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Quantification of Different drug substances in Pharmaceutical Formulations by Analytical HPLC

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ABSTRACT

The present study was conducted to develop and validate an analytical procedure for the determination of Busulfan, Bendamustine Hydrochloride and Clofarabine in Pharmaceutical Formulations. The analytical test attributesand evaluated as per the guidelines of ICH Q2 (R1). The method was validated for the determination of Assay in finished products of Busulfan, Bendamustine Hydrochloride and Clofarabine and the method validation parameters were evaluated for the analytical test attribute Busulfan, Bendamustine Hydrochloride and Clofarabine meets the acceptance criteria. The results obtained were within the specified limits and the samples were analyzed for test item concentration by High Performance Liquid Chromatography.

Keywords: Busulfan, Bendamustine Hydrochloride and Clofarabine, Validating the Assay, High Performance Liquid Chromatography, ICH Q2 (R1)

INTRODUCTION

In order promote a good public health; validation of analytical procedures is done to ensure quality, safety and efficacy of therapeutic drugs used for public health. It's very important to determine the content of Active Pharmaceutical Ingredient or drug content in the presence of recipients, Impurities or various inert substances that originate from raw materials, key starting materials, intermediates, by products, manufacturing process steps, impurities that are formed during drug recipient interactions, degradation impurities etc but not limited to. The validation of analytical procedures is done in order to assure that drug formulations are prepared in a most efficient and cost effective manner.

Busulfan is an antineoplastic agent with a cell-cycle nonspecific alkylating action (unlike that of the nitrogen mustards) that has a selective depressant action on the bone marrow. In small doses, it depresses granulocytopoiesis and to a lesser extent thrombocytopoiesis, but has little effect on lymphocytes. With larger doses, severe bone-marrow depression eventually ensues [1-4]. Intravenous administration of busulfan to rats for 1 year was reported to induce a variety of tumours in male rats, but the experiments could not be evaluated due to incomplete reporting [5-6].

Busulfan tablets on the market are available only in much smaller doses than those necessary for HCT conditioning [7], as the oral busulfan formulation was originally intended for the CML population [8-10]. Busulfan utilization has undergone dramatic progress in hematopoietic cell transplant (HCT) since its initial approval in 1954 [11]. Busulfan is an alkylating agent originally used in chronic myelogenous leukemia (CML), but it has progressively been recognized as a potent myeloablative agent in preparative regimens for hematopoietic cell transplantation (HCT) [12-13]. Busulfan-containing regimens have been widely accepted as a standard of care, and represent the most frequently used myeloablative regimens prior to HCT [14-15].

Bendamustine hydrochloride is a nitrogen mustardalkylating agent, structurally related to chlorambucil, which has been elaborated in 1962 in the former German Democratic

Republic, and since its very clinical introduction in 1969 has been used exclusively in this country up until the reunion of Germany [16-18].Bendamustine hydrochloride is among the first rationally designed alkylating drugs, whose structure comprises three pharmacophoremoieties: the bis-2-chloroethylamine alkylating group, a benzimidazolering serving as a purine base mimic (suggesting possible antimetabolite effects), and a butyric acid side chain to increase water solubility [19-21].The rapid degradation of the drug in serum and the extensive liver metabolism impair its cytotoxic action within a short period of time, necessitating application of relatively high doses [22].

Bendamustine bearing the name Treanda is achemotherapic medication used in the treatment of chronic lymphocytic leukemia, multiple myeloma, and non-Hodgkin's lymphoma. Bendamustine is a white, water soluble microcrystalline powder with amphoteric properties. It acts as an alkylating agent causing intra-strand and inter-strand cross-links between DNA bases. After intravenous infusion it is extensively metabolized in the liver by cytochrome p450 [23-27].

Clofarabine is a purine nucleoside analog indicated for treatment of relapsed or refractory acute lymphoblastic leukemia (ALL) in children [28].The drug is also increasingly used, outside of its Food and Drug Administration (FDA) approved indication, for treatment of relapsed or refractory acute myeloid leukemia (AML) in adults [29]. It acts by inhibiting DNA synthesis, the enzyme ribonucleotidereductase and repair and activation of mitochondrial repair processes [30]. We recently observed a case of acute kidney injury (AKI) associated with clofarabine treatment. We conducted a review of the literature and utilized the Food and Drug Administration Adverse Event Reporting System (FAERS)[31] to identify spontaneous reporting of renal adverse events with this drug.

