Selection of the Parameters in the Synthesis of Ethylenediamine-Folate Using the Plackett Burman Design

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Abstract: *This study is concerned with synthesizing ethylenediamine-folate (EDA-Folate), which will then be used as a precursor in synthesizing Gd-PEG-DOTA-Folate, a novel targeted-contrast agent for the diagnosis of cancer, employing the Magnetic Resonance Imaging method. This study aims to determine all the parameters affecting the synthesis of EDA-Folate using the Plackett Burman design. The synthesis method included activation of folic acid using dicyclohexylcarbodiimide and N-Hydroxysuccinimide to result in NHS-Folate, followed by conjugation of ethylenediamine with NHS-Folate to produce EDA-Folate. Analysis of the reaction product confirmed that the reaction product was EDA-Folate. From the resulted data, it can also be concluded that there were four significant parameters (out of the ten parameters studied) in the synthesis of EDA-Folate (with its value presented in the bracket), i.e., time inactivation of NHS-Folate (24 h), stirring rate inactivation of NHS-Folate (300 rpm), the mole of EDA (12 moles), and time of EDA-Folate (12 h). Moreover, the value or desirability of the experimental design was found to be 0.875 (which is < 1.0), meaning that the design will produce optimal conditions and thus the optimal yield of the reaction.*

Keywords: *ethylenediamine-folate; Plackett-Burman design; yield; contrast agent; Gd-PEG-DOTA-Folate*

■ INTRODUCTION

Ethylenediamine-folate (EDA-Folate) is one of the precursors required in the synthesis of a few targeted contrast agents, including Gd-PEG-DOTA-Folate [Gado linium-(Hexaglycerol Octaaminopropyl Polyoxy ethylene)-(1,4,7,10-Tetraazacyclodecane-1,4,7,10-Tetraace tic acid)-Folate], which is one of the topics of our research program. Contrast agents are, in general, chemical compounds used in the diagnosis of diseases employing the Magnetic Resonance Imaging (MRI) method. And Gd-PEG-DOTA-Folate is a targeted contrast agent and will be used to diagnose cancer. Magnetic Resonance Imaging (MRI) is the most flexible and powerful imaging modality available for clinical use in recent times. For almost five decades, the technology turned into improved, extended, and maintains to evolve, nonetheless no longer accomplishing the physical limits. Technical and physiological obstacles hamper the advancement and constrain physically feasible developments, making it an increasing number of challenges to innovate [1]. In the MRI method, contrast agents function to increase the difference between the image of sick body tissue and those of healthy one in a human body, so the diagnosis of the disease will be much more accurate [2].

Folic acid is a ligand that has a high affinity to folic receptors (Kd = 10^{-10}) found on the surface of various types of cancer. So, folic acid can be used as a carrier to bring about a contrasting compound directly to the target molecule, a folate receptor produced by cancer cells, to result in a clear enough imaging. Folate receptor is a glycosylphosphatidylinositol (GPI), a protein with an MW of 38–40 kDa, found on the surface of cancer cell membranes [3]. While their expression in normal cells is very limited, folate receptors are overexpressed in

epithelial, ovarian, breast, lung, kidney, and brain tumor tissues. When expressed in normal tissues, folate receptors are not located in the lungs, heart, and choroids because, in these tissues, folate receptors are limited to the surface of the apical membrane in epithelial tissue [4]. Folic acid is a type of B vitamin involved in the synthesis of purines. Most cells get folic acid needed through the anion channel or so-called reduced folate carrier, which has a low affinity to folate receptors. So, there is little or almost no expression of folate receptors in normal cells. However, cells that require a lot of folates, such as cancer cells and cells involved in embryonic development, will instead use a high affinity against these folate receptors. Therefore, folate receptors are overexpressed in cancer cells. Due to folate receptor differences between most normal cells and cancer cells, folate receptors are used to selectively target cancer cells for therapy or on compounds for imaging diagnosis [5].

To provide the need for contrast agents in all the hospitals in the country (Indonesia), the number of which is continuously increasing, contrast agents are still being fulfilled through import. In this way, the price is high, the continuity of availability is uncertain, and health service in hospitals is not ensured. Thus, research and development of contrast agents in the country have become a concern.

