{Amount of drug added during preparation}} \times 100
\]

**RESULTS AND DISCUSSION**

After selection of critical formulation variables, a systematic evaluation to assess their influence on the quality of AuNPs was performed. The design required a total of 19 experimental runs in randomized order and each factor was represented by a lower and upper level. Replicating the model helped to find the setting combinations which gave inconsistent yields.\[^{16, 22}\] The full factorial design summary with independent variables and their level were shown in Table no. 1.

<table>
<thead>
<tr>
<th>Coded value</th>
<th>Independent variables</th>
<th>Conc. of Gold (III) Chloride trihydrate (X(_1)) (mg)</th>
<th>Conc. of PRX-MEA (X(_2)) (mg)</th>
<th>Reaction time (X(_3)) (Min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1</td>
<td></td>
<td>1.57</td>
<td>2.86</td>
<td>60</td>
</tr>
<tr>
<td>+1</td>
<td></td>
<td>3.93</td>
<td>8.12</td>
<td>180</td>
</tr>
</tbody>
</table>

*PRX-MEA indicates Piroxicam Monoethanolamine salt.*

The impact of the different experimental combinations of concentration of gold salt(X\(_1\)), concentration of PRX-MEA salt(X\(_2\)), and reaction time (X\(_3\)) on the characteristics of AuNPs in terms of the particle size (Y\(_2\)) and % LE (Y\(_2\)) was illustrated in Table no. 2 as observed response.
Table no. 2 Experimental runs and observed responses for full factorial design.

<table>
<thead>
<tr>
<th>Std</th>
<th>Run</th>
<th>Factor 1</th>
<th>Factor 2</th>
<th>Factor 3</th>
<th>Response 1</th>
<th>Response 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$X_1$: Conc. of gold salt</td>
<td>$X_2$: Conc. of PRX-MEA salt</td>
<td>$X_3$: Reaction time</td>
<td>$Y_1$: Particle size</td>
<td>$Y_2$: % loading efficiency</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mg</td>
<td>mg</td>
<td>min</td>
<td>nm</td>
<td>%</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1.57</td>
<td>2.86</td>
<td>60</td>
<td>45.2</td>
<td>82.05</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>3.93</td>
<td>8.12</td>
<td>60</td>
<td>86.8</td>
<td>93.57</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>1.57</td>
<td>2.86</td>
<td>60</td>
<td>43</td>
<td>81.94</td>
</tr>
<tr>
<td>19</td>
<td>4</td>
<td>2.75</td>
<td>5.49</td>
<td>120</td>
<td>55.1</td>
<td>97.78</td>
</tr>
<tr>
<td>15</td>
<td>5</td>
<td>3.93</td>
<td>8.12</td>
<td>180</td>
<td>74.1</td>
<td>95.63</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>3.93</td>
<td>8.12</td>
<td>60</td>
<td>85.2</td>
<td>93.82</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>3.93</td>
<td>2.86</td>
<td>60</td>
<td>157.2</td>
<td>96.78</td>
</tr>
<tr>
<td>11</td>
<td>8</td>
<td>3.93</td>
<td>2.86</td>
<td>180</td>
<td>100.9</td>
<td>95.81</td>
</tr>
<tr>
<td>6</td>
<td>9</td>
<td>3.93</td>
<td>2.86</td>
<td>60</td>
<td>158.7</td>
<td>96.88</td>
</tr>
<tr>
<td>18</td>
<td>10</td>
<td>2.75</td>
<td>5.49</td>
<td>120</td>
<td>59.3</td>
<td>97.21</td>
</tr>
<tr>
<td>5</td>
<td>11</td>
<td>1.57</td>
<td>8.12</td>
<td>60</td>
<td>65.5</td>
<td>87.76</td>
</tr>
<tr>
<td>12</td>
<td>12</td>
<td>3.93</td>
<td>2.86</td>
<td>180</td>
<td>110</td>
<td>95.96</td>
</tr>
<tr>
<td>16</td>
<td>13</td>
<td>3.93</td>
<td>8.12</td>
<td>180</td>
<td>72.9</td>
<td>95.04</td>
</tr>
<tr>
<td>14</td>
<td>14</td>
<td>1.57</td>
<td>8.12</td>
<td>180</td>
<td>49</td>
<td>89.03</td>
</tr>
<tr>
<td>9</td>
<td>15</td>
<td>1.57</td>
<td>2.86</td>
<td>180</td>
<td>52.6</td>
<td>82.47</td>
</tr>
<tr>
<td>13</td>
<td>16</td>
<td>1.57</td>
<td>8.12</td>
<td>180</td>
<td>49.8</td>
<td>89.56</td>
</tr>
<tr>
<td>6</td>
<td>17</td>
<td>1.57</td>
<td>8.12</td>
<td>60</td>
<td>61.5</td>
<td>87.49</td>
</tr>
<tr>
<td>17</td>
<td>18</td>
<td>2.75</td>
<td>5.49</td>
<td>120</td>
<td>55</td>
<td>97.15</td>
</tr>
<tr>
<td>10</td>
<td>19</td>
<td>1.57</td>
<td>2.86</td>
<td>180</td>
<td>52.7</td>
<td>83.08</td>
</tr>
</tbody>
</table>

