Optimization of Process Conditions for Preparing an Iron-polysaccharide Complex by Response Surface Methodology

Y.-y. Zhang and J.-h. Liu*

Department of Pharmaceutics, Qingdao University of Science and Technology, 53 Zhengzhou Road, Qingdao, Shandong 266042, China

The synthetic process of an iron-polysaccharide complex drug substance was improved to make it more suitable for industrial scale production. The complex was synthesized by reacting $FeCl_3$ and syrup in basic solution followed by separation and purification by ethanol precipitation and low speed centrifugation. Process conditions were optimized using response surface methodology (RSM) based on the central composite design (CCD). A quadratic polynomial equation was developed, which indicated the effect of variables on the yield of the complex. The optimal conditions were 20 % Na_2CO_3 aqueous solution 51.7 g, concentration of $FeCl_3 \cdot 6H_2O$ $w = 30.3 \%$, and the temperature of the ferric hydroxide synthesis 6.5 °C. Under these conditions, the experimental yield was 40.1 %, which was agreed closely with the predicted value (38.9 %). The results of scale-up experiments indicated that the optimized process conditions could also improve the yield at larger scales.

Key words:
Iron-polysaccharide complex, drug substance, process conditions, response surface methodology

Introduction

In recent years, polysaccharides from plants, microorganisms (fungi and bacteria), algae, and animals have attracted much attention, due to their importance as bioactive substances or raw materials in several industries, in particular, the food and drug industries. Iron, Fe(III), is one kind of essential minor element in human body, which constitutes the core part of hemoglobin. Iron-saccharide complexes, such as iron-dextran, iron-sucrose, iron-gluconate, and iron-polysaccharide, have been used for the treatment of iron-deficiency anemia.[1,2] Certain types of polysaccharides are known to interact with Fe(III) to form iron-polysaccharide complex.[3] It is a complex of ferric iron and low-molecular-weight polysaccharide which is produced by extensive hydrolysis of starch catalyzed by enzymes.[4,5] It has been extensively used as an oral hematinic apparently without side-effects.[6,7] Compared with other iron supplements, such as ferrous sulfate associated with serious adverse events, the iron-polysaccharide complex has the advantages of generally good tolerability, good bioavailability and a high percentage of iron which will help to reduce the amount of material needed to be administered for a given dosage.[8]

However, there have been only a few reports on the synthesis of this iron-polysaccharide complex. The US patent 3821192 dealt with the process for preparing an iron-saccharide complex from the reaction of Fe^{3+} and a low molecular dextrin.[9] In this process, methanol was used as precipitator to precipitate the complex and high-speed centrifuge (25 000 rpm) was used to separate the precipitate. Bereman and Berg[10] described a method for preparing the complex by the neutralization of a $FeCl_3$-carbohydrate solution. Somsook *et al.*[11] also prepared the iron-rice starch, iron-sucrose, iron-dextran complexes using the reported methods by Bereman and Berg.[10] However, the above iron-saccharide complexes reported by Somsook *et al.*[11] are not the complexes of ferric iron and a low-molecular-weight polysaccharide. The disadvantages of the process described in US patent 3821192 are the high toxicity of methanol and high cost of the investment and operation using a high-speed centrifuge, which is not suitable for industrial scale production of the iron-polysaccharide complex according to the ICH guidelines (1997).[12] The aim of the present study is to develop a novel process for the efficient preparation of the complex to overcome the drawbacks and provide a satisfactory process for industrial scale. The work focuses on the optimization of the parameters for the synthesis of the complex using response surface methodology (RSM). This paper reports the relationships between the factors (20 % Na_2CO_3 aqueous

*Corresponding author. E-mail address: liujh@qust.edu.cn (J.-h. Liu).
Tel.: +86 532 84022755; Fax: +86 532 84023271.

solution, the concentration of $FeCl_3 \cdot 6H_2O$ and the temperature of the ferric hydroxide synthesis), and the response (the yield of the complex), as well as the optimum conditions by Box–Behnken central composite design (CCD) and RSM analysis.
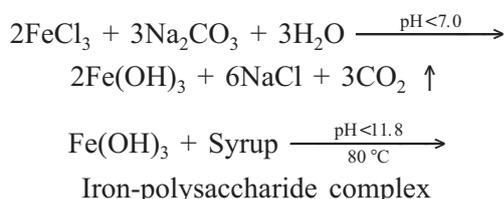
Materials and methods

Reagents

Syrup (composed of 26 % low-molecular-weight polysaccharide and 74 % water; produced by hydrolysis of starch catalyzed by enzymes) was obtained from Qingdao Monosodium Glutamate Manufactory (Qingdao, China); Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$, $M_W = 270.3$) was acquired from Shanghai Chemicals (Shanghai, China); sodium carbonate and sodium hydroxide were obtained from Sinopharm Chemical Reagent Co.Ltd (Shanghai, China). All chemicals used were of analytical grade.