Synthesis of a targeted contrast agent, i.e., Gd-DTPA-Folate [6] and Gd-DOTA-Poliamidoamine Generation 3-Trastuzumab [7], have recently been done. As a continuation of our research program, its PEG derivative - Gd-PEG-DOTA-Folate - is being studied to get a novel targeted contrast agent by conjugating Gd-DOTA-Folate with branch-structured derivative polyethyleneglycol. Using the novel targeted contrast agent, diagnosis with MRI will theoretically be better than using Gd-DOTA-Folate because the relaxivity and luminance of branch-structured PEG-Gd are higher than those of linear-structured PEG-Gd. Although the terminal Gd conjugation is different, it did not affect the longitudinal relaxivity and luminance in branch-structured PEG-Gds (the eight-arm PEG-Gd). The branch-structured PEG-Gds (eight-arm PEG-Gd) show the highest contrast efficiency and is even higher than that of commercially available contrast agents [8].

The synthesis of Gd-PEG-DOTA-Folate will be carried out through four reaction steps. The first step is the synthesis of EDA-Folate as a precursor. Synthesis of EDA-Folate can be done using one of the two synthesis methods, namely the direct- and indirect methods [9]. In the direct method, the synthesis is accomplished through two reaction steps: (1) Activation of folic acid using dicyclohexylcarbodiimide (DCC) and *N*-Hydroxysuccinimide (NHS) to produce ethylenediamine-Folate (EDA-Folate) [2-6], and (2) Conjugation of NHS-folate with ethylenediamine (EDA) to form EDA-Folate. Meanwhile, in the indirect method, the synthesis of EDA-Folate is carried out through five reaction steps, i.e., formations of: (1). pyrophoric acid, (2) petrol hydrazide, (3) petrol acid, (4) methyl ester of folic acid, and (5) EDA-Folate, as the final product [10].

In this study, the direct method has been chosen because it is simpler than the indirect method, in that the direct method consists of only two reaction steps. But the problem was that the yield of the reaction synthesis was low, and thus attempts should be made to increase the yield.

To obtain optimal rendement of the EDA-Folate product, a screening design is required to provide information on parameters that have significant effects in the synthesis process. The Plackett-Burman design is the most commonly used screening design because, with the design, estimation of all major effects with the same precision can be done. Another advantage of this fractional factorial design is that it minimizes the experimental process from a large number of variables to a smaller number of most significant factors to save time, cost, and chemicals [11]. The last three mentioned aspects are also important, especially if the experiment will be done later on a bigger scale. The Plackett-Burman design can only be used for experiments that are multiples of 4 with 8 as the starting point (N = 8, 12, 16, 20, 24, 28, 32, 36). A minimum of 4n experiments is needed for estimating the main effects for 4n–1 factors. For example, 4, 5, 6, or 7 factors would require 8 experimental runs, 8, 9, 10, or 11 would require 12 runs,

and so on [12]. In this study, the selection parameters of EDA-Folate were carried out, with 10 parameters being selected.

It has been reported that many parameters that affect the synthesis EDA-Folate parameters are lighting intensity, the addition of an organic base, ratio mole of folic acid and DCC, time inactivation of NHS-folate, temperature inactivation of NHS-Folate, stirring rate of NHS-Folate, ratio mole of EDA, and addition of pyridine [13-16]. This study aims to determine parameters that have significant effects in the synthesis of EDA-Folate through the direct method with Plackett Burman's design using the software Design Expert 10.0.1. The parameters studied included lighting intensity (dark/light), the addition of an organic base (DIPEA), the mole of DCC, time inactivation of NHS-Folate, temperature inactivation of NHS-Folate, stirring rate of NHS-Folate, the mole of EDA, the addition of pyridine, time of EDA-Folate, and stirring rate of EDA-Folate. Experiments in this study consisted of activation of folic acid using DCC and NHS, followed by conjugation of EDA with NHS-Folate to produce EDA-Folate. Characterization of the reaction product, i.e., EDA Folate, has been done using the mass spectrometric method, and determination of significant parameters has been done using Plackett Burman's design.

