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Development of stable formulations of pemetrexed

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ABSTRACT: This research has developed an improved lyophilized formulation for Pemetrexed to enhance its stability using various techniques such as amino acids, boric acid, and sugars. The preliminary screening identified sorbitol as the most suitable sugar and L-Arginine as most suitable amino acid due to low degradation rate and minimal change in reconstitution time after one month of storage under accelerated stability conditions. 3^2 factorial design was employed to optimize the formulation, considering the drug-to-boric acid ratio (X1 factor) and the drug-to-L-Arginine ratio (X2 factor). The Design-Expert® software was utilized to generate optimized formula based on the results of nine batches. The desired responses included the % assay of the lyophilized and reconstituted formulations, reconstitution time, and pH of the composition. The optimized batch exhibited results in-line with the software predictions. Stability testing of the optimized batch under accelerated conditions (for six months revealed no significant differences in the evaluation parameters. Furthermore, the optimized formulation outperformed the marketed formulation. Cell line studies conducted on Pemetrexed API and the formulated dosage form demonstrated enhanced efficacy of the formulation, indicated by a lower IC50 value compared to Pemetrexed API alone. These comprehensive studies confirmed the stability of the prepared dosage form.

KEYWORDS: Amino Acid; Anti-cancer agent; Lyophilized dosage form; Pemetrexed; Stability studies.

1. INTRODUCTION

Cancer is still a problem for the world's health and has to be treated effectively with novel approaches. A multi-targeted anti-folate drug called Pemetrexed has shown encouraging outcomes in the treatment of a number of cancers, especially mesothelioma and non-small cell lung cancer. Pemetrexed's inherent volatility, however, makes it difficult to formulate for pharmaceutical usage [1-5].

A lump of tissues or cells that resembles swelling is referred to be a tumor. Tumors typically fall into one of three categories. These tumors come in three different types: benign, pre-malignant, and malignant. The authors of the current study go into great detail about the physiology of the lungs to assist readers comprehend precisely where lung cancer develops. Lung cancer, also known as pulmonary cancer, develops from the cells of the lung as its name suggests. Lung cancer primarily comes in two different forms. Small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) are the two types. Around 80 to 85% of all lung cancers are NSCLC, depending on the kind. Adenocarcinoma, squamous cell carcinoma, and big cell (undifferentiated) carcinoma are the three primary subtypes of NSCLC [6-12].

The United States (US) granted its initial approval for Pemetrexed on February 4, 2004. On September 22, 2004, the European Union (which comprises around 28 countries) approved Pemetrexed, and on October 8, 2008, India did likewise. Anticancer medication Pemetrexed disodium operates on folate-dependent responses. These processes are necessary for cell development. Finally, the US Food and Drug Administration (USFDA) authorized Pemetrexed as the first medication for the management of the rare malignancy malignant pleural mesothelioma. Additionally, the USFDA granted Pemetrexed expedited approval for the second-line treatment of non-small-cell lung cancer. Pemetrexed has been authorized by the FDA under the trade name ALIMTA® and new drug application number N021462. An intravenous infusion of Pemetrexed is given over ten minutes [13-21].

The lyophilized form of the marketed formulation has a shelf life of only two years, and once reconstituted, the solution remains stable for a mere 24 hours. This presents a challenge for patients

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undergoing chemotherapy who must undergo a creatinine clearance blood test to qualify for dosing on a specific day. If a patient fails the test, they are retested after 24 hours. Consequently, the initially reconstituted solution would have lost its stability after this period, rendering it ineffective. Another issue with the current marketed formulation is its limited stability, attributed to the use of only one excipient, a sugar alcohol. Therefore, there was an unmet need for better stable formulation with novel mechanism that can improve both lyophilized and reconstituted stability of Pemetrexed formulation. This study sought to develop a better Pemetrexed lyophilized pharmaceutical dosage form, with an emphasis on improving stability by the addition of particular excipients with novel mechanism. Amino acids, boric acid, and sugar alcohols were only a few of the methods that were investigated to stabilize the formulation in combination of one another. Because of their capacity to participate in Lewis acid-base reactions, amino acids were chosen, whereas boric acid provided the possibility of donor-acceptor bonding. Additionally, sugar alcohols, renowned for their capacity to generate covalent bonds through anhydride production, were studied. Due to usage of this novel excipients, this study provided the lyophilized formulation with improved stability including of lyophilized formulation as well as reconstituted formulation than marketed formulation.