Reaction mechanism

The reaction mechanism for iron-polysaccharide complex synthesis is as follows:

$$2FeCl_3 + 3Na_2CO_3 + 3H_2O \xrightarrow{\text{pH}<7.0}$$
$$2Fe(OH)_3 + 6NaCl + 3CO_2 \uparrow$$

$$Fe(OH)_3 + Syrup \xrightarrow[80\,°C]{\text{pH}<11.8}$$
Iron-polysaccharide complex

Preparation of iron-polysaccharide complex

Preparation of ferric hydroxide

To 43 g of syrup a certain percentage (w/%) of aqueous solution of ferric chloride hexahydrate (containing 20 g of $FeCl_3 \cdot 6H_2O$) was added and stirred in a 1.0 L tank reactor. To this mixture a certain amount (g) of $w = 20$ % aqueous solution of sodium carbonate (Na_2CO_3) was added dropwise with intense stirring within 1–2 h, at a definite temperature (this temperature is defined as the temperature of the ferric hydroxide synthesis). The mixture was then stirred constantly for 15–30 min until the precipitation of ferric hydroxide was complete.

Complexation of ferric iron and polysaccharide

At room temperature, the above mixture was added a certain amount of sodium hydroxide (NaOH) solution (6 mol L^{-1}) dropwise with stirring until the pH of the solution was equivalent to or above 11.8. The mixture was then heated to 80 °C until a uniform solution of complex formed by the complexation reaction of syrup and ferric iron was observed.

Separation and purification

The reaction mixture was then cooled to room temperature and ethanol was added in an amount equal in volume to the amount of the mixture. This addition precipitated the complex and the mixture was then centrifuged at 5000 rpm (LXJ-IIB centrifuge, AnTing Scientific Instruments Co. Ltd., Shanghai, China) to separate the precipitate. Then 150 mL of 50–60 % ethanol was added and mixed with the precipitate to wash it at least three times. This mixture was centrifuged as before. The precipitate was dried by spray drying (LPG-25A High-Speed Centrifugal Spray Dryer, Changzhou Yibu Drying Equipment Co. Ltd) with inlet temperature 200 °C, outlet temperature 80–90 °C, rotation speed 25 000 rpm to produce a desired particle size (<0.45 mm).

Assay

Dissolve about 300 mg of iron-polysaccharide complex, accurately weighed, in 30 mL of water contained in a conical flask with stopper. Add 10 mL of hydrochloric acid test solution and heat at about 100 °C for 5 min, cool, and add 20 mL of potassium iodide, stopper, placed in the dark for 5 min and add 50 mL of water. Titrate with 0.1 mol L^{-1} sodium hyposulfite, adding 3 mL of starch indicator solution (1 %), determining the endpoint potentiometrically, and continue the titration until the blue color disappears. Perform a blank determination, and make any necessary correction. Each mL of 0.1 mol L^{-1} sodium hyposulfite is equivalent to 5.585 mg of Fe (iron).

Experimental design

Response surface methodology (RSM) is a collection of mathematical and statistical techniques that are useful for modeling and analysis of problems in which a response of interest is influenced by several variables.[13] It helps to optimize the effective parameters with a minimum number of experiments, and also to analyze the interaction between the parameters.[14,15] RSM has been successfully used for optimizing complex process, extraction technology, conditions of enzyme reaction, and so on.[16,17]

