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ORIGINAL PAPER

Screening and Optimization of the Reaction of Polymyxin B Sulphate with NBD-Cl for the Synchronous Spectrofluorimetric Determination of Polymyxin B Sulphate in Human Plasma

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Abstract An accurate and sensitive synchronous spectrofluorimetric method has been developed for the determination of Polymyxin B sulphate (Poly B) in human plasma. The method is based on the reaction of non-fluorescent Poly B with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) in borate buffer of pH 7 producing a yellow color with maximum relative fluorescence at 440 nm using a constant wavelength difference $\Delta\lambda$=80 nm. Reaction conditions and other analytical parameters were studied and optimized using factorial design. Three level factorial designs have been employed for the screening, optimization of all experimental variables and determination of their interactions on the final product formation. The variables under investigation were: pH of borate buffer, volume of buffer, volume of NBD-Cl, temperature, time of heating and volume of sulfuric acid. A linear plot between relative fluorescence and concentration was obtained over the concentration range 100.00–1200.00 ng mL^{-1}. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 10.31 and 31.24 ng mL^{-1}, respectively. The proposed method was validated according to ICH guidelines and successfully applied for the determination of Poly B in human plasma, where satisfactory results were obtained. The results obtained were statistically compared with those of a published method, where no significant difference was observed.

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Introduction

Poly B is a polycationic antibiotic isolated from various strains of Bacillus polymyxa and has bactericidal action against almost all gram-negative bacilli except the Proteus group. It is the drug of choice in the treatment of infections of the urinary tract, the eye, meninges, and blood stream [1]. It has a hydrophobic fatty acid moiety and a polar moiety of five unmasked amino groups (Fig. 1a). Under physiological pH conditions, these primary amine groups on the amino acids are ionized [2].

Several methods have been recorded for the analysis of Poly B in pharmaceutical dosage forms and biological fluids as thin-layer chromatography [3, 4], high-performance liquid chromatography with UV [5–7] fluorescence [8] mass spectrometric detection [9, 10], gas chromatographic [11], spectrophotometric [12, 13], capillary electrophoresis [14–16] and microbiological assays [17, 18].

The aim of the present study was to develop an accurate and sensitive method based on the synchronous spectrofluorimetric determination of the non-fluorescent Poly B through the formation of a fluorescent derivative using NBD-Cl allowing quantification of clinically relevant concentrations of Poly B in human plasma. The Lack of reliable assays for Poly B in human plasma is likely to be one of the major contributors to this study [8, 10].

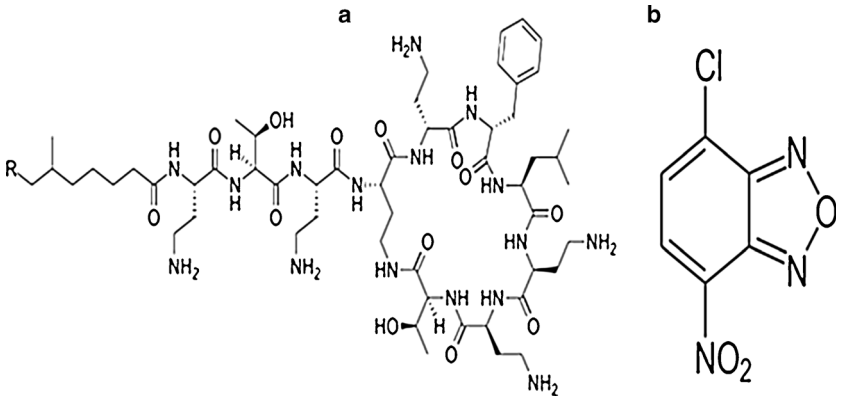

Fig. 1 Chemical structure of Polymyxin B (**a**) and NBD-Cl (**b**)

Synchronous spectrofluorimetry is based on measurement of the synchronous fluorescence intensity of the drug. Because of its sharp and narrow spectrum, it has superior advantages over conventional fluorescence spectroscopy as it results in simple spectra, low interference and high selectivity [19].

NBD-Cl (Fig. 1b), an electroactive halide reagent, was first introduced as an analytical reagent for the determination of some amines and amino acids [20]. It is non-fluorescent, and generates fluorescent products upon reacting with aliphatic amines or thiol compounds to form highly fluorescent compounds [21, 22].

A factorial design was used to identify the critical parameters of the reaction by delivering as much information as possible with a minimum of experimental and financial effort. Screening experiments were run in order to determine the variables to be investigated, optimization experiments were then used to evaluate the main effects as well as the interactions between the factors and therefore the best set of conditions can be selected [23, 24].

Experimental

Apparatus

Kontron Spectrofluorometer (Switzerland) Model SFM25 with 1 cm quartz cuvettes connected to IBM compatible computer fitted with WIND25 spectroscopy software for Windows. The PMT voltage was adjusted at 400 V and the optimum scan speed of 500 nm/s was used. The excitation and emission monochromators were scanned simultaneously with a constant difference $\Delta\lambda=80$ nm and a response time of 8 s. The slit width of both monochromators was 5 nm and the synchronous spectra were recorded in an excitation scale.