■ EXPERIMENTAL SECTION

Materials

Chemicals used were of analytical grade, purchased from Sigma Aldrich. They included dicyclohexyl carbodiimide (DCC), dimethylsulfoxide (DMSO), ethylenediamine (EDA), *N*-Hydroxysuccinimide (NHS), *N*,*N*-Diisopropyletnylamine (DIPEA), and pyridine.

Instrumentation

The equipment used in this study included glass apparatus, a magnetic stirrer (Heidolph MR 3002), a Mass Spectrometer (Waters Xevo Q-TOF MS), and the software Design Expert 10.0.1. The measurement condition of Mass Spectrometry was Capillary 3 kV, Sampling cone 40, Extraction cone 4.0, Sorce temp 100 °C, Desolvation temp 250 °C, and Desolvation gas 600 L/h. Inlet system was LC or Direct Probe, Ion Souce was ESI, Mass Analyzer was Quadrupole and Tof, and Detector type was Micro Chanel Plates (MCPs).

Procedure

Synthesis of ethylenediamine-folate

Folic acid (0.0882 g), DCC (0.418 g), and NHS (0.0460 g) were dissolved in 10 mL of DMSO, followed by the addition of DIPEA into the mixture, which was stirred overnight. A white precipitate (dicyclohexyl urea, as a side product) was formed and was removed by filtration. Into the filtrate containing NHS-Folate, ethylenediamine and pyridine were added. The mixture was then stirred using a magnetic stirrer until ethylenediamine-folate (EDA-Folate) as a final product of the reaction was formed. The product was then characterized by Mass Spectrometry. The resulted data were analyzed using the software Design Expert 10.0.1 to determine significant parameters.

Selection parameters of synthesis ethylenediamine-folate using Plackett Burman design

Table 1 shows the parameters (10) and a dummy (one), the upper level (+1), and the lower level (–1), which were determined by the Plackett-Burman design.

■ RESULTS AND DISCUSSION

The synthesis reactions of ethylenediamine-folate are shown in Fig. 1. As is shown in Fig. 1, the synthesis of ethylenediamine-folate was initiated with the activation of folic acid with dicyclohexylcarbodiimide (DCC) and *N*-Hydroxysuccinimide (NHS), followed by the conjugation of ethylenediamine with the activated folic acid (NHS-Folate), to produce ethylenediamine-folate (EDA-Folate). The mass spectrum of EDA-Folate produced is shown in Fig. 2.

Fig. 2. shows the spectrum of EDA-Folate ($C_{21}H_{25}N_9O_5$) theoretically has a molecular mass (m/z) of 483.4760. The molecular mass of the EDA-Folate compound resulting from the HR-TOFMS ES+ spectrum was 484.2191; the peak shows that the EDA-Folate with the addition of a proton. In all these experiments, EDA-Folate were successfully synthesized by using the direct method.

Table 1. Parameters and their levels to be determined using the Plackett Burman's design

No.	Parameter	Code	Unit	Level	
				Lower (–1)	Upper (+1)
1	Lighting intensity	A	-	dark	light
2	Addition of an organic base DIPEA	B	mL	without DIPEA	With DIPEA
3	Mole of DCC	C	mmol	1	2,25
4	Time inactivation of NHS-Folate	D	hours	12	24
5	Temperature inactivation of NHS-Folate	E	(°C)	room temp.	50
6	Stirring rate of NHS-Folate	F	rpm	100	300
7	Mole of EDA	G	mmol	8	12
8	addition of pyridine	H	(µL)	300	700
9	Stirring rate of EDA-Folate	I	rpm	100	300
10	Time of EDA-Folate	J	hours	6	12
11	Dummy	K	-	-1	+1