Clofarabine administered intraperitoneally had significant activity against a wide variety of human tumor xenografts implanted subcutaneously in athymic nude or severe combined immune deficiency mice [32]. Moderate to excellent sensitivity to tumour growth delays were seen in all eight human colon tumours, three out of four human renal tumours, all four non-small-cell lung tumours, and all three prostate tumours. This spectrum of widespread anticancer activity has been confirmed by other investigators in human tumour xenograft models in mice [33]. The anticancer activity of clofarabine was dose- and schedule-dependent, and greater antitumour activity was associated with more frequent administration [34].Clofarabine is a second generation purine nucleoside analog with antineoplastic activity. Clofarabine is phosphorylated intracellularly to the cytotoxic active 5'-triphosphate metabolite, which inhibits the enzymatic activities of ribonucleotidereductase and DNA polymerase, resulting in inhibition of DNA repair and synthesis of DNA and RNA [35-37].

ICH- international council for harmonization of technical requirements for pharmaceuticals for human use (ICH) is unique in bringing together the regulatory authorities and pharmaceutical industry to discuss scientific and technical aspects of drug registration.Q2 (R1) Validation of analytical procedures of methodology is document presents a discussion of the characteristics for consideration during the validation of the analytical procedures included as part of registration applications submitted within the EC, Japan and USA. This document does not necessarily seek to cover the testing that may be required for registration in, or export to, other areas of the world. Furthermore, this text presentation serves as a collection of terms, and their definitions, and is not intended to provide direction on how to accomplish validation. These terms and definitions are meant to bridge the differences that often exist between various compendia and regulators of the EC, Japan and USA. The objective of the analytical procedure should be clearly understood since this will govern the validation characteristics which need to be evaluated. Typical validation characteristics which should be considered are Accuracy, Precision, Repeatability, Intermediate Precision, Specificity, Detection Limit, Quantization Limit, Linearity, Range[38-39].

EXPERIMENTAL PROCEDURE

METHOD VALIDATION

The method for determination of different drug substances ware validated in terms of precision (System precision and Method precision), (Interference,Linearity), Stability of Analyte in solution, Filter compatibility and System Suitability

RESULTS – Overall Summary of Validation of Busulfan

Validation Parameters	Acceptance Criteria	Results	
Precision	➢ The relative standard deviation for Busulfan peak area from five replicate injection of standard solution should be not more than 2.0%	**Component name**	**% RSD**
		Busulfan	0.3%
	The relative standard deviation of assay results obtained from six sample preparations should not be more than 2.0%	Busulfan	0.1%
Specificity	**Interference** No Interference should be observed at the retention time of Busulfan peak in the chromatograms obtained from the diluent, Blank and placebo.	There is no interference is observed at the retention time of Busulfan peak in the chromatogram obtained from the diluent, Blank and placebo.	

Validation Parameters	Acceptance Criteria	Results			
		Drug Product (FP)			
	➢ Calculate the % degradation against as such test preparation for each condition, in any of one condition degradation should be achieved between 5.0% to 20.0%. ➢ Each degradation sample, purity angle should be less than the purity threshold for Busulfan peak.	**Array of Stress**	**% degradation**	**Purity Angle**	**Purity Threshold**
		As Such (Unstressed)	-	0.681	19.281
		Acid degradation	2.2	0.793	17.890
		Alkali degradation	8.6	0.796	18.528
		Oxidation degradation	2.2	1.069	66.090
		UV degradation	Not degraded	0.690	18.648
		Thermal degradation	2.6	0.678	19.149
		Neutral degradation	1.7	0.747	18.585
Linearity	➢Correlation coefficient should not be less than 0.999	Busulfan			
		Correlation coefficient (R)		0.999	