In this study, RSM was adopted to optimize the process conditions for maximum production of the iron-polysaccharide complex. The amount (g) of $w = 20$ % Na_2CO_3 aqueous solution (Z_1), the concentration (w/%) of a $FeCl_3 \cdot 6H_2O$ aqueous solution (Z_2) and the temperature of the ferric hydroxide synthesis (Z_3) were used as the independent variables to maximize the response (Y, the yield of the

complex). The code factors of the three independent variables above were expressed as X_1, X_2 and X_3, respectively. The variables and their respective levels are presented in Table 1. The experiments were designed according to the Box-Behnken central composite design (CCD) which included 15 experiments of three variables at three levels (–1, 0, +1) with 3 additional runs at the centre point level. A quadratic polynomial equation as given by eq. (1), was developed to study the effects of variables on the yield.

$$Y = A_0 + \Sigma A_i \, X_i + \Sigma A_{ii} X^2_{ii} + \Sigma\Sigma A_{ij} X_i X_j \quad (1)$$

where Y is the predicted response, A_0 is the constant and A_i, A_{ii}, A_{ij} are the regression coefficients of the model obtained by multiple regression (which represent the linear, quadratic and cross-product effects of the factors on the response, respectively), and X_i, X_j represent the independent variables.

T a b l e 1 – *Variables and experimental design levels for response surface*

Independent variables	Symbol		Coded levels		
	coded	uncoded	–1	0	1
Amount of 20 % Na_2CO_3 aqueous solution/g	X_1	Z_1	45	50	55
Concentration of $FeCl_3 \cdot 6H_2O$ aqueous solution (w/%)	X_2	Z_2	20	30	40
Temperature of the ferric hydroxide synthesis/°C	X_3	Z_3	0	5	10

The Design Expert software (version 8.0.1, Stat-Ease Inc., Minneapolis, USA) was used for regression and graphical of the data obtained. The fit of regression model was checked by the coefficient of determination (R^2) and the adjusted coefficient of determination (R^2_{Adj}). An analysis of variance (ANOVA), a regression analysis and the plotting of response surface were performed to fit quadratic polynomial equations for all response variables and obtain the optimum conditions for the production of the complex.

Results and discussion

Model fitting

The experimental values of the yield of the iron-polysaccharide complex at points based on the experimental design were shown in Table 2. The data obtained were used to calculate the coefficients of the quadratic polynomial equation, which were used to predict the yield. The best fitting model was

T a b l e 2 – *Box–Behnken central composite design and results for RSM*

Run	Variable levels			Yield[b]
	X_1[a]	X_2[a]	X_3[a]	Y/%
1	–1	–1	0	20.48
2	–1	1	0	27.17
3	1	–1	0	30.61
4	1	1	0	33.53
5	0	–1	–1	16.94
6	0	–1	1	34.73
7	0	1	–1	26.65
8	0	1	1	28.68
9	–1	0	–1	17.41
10	1	0	–1	22.87
11	–1	0	1	21.62
12	1	0	1	32.21
13	0	0	0	37.52
14	0	0	0	37.97
15	0	0	0	37.24

[a]$X_1 = (Z_1–50)/5.0$, $X_2 = (Z_2–30)/10$, $X_3 = (Z_3–5)/5.0$
[b]the yield is defined as the mass ratio of the iron-polysaccharide complex to the $FeCl_3 \cdot 6H_2O$

determined via regression using the Design Expert 8.0.1 software. By multiple regression analysis on the experimental data, the terms found to be significant were combined into the following fitted quadratic polynomial equation to predict the production of the complex:

$$Y = 37.58 + 4.07X_1 + 1.66X_2 + 4.17X_3 – 6.43X_1X_1 –$$
$$– 3.20X_2X_2 – 3.94X_2X_3 – 7.62X_3X_3 \quad (2)$$

The ANOVA for the response surface quadratic model is provided in Table 3. The goodness of the model fit was checked by determination coefficient (R^2).[18] In this case, the value of R^2 is 98.69 % and

T a b l e 3 – *Analysis of variance (ANOVA) for the fitted quadratic polynomial model*

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	725.09	9	80.56	41.81	0.0004[a]
Residual	9.63	5	1.93		
Total	734.72	14			

$R^2 = 0.9869$ $R^2_{Adj} = 0.9633$

[a]Significant at 1 % level.