2. RESULTS and DISCUSSIONS

2.1 Physical Compatibility Study

Physical compatibility study of the drug was determined. All the excipients with Pemetrexed showed no change in colour as well as physcial state change. From this physical interaction, it was concluded that there is no siginificant dfference between each of exipenints and concenration (drug: excipient = 1:1 to 1:3) thereof. After completion of study it was found that Pemetrexed was stable at accelerated condition of temperature and relative humidity condition with all the excipients, including Boric Acid, Sorbitol, Mannitol, Lactose Monohydrate, Glucose, L-Arginine, Phenylalanine and L-Lysine. Hence, all excipients were carried further for the excipient selection process.

2.2 Preliminary Screening Study

2.2.1 Preliminary screening of Sugars

The trials for selection of sugars were carried out as discussed in section 4.3.1 and showed results as mentioned in Table 1. Based on results showed in Table 1, it was observed that Sorbitol containing P1 batch had the lowest degradation rate and lowest change in reconstitution time after storage of one month at accelerated stability conditions, when compared to the other bathes P2 (containing mannitol), P3 (containing lactose monohydrate) and P4 (containing glucose). Hence, Sorbitol was finalized as the sugar excipient for further development of factorial design batches.

Table 1. Preliminary screening batches

Batch No.	Pemetrexed (mg)	Excipient name	Quantity (mg)	Results Interval			
				Initial		After 1 month	
				% assay (%)	Reconstitution time (sec)	% assay (%)	Reconstitution time (sec)
P1	100	Sorbitol	100	99.61 ± 0.03	62.47 ± 2.02	98.75 ± 0.04	63.91 ± 1.53
P2	100	Mannitol	100	99.45 ± 0.12	71.00 ± 2.11	98.42 ± 0.10	73.00 ± 2.88
P3	100	Lactose	100	99.07 ± 0.17	80.21 ± 2.32	98.19 ± 0.13	86.54 ± 2.58
P4	100	Glucose	100	99.51 ± 0.08	69.53 ± 1.68	98.28 ± 0.07	74.72 ± 2.13
P5	100	L-Arginine	100	99.64 ± 0.03	64.00 ± 2.21	99.27 ± 0.05	68.00 ± 2.87
P6	100	Proline	100	99.32 ± 0.11	81.00 ± 4.00	98.84 ± 0.12	86.33 ± 2.05
P7	100	Phenylalanine	100	99.18 ± 0.13	91.33 ± 3.51	98.53 ± 0.11	104.33 ± 4.02
P8	100	L-lysine	100	99.41 ± 0.05	73.00 ± 3.00	99.02 ± 0.06	78.33 ± 1.25

Results = mean ± SD; n = 3

2.2.2 Preliminary screening of Amino Acids

The trials for selection of Amino acids were carried out as discussed in section 4.3.2 and showed results as mentioned in Table 1. Based on results showed in Table 1, it was observed that L-Arginine containing P5 batch had the lowest degradation rate and lowest change in reconstitution time after storage of one month at accelerated stability conditions, when compared to the other bathes P6 (containing proline), P7 (containing phenylalanine) and P8 (containing L-Lysine). Hence, L-Arginine (P5) was finalized as the amino acid excipient for further development of factorial design batches.

2.3 Evaluation of 3^2 Factorial Design Batches

3^2 factorial design batches were taken as per the composition described in **Table 2** as F1 to F9. Lyophilized vials were initially checked for the results on evaluation parameters including % Assay of Lyophilized formulation, % Assay of reconstituted formulation, Reconstitution time and pH of reconstituted solution. The Lyophilized samples of these batches were then stored for one month in accelerated conditions at (40±2) ºC and (75±5) % RH and similarly, above analytical parameters were checked for initial samples as well as samples after one month. % Assay of reconstituted formulation was kept at room temperature until samples reaches to 90% drug concentration at regular interval of 1 day. Results are reported in Table 2.