<table>
<tr><td></td><td>for Busulfan.
➢Report the slope of regression line.
➢Report the Y-intercept of regression line.
➢Y-intercept bias at 100 % level should be between ± 5.0 % for Busulfan.</td><td colspan="3">slope of regression line</td><td>66205</td></tr>
<tr><td></td><td></td><td colspan="3">Y-intercept of regression line</td><td>104381</td></tr>
<tr><td></td><td></td><td colspan="3">Y-intercept bias at 100% level</td><td>3.0</td></tr>
<tr><td rowspan="3">Intermediate Precision</td><td rowspan="3">➢ The relative standard deviation results obtained from six sample preparations should not be more than 2.0%
➢ The cumulative %RSD of method precision and intermediate precision results obtained from twelve sample preparations (6 method precision and 6 intermediate precision) should not be more than 2.0%.</td><td colspan="3">Precision</td><td colspan="2">Intermediate Precision</td></tr>
<tr><td colspan="3">0.1%</td><td colspan="2">0.6%</td></tr>
<tr><td colspan="5">0.4%</td></tr>
<tr><td>Validation Parameters</td><td>Acceptance Criteria</td><td colspan="5">Results</td></tr>
<tr><td rowspan="5">Accuracy</td><td rowspan="5">➢ Recovery at each level and overall average recovery of assay results should be between 98.0% and 102.0%
➢ The RSD at each level and overall RSD of % recovery should not be more than 5.0%</td><td>Accuracy Level</td><td colspan="2">Average % Recovery</td><td>%RSD</td></tr>
<tr><td>50 %</td><td colspan="2">99.2</td><td>0.3</td></tr>
<tr><td>100 %</td><td colspan="2">100.2</td><td>0.2</td></tr>
<tr><td>150 %</td><td colspan="2">100.5</td><td>0.3</td></tr>
<tr><td>Overall % Recovery</td><td colspan="3">100.0 %</td></tr>
<tr><td>Overall % RSD</td><td colspan="3">0.6 %</td></tr>
<tr><td rowspan="2">Robustness</td><td rowspan="2">System suitability criteria defined in test procedure should meet in each condition.
➢ The Tailing factor for Busulfan should be NMT 2.0.
➢ The relative standard deviation for Busulfan peak from five replicate injections</td><td colspan="2" rowspan="2">Condition</td><td colspan="3">Busulfan</td></tr>
<tr><td>% RSD</td><td>Tailing factor</td><td>Theoretical plates</td></tr>
<tr><td colspan="2">As such (For Flow, Temperature, Organic composition,Derivatisation temperature,Derivatisation Time)</td><td>0.3</td><td>1.0</td><td>16290</td></tr>
</table>

	of standard solution should be NMT 2.0 %. The theoretical plates for Busulfan peak in standard solution should be not less than 2000.	Flow rate:1.3 mL/min	0.2	1.0	20283
		Flow rate:1.7 mL/min	0.3	1.0	19156
		Column oven temperature: 23°C	0.3	1.0	20075
		Column oven temperature: 27°C	0.1	1.0	20145
		Low organic composition(637 mL)	0.1	1.0	19366
		High organic composition(663 mL)	0.1	1.0	20976
		Derivatisation temperature: 50° C	0.7	1.0	19952
		Derivatisation temperature: 70° C	0.2	1.0	19793
		Derivatisation time: 10 min	0.4	1.0	20008
		Derivatisation time: 30 min	0.1	1.0	19837

Overall Summary of Validation Results of Bendamustine

<table>
<tr><th>Validation Parameters</th><th>Acceptance Criteria</th><th colspan="4">Results</th></tr>
<tr><td rowspan="5">Precision</td><td rowspan="2">System precision
The relative standard deviation for Bendamustine peak from five replicate injections of standard solution should be not more than 2.0%.</td><td colspan="2">Component name</td><td colspan="2">% RSD</td></tr>
<tr><td colspan="2">Bendamustine</td><td colspan="2">0.6</td></tr>
<tr><td rowspan="3">Method Precision
The RSD of results obtained from six sample preparations should not be more than 2.0%</td><td colspan="2">Strength</td><td colspan="2">% RSD</td></tr>
<tr><td colspan="2">25 mg/ vial</td><td colspan="2">0.5</td></tr>
<tr><td colspan="2">100 mg/ vial</td><td colspan="2">0.4</td></tr>
<tr><td rowspan="3">Specificity</td><td rowspan="3">Specificity by interference study
There should be no interference at the retention time of Bendamustine peak in the Chromatograms obtained from the diluent and the placebo solutions.
Specificity Forced degradation study
Calculate the % degradation against</td><td colspan="4">No interference observed at the retention time of Bendamustine peak in the chromatogram of blank, placebo and Known impurities</td></tr>
<tr><td colspan="4">Drug Product (FP)</td></tr>
<tr><td>Array of Stress</td><td>% degradation</td><td>Purity Angle</td><td>Purity Threshold</td></tr>
</table>

