International Journal for Pharmaceutical Research Scholars (IJPRS)

V-2, I-2, 2013 ISSN No: 2277 - 7873

RESEARCH ARTICLE

Analytical Method Development & Validation for Related Substances Method of Busulfan Injection by Ion Chromatography Method

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Manuscript No: IJPRS/V2/I2/00091, Received On: 04/05/2013, Accepted On: 09/05/2013

ABSTRACT

A new simple, accurate, precise and reproducible Ion chromatography method has been developed for the estimation of Methane sulfonic acid in Busulfan injectable dosage. The method which is developed is also validated in complete compliance with the current regulatory guidelines by using well developed analytical method validation techniques and tools which comprises with the analytical method validation parameters like Linearity, LOD and LOQ determination, Accuracy, Method precision, Specificity, System suitability, Robustness, Ruggedness etc. by adopting the current method the linearity obtained is near to 0.999 and thus this shows that the method is capable to give a good detector response, the recovery calculated was within the range of 85% to 115% of the specification limits.

KEYWORDS

HPLC, Busulfan, Methanesulfonic Acid.

INTRODUCTION

Busulfan is an alkylating antineoplastic agent that is used to condition patients prior to bone marrow transplantation. It is also indicated for palliative treatment of chronic myeloid leukemia. Busulfan is available 6 mg/mL solution for intravenous injection (Busulfex).

Busulfan (1,4-butanediol dimethanesulphonate) is a bifunctional alkylating agent belonging to the antineoplastic therapeutic category of alkanesulphonic acid esters. Two labile methanesulphonate groups are attached to the opposite ends of a butyl chain. Busulfan is known to undergo SN2-type nucleophilic substitutions of the N-7 position of guanine and of thiol groups[1]. When Busulfan hydrolyses in aqueous media, the methanesulphonate groups are released. The half-life of the intermediate, 4-methanesulphonyloxybutanol, is extremely short, which makes it unlikely that it is jointly responsible for the biological action of Busulfan[2].

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Figure 1: Busulfex

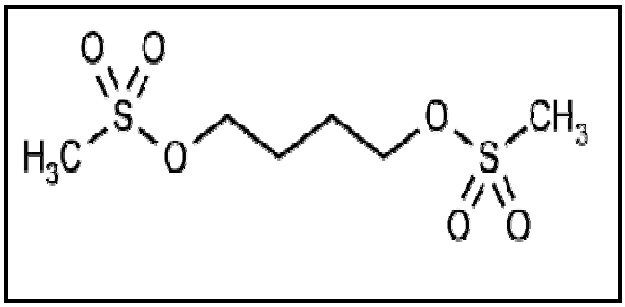

Figure 2: Busulfan

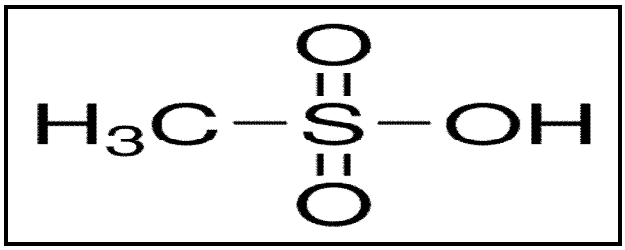

Figure 3: Methanesulfonic acid

Recently, intravenous Busulfan formulations were introduced on to the market, in order to minimize variations of inter- and intra-patient systemic exposure, and to provide complete dose assurance[3-5]. However, molecules of Busulfan are extremely insoluble in water and this property means that a critical formulation is required for parenteral administration. The intravenous (IV) formulation was initially commercially available on the US market as Busulfex. It is a clear, colorless concentrated solution of 6 mg/mL in ampoules; the Busulfan is dissolved in N,N-dimethylacetamide (DMA) 33% w/w and polyethylene glycol (PEG) 400 67% w/w, using the principle of co-solvency.

EXPERIMENTAL

Chemicals and Reagents

Pure Busulfan standard and Busulfan injection were procured from a reputed reference material supplier in India. Methanesulfonic acid, Sodium bicarbonate, Sulphuric acid, Water and Acetonitrile HPLC grade purchased from Merck chemicals. All the other chemicals used were of analytical grade.