Table 2. 3^2 Factorial design batches and evaluation results

Ingredients	Formulation batches (All quantities are in mg per tablet)								
	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9
Pemetrexed	100	100	100	100	100	100	100	100	100
Boric Acid (X_1)	10	30	50	10	30	50	10	30	50
L-Arginine (X_2)	10	10	10	30	30	30	50	50	50
Sorbitol	80	60	40	60	40	20	40	20	0
Total Weight	**200**	**200**	**200**	**200**	**200**	**200**	**200**	**200**	**200**

Evaluation results of 3^2 factorial design batches

Batch No.	% Assay results of lyophilized injection		Reconstitution time (sec)		pH of reconstituted solution		% Assay of reconstituted injection			
	Initial	After 1 month	Initial	After 1 month	Initial	After 1 month	Initial	After 1 day	After 2 days	After 3 days
F1	99.53 ± 0.05	98.96 ± 0.13	59 ± 1.25	65 ± 4.04	7.03 ± 0.02	7.05 ± 0.05	99.14 ± 0.17	94.35 ± 0.20	88.80 ± 0.10	86.15 ± 0.24
F2	99.65 ± 0.10	99.25 ± 0.05	54 ± 3.09	60 ± 5.05	6.88 ± 0.03	6.91 ± 0.06	99.25 ± 0.20	92.47 ± 0.10	87.53 ± 0.10	85.82 ± 0.10
F3	99.68 ± 0.06	98.91 ± 0.07	42 ± 2.87	54 ± 3.56	6.81 ± 0.12	6.85 ± 0.12	99.24 ± 0.10	92.72 ± 0.13	84.62 ± 0.13	84.49 ± 0.12
F4	99.62 ± 0.08	98.99 ± 0.12	73 ± 2.49	74 ± 2.94	6.91 ± 0.02	6.96 ± 0.04	99.40 ± 0.22	97.00 ± 0.11	94.11 ± 0.24	91.18 ± 0.13
F5	99.87 ± 0.05	99.54 ± 0.03	56 ± 2.16	59 ± 2.16	6.81 ± 0.05	6.84 ± 0.03	99.39 ± 0.09	94.99 ± 0.21	92.98 ± 0.43	90.19 ± 0.20
F6	99.91 ± 0.02	98.95 ± 0.06	50 ± 3.68	57 ± 0.82	6.76 ± 0.06	6.79 ± 0.14	99.56 ± 0.20	97.05 ± 0.12	93.69 ± 0.22	89.35 ± 0.19
F7	99.45 ± 0.08	98.68 ± 0.03	78 ± 2.87	82 ± 4.31	6.74 ± 0.04	6.76 ± 0.05	99.24 ± 0.20	93.08 ± 0.13	89.51 ± 0.10	86.61 ± 0.10
F8	99.85 ± 0.06	99.46 ± 0.10	90 ± 2.94	96 ± 4.50	6.73 ± 0.11	6.76 ± 0.07	99.15 ± 0.16	94.92 ± 0.20	90.59 ± 0.20	84.41 ± 0.13
F9	99.12 ± 0.06	98.88 ± 0.10	101 ± 2.62	102 ± 1.13	6.75 ± 0.15	6.77 ± 0.24	99.21 ± 0.09	93.07 ± 0.10	85.50 ± 0.14	86.25 ± 0.12

Results = mean ± SD; n = 3

2.4 Evaluation of Optimized Batch and Stability Study

Data of Table 2 were fed in Design-Expert® 11.1.2.0 (Trial Version) from Stat-Ease® Inc. to generate the response analysis as well as to generate the formula for optimized batch. Software generated overlay plot graph**.** According to overlay plot recommendations, optimized batch was taken and checked for the evaluation parameters including % Assay of lyophilized formulation, % Assay of reconstituted formulation and pH of reconstituted solution. Optimized batch was then stored for six months in accelerated conditions at (40±2) ºC and (75±5) % RH and similar analytical parameters were checked for initial samples as well as samples after one month to six-months. Formula for optimized and results thereof are reported in Table 3. Results reported in Table 3 proved that optimized batch was stable even after 6 months of stability study with respect to all above parameters. ANOVA stastical analysis methods were used to determine that the optimum formulation during storage at accelerated stability conditions did not show any significant changes.