Solid Phase Extraction: 12 Port Vacuum Extraction Manifold Assy (Phenomenex) with Vacuum Pump, Beco, Germany.

Jenway pH meter 3310 pH/mV/°C meter (UK) with combined glass electrode was used for pH adjustments.

Software

Data analysis was performed using Minitab® version 14.12.0 software.

Chemicals and Reagents

Pure standards

Poly B was kindly supplied by Egyptian International Pharmaceutical Industries Co., (EIPICO), Cairo, Egypt, and certified to contain 99.77 %.

Reagents and Solvents

Methanol, dimethyl formamide (DMF), acetone, acetonitrile and sulfuric acid were obtained from (ADWIC, Egypt). Borate buffer solution with pH ranges (6–8) was prepared by using boric acid and borax (ADWIC, Egypt) with appropriate concentrations. NBD-Cl was purchased from Sigma-Aldrich, Germany.

All chemicals, solvents and reagents used throughout this work were of analytical grade. Bi-distilled water was used throughout the whole work and was indicated by the word "water".

Standard Solutions

Freshly prepared stock standard solutions of Poly B and NBD-Cl, each having a concentration of 1.00 mg mL^{-1} were prepared in water and methanol, respectively. The Poly B stock solution was further diluted with the same solvent to give a concentration of 100.00 μg mL^{-1} to be used as working standard solution. These solutions were kept under refrigeration at 4 °C and remained stable for at least 3 months.

Procedure

Screening of Reaction Condition

Six parameters: pH of borate buffer, volume of buffer (mLs), volume of NBD-Cl (mLs), temperature, time of heating (min) and volume of conc. H_2SO_4 (mLs), were chosen as variables and tested at three levels. The three selected levels were: high and low levels were coded as 1 and −1, respectively, with level 0 being the center as shown in Table 1. The specified volumes of NBD-Cl and borate buffer with pH ranging from 6 to 8 were added to 10.00 μg mL^{-1} of Poly B in 10 mL test tubes. They were heated at different temperatures in a thermostatically controlled water bath and also the time of heating was tested. Then they were cooled and the required mLs of conc. H_2SO_4 were added. The contents of the test tubes were transferred into 10 mL volumetric flasks and the solutions were completed to the mark with methanol. The relative fluorescence of the obtained solution was recorded through synchronous fluorescence spectrum at 440 nm using a constant wavelength difference $\Delta\lambda$=80 nm and the blank spectra were drawn and subtracted by the software from the corresponding spectra.

Having six variables with low and high levels for each variable, there are 2^6, that is, 64 possible combinations in a full factorial design. Fractional factorial design allows reducing the number of experiments to 1/4. In this case we selected 2^{6-2} that is 16 experiments. Four center points were added in order to have an estimate of the experimental error. The coded fractional factorial design and responses for the total 20 experiments are shown in Table 2.

Design matrix using coded values was set up using Minitab version 14.12.0 software. Estimated effects and coefficients for response using coded units are shown in Table 3. An analysis of variance (ANOVA) test was used in order to identify the effects of individual factors and their second-order (2-way) and third-order (3-way) interactions as shown in Table 4. Higher order interactions were assumed to be negligible.

Table 1 Factors included in the fractional factorial design

Symbol	Parameter	Coded level		
		Low (−1)	Center (0)	High (1)
A	pH of borate buffer	6.0	7.0	8.0
B	Volume of buffer (mL)	0.5	1.25	2.0
C	Volume of NBD-Cl (mL)	0.5	1.5	2.5
D	Temperature (°C)	25	55	85
E	Time of heating (min.)	10	35	60
F	Volume of conc.H_2SO_4 (mL)	0.2	0.6	1.0

Table 2 Coded fractional factorial design and responses

Run	pH of (A)	mLs of buffer (B)	mLs of NBD-Cl (C)	Temp. (D)	Time (E)	mLs of H_2SO_4 (F)	Poly B rf
1	−1	−1	1	−1	1	1	2.16
2	−1	1	1	−1	−1	−1	2.37
3	−1	−1	−1	1	−1	1	24.50
4	1	1	−1	−1	−1	1	2.38
5	1	1	1	1	1	1	4.83
6	0	0	0	0	0	0	59.50
7	1	−1	−1	−1	1	−1	88.70
8	−1	1	−1	1	1	−1	125.60
9	1	−1	1	1	−1	−1	66.30
10	0	0	0	0	0	0	52.60
11	1	−1	−1	1	1	1	25.70
12	−1	1	1	1	−1	1	39.00
13	−1	−1	−1	−1	−1	−1	0.89
14	−1	−1	1	1	1	−1	54.00
15	1	1	1	−1	1	−1	78.60
16	1	−1	1	−1	−1	1	5.52
17	0	0	0	0	0	0	55.70
18	0	0	0	0	0	0	67.90
19	1	1	−1	1	−1	−1	58.70
20	−1	1	−1	−1	1	1	0.52