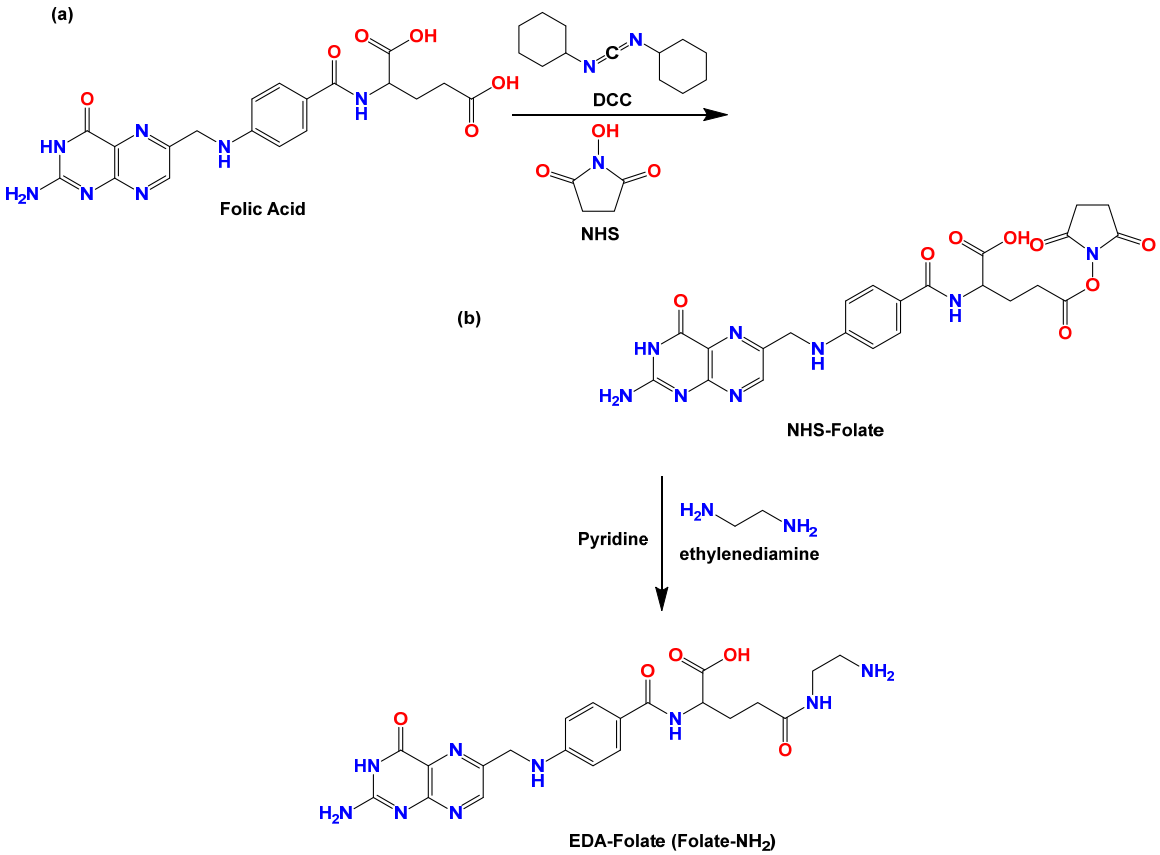

Fig 1. (a) Activation reaction of folic acid and (b) Conjugation reaction of NHS-Folate with ethylenediamine

Table 2 shows the resulted data on the responses determined in the synthesis of ethylenediamine-folate. The fractional factorial formula for the Plackett Burman is K= N–1, where N is a multiplication of 4, so the formula becomes K= 4n–1, where K is the parameter and n is runs. In this study, n = 3, and the number of parameters