Table 3. Formulation and evaluation of optimized batch

Composition of Optimized batch derived from with Design-Expert® 11.1.2.0 (Trial Version)					
Ingredients	Pemetrexed	Boric Acid (X1)	L-Arginine (X2)	Sorbitol	Total Weight
Quantity (mg)	100	22.35	26.81	50.84	200

Evaluation of Optimized batch derived from with Design-Expert® 11.1.2.0 (Trial Version)				
Evaluation Parameter	Time Interval			
% Assay of lyophilized formulation	Initial 99.68 ± 0.08	After 1 month 99.43 ± 0.07	After 3 months 99.34 ± 0.10	After 6 months 99.05 ± 0.05
pH of reconstituted solution	Initial 6.98 ± 0.02	After 1 month 6.92 ± 0.02	After 3 months 6.93 ± 0.02	After 6 months 6.87 ± 0.03
% Assay for reconstituted formulation	Initial 99.48 ± 0.08	After 1 day 96.27 ± 0.07	After 2 days 93.10 ± 0.08	After 3 days 91.30 ± 0.14

Results = mean ± SD; n = 3

Stastical Analysis using ANOVA for Optimized batch							
Responses	**Predicted Mean**	**Observed**			**95% PI low**	**Data mean**	**95% PI high**
% Assay Lyophilized	99.41	99.51	99.34	99.43	99.51	99.04	99.42
% Assay First reconstiuted solution	90.13	91.47	91.12	91.31	91.47	87.49	91.30
Reconstitution time	70	55	59	62	55	51.29	58.66
pH	6.89	6.89	6.93	6.94	6.89	6.83	6.92

Composition of Optimized batch derived from with Design-Expert® 11.1.2.0 (Trial Version)					
Ingredients	Pemetrexed	Boric Acid (X1)	L-Arginine (X2)	Sorbitol	Total Weight
Quantity (mg)	100	22.35	26.81	50.84	200

Evaluation of Optimized batch derived from with Design-Expert® 11.1.2.0 (Trial Version)				
Evaluation Parameter	Time Interval			
% Assay of lyophilized formulation	Initial 99.68 ± 0.08	After 1 month 99.43 ± 0.07	After 3 months 99.34 ± 0.10	After 6 months 99.05 ± 0.05
pH of reconstituted solution	Initial 6.98 ± 0.02	After 1 month 6.92 ± 0.02	After 3 months 6.93 ± 0.02	After 6 months 6.87 ± 0.03
% Assay for reconstituted formulation	Initial 99.48 ± 0.08	After 1 day 96.27 ± 0.07	After 2 days 93.10 ± 0.08	After 3 days 91.30 ± 0.14

Results = mean ± SD; n = 3

Stastical Analysis using ANOVA for Optimized batch							
Responses	**Predicted Mean**	**Observed**			**95% PI low**	**Data mean**	**95% PI high**
% Assay Lyophilized	99.41	99.51	99.34	99.43	99.51	99.04	99.42
% Assay First reconstiuted solution	90.13	91.47	91.12	91.31	91.47	87.49	91.30
Reconstitution time	70	55	59	62	55	51.29	58.66
pH	6.89	6.89	6.93	6.94	6.89	6.83	6.92

2.5 Comparative Study of Optimized Batch with Marketed Formulation

Comparative evaluation study of optimized batch as obtained from Design-Expert® software was done with marketed formulation. Evaluation parameters included % Assay of lyophilized formulation, % Assay of reconstituted formulation and pH of reconstituted solution. Lyophilized vials of both optimized batch and marketed formulation were initially checked for the results on above parameters. The Lyophilized samples of these batches were then stored for six months in accelerated conditions at (40±2) °C and (75±5) % RH and similarly, above analytical parameters were checked for initial samples as well as samples after six-months. Results obtained are reported in Table 4. From results reported in Table 4, it was concluded that optimized batch was more stable even after 6 months of stability study with respect to all above parameters against marketed formulation.

