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Analytical Method Development and Validation for Assay Method of Busulfan Injection by RP-HPLC Method

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ABSTRACT

A new simple, accurate, precise and reproducible RP-HPLC method has been developed for the estimation of Busulfan (1,4-butanediol dimethanesulfonate) in its inject able dosage. The method developed is Reverse Phase High Performance Liquid Chromatographic method using suitable C18 column (Length: 150mm, Diameter: 4.6mm, Particle size: 3µ) with isocratic elution and a simple Acetonitrile, Water and Tetrahydrofuran in the ratio of 66:32:2 (v/v/v) respectively as mobile phase. 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, Accuracy, Method precision, Specificity with forced degradation, 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 98% to 102% of the specification limits.

Keywords: HPLC, Busulfan, Methanesulfonic Acid

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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).

Figure 1: 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].

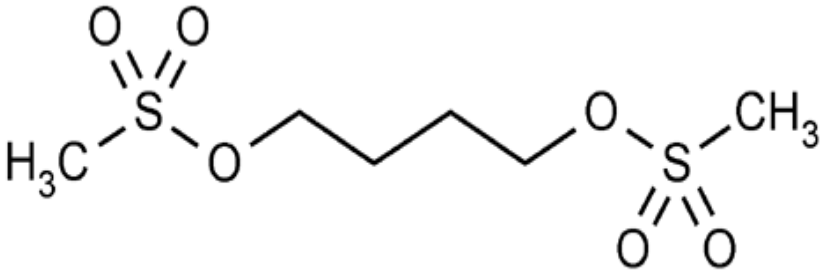

Figure 2: Busulfan

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.

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EXPERIMENTAL

Chemicals and Reagents

Pure Busulfan standard and Busulfan injection were procured from a reputed reference material supplier in India. Tetrahydrofuran, Sodium diethyldithiocarbamate, N,N Dimethylacetamide, Methanol, Water and Acetonitrile HPLC grade purchased from Merck chemicals. All the other chemicals used were of analytical grade.

Instrumentation and Conditions

Agilent HPLC separation module 1260series equipped with PDA detector was used for all the experiments performed for the method development and validation. Data acquisition was performed by EZ-Chrome software. Analysis was carried out at 280nm with a C18 column (150x4.6mm, 3µ) at 20°C temperature. The mobile phase was used is in the isocratic format, in the proportions of Acetonitrile: Water: THF (66:32:2). The flow rate was 1.0mL/min and the retention time was about 14 minutes. The mobile phase was degassed and filtered through 0.45µm membrane filter before pumping into the HPLC system.

Preparation of Solutions

Preparation of Mobile Phase

The Mobile phase was prepared by mixing Acetonitrile, Water and Tetrahydrofuran in the ratio of 66:32:2 (v/v/v) respectively. Diluent used is Methanol.

Preparation of Derivatising Solution

Weigh and transfer about 2.0gm of Sodium diethyldithiocarbamate into 50 mL volumetric flask, add 30mL of N,N-Dimethyl acetamide, Sonicate to dissolve and dilute to volume with N,N-Dimethyl acetamide.

Preparation of Standard Stock Solution

Weigh and transfer about 30.0 mg of Busulfan working Standard into 50 mL volumetric flask add 20mL of N,N-Dimethyl acetamide, Sonicate to dissolve and dilute to volume with diluent.

Preparation of Sample Stock Solution

Transfer 2.0mL of sample into 20mL of Volumetric flask, rinse pipette 2 times with diluent, further add 10mL of diluent sonicate for 10 minutes with swirling and vortex the sample for 10 minutes, dilute the volume with diluting solution and mix well.

Standard Preparation

Transfer 5.0mL of Sodium diethyldithiocarbamate stock solution in 25mL of Volumetric flask, keep Solution in water bath attain the solution at same temperature with shaking, add 2mL of Standard stock solution, shake and kept solution in water bath at 60°C for 15 minutes with swirling, allow to cool at room temperature, dilute to volume with diluent.

Sample Preparation

Transfer 5.0 mL of Sodium diethyldithiocarbamate stock solution in 25mL Volumetric flask, keep Solution in water bath to attain the solution at same temperature with shaking, add 2mL of Sample stock solution, shake and keep solution in water bath at 60°C for 15 minutes with swirling, allow to cool at room temperature, dilute to volume with diluent.

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 observations 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 Busulfan standard in the range of 50 to 150% of specification limit Recorded the area response for each level and calculated slope, intercept & correlation coefficient. Plotted a graph of Busulfan concentration (ppm) on X-axis and Area response on Y-axis. Stock solution of Busulfan standard was prepared and was diluted serially and then the derivatisation was done and then the linearity samples were injected and calculated. The correlation & regression coefficients are more than 0.998.

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 injecting the sample in mg added verses mg recovered, from 50 to 150% to the sample concentration. The experiment was performed in triplicate % recovery, mean % recovery, RSD (%) were calculated for each concentration.

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 response are calculated and are well within range of 1.0 % and 2.0 % respectively.

System suitability

System suitability was assessed by injecting six injections of the Busulfan 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.

Specificity

The analyte was subjected to forced degradation studies using photolytic, peroxide, thermal, acid and alkali treatments for demonstration of specificity of the method. Busulfan was analyzed under these conditions for purity, indicating that the developed HPLC method effectively separated the degradation products from the Busulfan standard peak. It was found that the Busulfan peak was well separated from diluent and placebo peaks. There is no any interference of any other peak with the peak of interest. The peak purity factor for the Busulfan peak was found to be 1.0

Robustness

The different variations are in flow rates by ±0.2ml/min, organic phase ratio ± 2.0%, and changes in the column temperature from developed HPLC conditions. The standard preparation was injected for six replicate injections and checked for the system suitability parameters. It is 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 derivatising agent and detection wavelength, Mobile phase selection 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.

