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Melphan as Anticancer and New Insights of Drug as Parenteral Dosage form against SARS-CoV-2 by Using Development and Validation

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ABSTRACT

A validated HPLC method was developed for the determination of Melphan (MPA) in pharmaceutical formulation. LC 20AT pump and UV-Visible detector with flexible wavelength programme and Rheodyne injector are used in this present problem. Phenomenex Synergi C18, 250 mm × 4.6 mm, 4μm or equivalent is used for this chromatography analysis. Column used in this measurement is Phenomenex Synergi C18,250mm×4.6mm,4μm or equivalent. Specificity (stressed condition) is confirmed by no co-elution of diluent peaks, Blank as diluent, excipients peaks, Monohydroxy MPA, Dihydroxy MPA, Isopropyl ester and dimer impurity were not interfere with MPA Peak along with each other. Obtained results are 1.2 and 270103 respectively. The method developed is simple and is better than the methods reported in the literature. With the help of different studies, therefore, we recommend pursuing an induction regimen of up to six cycles in all patients to postpone treatment with high-dose MPA. By using various studies, we recommend pursuing an induction regimen of up to six cycles in all patients to postpone treatment with high-dose MPA for SARS-CoV-2.

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INTRODUCTION

The Molecular formulae for MPA (MPA)is $C_{13}H_{18}Cl_2N_2O_2$·MPA is generally popular as L-phenylalanine mustard, phenylalanine mustard, L-PAM, or L-sarcolysin. It is acting as derivate for phenylalanine nitrogen mustard. MPA is represented as 4-[bis(2-chloroethyl)amino]-L-phenylalanine. MPA is readily accessible as tablet form also it used as oral administration. MPA stops synthesis of DNA along with synthesis of RNA. Recent advances in cancer immunotherapy have offered unprecedented opportunity to harness the power of the immune system to fight against cancer (Mellman *et al.*, 2011). Generally MPA utilized for treating different types of myeloma, (Facon *et al.*, 2007) ovarian cancer, AL amyloidosis along with rarely malignant melanoma. Structure Figure 1. For the estimation of MPA many type of instrumental techniques were reported in very natural drug form, pharma along with biological samples with help of electro kinetic chromatography, liquid chromatography, spcetrophotometry, HPTLC by unique nor blended arrangements. (Kumar and Nadh, 2011) proposed MPA in tablet formulation. Authors used Acetonitrile 70v/v, water 27v/v along with 1% orthophosphoric acid 3v/v composition is utilized as eluent. 1.00ml/min is rate of flow. For this determination column used as ODS C-18. 275nm is wavelength. Linearity range to MPA is 2.0μg/mL – 14.0μg/mL is measured. Both LOD and LOQ for MPA is identified within 0.5μg/ml and 1.5μg/ml respectively. (Brightman *et al.*, 1999)

proposed MPA. Linear gradient initiated from5% and ended with 60% acetonitrile as combination of water 0.5ml of acetic acid, 0.1ml of triethylamine and 0.05% w/v ammonium acetate are applied in time range as 20 min. (Malecki *et al.*, 1993) developed a MPA known as compound C.B.3025 is the phenylalanine derivate of nitrogen mustard. It has been synthesized in 1953. Chemically, it is p-(di-2-chloroethyl) amino-L-phenylalanine. Various authors are published their work for measurement of MPA those includes VIS-spectrophotometry, electrochemistry, GC-MS along with HPLC by UV, fluorescence and volt- amperometric measurement. In this present work authors are tried to explain about very rapid, more sensitive measurement of MPA by using tablets also injections RP-HPLC by using UV detection.

Very High-dose MPA along with autologous haematopoietic cell transplantation (AHCT) (Malard and Mohty, 2020) remains actual of care to fit patients by newly diagnosed multiple myeloma. The Risk factors for COVID-19 severity along with death includes increased age also presence of comorbidities in combination of diabetes, hypertension, or cardiac diseases. (Onder *et al.*, 2020) In addition to this information by China suggested as patients with cancer have a significantly higher incidence of severe events after contracting SARS-CoV-2 than patients without cancer (seven [39%] of 18 patients with cancer *vs* 124 [8%] of 1572 patients without cancer.