Instrumentation and Conditions

Metrohm ion chromatographic system equipped with conductivity detector was used for all the experiments performed for the method development and validation. Data acquisition was performed by chromeleon software. Analysis was carried out with conductivity detection with a Metrosep A Supp 5 x 250 mm Column at 25°C temperature. The mobile phase was used is in the isocratic format. The flow rate was 0.5mL/min with a maximum pressure of 15 Mpa and the run time for the test was about 25 minutes, the sample volume injected was 100µL. The mobile phase was degassed and filtered through 0.45µm membrane filter before pumping into the HPLC system.

Preparation of Solutions

Preparation of Diluent

3.2 mmol / L sodium carbonate + 1 mmol / L sodium bicarbonate in Ultra pure water.

Preparation of Supressor Solution

Ultra pure water and 50 mmol / L sulphuric acid.

Standard Preparation

15 mg of Methane Sulphonic acid standard in 100 mL of Ultra pure water.

Sample Preparation

Weigh and transfer accurately about 25 mg of sample into a clean 10 mL volumetric flask, dissolve and dilute to the volume with Acetonitrile. Filter the solution through 0.2 µm membrane filter and inject into the ion chromatograph.

Experimental Procedure for Method Validation

The method was validated according to International Conference on Harmonization (ICH) guidelines for validation of analytical procedures [6-7].

Linearity

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. Performed the linearity with Methane sulphonic acid in the range of LOQ to 150% of specification limit.

Recorded the area response for each level and calculate slope, intercept & correlation coefficient. Test the intercept for statistical equivalence to zero. Stock solution of methane sulfonic acid was prepared and diluted serially and were injected into the chromatographic system and calculated. Plotted a graph of Methane sulphonic acid concentration (ppm) on X-axis and Area responses on Y-axis. The correlation & regression coefficients are more than 0.995.

LOD and LOQ Determination

Limit of detection is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. Limit of Quantitation is the lowest amount of analyte in a sample that can be quantitated with acceptable accuracy and precision, under the stated experimental conditions. Calculate slope and the residual standard deviation from the linearity curve. From the slope and residual standard deviation, calculated LOD and LOQ.

Calculate limit of detection from linearity curve as per formulae given below.

$$LOD = \frac{3.3 \times \text{Residual Standard deviation}}{\text{Slope}}$$

Calculate limit of Quantitation from linearity curve as per formulae given below.

$$LOQ = \frac{10 \times \text{Residual Standard deviation}}{\text{Slope}}$$

To confirm the LOD and LOQ calculate theoretically from the above; prepare freshly Methane sulphonic acid standard at LOD and LOQ concentration and inject into the chromatograph (LOD level single and LOQ level 6 injection).Record the area responses and calculate the RSD. Calculate the percentage of LOD and LOQ with respect to sample concentration. The estimation of LOD and LOQ can also be performed by the serial dilution methods. The acceptance criteria states that distinct visible peak shall be observed at LOD level concentration and the %RSD of area response from 6 injections (LOQ level) shall be NMT 10.0.

Accuracy

The accuracy of an analytical method is the closeness of test Observations obtained by that method to the true value (standard value). Accuracy was performed by spiking known quantity of Methane sulphonic acid at LOQ level, 50%, 100% and 150% of specification limit into the sample. Analyzed these samples in triplicate for each level. Calculated the % recovery from the observations of Accuracy.

Precision of the Method

Precision was measured in accordance with ICH recommendations. The precision study was carried out by injecting sample preparation six times into the chromatographic system. The % RSD for Retention time and area responses are calculated and are well within range of 2.0 % and 5.0 % respectively.

System Suitability

System suitability was assessed by injecting six injections of the Methane sulfonic acid standard solution into the chromatographic system and the chromatogram with the area response was obtained. The system suitability parameters such as tailing factor, theoretical plate count and reproducibility (%RSD) of analyte retention time and area of the six replicates calculated from the chromatogram.

Stability of Analyte in Solution

Evaluate the stability in analytical solution by injecting the standard preparation and sample preparation at regular interval at specified temperature. The Acceptance criteria states that the % difference of assay for the peaks in standard preparation and sample preparation should be within ± 2.0 from initial assay after specified period.

