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Evaluation of the Release of Nanoconjugate Recombinant Streptokinase Using State-Ease Software Application

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Abstract

Controlled drug release is a process in which a nanoparticle specifically incorporated into a medicine is separated from the medicine in a predetermined and desirable manner and the retention time of the drug in the biological system is increased. Therefore, estimation of the lifespan of conjugated nanodrug relative to the retention time of the drug itself is of great importance. In the present study, nanoconjugate release was designed based on RSM statistical method and was tested at different temperatures, pH values, and rotation times. Finally, samples were tested by two methods including high-performance liquid chromatography (HPLC) and absorption concentration reading. The resulting data were analyzed and the drug release stability was calculated in order to obtain the optimum time.

Keywords: Test Design, Nanodrug, RSM, HPLC

Introduction

In order to cover desire effects and eventually leave the body at the right time, a medicine should have sufficient half-life it is occasionally observed that some drugs do not have sufficient half-life and are removed and excreted by defensive systems after entering the biological system (Turece et al., 2006) In therapeutic processes, the drug dosage in the circulation should be enough for covering the need of patients after administration until the next dose arrived. It has been observed that reducing the impact level of drugs influences their effectiveness. In fact, after the drug enters the patient body, its concentration increases in the circulatory system or the administration site. This can be problematic about toxic drugs. That's why the technology of slow and controlled release of drug can be greatly helpful in this regard (Davidson et al., 2015) However, the use of these systems also causes some limitations, such as the occurrence of new poisonings due to the administration of new materials along with the drug, delay in drug dispersion, and the need for further testing of nanodrugs (Pundir et al., 2017; Kamaly et al., 2016). Therefore, the drug release and optimization of this process are very important.

Streptokinase (SK) is the first thrombolytic drug that has been used to treat acute myocardial infarction for over 45 years. With a molecular weight of 47 kDa, streptokinase is a single-chain polypeptide composed of 414 amino acids. Streptokinase connects to and reacts with plasminogen through its multiple domains (Kunamneni &. Durvasula, 2014) Activation of plasminogen and its transformation into plasmin is the main phenomenon that leads to fibrin degradation through proteolysis. It is noteworthy that streptokinase is excreted from the body within 4 hours; hence, extending its half-life in the circulation can be important (Kumar et a;., 2017). Dendrimers are nanoparticles with unique characteristics such as spherical structure, a molecular weight less than 100 Daltons, and, most importantly, multivalence. These characteristics caused them to be gradually introduced as drug release tools (Kambhampati, & Kannan, 2013) Determination of half-life of streptokinase conjugated with g2 dendrimer was aim of this study In this study,

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nanoparticles containing recombinant streptokinase (NRSK) were prepared using the polyethylene glycol-citrate negatively-charged linear-spherical dendrimer (Roohvand, 2018; Sawhney et al., 2016). Since the presence of nanoparticles can affect the behavior and stability of the target drug, these characteristics are therefore required to be investigated subsequent to the development of the nanoparticles (Hughes, 2017). The objective of this study was to increase the stability of conjugated nanodrug using a second-generation dendrimer and the experiments design was done at three levels of temperature, pH, and rotation time using State-Ease software application. In this study the lifespan of the drug inside the body can be increased by using a PEGylated nanoparticle along with the drug (Zhang et al., 2012; Song et al., 2015)

Materials and Methods:

The second-generation of anionic dendrimer nanoparticles was synthesized and the synthesis was confirmed using dynamic light scattering (DLS). RSK was uploaded onto the nanoparticle surface and the preparation was several times washed and dialyzed for effectiveness studies. The uploading site was confirmed by the FTIR spectrum. For optimizing the drug release in the next step, RSK uploaded on the second-generation anionic dendrimer nanoparticle was tested for the stability at three levels of time, pH, and rotation. The laboratory method for the release of recombinant streptokinase (RSK) from NRSK was as follows. First of all, 500 µl of the lyophilized NRSK was transferred into a dialysis membrane (Sigma, Dorset, UK) with 12 kDa cut off and the dialysis process was performed against 500 ml of 10 mM phosphate buffer saline, at different pH values of 2, 4, and 7for 3.5, and 7 days. The drug release was investigated at 37°C on a magnetic stirrer, with stirringat 100, 200 and 300 rpm. The drug concentration on the samples was measured using reverse-phase high-performance liquid chromatography (RP-HPLC) using c18 column (Alliance2695, Waters, USA) and a spectrophotometer (Rigoly, China). Optimization of drug release from nanoparticles was done based on the experimental design by State-Ease software.