Validation Parameters	Acceptance Criteria	Results			
	as such test preparation for each condition, in any of one condition degradation should be achieved between 5.0% to 20.0%. Each degradation sample, purity angle should be less than the purity threshold for Bendamustinepeak.	As Such (Unstressed)	NA	0.107	0.218
		Acid degradation	5.5	0.113	0.215
		Alkali degradation	12.5	0.112	0.228
		Peroxide degradation	0.6	0.106	0.218
		Photolytic degradation	0.4	0.101	0.225
		Thermal degradation	1.3	0.115	0.231

<table>
<tr><th>Validation Parameters</th><th>Acceptance Criteria</th><th colspan="3">Results</th></tr>
<tr><td rowspan="4">Linearity</td><td rowspan="4">• Correlation coefficient should not be less than 0.999 for Bendamustine Hydrochloride.
• Report the slope of regression line.
• Report the Y-intercept of regression line.
• Y-intercept bias at 100 % level should be between ± 5.0 % for Bendamustine Hydrochloride.</td><td colspan="3">Bendamustine Hydrochloride</td></tr>
<tr><td>Correlation coefficient</td><td colspan="2">0.9999</td></tr>
<tr><td>Slope of regression line</td><td colspan="2">27800</td></tr>
<tr><td>Y-intercept of regression line</td><td colspan="2">7930.9</td></tr>
<tr><td>Intermediate Precision</td><td>• The relative standard deviation of results obtained from six sample preparations should not be more than 2.0%
• The cumulative relative standard deviation of method precision and intermediate precision results obtained from twelve sample (6 methods precision and 6 intermediate precision) preparations should not be more than 2.0%.</td><td>% RSD</td><td colspan="2">Cumulative % RSD</td></tr>
<tr><td></td><td>0.6</td><td colspan="2">0.5</td></tr>
<tr><td rowspan="5">Accuracy</td><td rowspan="5">% Recovery at each level and overall % recovery should be between 98.0 and 102.0 for BendamustineHCl.
The %RSD at each level and overall recovery should not be more than 2.0.</td><td>Accuracy Level</td><td>Average %</td><td>%RSD</td></tr>
<tr><td>50 %</td><td>100.5</td><td>0.1</td></tr>
<tr><td>100 %</td><td>99.4</td><td>0.9</td></tr>
<tr><td>150 %</td><td>99.1</td><td>0.3</td></tr>
<tr><td>Overall % Recovery</td><td colspan="2">99.7</td></tr>
<tr><td></td><td>Overall % RSD</td><td colspan="2">0.8</td></tr>
<tr><td>Range</td><td>NA</td><td colspan="3">Based on the Linearity, Method precision and Accuracy data Range of the method is 50 to 150% of test concentration</td></tr>
</table>