Specificity

Specificity is the ability of analytical method to assess unequivocally the analyte in the presence of component that may be expected to be present in the drug product, such as impurities, degradation products and matrix components. Performed the specificity parameter of the method by injecting diluent, Methane sulfonic acid standard, sample preparation and placebo preparation into the chromatographic system and recorded the retention times of diluent, standard preparation, sample preparation and placebo preparation.

Robustness

The parameter that was varied for the robustness was change in the flow rates by ±0.1ml/min. The standard preparation was injected for six replicate injections and checked for the system suitability parameters. It was found that the method is robust. The system suitability parameters were well within the acceptance criteria.

Ruggedness

The ruggedness of the method was demonstrated by analysis of the sample as for precision study by a second analyst.

RESULTS AND DISCUSSION

Method Development and Optimization

The parameters like Solubility, Selection of the chromatographic system, suppressor solution, selection of diluent and column selection were studied as a part of method development and based on the outcome of the final parameters the method validation activity was initiated.

Method Validation

Linearity

The calibration curve constructed was evaluated by using correlation coefficient. The peak area of the Methane sulfonic acid was linear in the range of LOQ to 150%. The area for each of the concentration obtained was plotted against the concentration of the analyte; the correlation coefficient (R^2) is 0.999. Based on the linearity values obtained from the above table it was observed that the LOQ level can be still lower as compared. So in order to optimize the LOQ values the linearity was again injected with the

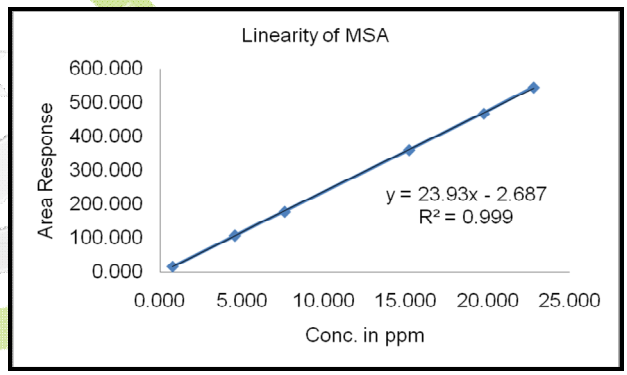

Figure 4: Linearity of Methane sulfonic acid

Table 1: Observations for Linearity study

S. No	Conc. Levels	Conc. (ppm)	Peak Area of Methane sulphonic acid		Mean Peak Area
			Injection 1	Injection 2	
1	**LOQ**	0.760	15.178	18.227	16.703
2	**30%**	4.561	110.222	105.658	107.940
3	**50%**	7.602	175.332	178.229	176.781
4	**100%**	15.204	360.243	360.125	360.184
5	**130%**	19.765	465.887	470.986	468.437
6	**150%**	22.806	550.222	542.238	546.230

concentrations lower than the LOQ level and the data is captured in the below table. And based on the results obtained the values for LOQ and LOD are established and the LOD verified as per requirements and the precision for LOQ level is also performed by injecting 6 injections of the optimized LOQ concentration.

Table 2: Linearity for determination of LOD/LOQ

S. No	Concentration (ppm)	Peak Area
1	0.800	18.012
2	0.500	11.258
3	0.100	2.252
4	0.050	0.967
5	0.020	0.476
6	0.010	0.315
7	0.005	0.253
Correlation	**1.000**	
STEYX	**0.1011**	
Slope	**22.4700**	
LOD in ppm	**0.013**	
LOQ in ppm	**0.044**	

Table 3: Verification of the LOD area

S. No.	Peak Area of Methane Sulphonic acid
1	0.337
2	0.322

Table 4: Precision at LOQ level

S. No	Peak Area of Methane Sulphonic acid
1	1.011
2	1.090
3	1.068
4	1.008
5	1.024
6	0.982
Mean	**1.031**
SD	**0.0406**
% RSD	**3.9**

Accuracy

Accuracy of the method was expressed in terms of recovery of added Methane sulfonic acid concentration into the sample. Percentage recovery was calculated by multiplying the ratio of the measured concentration with 100. Mean % recovery and %RSD were calculated and were found to be within 85% to 115% respectively. It can be obtained from the below table that the developed method for Methane sulfonic acid is accurate.