Experimental design assay for optimization of streptokinase release from G2-dendrimer in State-Ease:

After obtaining the results of absorption by a spectrophotometer, the data were analyzed by State-Ease software. Because the data limit was between 0.1 and 0.86 and the Ratio of maximum to minimum data was 4.6 (i.e., the number below 10), there was no need for transformation (Belwal et al., 2017). As a result, the fit summary situation was investigated. The basis for selecting of the model was the highest polynomial degree, given that p-value was small (i.e. significant and not to be Aliased), and the selected model was 2FI (Adeyanju et al., 2016). The relationship between the three variables A, B, and C was examined according to the following equation:

$$C = +8.5 \times 10^{-3} + 0.018 \times pH + 1.99 \times 10^{-3} \times mix\ speed + 0.04 \times Time - 2.28 \times 10^{-4} \times pH \times mix\ speed - 1.2 \times 10^{-3} \times pH \times Time - 3.2 \times 10^{-6} \times mix\ speed \times Time$$

Result and Discussion:

Data from HPLC at different pH values and times:

The drug release from the nanoparticles was studied at different pH values and agitation rates, at 37°C and the drug concentration was quantified using RP-HPLC and optical density (OD) absorption at 280 nm. The results are shown in tables 1 and 2. The release of the dendrimers at low pH values was very fast. In contrast at pH=7.0 the interaction between the streptokinase and G2-dendrimer (NSK) was significantly enhanced and this pH value decelerated the release of the drug.

Table 1: Measurement of OD at different dialysis periods, pH values, and agitation rates, at 37°C

pH	Agitation rate (rpm)	Dialysis time (Day)	OD (280 nm)
2	100	0	0.435
		3.5	0.53
		7	0.54
	200	0	0.35
		3.5	1.62
		7	1.64
	300	0	0.144
		3.5	0.271
		7	0.48
4.7	100	0	0.43

		3.5	0.44
		7	0.45
	200	0	0.33
		3.5	0.5
		7	0.51
	300	0	0.27
		3.5	0.48
		7	0.74
7.4	100	0	0.43
		3.5	0.54
		7	0.544
	200	0	0.18
		3.5	0.52
		7	0.53
	300	0	0.35
		3.5	0.48
		7	0.56

Table 2: The percentage of streptokinase surface curve to the dendrimer isolated from the conjugated drug

pH	Agitation rate (rpm)	Dialysis time (Day)	Ratio= (The area under the SK curve / The area under the G2curve)
2	100	0	0.2
		3.5	1.08
		7	0.21
	200	0	0.22
		3.5	0.144
		7	0.1
	300	0	12.5
		3.5	0.84
		7	0.95
4.7	100	0	0.2
		3.5	0.21
		7	0.31
	200	0	0.19
		3.5	0.19
		7	0.15
	300	0	1.37
		3.5	9.3
		7	0.29
7.4	100	0	0.2
		3.5	0.26
		7	0.217
	200	0	0.18
		3.5	0.48
		7	0.14
	300	0	2.1
		3.5	0.9
		7	0.39

According to the data of these tables, the drug release is greater in acidic pH values. According to existing references, the G2 dendrimer is anionic and presents a better release when it is incorporated into a drug in acidic conditions after conjugation (Adeyanju et al., 2016; Caminade et al., 2015).To determine the compliance of this model with our method, lack of fit test should be examined. In the multiple regression analysis, the "Adjusted R squared" gives an idea of how the model generalizes. In an ideal situation, it is preferable that its value is as close as possible to the value of "R squared". So, the proportion of the variance explained by the multiple regression model is indicated by "R squared". If a hierarchical regression has been conducted, then the improvement of the model can be assessed at each stage of the analysis by looking at changes in "R squared" and assessing the significance of such change. Since the difference between

the adjusted R-squared and the Predicted R-squared is low (about 0.2), it confirms the 2FI model (Bates et al., 2014; Jelokhani-Niaraki et al., 2016).