EXPERIMENTAL

Equipment

LC 20AT pump and UV-Visible detector with flexible wavelength programme and Rheodyne injector are used in this present problem. Phenomenex Synergi C_{18}, 250 mm $\times$ 4.6 mm, 4μm or equivalent is used for this chromatography analysis. With the help of Loba ultrasonic bath sonicator mobile phase containing the gas was separated. The common balance for measuring the drugs is employed.

Chemicals and Solvents

From local market sample MPA is purchased. Trifluoroacetic acid along with Orthophosphoric acid are AR grade, Acetonitrile and water collected as HPLC grade and used. Exactly 10.00g ammonium acetate is transferred in 100.00mL standard flask. To this 50.00mL water is added after that subjected to dissolution. Again, for this solution 10.00mL glacial acetic acid, 2.00mL of triethylamine is added. Mobile Phase A is prepared by mixing exact amount with 5.00ml buffer solution is taken and diluted in 1000ml of water. After that filtered and subjected to degasation. Mobile Phase is prepared by mixing ACN, Methanol with Buffer in the combination 80.0:20.0:0.5 v/v/v and sonicated for degassing.

Preparation of Solutions

Regular solution is prepared by taking 10.00mg MPA and taken in 100.00ml standard flask. This gives 0.1 mg/mL solution. Diluted Standard as 10ppm is prepared by taking2.00mg MPA regular in 20.00ml standard flask. This preparation gives equivalent to 0.01 mg/mL. Sample solution is prepared by reconstituting 2 vials each by 5.00mL diluent after that transferred in 50.00mL standard flask. Rinse vial at least more than two times by diluent after that transferred total contents of vial in same standard flask after that diluted to required volume up to mark by suitable diluent. Placebo is prepared by reconstituting 1 vials each by 5.00mL diluent after that transferred in 25.00mL standard flask. Rinse vial at least more than two times by diluent after that transferred total contents of vial in same standard flask.Separately injected Blank (diluent) (1 injection), Diluted Standard solution (6 injections) into chromatography and check the different parameters. Theoretical plate count MPA HCl peak from diluted standard solution is not minimum to 2000. % RSD for total 6 measurements of regular solution is should not exceed to value 5.

Method Development

Various parameters were studied and considered for this analytical method validation of related substances in the drug product MPA injection concentrate 20mg/ml. For 10ppm solution of MPA by using UV spectrophotometer the spectrum in Acetonitrile was recorded separately. The wavelength is measured as 260nm. Column used as Phenomenex Synergi C18, 250 mm $\times$ 4.6 mm, 4μm or equivalent column. Acetonitrile as 80v/v, methanol as 20v/v along with buffer as 1.00ml is consumed as eluent. From the observation of the reaction 1.00mL/min flow rate is acceptable for effective separation of analyte. The circumstances for the chromatographic analysis of MPA were optimized, and they are exhibited in Table 1. These are use full for the measurement of MPA in overall samples and in its formulation and for studies of SARS-CoV-2.

Proposed Method And Requirements For Validation

System Suitability

Injected blank as 1 injection, system Suitability solution as 1injection, sensitivity solution as 1 injection, diluted regular solution as 6 measurements. Results obtained were finalized as system should favorable

for analytical method corroboration. Results are tabulated in Table 2.

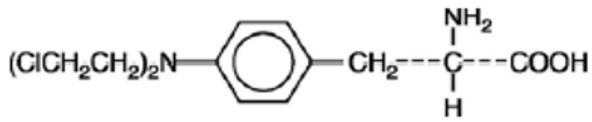


Figure 1: Structure of MPA

Specificity

Conducted specificity criteria of method by introducing Blank (diluent), Placebo preparation, s regular solution, Monohydroxy MPA, Dihydroxy MPA, MPA isopropyl ester, MPA Dimer, Sample Preparation, Spiked sample with known impurities into the chromatographic system and recorded the retention times. The peaks of Blank (diluent), Placebo, Monohydroxy MPA, Dihydroxy MPA, MPA isopropyl ester and MPA dimer should not interfere MPA peak and each other. From the below results, it can be concluded that no retardation of other peaks of placebo & impurity peaks including MPA peak. Carried out this experiment by passing Blank injecting blank as diluent, placebo preparation, Diluted standard solution, Monohydroxy, Dihydroxy, isopropyl ester, dimer of MPA, prepared sample, spiked sample with known impurities and documented different retention times. Results are tabulated in Table 3.