Table 4. Comparative study of optimized batch with marketed formulation

Evaluation Parameter	Time Interval			
% Assay of lyophilized formulation	**Initial**	**After 1 month**	**After 3 months**	**After 6 months**
Optimized Batch	99.68 ± 0.08	99.43 ± 0.07	99.34 ± 0.10	99.05 ± 0.05
Marketed formulation	99.64 ± 0.09	98.88 ± 0.06	98.29 ± 0.09	97.72 ± 0.08
pH of reconstituted solution	**Initial**	**After 1 month**	**After 3 months**	**After 6 months**
Optimized Batch	6.98 ± 0.02	6.92 ± 0.02	6.93 ± 0.02	6.87 ± 0.03
Marketed formulation	7.01 ± 0.02	6.98 ± 0.02	6.95 ± 0.02	6.88 ± 0.02
% Assay for reconstituted formulation	**Initial**	**After 1 day**	**After 2 days**	**After 3 days**
Optimized Batch	99.48 ± 0.08	96.27 ± 0.07	93.10 ± 0.08	91.30 ± 0.14
Marketed formulation	99.55 ± 0.06	90.58 ± 0.09	88.22 ± 0.12	85.63 ± 0.18

Results = mean ± SD; n = 3

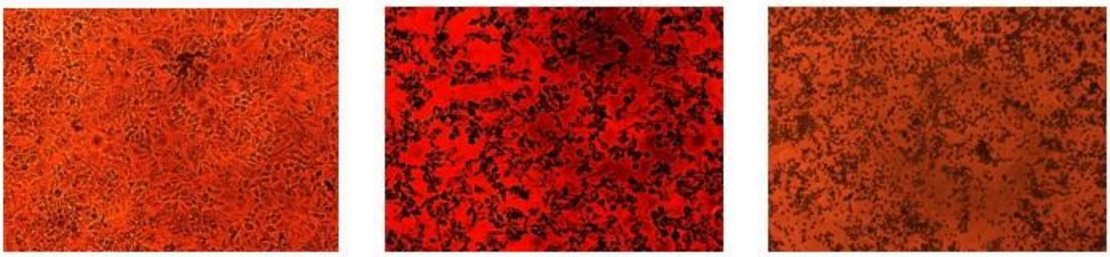
2.6 Cell Line Study for Pemetrexed API and Optimized Formulation Thereof

Cell line study was performed as discussed in section 2.9. Results obtained are reported in **Table 5**.

Table 5. Results for cell line study for Pemetrexed API and optimized formulation thereof

Drug Con (μm)	0	1.5	3	6	12	24	30
Pemetrexed API							
Absorbance	0.611	0.557	0.510	0.482	0.431	0.402	0.316
% Survival	100	91.11	83.47	78.84	70.61	65.75	51.79
IC_{50}	**6.92**						
Optimized Formulation							
Absorbance	0.611	0.514	0.494	0.478	0.445	0.424	0.319
% Survival	100	84.18	80.91	78.18	72.79	69.35	52.23
IC_{50}	**4.36**						

The IC50 value of Pemetrexed alone was found to be 6.92 ± 0.658 μM (n=3). This indicates that a concentration of 6.92 ± 0.658 μM of Pemetrexed is necessary to achieve a 50% inhibition of the biological process in a laboratory setting. The formulation containing Pemetrexed has an IC50 value of 4.36 ± 0.485 μM. The IC50 of pemetrexed alone and in formulation exhibit a significant difference at a 5% level of significance, as indicated by a t-statistic of 4.93 and a p-value of 0.0078. This implies that a reduced concentration of the formulation is required to attain an equivalent degree of inhibition in comparison to using pemetrexed alone. The formulation's lower IC50 value suggests that it may have increased effectiveness in comparison to pemetrexed alone. The formulation has the potential to be more powerful or have enhanced drug transport capabilities, resulting in greater toxicity against the A549 lung cancer cell line. The morphology of untreated and treated cells are illustrating in **Figure.**



(A) (B) (C)

Figure. Cell line study: (A) is untreated; (B) is MTT assay for Pemetrexed API; (C) is MTT assay for optimized formulation