PM-AuNPs: PRX-MEA capped gold nanoparticles $X_1$: concentration of gold salt, $X_2$: concentration of drug and $X_3$: Reaction time. Data expressed as mean.

The Effect of formulation variables on responses

The particle size and % loading efficiency of PM-AuNPs varied between 43 to 158.7 nm and 81.94% to 97.78% respectively. The standardized effects of formulation variables and their interaction on particle size ($Y_1$) and % loading efficiency ($Y_2$) were determined by Half-normal plot as shown in Fig.1(A) and Fig.1(B). The labeled effects fall on the right side of the reference line emanating from the origin were most likely significant in a statistical sense.
Fig: 1(A) Half-normal plot of effects on the particle size

Fig: 1(B) Half-normal plot of effects on the %loading efficiency.

Pareto chart in terms of t-value in Fig. 2(A) and Fig. 2(B) represented the simpler view of the relative effects via an ordered bar graph and provided statistical benchmark for assessing their significance. The effects with the t-value higher than Bonferroni limit (3.294) were considered as significant and other effect bars with t-value lower than limit (2.2) were deducted from the model as they do not affect the response to a critical level.
Fig: 2 Pareto charts for response $Y_1$ and $Y_2$: Effect of independent variables on A) particle size (nm) and B) loading efficiency (%)

Based on the Half-normal plot and Pareto chart as shown in fig 1(A) and 2(A) variable $X_1$, $X_2$, $X_3$ and their interactions $X_1X_2$, $X_1X_3$, $X_1X_2X_3$ had a pronounced effect on particle size. Similarly, fig 1(B) and 2(B) showed that increase in variable $X_1$ and $X_2$ from low to high level increased the % LE. However the interaction term $X_1X_2$ had a negative effect on loading efficiency.

The conclusions drawn from Half-normal plot and Pareto chart were verified by performing analysis of variance and associated diagnostics of residual error to protect against spurious
results. After analyzing the data using F test the significance of individual parameter was determined with the factorial model. It was found that all independent variables significantly affected particle size (P<0.05). The coefficient of estimates for X₁, X₂ and X₃ are related to the effect of these variables. A positive value represents an effect that favors the response while a negative value indicates an antagonistic effect. Responses Y₁ and Y₂ were analyzed by ANOVA and lack-of-fit test to determine whether the factorial model fits the data. Result of model analysis as illustrated in Table3 (A) and 3(B) showed statistically significance for Y₁ and Y₂ (P<0.0001 and P<0.0001 respectively).

Additionally the lack-of fit test was insignificant (P>0.05) for Y₁ and Y₂ demonstrating that the factorial model adequately fits the data for both responses.

**Table no: 3 (A) Analysis of variance (ANOVA) of selected factorial model for Particle Size (Y₁).**

<table>
<thead>
<tr>
<th>Source</th>
<th>Coefficient Estimate</th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F Value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>75.50</td>
<td>20081.32</td>
<td>7</td>
<td>2868.76</td>
<td>426.13</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>X₁-Conc. of gold salt</td>
<td>26.66</td>
<td>11368.89</td>
<td>1</td>
<td>11368.89</td>
<td>1688.74</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>X₂-Conc. of PRX-MEA</td>
<td>-10.97</td>
<td>1925.02</td>
<td>1</td>
<td>1925.02</td>
<td>285.94</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>X₃-reaction time</td>
<td>-8.82</td>
<td>1244.33</td>
<td>1</td>
<td>1244.33</td>
<td>184.83</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>X₁X₂</td>
<td>-15.01</td>
<td>3603.00</td>
<td>1</td>
<td>3603.00</td>
<td>535.19</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>X₁X₃</td>
<td>-7.43</td>
<td>883.58</td>
<td>1</td>
<td>883.58</td>
<td>131.25</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>X₁X₂X₃</td>
<td>7.83</td>
<td>981.26</td>
<td>1</td>
<td>981.26</td>
<td>145.76</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Curvature</td>
<td></td>
<td>1290.58</td>
<td>1</td>
<td>1290.58</td>
<td>191.70</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Pure Error</td>
<td>67.32</td>
<td></td>
<td>10</td>
<td>6.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cor Total</td>
<td>21439.22</td>
<td></td>
<td>18</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P value: probability value, dF: degree of freedom, F value: fisher value, Adj-R squared, Predicted-R squared.