Stressed Condition Studies

Stressed condition of MPA injection shall be done for finalizing stability information and also degradation path way or through its shelf life or any non-persistent substance is identified that may not combined by using MPA injection peak. Various solutions are prepared for this study like sample, placebo, Acid Stressed sample (1.0N HCl), Alkali Stressed sample (1.0N NaOH), 3.0% w/v Peroxides of Hydrogen Stressed (3.0%w/v H2O2), Neutral Stressed, UV light exposed, Photo stability exposed Sunlight exposed, Thermal stressed (Dry heat) sample.

For repeated analysis alkali stressed sample (1.0N NaOH) at 25°C for 5 minutes is measured. Neutral stressed sample for 2 hours. Finally, concluded that Sample is found to be degrading in alkali condition and Neutral condition. By using sunlight it is slightly undergoing degradation in Hydrogen peroxide. No degradation is observed in Photo stability, acidic, thermal and UV light stressed condition. However, unknown impurities, related compounds along with degradation impurity are well scattered from MPA peak. MPA peak is pure, which was confirmed by Chromeleon software. Hence, this related substance procedure is considered for product release ad shelf-life.

Precision

System Precision

The Retention Time (Rt) and area for total 6 determinations are calculated along with that % RSD may also calculated. Injected diluent as blank and standard into system. Recorded the % RSD for Rt and response of peak for MPA. It is evident that from the obtained data Rt and peak responses are same which is supported by RSD. Due to this reason, finalized that precision of system reaches exactness of method validation. The results obtained are showed in the in Table 4.

Method Precision

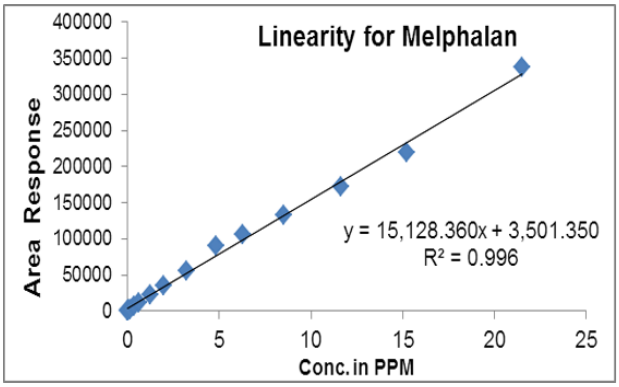
Consistence substance of a pointed batch is subjected to analysis to 6 times as total. Therefore, the conclusion from the above procedure is precise. % RSD of impurities 0.05% and above for total 6 measurements is 15.0.%RSD of total impurities for total 6 measurements is 10.0. From below values, it is finalized as this method is precise. Results are tabulated in Table 5.

Intermediate Precision

Calculated the Percentage of impurities compared the results obtained in method of precision and Precision of Intermediate, calculated % RSD. % RSD of impurities 0.05% and above for total 6 measurements is NMT 15.0.% RSD of Total impurities for 6 measurements is NMT 10.% RSD of the related compounds 0.05% and above for 12 preparation NMT is 15.0. Impurity below LOQ is not considered in total impurity. Comparison between Method Precision and Intermediate Precision results are tabulated in Table 6.

Stability In analytical Solution

Percentage Difference in range response for peak in regular & unknown preparative should be in the range $\pm$ 5.0 from beginning area and area after specified period.% variations of area response for MPA peaks in diluted standard preparation is within $\pm$ 5.0 from the initial area to after period. For Impurity 0.05% above and total impurity, % variation in the area counts between started time and ended time. Difference in area response as initial also after specified period is within $\pm$ 15.0 and 10.0. Monohydroxy MPA and MPA Dimer impurities are above LOQ. Other known and unknown impurities are below LOQ. The Diluted solution of standard is stable to about 51 hours at temperature of 5°C. The sample solution is stable upto 17hours in 5°C (% difference of monohydroxy MPA along with MPA dimer is 0.3& 2.0). For total impurity sample solution is