Table 3 Estimated effects and coefficients for rf using coded values

Term	Poly B		
	Coeff.	SE of Coeff.*	P
Constant (I)	36.24	2.155	0.000
Block	−2.12	1.928	0.352
A	5.11	2.155	0.099
B	2.76	2.155	0.290
C	−4.64	2.155	0.120
D	13.59	2.155	0.008
E	11.28	2.155	0.014
F	−23.16	2.155	0.002
A*B	−7.98	2.155	0.034
A*C	2.11	2.155	0.400
A*D	−16.05	2.155	0.005
A*E	−3.16	2.155	0.239
A*F	−8.57	2.155	0.028
B*D	4.44	2.155	0.132
B*F	−4.16	2.155	0.149
A*B*D	−6.34	2.155	0.060

* *SE of Coeff.* Standard error of coefficient

Table 4 Analysis of variance (ANOVA) test for response (up to 3-way interactions)

Source	Poly B		
	DF	F	P
Blocks	1	1.21	0.352
Main effects	6	32.41	0.008
2-way interactions	7	13.72	0.027
3-way interactions	1	8.66	0.060
Curvature	1	22.16	0.018
Residual error	3		
Total	19		

Optimization of Reaction Condition Using Response Surface Methodology (RSM)

The central composite design was performed using five parameters as variables and tested at three levels. The parameters were pH of borate buffer, volume of buffer (mLs), temperature, time of heating (min) and volume of conc. H_2SO_4 (mLs). The three selected levels were: high and low levels were coded as 1 and −1, respectively with level 0 being the center as shown in Table 5.

NBD-Cl (0.5 mL) and certain volumes of borate buffer with pH ranging from 6 to 8 were added to 10.00 μg mL^{-1} of Poly B in 10 mL test tubes. They were heated at different temperatures in a thermostatically controlled water bath and also the time of heating was tested. Then they were cooled and the required mLs of conc. H_2SO_4 were added. The contents of the test tubes were transferred into 10 mL volumetric flasks and the solution was completed to the mark with methanol. The relative fluorescence of the obtained solution was recorded and the blank spectra were drawn and subtracted by the software from the corresponding spectra.

Having five variables with three levels for each variable, there are 33 experiments were performed and divided as follows, 16 factorial experiments, 10 axial points and 7 replicates at center points. The coded Central Composite Design and responses for the total 33 experiments are shown in Table 6.

Table 5 Factors included in the central composite design

Symbol	Parameter	Coded level		
		Low (−1)	Center (0)	High (1)
A	pH of borate buffer	6.0	7.0	8.0
B	Volume of buffer (mL)	0.5	1.25	2.0
C	Temperature (°C)	25	55	85
D	Time of heating (min.)	10	35	60
E	Volume of conc.H_2SO_4 (mL)	0.2	0.6	1.0

Table 6 Coded central composite design and responses

Run	pH (A)	mLs of buffer (B)	Temp. (C)	Time (D)	mLs of H_2SO_4 (E)	Poly B rf
1	0	0	0	0	0	45.000
2	0	0	0	0	0	44.600
3	1	1	−1	1	−1	27.000
4	−1	1	1	−1	1	23.400
5	0	0	0	0	0	57.600
6	−1	1	1	1	−1	103.600
7	−1	−1	−1	1	−1	0.720
8	1	−1	1	−1	1	23.100
9	0	0	0	0	0	53.300
10	1	−1	−1	1	1	18.800
11	1	−1	−1	−1	−1	13.300
12	1	1	1	−1	−1	39.900
13	−1	−1	−1	−1	1	0.355
14	0	0	0	0	0	44.800
15	−1	1	−1	1	1	0.371
16	1	1	1	1	1	10.293
17	0	0	0	0	0	56.300
18	−1	1	−1	−1	−1	0.190
19	1	1	−1	−1	1	1.130
20	−1	−1	1	−1	−1	31.200
21	1	−1	1	1	−1	32.900
22	−1	−1	1	1	1	20.400
23	0	0	0	1	0	55.100
24	0	0	0	−1	0	18.300
25	−1	0	0	0	0	15.800
26	0	0	0	0	−1	78.000
27	0	0	−1	0	0	5.068
28	0	0	0	0	1	26.900
29	0	0	0	0	0	46.000
30	0	−1	0	0	0	57.000
31	0	0	1	0	0	21.200
32	0	1	0	0	0	30.400
33	1	0	0	0	0	39.500

Design matrix using coded values was set up using Minitab version 14.12.0 software. Estimated effects and coefficients for response using coded units are shown in Table 7 and the analysis of variance (ANOVA) test is presented in Table 8.

Synchronous Spectrofluorimetric Method

Aliquots of Poly B standard working solution were transferred into a series of 10 mL test tubes, 2.0 mL borate buffer of pH 7 and 0.5 mL of NBD-Cl were added. The test tubes were heated at 60 °C in a thermostatically controlled water bath for 45 min, cooled and acidified by adding 0.2 mL of conc. H_2SO_4. Then they were transferred into 10 mL volumetric