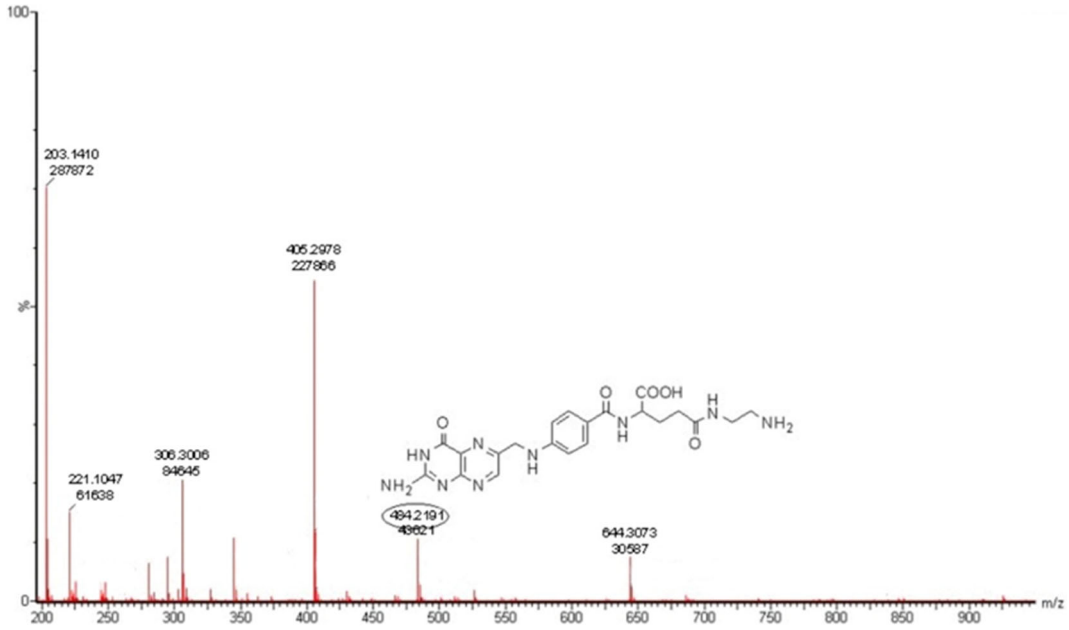

Fig 2. Mass spectrum of EDA-Folate

Table 2. Matrix of the Placket-Burman design containing resulted data on the responses in the synthesis of ethylenediamine-folate

		A	B	C	D	E	F	G	H	I	J	K	R
Std	Run	Lighting intensity	DIPEA (addition of 0.1 mL)	Mole of DCC (mmol)	Time of NHS-Folate (h)	Temp. of NHS-Folate (°C)	Stirring rate of NHS-Folate (rpm)	Mole of EDA (mmol)	addition of pyridine (µL)	Stirring rate EDA-Folate (rpm)	Time of EDA-Folate (h)	Dummy	Peak Intensity
2	1	dark	with	2.25	12	50	300	12	300	100	6	+1	3290
10	2	dark	with	2.25	24	room temp	100	8	700	300	6	+1	4378
7	3	light	without	1	12	50	100	12	700	300	6	+1	775
8	4	light	with	1	12	room temp	300	8	700	100	12	+1	7321
6	5	dark	without	1	24	room temp	300	12	300	300	12	+1	43261
4	6	dark	with	1	24	50	100	12	700	100	12	-1	2973
9	7	light	with	2.25	12	room temp	100	12	300	300	12	-1	2939
12	8	dark	without	1	12	room temp	100	8	300	100	6	-1	1776
11	9	light	without	2.25	24	50	100	8	300	100	12	-1	4292
3	10	light	without	2.25	24	room temp	300	12	700	100	6	-1	27043
1	11	light	with	1	24	50	300	8	300	300	6	-1	3849
5	12	dark	without	2.25	12	50	300	8	700	300	12	-1	14055

to be selected were ten. It was necessary to have the dummy meet the formula, with the number of experiments (4n) being 12 (see Table 2).

In the Plackett-Burman design, the upper level (+1) and lower level (–1) were determined to evaluate which parameters significantly impacted the experiment. As can be seen in Table 1, the upper and the lower values for each parameter were selected based on secondary data [13-16]. Furthermore, these parameters were incorporated, and the Plackett-Burman design matrix with encoding for 12 experiments to be conducted along with the peak intensity mass spectrometry shown in Table 2.

The equation derived from the Plackett-Burman design is presented in Eq. (1)

$$Y= 9662.67 - 1959.50X_1 - 5537.67X_2 - 329.83X_3 + 4636.67X_4 - 4790.33X_5 + 6807.17X_6 + 3717.50X_7 - 238.50X_8 + 1880.17X_9 + 2810.83X_{10} + 890.17X_{11} \quad (1)$$

where: X_1 (lighting intensity); X_2 (addition of an organic base DIPEA); X_3 (mole of DCC); X_4 (time in activation

of NHS-Folate) X_5 (temperature in activation of NHS-Folate); X_6 (stirring rate of NHS-Folate); X_7 (mole of EDA); X_8 (addition of pyridine); X_9 (stirring rate of EDA-Folate); X_{10} (time of EDA-Folate); X_{11} (Dummy).