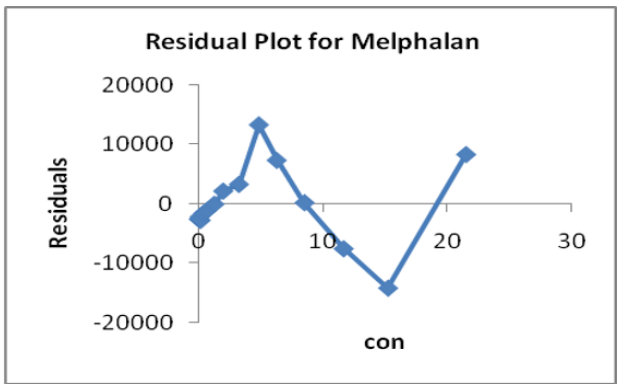
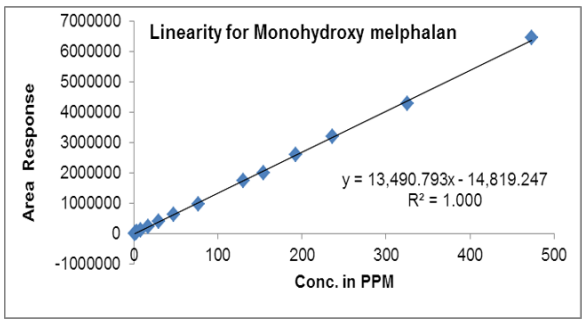


Figure 2: Results for Linearity of Monohydroxy MPA



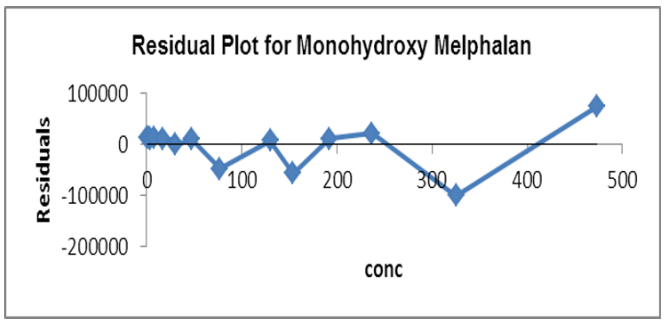
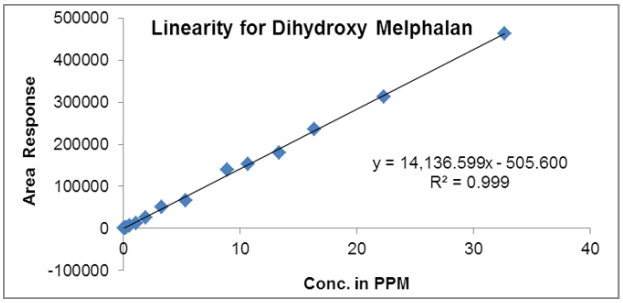


Figure 3: Linearity range graph for Monohydroxy MPA



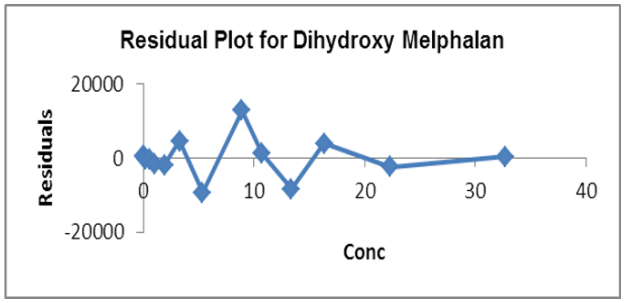
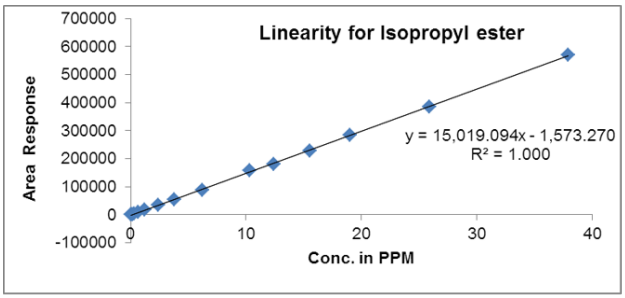