Table 7 Estimated effects and coefficients for rf using coded values

Term	Poly B		
	Coeff.	SE of Coeff.*	P
Constant (I)	45.7135	3.720	0.000
Block	4.7201	2.951	0.138
A	0.5493	3.080	0.862
B	2.1394	3.080	0.502
C	13.2811	3.080	0.001
D	6.5727	3.080	0.056
E	−11.2256	3.080	0.004
A*A	−12.8428	8.358	0.153
B*B	3.2072	8.358	0.708
C*C	−27.3588	8.358	0.007
D*D	−3.7928	8.358	0.659
E*E	11.9572	8.358	0.180
A*B	−5.2914	3.267	0.134
A*C	−8.1876	3.267	0.029
A*D	−3.6489	3.267	0.288
A*E	1.9629	3.267	0.560
B*C	4.6298	3.267	0.184
B*D	4.4862	3.267	0.197
B*E	−7.5019	3.267	0.042
C*D	1.1048	3.267	0.742
C*E	−6.8658	3.267	0.059
D*E	−4.8594	3.267	0.165

* *SE of Coeff.* Standard error of coefficient

flasks and the solution was completed to the mark with DMF. The synchronous fluorescence spectrum was recorded at 440 nm using a constant wavelength difference Δλ=80 nm, response=8 s and voltage=400 mV. The blank spectra were drawn and subtracted by the software from the corresponding spectra, the calibration curve was constructed by plotting relative fluorescence versus the corresponding concentration and the regression equation was computed.

Table 8 Analysis of variance (ANOVA) test for response in central composite design

Source	Poly B		
	DF	F	P
Block	1	2.56	0.138
Regression	20	4.78	0.005
Linear	5	7.39	0.003
Square	5	6.43	0.005
Interaction	10	2.64	0.063
Residual error	11		
Total	32		

Stoichiometry of the Reaction

The stoichiometry of the reaction was studied adopting job's method of continuous variation [25]. The concentration of Poly B and NBD-Cl were 100 μM and ten solutions containing Poly B and NBD-Cl were used in various molar ratios so their volume was constant at 1 mL and the above mentioned procedure was adopted.

Assay of Poly B in Human Plasma

The suggested procedure was applied for the analysis of Poly B in human plasma with peak plasma concentrations ranged from 2.38 to 13.9 mg/L after an intravenous infusion [26].

10.0, 20.0 and 30.0 μL of standard working solution were added separately in three 10 mL stoppered shaking tubes each containing 0.5 mL plasma and 2.0 mL acetonitrile. After vortex-mixing for 2 min and centrifugation (8000 g for 30 min), all of the supernatant was transferred to SPE cartridge which has been conditioned with methanol and equilibrated with water. Poly B was eluted with 0.5 mL of water. Then the described procedure was applied to the eluted samples.

Concentrations were calculated using the corresponding regression equation; the mean recovery % and standard deviations were then calculated.

Results and Discussion

Screening of Reaction Conditions

Fractional factorial design was used for the screening of factors affecting the reaction of Poly B with NBD-Cl to identify factors that are more influential on the response through the determination of their coefficients.

The addition of replicate experiments at center points gives more informative screening than using factorial design only.

In order to evaluate these coefficients, the data in Table 3 were graphically presented in a Pareto chart as shown in (Fig. 2). This chart shows the different factors evaluated and their interactions and the calculated standardized effects in the vertical axis and horizontal axis; respectively.

The model contains six main effects. ANOVA table, Table 4, shows that p-value for the set of main effects is 0.008.

Statistical significance can be assessed by comparing *p*-value with α-level (0.05):

- If the p-value is less than or equal to α-level, the effect is significant.
- If the p-value is greater than α-level, the effect is not significant.

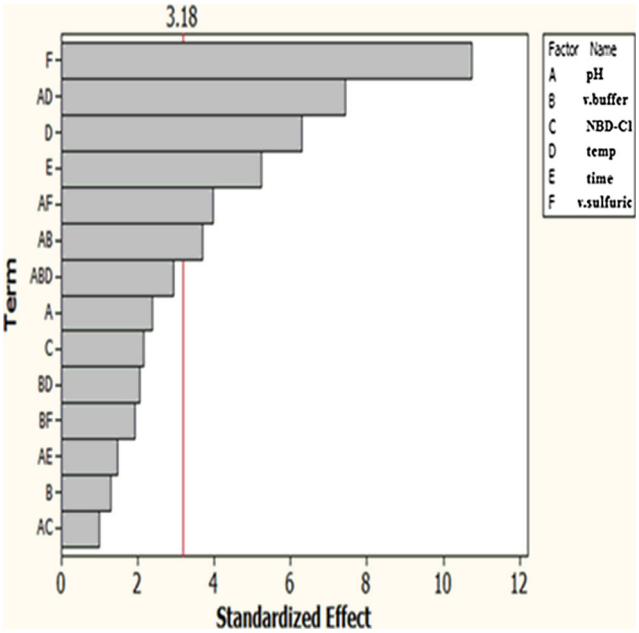

Fig. 2 Graphical presentation of coefficients of fractional factorial design by Pareto chart of the standardized effects for Polymyxin B

Since the resulted p-value for the set of main effects is 0.008, that is, less than 0.05, there is an evidence of a significant effect.

As shown in (Fig. 2), the most important factors are temperature, time of heating and volume of conc. H_2SO_4. The significance of these main effects is proven statistically by comparing their corresponding p-values shown in Table 3 with α-level (0.05). They all have p-value less than 0.05 indicating significant influence of these factors on the reaction.