the value of R^2_{Adj} (96.33 %) is also high to advocate that only 3.67 % of the complex synthesis was not explained by the model. It indicated that the fitted quadratic model accounted for more than 96.33 % (R^2_{Adj}) of the variations in the experimental data, which were found to be highly significant ($p < 0.001$). An F-value test was also conducted to evaluate the goodness of the model. The F-ratio of quadratic regression model was 41.81. On this basis, it can be concluded that the model is well accurate for predicting the yield. The results of analysis showed that the experimental values were significantly in agreement with the predicted values and also suggested that the model of eq. (2) was satisfactory and accurate (Fig. 1). It was considered reasonable to use the regression model to analyze trends in the responses.
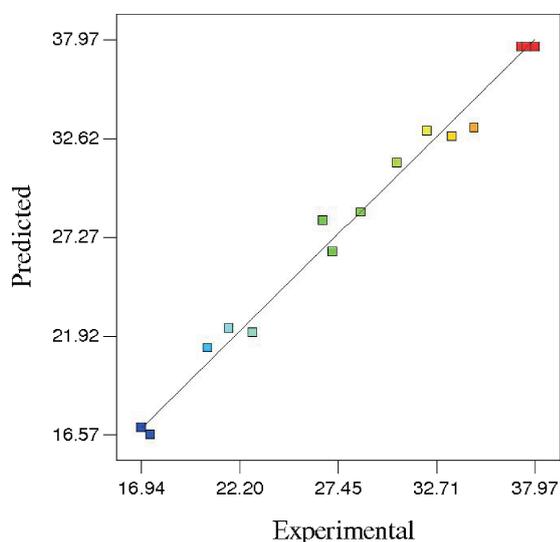

F i g . 1 – *Model (predicted) vs. experimental values in the synthesis of iron-polysaccharide complex*

Statistical analysis of effect estimates for response surface quadratic model was determined by F-value and p-value, which is shown in Table 4. For any of the terms in the model, a large F-value and a small p-value would indicate a more significant effect on the response variable.[19] Thus, the variable with the largest effect on the yield was the quadratic term of X_3, followed by the quadratic term of X_1 and the linear terms of X_1 and X_3 ($p < 0.001$); the linear term and the quadratic term of X_2 as was one cross-product ($X_2 X_3$) were significant at 5 % level ($p < 0.05$). The effect of the remaining terms was insignificant ($p > 0.05$) (Table 4).

Analysis of response surfaces

To visualize the effect of the independent variables on the dependent variable, surface response and contour plots of the quadric polynomial model

T a b l e 4 – *Significance test of regression coefficient of model*

Parameter	Estimate	Standard Error	F-value	p-value
Intercept	37.58	0.80	41.18	<0.0001[b]
X_1	4.07	0.49	68.69	0.0004[b]
X_2	1.66	0.49	11.42	0.0197[a]
X_3	4.17	0.49	72.23	0.0004[b]
$X_1 \cdot X_1$	−6.42	0.72	79.12	0.0003[b]
$X_1 \cdot X_2$	−0.94	0.69	1.84	0.2326
$X_1 \cdot X_3$	1.28	0.69	3.41	0.1239
$X_2 \cdot X_2$	−3.20	0.72	19.66	0.0068[a]
$X_2 \cdot X_3$	−3.94	0.69	32.22	0.0024[a]
$X_3 \cdot X_3$	−7.62	0.72	111.36	0.0001[b]

[a]Significant at 5 % level.
[b]Significant at 1 % level.

were generated by varying two of the independent variables within the experimental range while holding the other constant at the centre point using the Design Expert 8.0.1 software, which are depicted in Fig. 2. These response surface plots and their respective contour plots provide a visual interpretation of the interaction between two variables and are used to obtain the optimum conditions for the production of the complex. The shapes of the contour plots provide a measure of the significance of the mutual interactions between the variables. An elliptical contour plot indicates a significant interaction between variables.