Figure 4: Linearity range graph for Dihydroxy MPA



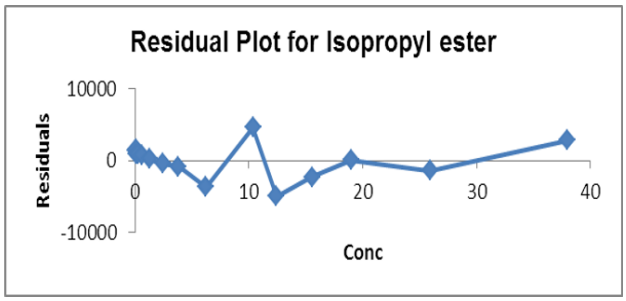
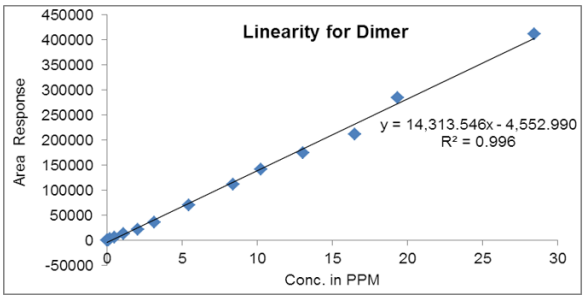


Figure 5: Linearity range graph for Isopropyl ester of MPA



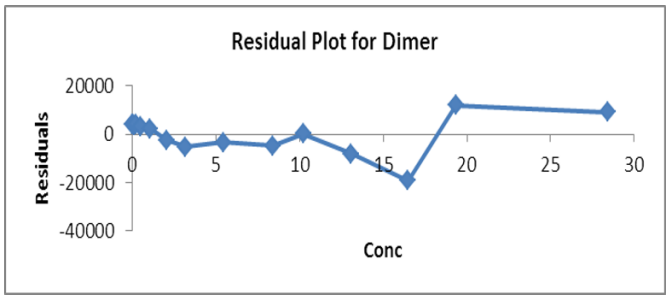


Figure 6: Linearity range graph for Dimer of MPA

Table 1: Gradient program

Time (in minutes)	Mobile Phase A (in %)	Mobile Phase B (in %)
0.00	100.0	0.0
35.0	65.0	35.0
45.0	58.0	42.0
55.0	45.0	55.0
60.0	35.0	65.0
68.0	35.0	65.0
69.0	100.0	0.0
75.0	100.0	0.0

Table 2: Results for System suitability

Injection No.	Retention Time (In minute)	Area response
1	37.388	173824.224
2	37.410	174792.889
3	37.411	176874.030
4	37.401	173409.382
5	37.407	173937.142
6	37.402	174201.921
Mean	37.403	174506.598
% RSD	0.0	0.7

Table 3: Results for specificity

Name of solution		Retention Time
Blank (diluent)		-
Diluted standard solution		36.903
Sample solution		36.923
Placebo preparation		-
Monohydroxy MPA		21.877
Dihydroxy MPA		11.707
MPA Isopropyl ester		55.443
MPA Dimer		46.277
Sample+ impurities spiked sample	Monohydroxy MPA	21.917
	Dihydroxy MPA	11.700
	MPA Isopropyl ester	55.427
	MPA dimer	46.260
	MPA	36.927

stable upto 17 hours. % difference for total impurity is -1.2.

Linearity

Plotted a graph of MPA, Monohydroxy MPA, Dihydroxy MPA, Isopropyl ester and MPA dimer strength as ppm with X-axis along with area response by using Y-axis. Both coefficients of correlation along with coefficient of regression are minimum to 0.995. % intercept is nearly equal to value 5.0. For this study MPA Linearity regular standard stock, Monohydroxy MPA Linearity regular stock, Dihydroxy MPA Linearity stock solution, MPA Isopropyl ester Linearity stock solution, MPA Dimer Linearity regular stock are prepared. With help of statistical situation for linearity values for MPA, Monohydroxy MPA, MPA, Monohydroxy MPA, Dihydroxy MPA, MPA isopropyl ester and MPA dimer. It clear that MPA, Monohydroxy MPA, Dihydroxy MPA, MPA isopropyl ester and MPA dimer is linear, between LOQ levels to 300% area of limit of specification. Both corre-

Table 4: Results for system precision

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5	37.407	173937.142
6	37.402	174201.921
Mean	37.403	174506.598
% RSD	0.0	0.7