Calculation of the response or acquisition of intensity (R_1) can statistically be done by using Eq (2).

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_5X_5 + \beta_6X_6 + \beta_7X_7 + \beta_8X_8 + \beta_9X_9 + \beta_{10}X_{10} + \beta_{11}X_{11} \quad (2)$$

where Y is a response, β_0 is a constant, and β_1 and β_n are coefficient of response values.

Using Eq. (1), parameters having positive and negative influences on the response have been determined. The parameters with positive or negative effects were determined using coefficient calculations (β) for each parameter in the experimental data. The eleventh coefficient of parameters (β_1, β_2, β_3,…, β_{11}) can be calculated from the multiplication of each result with the value of each parameter (+1/−1) for each experiment. The results of the coefficient of parameters were then divided by the number of experiments. For example, for parameter 1, which is exposure, the value of the coefficient was:

β_1 = (+2939 – 2973 – 1776 – 43261 +775 – 14055 – 4378 + 27043 + 4292 +7321+ 24.073 + 3849 – 3290)/12= –1959.5

The result of coefficient β_1 was negative, which means that from the average of 12 experiments conducted, the acquisition of EDA-Folate intensity will decrease if this synthesis is made in a bright place. It is because folic acid undergoes decomposition [13,16]. The negative value of a parameter coefficient (β) indicates that increasing the value of the parameter coefficient will decrease the peak intensity of the EDA-Folate. In contrast, a positive value of parameter coefficient (β) indicates that increasing the value will increase the peak intensity of EDA-Folate.

Fig. 3 is a Normal Plot Distribution and Pareto chart of the relevant parameters being studied. As is shown in the figure, six parameters were significant to the normal percent probability and T-value. The six parameters were parameter B (addition of the organic base DIPEA), parameter D (time inactivation of NHS-Folate), parameter E (temperature of NHS-Folate), parameter F (stirring rate of NHS-Folate), parameter G (mole of EDA), parameter J (time of EDA-Folate). Of the six significant parameters, four parameters, i.e., parameter D (time inactivation of NHS-Folate), parameter F (stirring rate inactivation of folic acid), parameter G (mole of EDA), and parameter J (time of EDA-Folate), showed positive effects. Meanwhile, two parameters, i.e., parameter B (addition of the organic base DIPEA) and parameter E (temperature of NHS-Folate), showed negative effects on normal percent probability and T-values.

From the normal plot distribution (Fig. 3(a)), the stirring rate of NHS-Folate was farthest from the response line. At the same time, the addition of organic base (DIPEA) had the highest negative effect on the intensity of synthesis EDA-Folate because it was at the lowest from the response line. As shown in the Pareto chart (Fig. 3(b)), it can also be seen that the stirring rate of NHS-Folate had the highest positive effect on the intensity of EDA-Folate synthesis.

The graphs shown in Fig. 3 indicate that only four parameters' responses can be used for the next stage of optimization. They are stirring time inactivation of NHS-Folate (24 h.), stirring rate activation of NHS-Folate (300 rpm), a mole of EDA (12 mmol), and stirring time of EDA-Folate (12 h). With these four parameters, a desirability value of 0.875 and a minimum intensity of EDA-Folate of 37962.8 could be obtained.

Desirability value is a function of optimization value that demonstrates the ability of programs to desire based on criteria set on the final product. The range of the value of desirability is from 0.0 to 1.0. A desirability value getting closer to 1.0 indicates that the design or program can produce an optimal product. An optimization goal is not to get a desirability value of 1.0 but to find the best condition that brings all optimal objective functions [17]. Fig 4. Represents the graphs of the parameter of optimum response based on the Plackett-Burman design. The addition of DIPEA and temperature during stirring is zero. It was due to the pH value at the time of addition of DIPEA was not achieved or even exceeding the pH in the process of activation of folic acid (i.e., 4.5–7.5) [18]. It will be noted that activation of folic acid is better to be conducted in a room