Fig. 2(a) represents the effects of different amount of 20 % Na_2CO_3 aqueous solution (X_1), the concentration of $FeCl_3 \cdot 6H_2O$ aqueous solution (X_2) on the complex synthesis at a constant temperature of the ferric hydroxide synthesis (X_3) of 5 °C. It is clear from the figure that both higher and lower X_1 or X_2, decreased the yield. An appropriate amount of 20 % Na_2CO_3 solution was critical to provide a suitable pH for forming more $Fe(OH)_3$ which may result in increasing the complex. Less or excessive Na_2CO_3 could lead the system pH apart from the appropriate pH on which the form of $Fe(OH)_3$ is dependent. The addition of the Na_2CO_3 solution brought the pH of reaction solution from strongly acidic to weakly acidic. Moreover, the pH of the solution is still acidic (below pH 7.0) until $Fe(OH)_3$ is complete. This observation is in agreement with previous investigation. Sipos *et al.*[20] researched the rod-like Fe(III) oxyhydroxide particles in Fe(III)-polysaccharide solutions. They also indicated that the
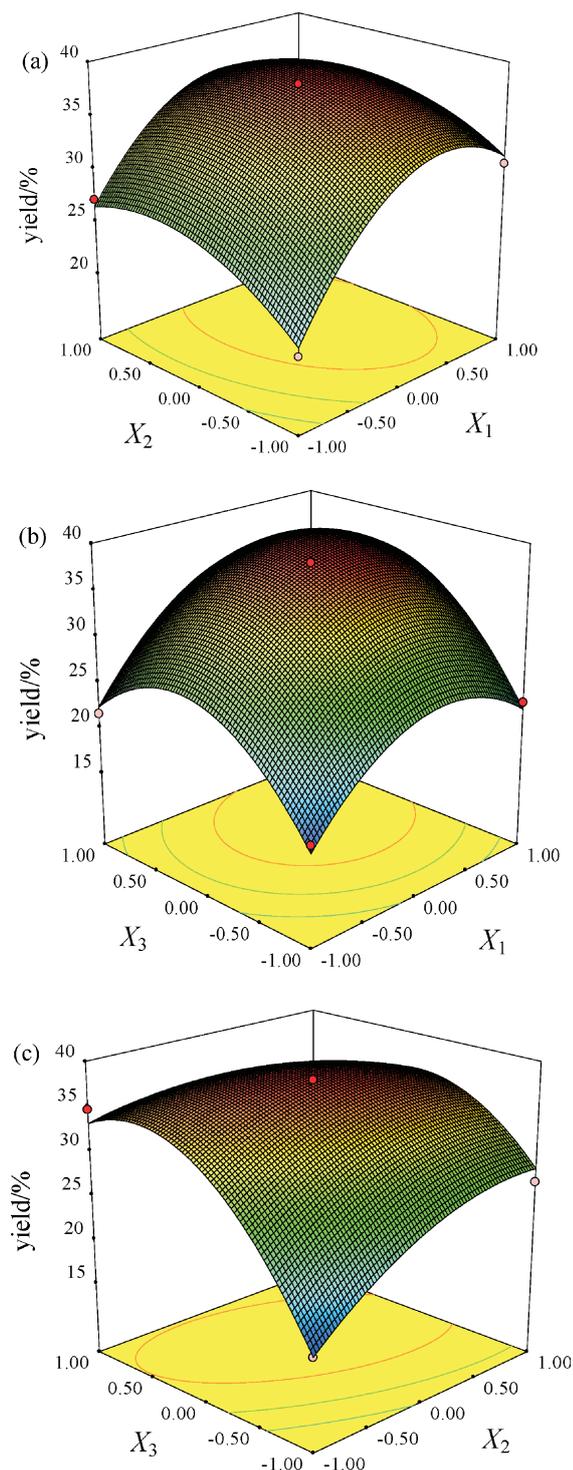
F i g . 2 – *Surface plot and contour plot of the combined effects of X_1 and X_2 (a); X_1 and X_3 (b); X_2 and X_3 (c) on the yield of iron-polysaccharide complex at another coded level of zero*

metal binding started in the acidic pH region with the $Fe(OH)_3$ precipitating at $3.5 \leq pH \leq 6$ and the precipitate dissolved at pH > 6. At any designed amount of 20 % Na_2CO_3 solution, the yield was similarly affected by $FeCl_3 \cdot 6H_2O$ concentration. Excess of $FeCl_3$ may cause the augmentation of

$Fe(OH)_3$ and result in the increase of the system viscosity which is unfavorable for the complexation of ferric ion and syrup. The increase in the yield was found to be very sharp with change of the Na_2CO_3 amount and the $FeCl_3 \cdot 6H_2O$ concentration (Fig. 2(a)). Fig. 2(b) shows the effects of the 20 % Na_2CO_3 aqueous solution (X_1) and the temperature of the ferric hydroxide synthesis (X_3) on the complex synthesis at a constant concentration of $FeCl_3 \cdot 6H_2O$ aqueous solution (X_2). X_3 also exerted significant influence on the synthesis of the complex. It was observed that that the yield increased as X_3 increased, reached the maximum value, and then gradually decreased. Fig. 2(c) depicts the effects of the concentration of $FeCl_3 \cdot 6H_2O$ aqueous solution (X_2) and the temperature of the ferric hydroxide synthesis (X_3), and their mutual interaction on the complex synthesis at a constant amount of 20 % Na_2CO_3 aqueous solution (X_1). The yield of the complex passed through a maximum and then decreased with the increasing of X_3. As indicated, the interaction between X_2 and X_3 was significant ($p < 0.05$).