3. CONCLUSION

The aim of the research was to prepare improved lyophilized pharmaceutical dosage form of Pemetrexed, achieving improved stability compared to marketed formulations. To stabilize the pharmaceutical dosage form, various techniques that include usage of amino acids (acts as Lewis base and form Lewis acid-base reaction), boric acid (donor-acceptor type of bond), and sugar (weak covalent bond through anhydride formation), were used. Sorbitol, chosen as the sugar alcohol excipient, provided the lowest degradation rate and lowest change in reconstitution time during accelerated stability conditions. Similarly, L-Arginine, selected as amino acid excipent, demonstrated the lowest degradation rate and lowest change in reconstitution time during accelerated stability conditions. 3^2 full factorial design incorprated drug: boric acid as X1 factor and drug: L-Arginine as X2 factor. The optimized batch, generated from Design-Expert® 11.1.2.0 (Trial Version), aligned closely with predicted results for % Assay of lyophilized formulation, % Assay of reconstituted formulation, reconstitution time and pH of the composition. Accelerated stability study at 40°C and 75% RH for 6 months showed that no significant difference in evaluation parameters for the optimized batch. Further, comparative analysis with the marketed formulation faovured the optimized formulation. In addition, cell line study on Pemetrexed formulation showed lower IC50 value than Pemetrexed API indicated enhanced efficacy compared to Pemetrexed API alone. Collectively, these results confirm the stability of the prepared dosage form of Pemetrexed.

4. MATERIALS AND METHODS

4.1 Materials

Pemetrexed was obtained from B D R Lifesciences Pvt. Ltd., India, India. Mannitol, Sorbitol, Lactose, Glucose, L-Arginine, Proline, Phenylalanine, L-Lysine and Boric Acid were procured from Sisco Research Laboratories Pvt. Ltd., India

4.2 Physical Compatibility study

Compatibility study was carried out to investigate any existing interaction between the drug and the excipients used in the formulation. 100 mg of Pemetrexed was uniformly mixed individually with excipients like sorbitol, mannitol, lactose monohydrate, glucose, boric acid, L-arginine, proline, phenyl alanine and L-lysine in different ratios of 1:1, 1:2 and 1:3. These physical mixtures were filled in respective vials. These vials were stored for 1 week in accelerated stability studies at (40±2) ºC and (75±5) %RH and changes in physical appearance was checked.

4.3 Preliminary Screening Study

4.3.1 Preliminary Screening of Sugars

Sugars used in parenteral composition like sorbitol, mannitol and lactose monohydrate in different concentrations were mixed individually with the drug for the screening of sugars. These batches are shown in Table 1. These batches were placed under accelerated conditions at (40±2) ºC and (75±5) % RH for one month and analyzed for % assay and reconstitution time initially as well as after one month. From the observation of data, or sugar was finalized for the further developmental batches.

General formulation Procedure for preliminary screening study: About 7.5 ml Purified Water for injection was taken in a beaker and heated at 40-45°C. 100 mg Pemetrexed was slowly then added into above Purified Water for injection to form a clear solution on continuous stirring about 300 RPM. 2.5 ml of Purified Water for injection was added into the above solution in continuous stirring. To the above solution, 100 mg of sugars (as per batches shown in Table 1) was slowly added on continuous stirring. Stirring was continued for about 20 minutes to get clear solution. Above solution was then cooled down to the room temperature and was filtered using 0.2 micron membrane filter using a syringe. 10 ml solution prepared as per above step was filled into vial through syringe and was closed with half stopper grey bromo butyl rubber stopper. This vial was placed in a lyophilizer for the lyophilization cycle as mentioned in Table 6. Lyophilized vials were stored for one month in accelerated conditions at (40±2) ºC and (75±5) % RH and analytical parameters like % assay and reconstitution time were checked for initial samples as well as samples after one month [22-24].

Table 6. Lyophilization cycle

Process Step	Set Temperature (°C)	Vacuum (Pascal)	Time (Minutes)	
			RAMP	Hold
Freezing	-10	-	90	90
	-25	-	90	90
Primary Drying	-10	100	180	900
	5	100	120	240
Secondary Drying	15	100	180	360
	25	100	180	1500

4.3.2 Preliminary Screening of Amino Acids

Amino acids used in parenteral composition like L-arginine, proline, phenyl alanine and L-lysine in different concentrations were mixed individually with the drug for the screening of Amino acids. These batches are shown in Table 1. These batches were placed under accelerated conditions at (40±2) ºC and (75±5) % RH for one month and analyzed for % assay and reconstitution time initially as well as after one month. From the observation of data, sugar was finalized for the further developmental batches. Similar general procedure as mentioned in section 4.3.1 was followed to prepare the batches.