The ANOVA table, Table 4, shows that there are significant 2-way interactions with p-value 0.027 which is less than 0.05 and insignificant 3-way interactions with p-value 0.06 which is more than 0.05.

As shown in (Fig. 2) the most important 2-way interaction coefficients are A*B, A*D, A*F. All of them have p-value less than 0.05 as shown in Table 3.

The addition of center points allowed the detection of curvature which is non-linear relationship between factors and response. Since the resulted p-value of curvature is 0.018, which is less than 0.05 as shown in

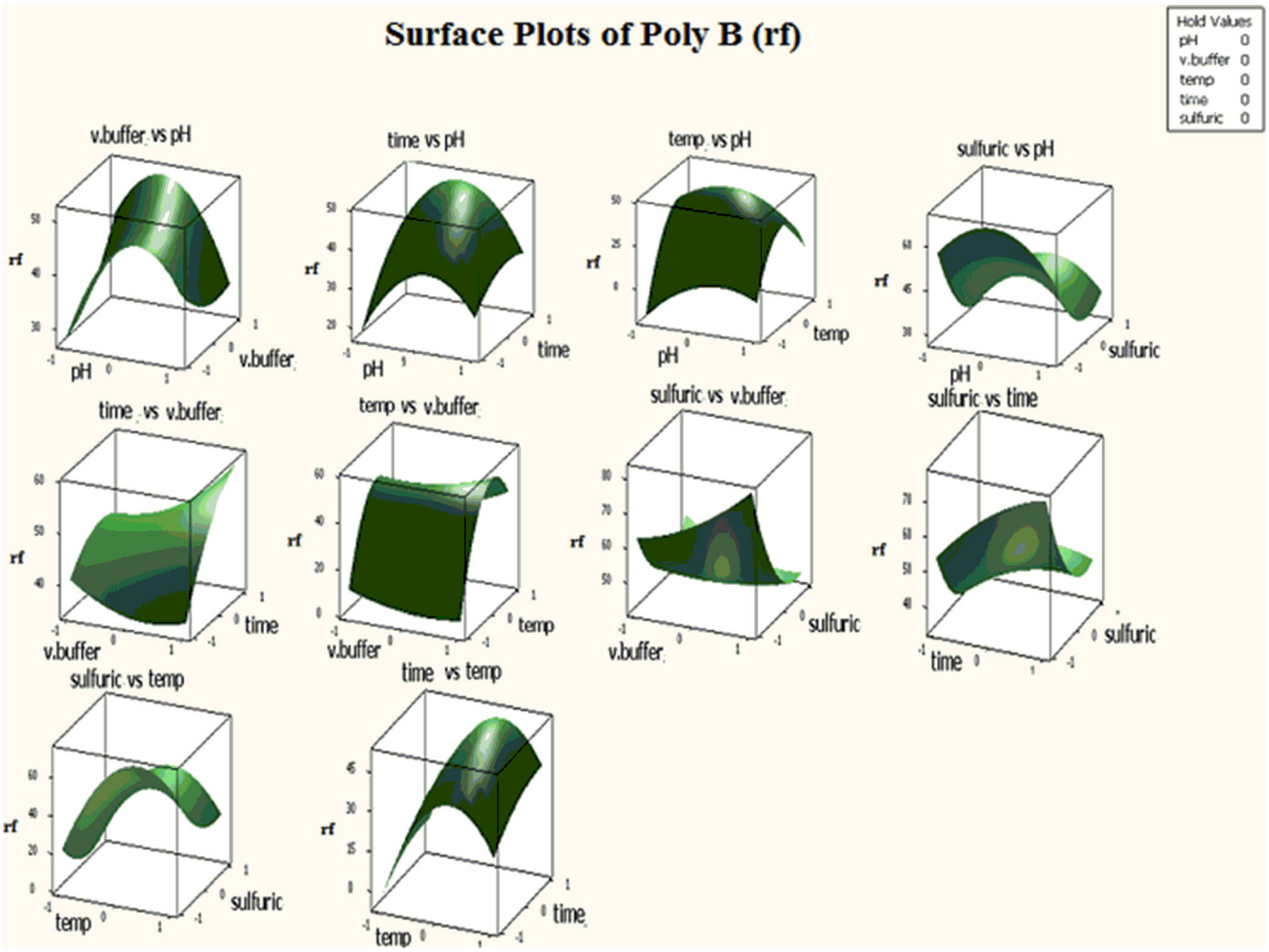

Fig. 3 Response surfaces for Polymyxin B relative fluorescence versus different factors