The optimum values of the selected variables were obtained by solving the regression equation (eq. (2)) using Matlab software (Version 6.5.0, Relase13, the Math Works Inc., Natick, MA). The optimum values of the test variables in the coded units were as follows: $X_1 = 0.344$, $X_2 = 0.026$, and $X_3 = 0.296$. The natural values obtained by putting the respective values of X_j into $X_j = (Z_j–Z_{0j})/\Delta_j$ (see Table 2) were: the amount of 20 % Na_2CO_3 aqueous solution = 51.7 g, the concentration of a $FeCl_3 \cdot 6H_2O$ aqueous solution w = 30.3 %, and the temperature of the ferric hydroxide synthesis = 6.5 °C, with the predicted yield of the iron-polysaccharide complex of 38.9 %.

In order to verify the prediction of the model, the optimum process conditions were applied to three independent replicates for the iron-polysaccharide complex synthesis. The average yield is 40.1 %, which is in good agreement with the predicted value (38.9 %). This indicates that the model can be considered quite reliable for predicting the effect of each factor on the yield of the complex.

Scale-up experiment

After obtaining the optimum process conditions using RSM, we tested the feasibility of scale-up. Tests were performed in both a 5 L reactor and a 150 L reactor. At the 5 L scale, $FeCl_3 \cdot 6H_2O$ feeding was increased from 20 g to 0.36 kg, 18 times enlarged. Table 5 shows the experimental results. The yields of the complex at this scale is in agreement with that at 20 g of $FeCl_3 \cdot 6H_2O$ feed-

T a b l e 5 – *Scale-up test results*

Run	FeCl$_3$ · 6H$_2$O/kg	Iron-polysaccharide complex/kg	Yield/%
1	0.36	0.1442	40.05
2	0.36	0.1458	40.50
3	0.36	0.1509	41.92
4	20.0	8.0660	40.34
5	20.0	8.0821	40.41
6	20.0	8.0279	40.14

ing. Then, based on the results of 5 L scale, production of the complex was carried out at a pilot-scale with a 150 L reactor. Ethanol instead of methanol was used to precipitate the complex because ethanol is more favorable than methanol which is with higher toxicity in the production of the drug substances. A low-speed industrial three-column filtering centrifuge (SS450-type, Liuzhou Zinc Products Co., Ltd., China) was employed to separate the complex precipitate at 2000 rpm with maximum handling capacity of 3000 L h^{-1}. The low-speed industrial centrifuge rather than a high-speed centrifuge with maximum speed of 20 000 rpm and maximum handling capacity of 12 L h^{-1} was used here, which extremely reduced the cost of the investment and operation of the separation and purification process, by about 90 %. At the 150 L scale, FeCl$_3$ · 6H$_2$O feeding was increased from 0.36 kg to 20 kg, about 56 times enlarged. In order to enhance the product yield per reactor volume, the concentration of FeCl$_3$ was increased in the scale-up experiment, thus, the higher amounts of FeCl$_3$ were fed in. The experimental results indicate that it is feasible. Shown in Table 5, the yields at 20 kg of FeCl$_3$ · 6H$_2$O feeding reached higher than 40 %, indicating that the process developed here is flexible and stable and can be applied in the industrial production of the complex. The detection results of three batches of iron-polysaccharide complex obtained from 150 L scale-up experiment are described in Table 6.