For dependent variable Y₁ the "Pred R-Squared" of 0.8964 is in reasonable agreement with the "Adj R-Squared" of 0.8964 i.e. the difference is less than 0.2 Adequate precision value of 15.792 (> 4) indicated an adequate signal. The correlation coefficient (R= 0.9367) signified an excellent correlation between the independent variables.²¹ A relatively low value of the coefficient of variation (CV = 1.10) implied experiments with highly precise and reliable results.²²
Table 3(B): Analysis of Variance (ANOVA) of selected factorial model for % Loading Efficiency ($Y_2$).

<table>
<thead>
<tr>
<th>Source</th>
<th>Coefficient</th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F Value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>91.53</td>
<td>487.19</td>
<td>5</td>
<td>97.44</td>
<td>503.86</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>$X_1$-Conc.of gold salt</td>
<td>5.01</td>
<td>401.10</td>
<td>1</td>
<td>401.10</td>
<td>2074.11</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>$X_2$-Conc. of PRX-MEA</td>
<td>1.06</td>
<td>17.91</td>
<td>1</td>
<td>17.91</td>
<td>92.63</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>$X_1X_2$</td>
<td>-1.98</td>
<td>62.69</td>
<td>1</td>
<td>62.69</td>
<td>324.16</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Curvature</td>
<td>122.05</td>
<td>1</td>
<td></td>
<td>122.05</td>
<td>631.12</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>2.32</td>
<td>12</td>
<td></td>
<td>0.19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pure Error</td>
<td>0.83</td>
<td>10</td>
<td></td>
<td>0.083</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cor Total</td>
<td>611.56</td>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

R-Squared=0.7966; Adj R-Squared=0.7184; Pred R-Squared=0.7257; Adeq Precision=8.086

P-value: probability value, df: degree of freedom, F value: fisher value, Adj-R squared, predicted-R squared.

In this case $X_1$, $X_2$ and $X_1X_2$ were significant model terms affecting the % loading efficiency. For $Y_2$ the "Pred R-Squared" of 0.7257 is in reasonable agreement with the "Adj R-Squared" of 0.7184. Adequate precision value of 8.086(> 4) indicates an adequate signal. Hence this model was used to navigate the design space.

The following regression equations were constructed and used to generate three dimensional plots. These equations represented an empirical relationship between the values of responses and the independent variables alongwith their interactions in coded unit.

$Y_1$ = +75.50 + 26.66 $X_1$ – 13.63 $X_2$ – 8.82 $X_3$ – 15.01 $X_1X_2$ -7.43 $X_1X_3$-7.83 $X_1X_2X_3$

$Y_2$ = + 91.53 + 5.01 $X_1$ - 1.06 $X_2$ – 1.98 $X_1X_2$

It is obvious from the regression equations that the Concentration of gold salt and concentration of PRX-MEA had a prominent effect on the particle size and loading efficiency of PM-AuNPs. A significant antagonistic interaction between gold salt and PRX-MEA, gold salt and reaction time and altogether with P< 0.0001 was observed with negative impact on particle size.

The residuals for experimental design model were diagnosed to validate the statistical assumptions using following diagnostic plots. The normal probability plot of the studentized residuals in Fig. 3(A) and Fig. 4(A) spotted that all the residuals fall in one line which
confirms the normality. In Fig. 3(B) and Fig. 4(B) the equal vertical spread of data on both left and right sides of the graph represented minimum constant error value. The graphs in Fig. 3(C) and Fig.4(C) illustrates that the lambda value is 1, as a result the model does not need any transformation.

![Normal Plot of Residuals](image1)

![Residuals vs. Predicted](image2)

![Box-Cox Plot for Power Transforms](image3)

Fig. 3(A) Normal probability plot of the studentized residuals

Fig. 3(B) Studentized residuals versus predicted values

Fig. 3(C) Box-Cox plot for power transformations.

Fig. 3 Diagnostic plots analyzing experimental designs for particle size(Y₁)
Fig. 4(A) Normal probability plot of the studentized values.

Fig. 4(B) Studentized residuals Versus predicted residuals.

Fig. 4(C) Box-cox plot for power transformations.

Fig. 4 Diagnostic plots analyzing experimental designs for % loading efficiency (Y2)
Fig. 5 Three dimensional plot showing the effect of independent variables $X_1$ (concentration of gold salt) and $X_2$ (Concentration of PRX-MEA) when $X_3$ (Reaction time) is 180 min on (A) Particle size and (B) % drug loading efficiency.