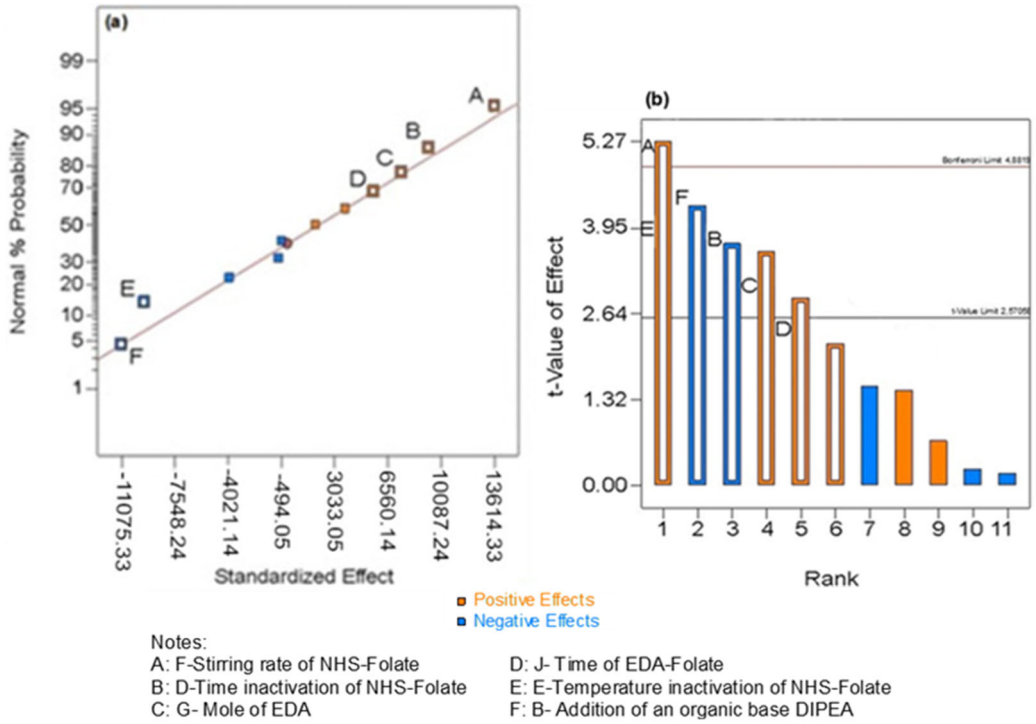

Fig 3. (a) Normal plot distribution and (b) Pareto chart of the eleven parameters

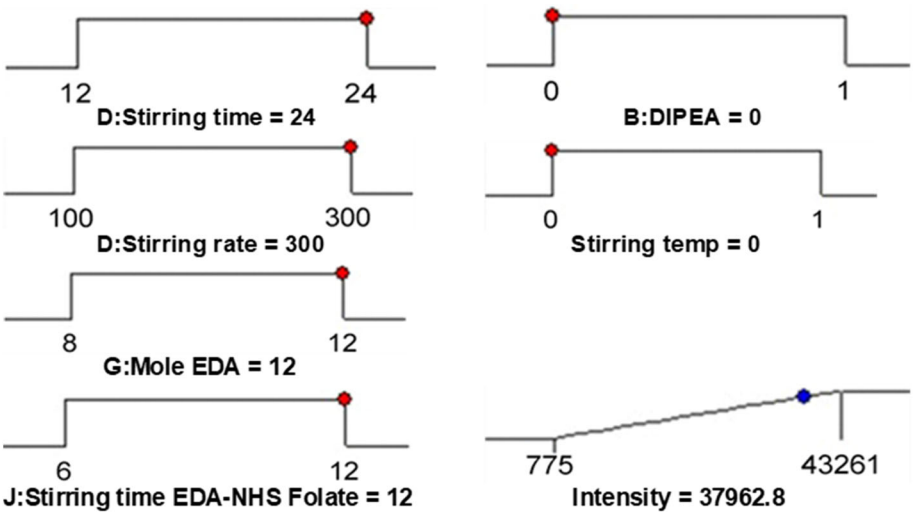

Fig 4. Graphs of the parameter of optimum response based on the Plackett-Burman design

temperature (without heating) because folic acid is undergoing decomposition at high temperatures.