Evaluating the statistical validity of the proposed model of drug release based on the response level

The method of analysis of variance was used to evaluate the adequacy of the model and to confirm the significance of the factors. In general, according to the variance analysis table, the validity of the proposed model can be realized (Table 3). The results of ANOVA evaluated the coefficients and examined the p-value of parameters taking into account the 95% confidence level and the significance of the model was confirmed (Maran et al., 2017). According to analysis of variance table (table 3), time and pH factors have more impact on drug release. Also, according to curve 1, data distribution is linear**.**

Table 3: Analysis of variance of data

Analysis of variance table [Partial sum of squares - Type III]						
	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Probe > F	
Model	0.3894	6	0.0649	37.68301	< 0.0001	significant
A-pH	0.07259	1	0.07259	42.14835	< 0.0001	
B-mix speed	0.083174	1	0.083174	48.29376	< 0.0001	
C-Time	0.202208	1	0.202208	117.4088	< 0.0001	
AB	0.030381	1	0.030381	17.64027	0.0010	
AC	0.001035	1	0.001035	0.601027	0.4521	
BC	1.01E-05	1	1.01E-05	0.005879	0.9401	
Residual	0.022389	13	0.001722			
Lack of Fit	0.018839	8	0.002355	3.315866	0.1013	not significant

Based on the data in Table 3, p-value for coefficients of AC and BC is less than 0.05, which indicates the significance of the coefficients. Curves 1, 2, 3, and 4 all prove the validity of the model**.**

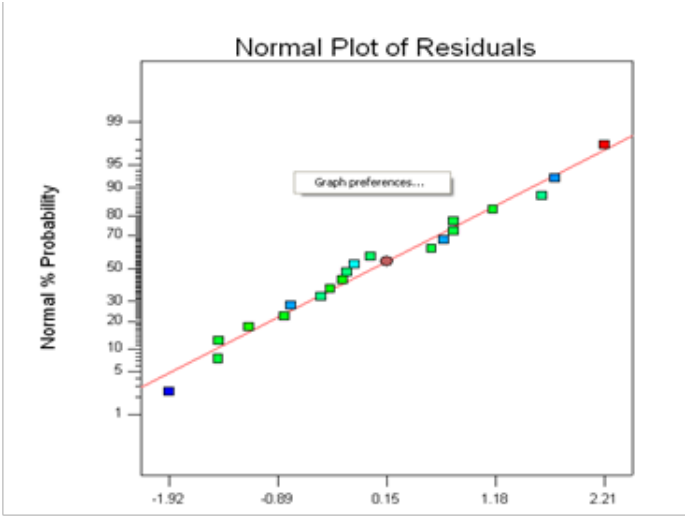


Fig. 1:

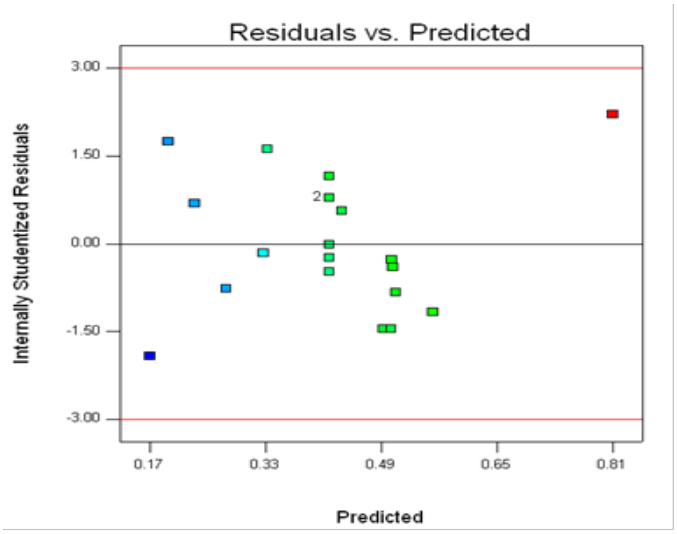


Fig. 2: Residuals versus predicted response

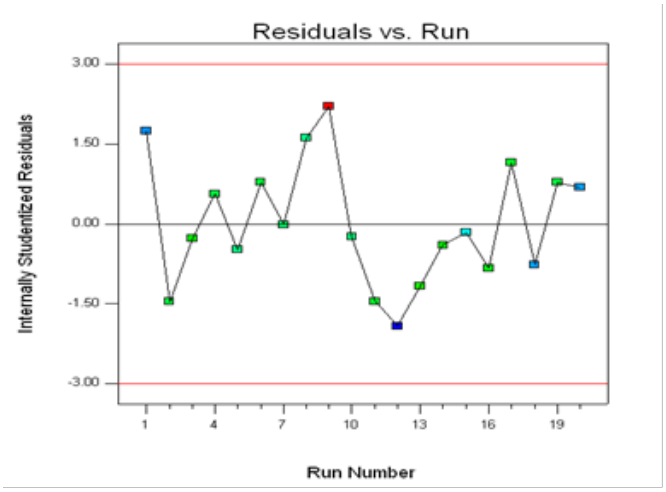


Fig. 3: Residuals versus the test number

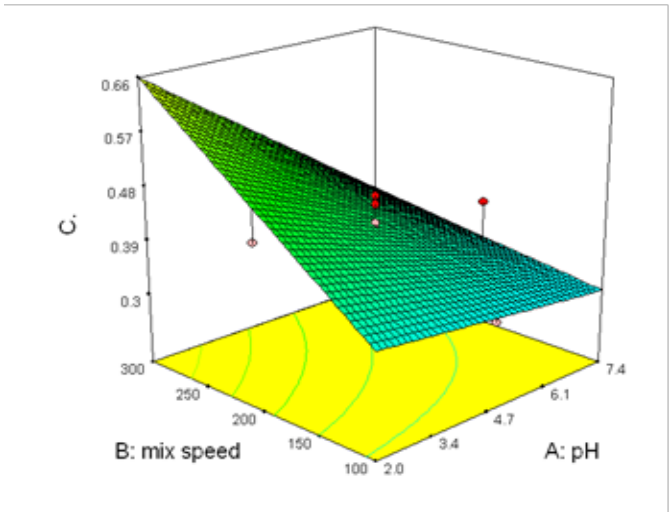
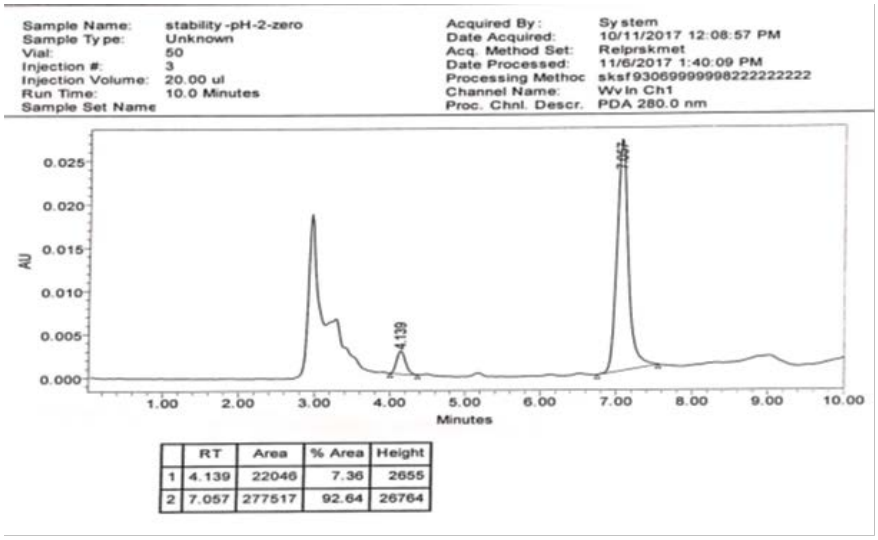