Table 5: Results for Method precision

Sample set No.	Monohydroxy MPA impurity (in % w/w)	MPA dimer Impurity (in % w/w)	Maximum single unknown impurity	Total Impurities (in % w/w)
1	1.07	0.103	0.048	1.173
2	1.05	0.103	0.049	1.153
3	1.07	0.103	0.048	1.173
4	1.05	0.104	0.051	1.154
5	1.08	0.104	0.051	1.184
6	1.06	0.102	0.049	1.162
Mean	1.06	0.103	0.049	1.170
%RSD	1.1	0.7	2.8	1.0

Table 6: Results for comparison of MP & IP

	Sample set No.	Monohydroxy MPA impurity as % w/w	MPA dimer Impurity (in % w/w)	Relative Rt at 0.50	Entire Impurities in % w/w
Method Precision	1	1.07	0.103	0.048	1.173
	2	1.05	0.103	0.049	1.153
	3	1.07	0.103	0.048	1.173
	4	1.05	0.104	0.051	1.154
	5	1.08	0.104	0.051	1.184
	6	1.06	0.102	0.049	1.162
Intermediate Precision	1	1.0718	0.1233	0.0595	1.195
	2	1.0686	0.1201	0.0599	1.189
	3	1.0862	0.1237	0.0529	1.210
	4	1.0846	0.1251	0.0522	1.210
	5	1.0757	0.1247	0.0609	1.200
	6	1.0680	0.1253	0.0577	1.193
	Mean	1.0696	0.1134	0.0533	1.180
	%RSD	1.1	9.5	9.2	1.7

Table 7: Relative retention times of MPA and related compounds

	RT	RRT	SLOPE	RF	RRF
MPA	36.867	1.00	15128.360	1	1
Monohydroxy MPA	21.86	0.59	13490.794	1.12	0.89
Dihydroxy MPA	11.703	0.32	14136.599	1.07	0.93
MPA isopropyl ester	55.433	1.50	15019.094	1.01	0.99
MPA dimer	46.253	1.25	14313.546	1.06	0.95

Table 8: Confirmed LOD & LOQ level

	Monohydroxy MPA	Dihydroxy MPA	MPA isopropyl ester	MPA dimer	MPA
LOD(PPM)	0.46	0.41	0.48	0.50	0.43
LOQ(PPM)	1.39	1.24	1.46	1.52	1.29
LOD (%)	0.023	0.021	0.024	0.025	0.022
LOQ (%)	0.07	0.062	0.073	0.076	0.065

Table 9: Results for robustness

		Theoretical Plates NLT 2000	Tailing factor is 2.0	%RSD is 5.0
Original parameters		279258	1.12	0.7
variation in flow rate	-0.2 ml/min	297972	1.12	0.5
	+0.2 ml/min	263297	1.10	0.6
Temperature	-5°C	253508	1.17	0.7
	+5°C	285888	1.03	0.4
Wavelength	-5nm	259720	1.21	2.6
	+5nm	259082	1.26	1.0
Change in organic (Methanol)	+2%	295891	1.15	3.6
	-2%	295931	1.13	1.0
Change in organic (Acetonitrile)	+2%	268148	1.12	0.3
	-2%	268197	1.12	0.3

lations along with regression coefficient were more than to value 0.095. Value of % of intercept is may not ±5.0% of the area count. Results are tabulated in Table 7, graphs are figured from Figures 2, 3, 4, 5 and 6.

Limit of Detection and Quantitation

To confirm LOD along with LOQ calculated theoretically from above; prepared freshly solution of MPA, Monohydroxy MPA, Dihydroxy MPA, Isopropyl ester and MPA dimer at LOD and LOQ concentration and inject into the chromatograph (LOD range single and LOQ range 6 injections). Calculated % RSD and calculated percentage of LOD and LOQ with respect to working strength. % RSD of area response obtained from 6 injections at LOQ range value is NMT10.0. The results are tabulated in Table 8.

Confirmed LOD & LOQ Level

Accuracy

Spiked known quantity of Monohydroxy MPA, Dihydroxy MPA, Isopropyl ester and MPA dimer standard into sample. Spike the known quantity of MPA into placebo at specification level of unknown impurity. Analyzed these samples intriplicate for each range. From values obtained calculated accuracy and range parameters.