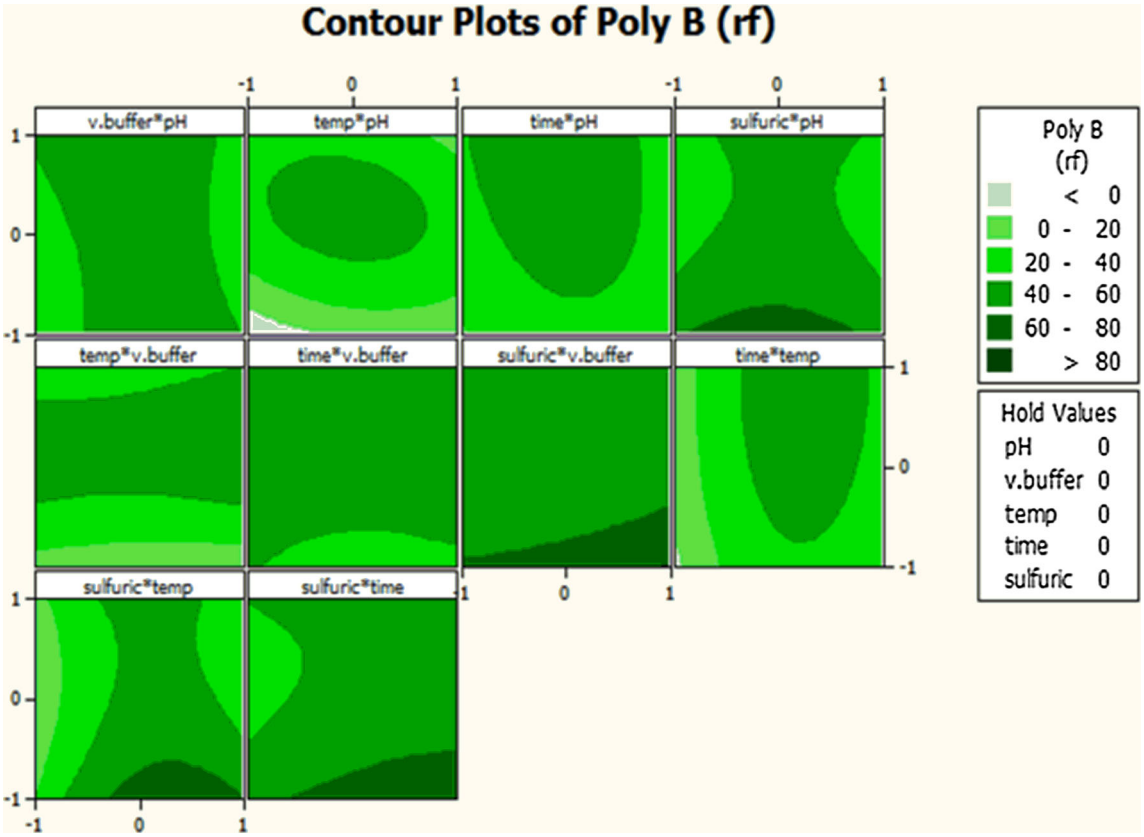

Fig. 4 Contour plots for Polymyxin B relative fluorescence versus different factors

Table 4; there is a significant curvature which means that the relationship between at least one factor and response does not follow a straight line but rather a curved line. Also the residual error is negligible as shown in Table 4.

So by using the fractional factorial design, five factors were found to be the most significant to give the maximum response. They are pH of borate buffer, volume of buffer, temperature, time of heating, and volume of conc. H_2SO_4.

Optimization of Reaction Condition Using Response Surface Methodology (RSM)

The central composite design was used for optimization of reaction condition to determine the best combination of the chosen factors through the determination of their coefficients shown in Table 7.

The model contains five linear effects. ANOVA table, Table 8, shows that p-value for the set of main effects is