Fig. 4: The three-dimensional graph of drug release

Data analysis using RP-HPLC results

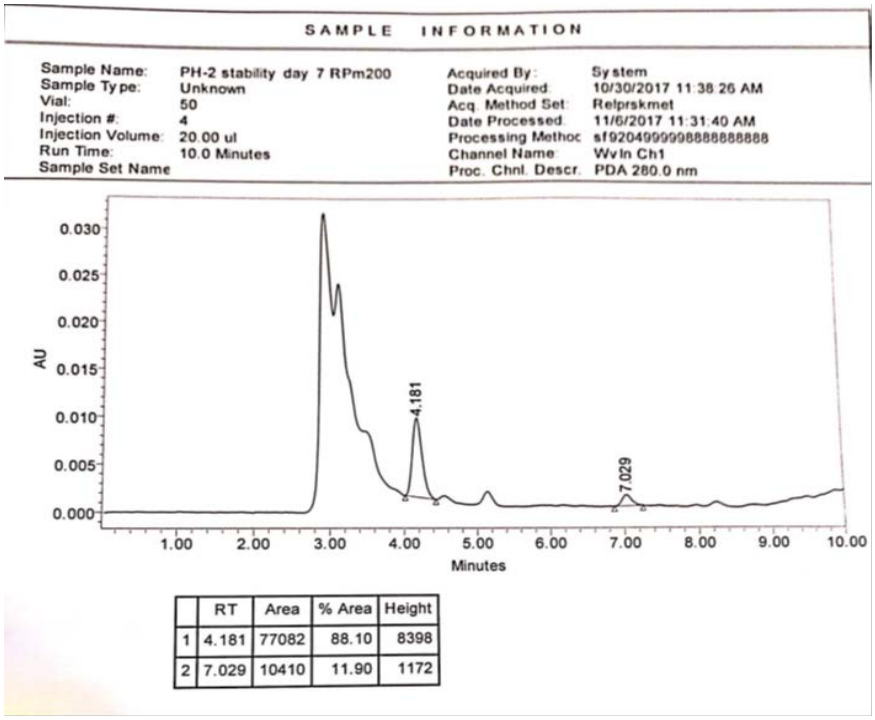
Similar to the steps taken in drug stability to measure the drug concentration throughout release at different dialysis periods, pH values, and agitation rates, the drug-conjugated nanoparticles were tested by chromatography under the same conditions (Wei et al., 2015) In each sample, the peak produced by the drug to the release rate of the nanoparticle ratio was measured and ultimately, the maximum and minimum releases were obtained in the 1st and 2nd peaks.



	RT	Area	% Area	Height
1	4.139	22046	7.36	2655
2	7.057	277517	92.64	26764

Peak 1: The maximum of drug release from the nanoparticle

According to the peak produced by RP-HPLC, the highest drug release (highest peak) was at pH = 2, t=0 and agitation 300 rpm. The 7-minute period was related to the recombinant Streptokinase drug and the 4-minute period was related to dendrimer.



	RT	Area	% Area	Height
1	4.181	77082	88.10	8398
2	7.029	10410	11.90	1172

Peak 2: The minimum of drug release from the nanoparticle

According to the 2nd peak, the lowest drug release was at pH = 2 and day 7 with a rotation of 200 rpm. The 7-minute period was related to the recombinant Streptokinase and 4-minutes period was related to dendrimer.

Conclusion:

Basically, RSM has been developed to model experimental responses (Box & Draper, 1987) and then led to the modeling of numerical experiments. In physical experiments, mistakes in experiments can occur in different ways, for example, for error evaluation when an inconvenience or error is due to a misunderstanding of the convergence (for example, the drowsiness or fatigue of the experimenter or the heterogeneity of the test material) or when continuous physical phenomenon is discussed as discrete while it is not possible to do this. In RSM, errors are assumed to be random. RSM is used to optimize the design to reduce the cost of expensive analytical methods and their associated numerical disorders (such as CFD or finite element analysis). RSM converges towards an optimal element because it reduces the effects of disorder factors (Asfaram et al., 2015; Rahimi-Gorji et al., 2015).

The purpose of the design with state-ease software was to estimate the proper time, pH, and agitation to obtain conjugated nanodrug stability because this software helps to estimate the right result within the specified time with the minimal test. According to the design, this test was designed at three levels of time, pH and rotation and was exposed to different conditions based on the design of the drug and the results were analyzed by two sample absorption assay method including spectrophotometry and liquid phase chromatography. The advantage of using this method is that it can measure the release of the drug in areas where the test cannot be performed and the effect of each factor and the interactions between variables is also determined. Since the predictive power of response has three factors in different values, it is a proper way to design and obtain the release time of the drug.

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