Range

Percentage RSD obtained for all accuracy range determinations is NMT 10.0. Correlation and regression coefficient is minimum to 0.995 for Linearity and accuracy range parameter. By using obtained values finally it is finalized that method proposed is laying LOQ Level to 300% of target strength to monohydroxy MPA, Dihydroxy MPA, Isopropyl ester along with MPA. Range of method is varying LOQ to

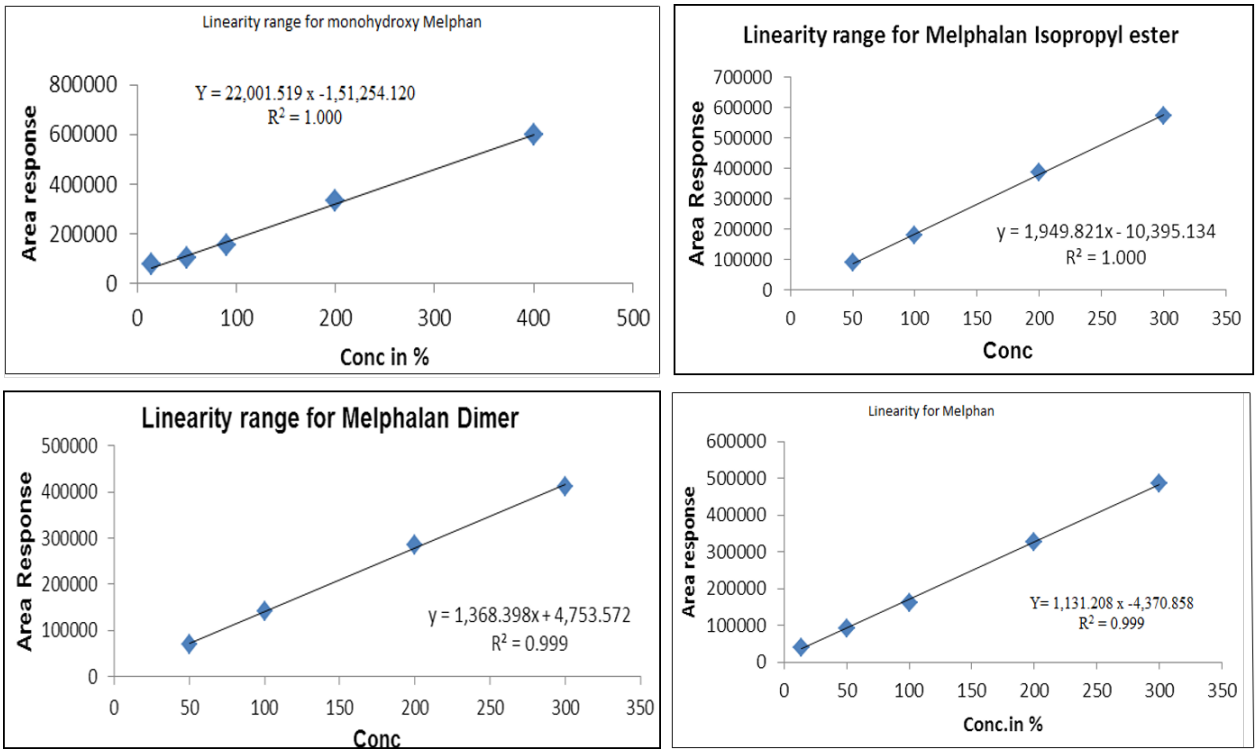

Figure 7: Linearity Range for MPA and its Impurities

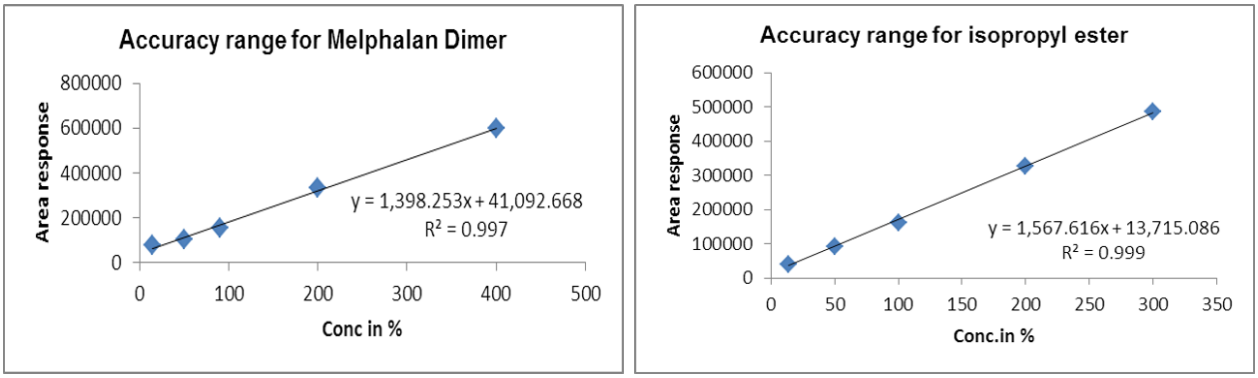

Figure 8: Accuracy range for MPA and its impurities graphs

400% of target strength to MPA dimer. Graphs are shown in Figures 7 and 8.

Linearity range graphs

Robustness

Changed oven temperature to ±5°C. From results, this method is finalized that process is robust towards small variation in process parameters. By results obtained finally it is finalized that proposed process is robust for very small changes in different parameters of method. The results are tabulated in Table 9.

Melphan as a treatment of SARS-CoV-2

Other critical services to patients that have high-dose MPA along with AHCT that could be negatively concerned by SARS-Cov-2 includes connection of blood products, as number of blood donors might minimum by guidance to self-isolate, which may leads to minimum blood and platelet products (Ranney *et al.*, 2020; Pagano *et al.*, 2020). Finally, deciding whether utilizing high-dose MPA along with AHCT are the right treatments for patients by multiple myeloma could be very carefully decided in current SARS-Cov-2 pandemic situation. For patients with high-risk cytogenetics, the situation is more complex. These patients might need to have high-dose MPA and AHCT as first-line treatments. Still, approximately 18% of patients have asymptomatic SARS-CoV-2 infection, we recommend testing patients for SARS-CoV-2 before transplant. After intravenous administration, mean plasma $t_{0.5}\alpha$ was 8.0±2.3 min, $t_{0,5}\beta$ was 63.3±8.7 min, and total systemic clearance is identified as 510.4 ± 57.9 ml/min (Woodhouse *et al.*, 1983).

RESULTS AND DISCUSSION