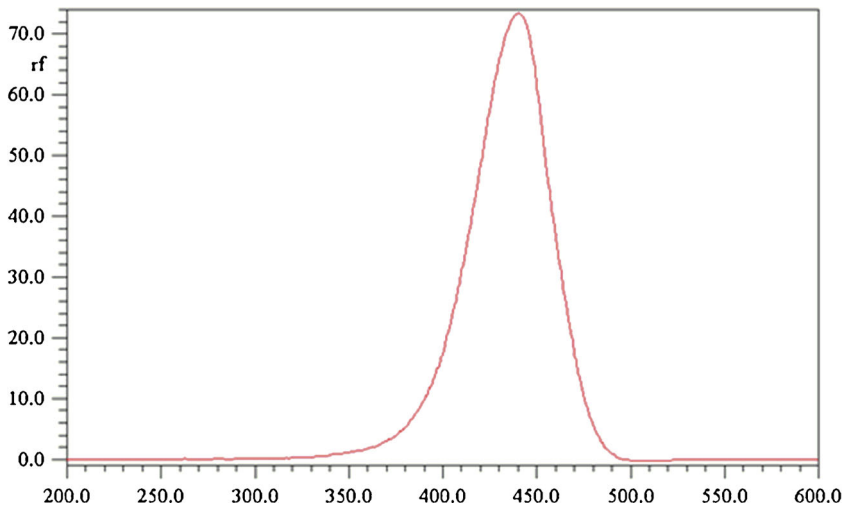

Fig. 5 Synchronous fluorescence spectrum of 800.00 ng mL^{-1} Polymyxin B

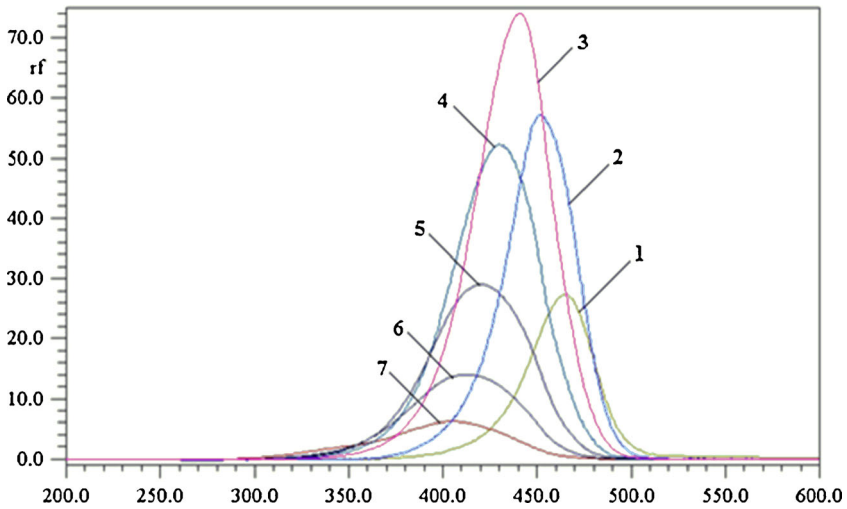

Fig. 6 Synchronous spectra showing the effect of the constant wavelength difference (Δλ) on the synchronous spectrum of Polymyxin B (1)30, (2)50, (3)80, (4)100, (5)120, (6)140, (7)160 nm

0.003. Therefore, there is evidence of a significant effect. The most important factors are temperature and volume of conc. H_2SO_4. The significance of these main effects is proven statistically by comparing their corresponding p-values shown in Table 7 with α-level (0.05). They all have p-value less than 0.05 indicating significant influence of these factors on the reaction.

The model contains five quadratic (square) effects. ANOVA table, Table 8, shows that p-value for the set of quadratic effects is 0.005. Therefore, there is evidence of a significant effect. The most important quadratic effect is the temperature. The significance of the quadratic effect is proven statistically by comparing to the p-value shown in Table 7 which is less than (0.05). Significant quadratic effect means that the relationship between this factor and response does not follow a straight line but rather curved line.

The model contains ten interaction effects. ANOVA table, Table 8, shows that p-value for the set of quadratic effects is 0.063. Therefore, there is evidence of insignificant effect. However, A*C and B*E show significant interactions which are proven statistically by comparing to their p-values shown in Table 7 which are less than (0.05).

The different interactions and their influence on the response are illustrated in the response surface plots (Fig. 3).

For the optimization, the contour plots (Fig. 4) were used to show the effect of factors on the response. There are numbers of combination of variables that could give maximum absorbance as contour plot of pH versus volume of buffer indicating that when the reaction is done at moderate pH (7) and high volume of buffer (2.0 mL) maximum fluorescence was obtained. Also pH versus time of heating contour shows that maximum response is obtained at moderate pH (7) and moderate time of heating (45 min). Time of heating versus volume of conc. H_2SO_4 shows that maximum response is obtained at moderate time of heating (45 min) and low volume of conc. H_2SO_4 (0.2 mL). Temperature versus volume of conc. H_2SO_4

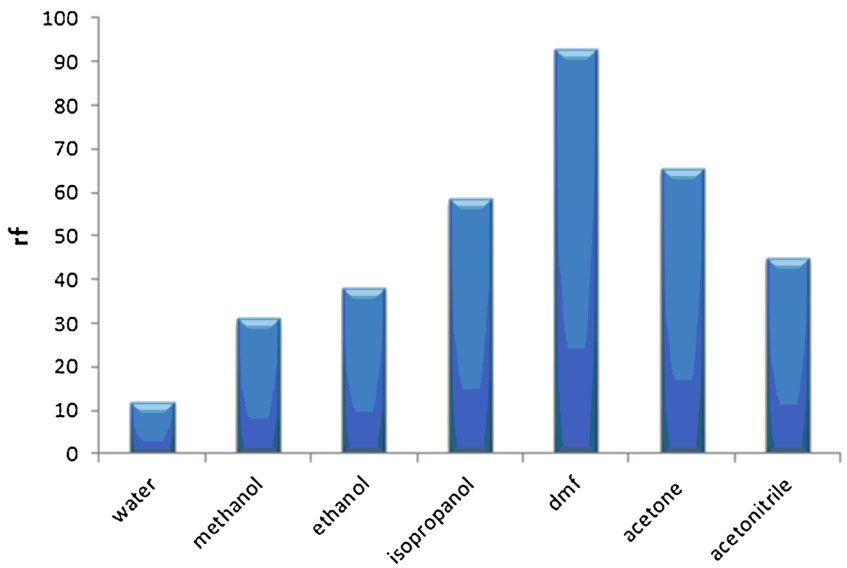

Fig. 7 Effect of diluting solvents on the fluorescence intensity of 1000.00 ng mL^{-1} of Polymyxin B

Table 9 Results of assay validation parameters of the proposed method for the analysis of Poly B in pure form

Parameter	Poly B
Accuracy (mean±SD)	100.23±1.13
Precision (SD)	
Repeatability[a]	0.93
Intermediate precision[b]	1.37
Range (ng mL^{-1})	100.00–1200.00
Linearity	
Intercept	−4.0690
Slope	0.0962
Correlation coefficient	0.9995
LOQ (ng mL^{-1})	10.31
LOD (ng mL^{-1})	31.24

[a] The intraday and [b] the interday standard deviations of samples of concentration 100.00, 300.00 and 1000.00 ng mL^{-1} performed as triplicates

shows that maximum response is obtained at moderate temperature (60 °C) and low volume of conc. H_2SO_4 (0.2 mL).