4.4 Formulation of Batches by 3^2 Factorial Design

The 3^2 factorial design applied in this study involved evaluation of two factors, each at three levels. Experiments were performed at all nine possible combinations. ratio of concentration of drug: concentration of boric acid (X1) and ratio of concentration of drug: concentration of L-Arginine (X2) were selected as independent variables. % Assay of lyophilized formulation (Y1), % Assay of reconstituted formulation (Y2), reconstitution time (Y3) and pH of reconstituted solution (Y4) were selected as dependent variables.

4.4.1 Design Layout According to 3^2 Factorial Design

Table 2 (Batches F1–F9) incorporates design layout for nine batches according to 3^2 factorial design. Ratio of concentration of drug: concentration of Boric Acid was selected in the range of 1: 0.1, 1: 0.3 and 1: 0.5. Similarly, ratio of concentration of drug: concentration of L-Arginine was used. Different concentrations of both factors were used to design the factorial design matrix. Here, 1 means 100 mg, which is standard dose of API Pemetrexed. Method of preparation was same as below general formulation procedure.

General formulation Procedure for 3^2 factorial design: About 7.5 ml Purified Water was taken in a beaker as was heated to 40-45˚C. 100 mg Pemetrexed was slowly then added into above water for injection to form a clear solution on continuous stirring about 300 RPM. 2.5 ml of Purified Water was added into the above solution in continuous stirring. To the above solution, above quantity of L-Arginine, Boric Acid and Sorbitol (as per batches mentioned in Table 2) were slowly added on continuous stirring. Stirring was continued for about 20 minutes to get clear solution. Above solution was then cooled down to the room temperature and was filtered using 0.2 micron membrane filter using a syringe. 10 ml solution prepared as per above step was filled into vial through syringe and was closed with half stopper grey bromo butyl rubber stopper. Above vial was placed in a lyophilizer for the lyophilization cycle as mentioned in Table 6. Lyophilized vials were stored for one month in accelerated conditions at (40±2) ºC and (75±5) % RH and analytical parameters like % assay and reconstitution time were checked for initial samples as well as samples after one month.

4.5 Optimization of Formula by Design Expert® 11.1.2.0 (Trial Version)

Optimization of formula was investigated with Design-Expert® 11.1.2.0 (Trial Version) from Stat-Ease® Inc. by identification of influencing factors. Results obtained after one-month stability study of all 3^2 factorial design batches were fed into above software. In this software following were considered as dependent factors which are responsible for making change in the response result. Concentration (mg) of Boric Acid was considered as Factor-1 and Concentration (mg) of L-Arginine was considered as Factor-2. Responses were included as follows: % Assay for lyophilized formulation results after one month of stability study (Response-1), % Assay for first reconstituted formulation results after one week of stability study (Response-2), Reconstitution time (sec) results after one month of stability study (Response-3) and pH of Reconstituted solution results after one month of stability study (Response-4).

4.6 Evaluation Parameters for Factorial and Optimized Batch

Various critical parameters evaluated during research work are as follows for factorial design batches as well as optimized bathes.