For various validation criteria of HPLC, different elute ratios are verified. Satisfactory separation with good peak symmetry is measured in combination of both elute A and B. Column used in this measurement is Phenomenex Synergi C18, 250 mm $\times$ 4.6 mm, 4μm or equivalent (P.No. 673882-1). Specificity(stressed condition) the peaks of Blank (diluent), excipeients peaks, Monohydroxy MPA, Dihydroxy MPA, Isopropyl ester and Dimer impurity were not interfere with MPA Peak along with each other. Match factor is more than 0.99 /990.0. For system criteria symmetry factor for MPA peak is 2. Obtained results are 1.2 and 270103 respectively. Relative regular deviation (% RSD) for total 6 measurements of regular solution is maximum to value 5. % RSD of retention time for MPA peak measured from total 6 measurements of diluted regular solution is 1.0 with % RSD of the area response for MPA peak measured from total 6 measurements of diluted regular solution is 5.% RSD for sum of impurities from total 12 measurements (method precision & intermediate precision) is 10.0 which is identified as 1.7. % difference in area response from minimum to specified period from sample solution is -1.2 at 25°C. Distinct visible peak is measured at LOD level strength. Proposed HPLC method for estimation of related substance in drug product MPA for injection is validated as per analytical method validation. Accuracy of this method is established for MPA for injection 50mg/ml. Intermediate precision, LOD along with LOQ are performed. System suitability test is established including related parameters. Taking into account, that severe cases of SARS-Cov-2 are characterized by hyperergic inflammatory counter it can be counterfeit that inhalation utilization of very low-doses of MPA due to its anti-inflammatory characteristic can be adequate treatment to patients those who are facing problem with SARS-Cov-2 -associated pneumonia.

CONCLUSION

Proposed method is specific with linear in the specified range method is also indicating as evidenced by stressed studies. This proposed method is found to be linear in the specified range for MPA and its related impurities. Hence, by using this process finally it is concluded that this method stands validated it can be used for routine with stability analysis. Therefore, we recommend pursuing an induction regimen of up to six cycles in all patients to postpone treatment with high-dose MPA for treatment of SARS-CoV-2.

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Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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