So by using the central composite design, the best combination of factors that give maximum relative fluorescence is: pH=7, volume of buffer=2.0 mL, temperature=60 °C, time of heating=45 min and volume of conc. H_2SO_4=0.2 mL.

The synchronous fluorescence spectrum was at 440 nm using a constant wavelength difference Δλ=80 nm response=8 s and voltage=400 mV (Fig. 5).

The proposed method was successfully applied for determination of Poly B in pure form and in human plasma by applying synchronous spectrofluorimetric technique. Several wavelength differences were tried as shown in (Fig. 6) and Δλ=80 nm was chosen for the optimum results. Also from different diluting solvents including water, methanol, acetone, DMF and acetonitrile, DMF gave the highest synchronous fluorescence intensities compared to the other solvents (Fig. 7).

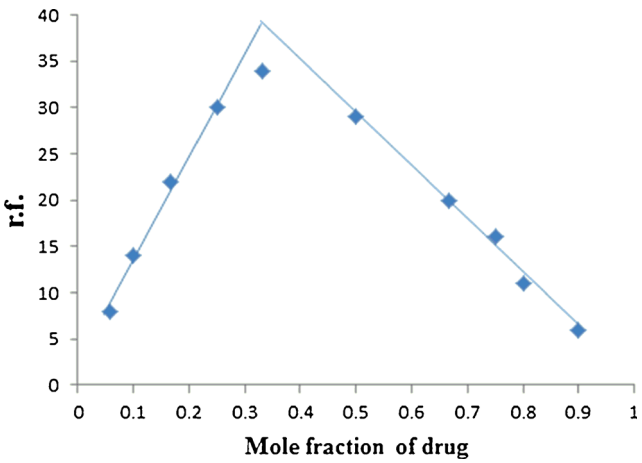

Fig. 8 Job's plot for the stoichiometry of the reaction Poly B and NBD-Cl

Table 10 Average recovery of Poly B in spiked human plasma sample by the proposed method

Added (ng mL^{-1})	Found* (ng mL^{-1})	Recovery %
100.00	105.74	105.74
200.00	210.44	105.22
300.00	317.73	105.91
Mean±SD		105.62±0.36

*Average of three determinations

Method Validation

Range and Linearity

A linear correlation was obtained between the relative fluorescence (at Δλ=80 nm, response=8 s and voltage=400 mV) and the concentration of the pure drug in the range of 100.00–1200.00 ng mL^{-1} for Poly B. The developed method showed good correlation coefficient close to unity, indicating good linearity, as shown in Table 9.

Accuracy

To check the accuracy of the proposed method, the described procedure was repeated three times for determination of different concentrations of Poly B as shown in Table 9.

Precision

The intraday and the interday precision for Poly B were evaluated by assaying variable prepared samples within one day and for three successive days. Low values of SD variations by the proposed method for intraday (0.93) and interday (1.37) suggest an excellent precision.

Table 11 Statistical comparison between the results obtained by applying the proposed method and the published method [13] for the analysis of Poly B in pure form

Parameter	Average recovery of Poly B	
	Proposed method	Published method**
Mean	100.23	100.56
SD	1.13	1.20
Variance	1.28	1.44
n	9	5
t	0.51 (2.18)*	–
F	1.11 (3.84)*	–

*The values between parentheses are the corresponding theoretical values of **t** and **F** at the 95 % confidence level

**Multivariate partial least square (PLS-1) chemometric approach using direct spectra signal for Polymyxin B determination

LOD and LOQ

LOD value was found to be 10.31 and LOQ was found to be 31.24 ng mL^{-1}. These values indicate great sensitivity of this method in comparison with other techniques used for Poly B determination as thin-layer chromatography [4], high-performance liquid chromatography [8] and capillary electrophoresis [14, 16]. Results of assay validation parameters are presented in Table 9.

Stoichiometry of the Reaction

The type of the reaction between Poly B and NBD-Cl is nucleophilic substitution reaction. Job's method plot reached maximum relative fluorescence at a mole fraction of 0.33 which indicated a reaction ratio of 1:2 (Poly B:NBD-Cl) as shown in (Fig. 8).

Application to Human Plasma

The proposed method was applied to the analysis of Poly B in human plasma as shown in Table 10. The results obtained by the adopted method was compared with that of the published one for Poly B [13] where no significant difference between both methods was observed, as shown in Table 11.

Conclusion

A new method was developed and optimized for the determination of the non-fluorescent Poly B with a sensitive fluorimetric method. The low detection limit obtained is important for the analysis of this drug in plasma. Thus the proposed method can be successfully applied for the analysis of Poly B in pure form and in biological fluids as plasma which is of great importance for the pharmaceutical quality control as well as clinical studies. The results show good selectivity, accuracy and precision demonstrating that the developed method is rapid, selective, sensitive, economic and reproducible.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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