Precision Study

The precision of an analytical method is the degree of agreement among individual test Observations when the method is applied repeatedly to multiple sampling of homogeneous sample. The precision of analytical method is usually expressed as the standard deviation or relative standard deviation (Coefficient of variation) of series of measurements.

Table 5: Observations for recovery study

Sr. No.	Level	mg/mL Added	mg/mL Recovered	% Recovery	Mean % Recovery	% RSD
1	LOQ	0.0563	0.0540	95.9	98.9	2.7
2		0.0563	0.0570	101.2		
3		0.0563	0.0560	99.5		
4	50%	0.5625	0.5798	103.1	101.2	1.6
5		0.5625	0.5652	100.5		
6		0.5625	0.5630	100.1		
7	100%	1.1250	1.1300	100.4	100.6	1.9
8		1.1250	1.1102	98.7		
9		1.1250	1.1535	102.5		
10	150%	1.6875	1.7502	103.7	101.3	2.1
11		1.6875	1.6984	100.6		
12		1.6875	1.6812	99.6		

Table 6: Observations for system precision

Inj. No.	1	2	3	4	5	6	Mean	%RSD
RT (minutes)	5.123	5.125	5.122	5.129	5.128	5.125	5.125	0.1
Area	360.123	359.587	355.128	366.987	360.875	359.222	360.320	1.1

System Precision

The system precision is checked by using standard chemical substance to ensure that the analytical system is working properly. The retention time and the area response of six determinations should be measured and calculate relative standard deviation. Injected blank one injection and standard preparation six injections into the chromatograph. Recorded and calculated relative standard deviation.

Method Precision

In method precision, a homogeneous sample of a single batch should be analyzed six times. This indicates whether a method is giving consisting Observations of a single batch. Analyzed the samples of Busulfan for injection six times of a same batch as per analytical procedures. From the values observed calculated the % content of Methane sulfonic acid.

Table 7: Observations for method precision

Sample	% methane sulphonic acid
1	4.51
2	4.61
3	4.52
4	4.59
5	4.48
6	4.53
Mean	**4.54**
% RSD	**1.1**

The results obtained from the method precision study are well within the acceptance criteria of, the % RSD of amount of methane sulphonic acid in six preparations should be not more than 10.0. From the above Observations, it can be concluded that the method is precise.

System Suitability

The %RSD of the peak area and retention time of Busulfan were within 2%. The efficiency of column is expressed by the number of theoretical plates for six replicate injections were found to be 8761 and the tailing factor was 1.12.

Stability of Analyte in Solution

Evaluate the stability in analytical solution by injecting the standard preparation and sample preparation at regular interval at specified temperature. From the values calculated, it can be concluded that the Standard preparation and Sample preparation are stable for 24 Hours at 25°C.

Specificity

The sample preparation, standard preparation, diluent and placebo were injected into the chromatographic system and based on the elution pattern observed it can be concluded that the diluent peaks and placebo peaks were not interfered with Methane sulfonic acid peak.

Robustness

A study was conducted to know the effect of deliberate variations in the flow rate. As per the proposed method, standard preparations were injected into the chromatographic system. The system suitability parameters were evaluated.

In all the cases the results were well within the acceptance criteria. From the above study the proposed method was found to be robust.

Ruggedness

The results were well within acceptable limits these results indicate that the developed ion chromatographic method was rugged.

Table 8: Ruggedness results

Study	% methane sulphonic acid impurity	
	Method Precision	**Intermediate precision**
1	4.51	4.68
2	4.61	4.55
3	4.52	4.59
4	4.59	4.69
5	4.48	4.58
6	4.53	4.63
Mean	**4.58**	
% RSD	**1.43**	

The results obtained from the ruggedness study are well within the acceptance criteria of; the % RSD of amount of methane sulphonic acid in six preparations should be not more than 10.0. And the cumulative % RSD for 12 sample preparations (Method precision & Intermediate precision) should be not more than 10.0. From the above Observations, it can be concluded that the method is rugged.

CONCLUSION

In this study a simple, fast and reliable Ion chromatographic method was developed and validated for the related substances in Busulfan injection in vial dosage form. The developed method by Ion chromatography technique was successfully applied for the analysis of Methane sulfonic estimation. The method shows a good Performance with respect to linearity, accuracy, precision, specificity etc. So the proposed method can be used in routing quality control laboratories.

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