Fig. 5 is the linear regression curve between actual responses and those predicted using the Plackett Burman design. Analysis of this design using ANOVA (Table 3) has resulted in a value of R^2 (coefficient of determination) of 0.9450 that close to one. So, the design was acceptable. This value indicates a match between the predicted value and the actual value. A slight difference between the measured and calculated values indicates the higher suitability of the model [19-20].

The reliability level of a model is acceptable if the value of P is less than 0.05 (significant). In this experiment, the value of P calculated from the Plackett Burman design was found to be 0.0052. This value of P is therefore acceptable. This design also produces Adeq

Table 3. ANOVA table for the selected factorial model

ANOVA for the Selected Factorial Model Analysis of Variance Table [Partial sum of squares-Type III]						
Source	Sum of squares	df	Mean square	F value	p-value Prob > F	
Model	1.718 E + 09	6	2.863 E + 08	14.32	0.0052	(significant)
B-DIPEA	3.680 E + 08	1	3.680 E + 08	18.40	0.0078	
D-Stirring time	2.580 E + 08	1	2.580 E + 08	12.90	0.0157	
E-Stirring temp	2.754 E + 08	1	2.754 E + 08	13.77	0.0138	
F-Stirring rate	5.561 E + 08	1	5.561 E + 08	27.80	0.0033	
G-Mole EDA	1.658 E + 08	1	1.658 E + 08	8.29	0.0346	
J-Stirring time EDA-NHS Folate	9.481 E + 07	1	9.481 E + 07	4.74	0.0814	
Residual	9.999 E + 07	5	2.000 E + 07			
Cor Total	1.818 E + 09	11				
Standard Deviation	4471.98		R-Squared	0.9450		
Mean	9662.67		Adj. R-Squared	0.8790		
C.V. %	46.28		Pred. R-Squared	0.6832		
PRESS	5.76 E+ 08		Adeq Precision	11.1520		
-2 Log Likelihood	225.28		BIC	242.6773		
			AICc	267.2830		

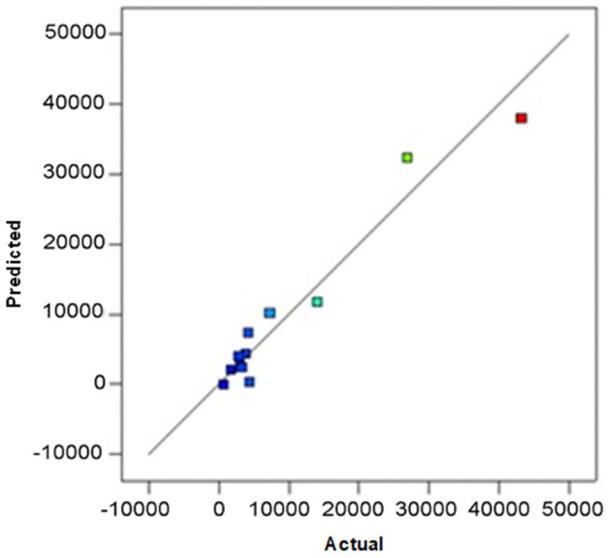

Fig 5. Linear regression curve between actual response and prediction of Plackett Burman

Precision which measures the signal-to-noise ratio (S/N). The ratio is expected to be greater than 4. In this study, the resulting S/N was 11.152.

■ CONCLUSION

Based on the mass spectrum of the reaction product, it can be concluded that EDA-Folate has been successfully synthesized by using the direct method. The method consisted of two reaction steps, i.e., inactivation of folic acid using DCC (dicyclohexylcarbodiimide) and NHS (*N*-Hydroxysuccinimide) to result in NHS-Folate, followed by conjugation of EDA (ethylenediamine) with NHS-Folate to produce EDA-Folate. Based on the Plackett-Burman experimental design, it can also be concluded that there were four significant parameters in the synthesis of EDA-Folate, which were time inactivation of NHS-Folate, stirring rate inactivation of NHS-Folate, a mole of EDA, and time of EDA-Folate. Moreover, the value or desirability of the experimental design was calculated to be 0.875, a value getting closer to 1.0, indicating that the resulting design will be able to produce optimal reaction conditions and, accordingly, optimal reaction products, both qualitatively and quantitatively. EDA-Folate will be used as a precursor in synthesizing the Gd-PEG-DOTA-Folate targeted contrast agent.

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