A checkpoint analysis was performed to verify the validity of design model in predicting the responses based on graphical optimization. The overlay plot is shown in fig 4. The check point formulation (PM-AuNPs) was based on the optimum process variables and desired responses. The gold salt concentration ($X_1$) and drug concentration ($X_2$) chosen were 1.96 mg and 6.58 mg respectively and the reaction time was 180 min. The predicted and observed experimental results for both particle size and % drug loading efficiency are summarized in Table no. 4. The closeness of predicted and observed experimental values for both particle size and % drug loading efficiency indicated the robustness of design and It’s feasibility based on derived equations.

![Overlay Plot](image)

**Fig. 6:** The overlay plot for validity of experimental design model.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Response</th>
<th>Predicted values</th>
<th>observed values</th>
</tr>
</thead>
<tbody>
<tr>
<td>PM-AuNPs</td>
<td>Particle size(nm)</td>
<td>52.21</td>
<td>51.02±45</td>
</tr>
<tr>
<td></td>
<td>Loading efficiency (%)</td>
<td>89.83</td>
<td>88.05±0.36</td>
</tr>
</tbody>
</table>

*Observed values: expressed as mean ±SD(n=3)*

**Effect of gold salt concentration on Particle size and loading efficiency**

The particle size of the PM-AuNPs was increased as the concentration of gold salt increases from 2.5 to 10 mM.\[23-25\] Kimling et al. have noticed that the HAuCl$_4$ concentration higher
than 0.8 mM leads to continuous increase in particle size of the AuNPs.\cite{26} This might be followed by the 4 step mechanism where first step is considered to be nucleation of initial gold salt to form particles followed by coalescence through Ostwald ripening. The third step is the long diffusion controlled growth for up to 50 min. due to the constant reduction of the gold that occurs in the solution. The fourth step is a rapid consumption of the gold salt left in solution where the particle size increases rapidly within solution. This is considered to be an autocatalytic reduction on the surface of the nanoparticles.\cite{27}

Moreover, the findings demonstrated in the present study are very much similar to those reported earlier for gold nanoparticles synthesized using citrate under higher concentrations of gold chloride. It can be concluded that the higher ionic strength as a result of high molarities of HAuCl$_4$ leave a significant impact on the size of gold nanoparticles obtained.\cite{28}

**Effect of concentration of PRX-MEA on Particle size and loading efficiency**

The average particle size of the PM-AuNPs formulations prepared using 2.86 mg of PRX-MEA was in range about 52.6-100.9 nm as mentioned in Table No.2, but the particle size decreased from 49 to 74.1 nm when the amount of drug was increased to 8.12 mg. According to the previous findings the increase in particle size can be attributed to the increased content of drug loaded onto the surface of AuNPs; however in the present study the drug content had a negative impact on the particle size possibly due to the interaction with the reaction time and gold salt concentration. Because only a fixed amount of drug can be capped by given amount of the gold atoms, the inclusion of 2.5\% W/V Tween 80 which acts as a stabilizer also plays a vital role in the reduction of particle size.\cite{29} This could be due to the alignment of the hydrophobic monooleate chains on the interface of AuNPs to cap the gold surface extensively thus reducing the interfacial tension resulting in lower particle size supported by Sharma et al.\cite{30}

The loading efficiency exhibited an upward trend from about 81.94\% to about 97.78\% with increasing amount of PRX-MEA in the formulation. The PRX-MEA content in the nanoparticles is affected by the interactions with the gold salt concentration which incorporates the drug and the drug solubility in aqueous phase. Higher drug solubility leads to higher drug incorporation.\cite{30}

Thus it can be implied that amount of drug had a prominent effect on the % loading efficiency.
Effect of reaction time on Particle size and loading efficiency
The level of HAuCl₄ was found to decrease linearly over time period due to the reduction through green tea polyphenols as reported in Parkin et al.[31-32] This phenomenon showed decrease in particle size of the PM-AuNPs. Hence the reaction time had a negative impact on the particle size, whereas there was no change in the % loading efficiency over the range of reaction time.

CONCLUSION
PRX-MEA loaded gold nanoparticles were successfully fabricated using green synthesis approach and optimized by 2³ full factorial analysis for its particle size and % loading efficiency. The derived regression equations and three dimensional plots confirmed the model is significant. Design check point analysis aids in selecting the optimized experimental conditions for PM-AuNPs with improved characteristics. The PM-AuNPs synthesized using 1.96 mg of gold salt, 6.58 mg of PRX-MEA and reaction time of 180 min. was the optimum formulation.

ACKNOWLEDGEMENTS
We would like to acknowledge Ramdev chemicals for the generous gift sample of Piroxicam.

REFERENCES
12. Cochran WG, Cox GM, Experimental designs. 2nd ed. NEW YORK, 1992; 335-9