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Method Validation

Linearity

The calibration curve constructed was evaluated by using correlation coefficient. The peak area of the drug was linear in the range of 50% to 150%. The area for each of the concentration obtained was plotted against the concentration of the analyte The correlation coefficient (R^2) is 1.000.

Figure 3: Linearity of Busulfan

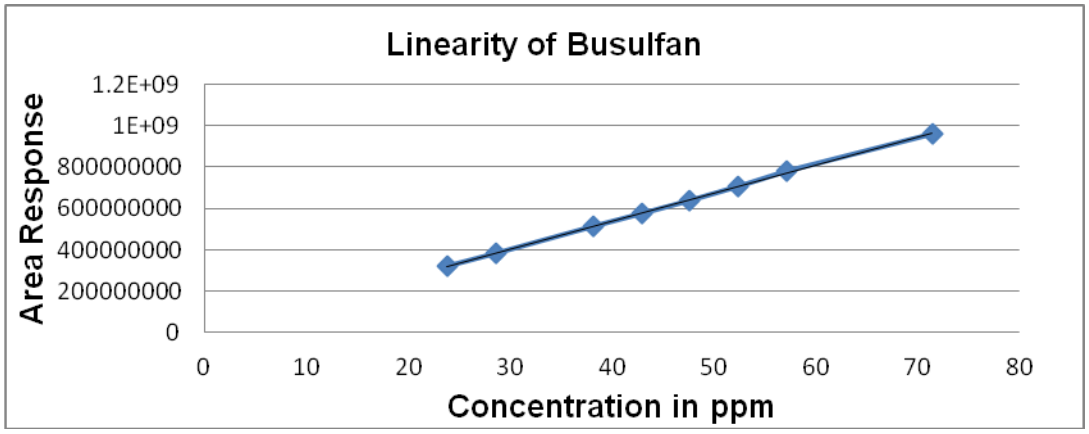

Table 1: Observations for Linearity study

Level	Concentration in ppm	Area Response
1	23.814	320097012
2	28.577	384116413
3	38.102	512155218
4	42.865	576174621
5	47.628	640194023
6	52.391	704213424
7	57.154	782328279
8	71.442	960291034
Correlation coefficient		**1.000**
Regression coefficient		**1.000**
Slope		**13441547.47**
Intercept		**0.0**
% Intercept		**0.0**

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Accuracy

Accuracy of the method was expressed in terms of recovery of added compound into the placebo. 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 98% to 102% respectively. It can be obtained from the below table that the developed HPLC method for Busulfan is accurate.

Table 2: Observations for recovery study

Sr. No.	Level	mg/mL Added	mg/mL Recovered	% Recovery	Mean % Recovery	% RSD
1	50%	0.0480	0.0476	**99.2**	**99.6**	**0.7**
2		0.0480	0.0476	**99.2**		
3		0.0480	0.0482	**100.4**		
4	100%	0.0960	0.096	**100.0**	**99.9**	**0.3**
5		0.0960	0.0956	**99.6**		
6		0.0960	0.0962	**100.2**		
7	150%	0.1440	0.143	**99.3**	**99.9**	**0.8**
8		0.1440	0.1452	**100.8**		
9		0.1440	0.1434	**99.6**		

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.

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.

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Table 3: observations for system precision

Inj. No.	1	2	3	4	5	6	Mean	%RSD
RT in minutes	13.420	13.427	13.413	13.420	13.427	13.420	**13.421**	**0.0**
Area	644420972	644577070	644335415	644081787	644029548	644249077	**644282312**	**0.0**

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 % Assay of Busulfan.

Table 4: observations for method precision

Sample	% Assay of Busulfan
1	98.1
2	98.7
3	98.7
4	98.9
5	97.6
6	98.7
Mean	**98.5**
% RSD	**0.5**

The results obtained from the method precision study are well within the acceptance criteria of, the %RSD of the calculated Assay observations for six determinations should be not more than 2.0. 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 13245 and the tailing factor was 1.01.

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Specificity

Accelerated degradation studies under different conditions viz., acid treatment; base treatment, peroxide, thermal were conducted to demonstrate the specificity. The Sample was found to be degraded in acid, alkali, and peroxide stressed conditions. However, unknown impurities are well separated from Busulfan peak and impurities. The Busulfan peaks are pure. Hence, the Assay method was considered specific & stability indicating.

Robustness

A study was conducted to know the effect of deliberate variations in the mobile phase Composition, flow rate and column temperature. As per the proposed method, standard preparations were injected into the HPLC 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 HPLC method was rugged.

Table 5: Ruggedness results

Study	% Assay of Busulfan	
	Method Precision	Intermediate precision
1	98.1	98.7
2	98.7	98.4
3	98.7	98.5
4	98.9	97.6
5	97.6	98.3
6	98.7	98.4
Mean	98.4	
% RSD	0.4	

The results obtained from the ruggedness study are well within the acceptance criteria of, the %RSD of the calculated Assay observations for six determinations should be not more than 2.0, and also the twelve results generated from the precision and the ruggedness study are also within the acceptance criteria of the %RSD of the calculated Assay Observations for twelve determinations should be not more than 2.0. From the above Observations, it can be concluded that the method is rugged.

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CONCLUSION

In this study a simple, fast and reliable HPLC method was developed and validated for the determination of Busulfan content in Busulfan injection in vial dosage form. The developed method was successfully applied for the analysis of Busulfan. 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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