Conclusions

The synthesis of the iron-polysaccharide complex was successfully optimized using RSM, whereas a satisfactory quadratic polynomial model was derived and demonstrated. The predicted model fits well with the experimental results, which is sufficient to describe and predict the responses. The amount of 20 % Na$_2$CO$_3$ aqueous solution, 51.7 g; concentration of the FeCl$_3$ · 6H$_2$O aqueous solution, w = 30.3 %; and temperature of the ferric hydroxide synthesis, 6.5 °C were found to be the optimum conditions for achieving the maximum yield of the complex. Under the optimum conditions, the average yield of product reached 40.1 %, which is in good agreement with the predicted yield, and suggests that the model was satisfactory and accurate. The results of scale-up experiment indicate that the optimized process conditions could also improve the yield of the complex at larger scales. The use of the low-speed industrial centrifuge extremely reduced the cost of the investment and operation, at least 90 %. Thus, the process developed here has been shown to be flexible and stable, cheaper, environment-friendly, and can be applied in the industrial production of the complex.

List of symbols

w – mass fraction, %
M_W – molecular weight
R^2 – determination coefficient
R^2_{Adj} – adjusted coefficient of determination

References

1. *Hudson, J. Q., Comstock, T. J.,* Clin. Ther. **23** (2001) 1637.
2. *Kane, R. C.,* Curr. Ther. Res. **64** (2003) 263.
3. *Ma, Y., Liu, M., Yue, X.-L., Zha, Z.-B., Dai, Z.-F.,* Int. J. Biol. Macromol. **46** (2010) 109.
4. *Coe, E. M., Bowen, L. H., Speer, J. A., Bereman, R. D.,* J. Inorg. Biochem. **57** (1995) 287.
5. *Coe, E. M., Bowen, L. H., Speer, J. A., Wang, Z., Sayers, D. E., Bereman, R. D.,* J. Inorg. Biochem. **58** (1995) 269.
6. *Zhang, Y.-Y., Liu, J.-H., Su, F., Lui, Y.-T., Li, J.-F.,* Curr. Ther. Res. **70** (2009) 104.

T a b l e 6 – *The detection of the obtained iron-polysaccharide complex product*

No.	Iron content/%	Impurity content/%				Water/%
		Na$^+$	Cl$^-$	Fe^{3+}	Polysaccharide	
1	41.36	2.92	1.34	<0.2	<0.5	5.31
2	41.84	2.81	1.49	<0.2	<0.5	4.47
3	42.07	2.85	1.27	<0.2	<0.5	4.83

7. *Zheng, J., Yue, X.-L., Dai, Z.-F., Wang, Y., Liu, S.-Q., Yan, X.-F.,* Acta Biomater. **5** (2009) 1499.

8. *Raghuveer, T. S., McGuire, E. M., Martin, S. M., Wanger, B. A., Rebouché, C. J., Buettner, G. R., Widness, J. A.,* Pediatr. Res. **52** (2002) 964.

9. *Kenneth, O. M., Chandrakant, R. J.,* Process for preparing an iron-saccharide complex, USP3821192, 1974.

10. *Bereman, R. D., Berg, K. A.,* Inorg. Chim. A. **155** (1989) 183.

11. *Somsook, E., Hinsin, D., Buakhrong, P., Teanchai, R., Mophan, N., Pohmakotr, M., Shiowatana, J.,* Carbohyd. Polym. **61** (2005) 281.

12. International Conference on Harmonization (ICH), Technical requirements for the registration of pharmaceuticals for human use, Q3C: impurities: guideline for residual solvents, 1997, Step 4.

13. *Montgomery, D. C.*, Design and Analysis of Experiments, 5th ed., John Wiley and Sons, New York, USA, 2001.

14. *Azargohar, R., Dalai, A. K.,* Micropor. Mesopor. Mat. **85** (2005) 219.

15. *Liu, J.-H., Zhang, Y.-Y., Xia, Y.-M., Su, F.,* Chem. Biochem. Eng. Q. **24** (2010) 203.

16. *Guo, X., Zou, X., Sun, M.,* Carbohyd. Polym. **80** (2010) 344.

17. *Sun, Y.-X., Li, Y.-J., Li, M.-Q., Tong, H.-B., Yang, X.-D., Liu, J.-C.,* Carbohyd. Polym. **75** (2009) 575.

18. *Celebi, N., Yildiz, N., Demir, A. S., Calimli, A.,* J. Supercrit. Fluids **47** (2008) 227.

19. *Yuan, Y., Gao, Y.-X., Mao, L., Zhao, J.,* Food Chem. **107** (2008) 1300.

20. *Sipos, P., Stpierre, T. G., Tombacz, E., Webb, J.*, J. Inorg. Biochem. **58** (1995) 129.