4.6.1 % Assay Determination by HPLC Method

Buffer preparation was done with 0.17 per cent v/v of glacial acetic acid in water adjusted to pH 5.3 with 50 per cent sodium hydroxide solution. This buffer and acetonitrile in the ratio 65:35 v/v was used as Mobile phase. Water was used as diluent. Standard solution was prepared by transferring about 5 mg of Pemetrexed in 500 ml of volumetric flask. About 100 ml of water was added and sonicated for about 30 sec. Volume was made with water. Sample solution for lyophilized injection was prepared by transferring 5 mg of Pemetrexed from 1 vial (10 mg lyophilized Injection) to 500 ml volumetric flask. About 100 ml of water was added and sonicated for about 30 sec. Volume was made with water. (0.01% w/v concentration). Sample solution for reconstituted injection was prepared by transferring 1 ml from 4 ml of reconstituted Pemetrexed injection from 1 vial to 25 ml volumetric flask. About 10 ml of water was added and sonicated for about 30 sec. Volume was made with water. 1 ml from this solution was accurately transferred to 100 ml volumetric flask. Volume was made with water. (0.01% w/v concentration). Limit as per Indian Pharmacopoeia for Pemetrexed Injection contains not less than 90.0 per cent and not more than 110.0 per cent of the stated amount of Pemetrexed [25-27].

4.6.2 Reconstitution Time

1 vial was reconstituted with 4 mL of 0.9% Sodium Chloride. The reconstituted product was checked for clarity and colorlessness of the solution [27].

4.6.3 pH of Reconstitution Time

1 vial was reconstituted with 4 mL of 0.9% Sodium Chloride. The reconstituted product was checked for pH of the solution [27].

4.7 Stability Study for Optimized Batch

Adequate stability data of dosage form is essential to prove the quality, purity, safety and effect of time during storage. Hence, optimized batch was subjected for stability study for 6 months at (40 ± 2) ºC and (75 ± 5) % RH.

4.8 Comparative Study of Optimized Batch with Marketed Formulation

Comparative study of optimized batch as obtained from Design-Expert® 11.1.2.0 (Trial Version) from Stat-Ease® Inc. software with marketed formulation was performed and similar evaluation parameters were checked which includes % Assay of lyophilized formulation, % Assay of reconstituted formulation and pH of reconstituted solution. Lyophilized vials were initially checked for the results on above parameters. The Lyophilized samples of these batches were then stored for six months in accelerated conditions at (40±2) ºC and (75±5) % RH and similarly, above analytical parameters were checked for initial samples as well as samples after six-months. Results of the optimized batch were compared with marketed formulation of Pemetrexed.

4.9 Cell Line Study for Pemetrexed API and Optimized Formulation Thereof

To investigate the IC50 value of Pemetrexed formulation, Cytotoxicity (MTT assay) study of formulation was performed on lung cancer cell line A549. IC50 value is a quantitative measure that indicates how much of a particular drug is needed to inhibit biological process by 50% in vitro. Cell line was procured from National Centre for Cell Science (NCCS), Pune.

4.9.1 Day 1: Procedure for Cytotoxicity Study

Culture flask with 80-90% confluent cells were taken and cells were washed with with 1ml Phosphate buffered saline (PBS) twice. PBS was then removed and cells were Trypsinized by adding 1ml Trypsin-EDTA solution. Culture flask was then incubated at 37°C in CO_2 incubator for 7-8 minutes at 5% CO_2. 1 ml of cell suspension was transferred to 1.5 ml microcentrifuge tube and cells were centrifuged at 500g for 10 minutes at 25°C. Media was removed carefully. Then 1ml of PBS was added in each vial and was mixed gently to remove cell clumps. 1ml media was then added and mixed gently. 10µl of cell suspension was taken and cells were counted. Around 1000 cells in each well of 96-well plate were added according to cell count. Plate was then incubated at 37°C for 24 hours.

4.9.2 Day 2: Procedure for Cytotoxicity Study: Drug Treatment Phase

After 24 hours, media was removed from each well. Drug was added at different concentration (1.5, 3, 6,12, 24, 30 and 60 µM) in triplicate and make up the volume of each well up to 300 µl with media. One set of the three wells were kept as untreated that will serve as controls. This well-plate was incubated again for 24 hours at 37°C in CO_2 incubator.

4.9.3 Day 3: Procedure for Cytotoxicity Study: MTT Assay

Fresh solution of MTT (5 mg/ml) in PBS was prepared and filtered using 0.22µ filter. 25 µl of freshly prepared MTT solution was added in each well and incubated it at 37°C for 2-3 hours. Media was then

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removed and 100µl DMSO was added and mixed gently by pipetting. Plate was then incubated at 37°C overnight. Absorbance at 570 nm was read after overnight. Obtained results were fed to into Prism software to calculate IC50 value.

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