<table>
<tr><th>Validation Parameters</th><th>Acceptance Criteria</th><th>Results</th></tr>
<tr><td>Robustness</td><td>➢ The Tailing factor for Bendamustine peak from first injection of standard solution should be not more than 2.0.
➢ Theoretical Plates for Bendamustine peak from first injection of standard solution should be not less than 2000.
➢ The relative standard deviation for Bendamustine peak from five replicate injections of standard solution should be not more than 2.0%.</td><td>
<table>
<tr><th rowspan="2">Condition</th><th colspan="3">Bendamustine</th></tr>
<tr><th>Tailing</th><th>Theoretical</th><th>% RS</th></tr>
<tr><td>Flow rate 1.3 mL/min</td><td>1.2</td><td>5598</td><td>0.2</td></tr>
<tr><td>Flow rate 1.7 mL/min</td><td>1.1</td><td>4842</td><td>0.2</td></tr>
<tr><td>Column oven temperature 23°C</td><td>1.2</td><td>4999</td><td>0.5</td></tr>
<tr><td>Column oven temperature 27°C</td><td>1.2</td><td>5314</td><td>0.1</td></tr>
<tr><td>Mobile phase composition (68:32)</td><td>1.2</td><td>5603</td><td>0.8</td></tr>
<tr><td>Mobile Phase composition (72:28)</td><td>1.2</td><td>4568</td><td>0.2</td></tr>
</table>
</td></tr>
<tr><td>Stability of analyte in solution</td><td>➢ % Difference of BendamustineHCl assay obtained from standard solution at each time point should not be more than ± 2.0 from the initial assay.
➢ % Difference of BendamustineHCl assay obtained from sample solution at each time point should not be more than ± 2.0 from the initial assay.</td><td>
% Difference of Assay
<table>
<tr><th rowspan="2">Time Interval</th><th colspan="2">Standard Solution at</th></tr>
<tr><th>RT</th><th>2-8°C</th></tr>
<tr><td>Initial</td><td>NA</td><td>NA</td></tr>
<tr><td>24 hrs.</td><td>-0.3</td><td>1.2</td></tr>
<tr><td>48 hrs.</td><td>-1.2</td><td>1.3</td></tr>
</table>
% Difference of Assay
<table>
<tr><th rowspan="2">Time Interval</th><th colspan="2">Sample Solution at</th></tr>
<tr><th>RT</th><th>2-8°C</th></tr>
<tr><td>Initial</td><td>NA</td><td>NA</td></tr>
<tr><td>24 hrs.</td><td>-1.5</td><td>0.8</td></tr>
<tr><td>48 hrs.</td><td>-1.2</td><td>-0.7</td></tr>
</table>
</td></tr>
<tr><td>Filter</td><td>% Difference of BendamustineHCl</td><td>% Difference of Assay</td></tr>
</table>

Validation Parameters	Acceptance Criteria	Results			
variability	assay obtained from unfiltered sample solution and filtered sample solutions should not be more than ± 2.0.	**PVDF filter**		**Nylon filter**	
		1.0		0.9	
System suitability overall summary	The Tailing factor for Bendamustine peak from first injection of standard solution should be not more than 2.0. Theoretical Plates for Bendamustine peak from first injection of standard solution should be not less than 2000. The relative standard deviation for Bendamustine peak from five replicate injections of standard solution should be not more than 2.0%.	**Bendamustine**			
		Parameter	**Minimum**	**Maximum**	**Average**
		Tailing factor	1.1	1.2	1.2
		Theoretical plates	3922	5603	4960
		% RSD	0.1	0.7	0.4

Overall Summary of Validation Results of Clofarabine

Validation Parameters	Acceptance Criteria	Results	
Precision	**1.1 System precision** The RSD of results obtained from six standard NMT 2.0%	**Component name**	**% RSD**
		Clofarabine	0.02%
	1.2 Method Precision The relative standard deviation results obtained from six sample preparations should not be more than 2.0%	**Component name**	**% RSD**
		Clofarabine	0.08%

Validation Parameters	Acceptance Criteria	Results	
Specificity	**2.1 No interference from diluent, placebo and known impurities** No Interference should be observed at the retention time of Clofarabine peak in chromatograms obtained from the diluent, placebo and the impurities	There is no interference is observed at the retention time of Clofarabine peak in the chromatogram obtained from the diluent, placebo and known impurities.	
	2.2 Forced degradation study a. Calculate the % degradation against as such test preparation for each condition in any one of condition degradation should be achieved between 5.0% to 20.0%. b. For each degradation sample, purity angle should less	**Drug Product (FP)**	
		As Such (Unstressed)	0.0
		Acid degradation	-1.2
		Alkali degradation	8.0

	than the purity threshold for Clofarabine peak.	Peroxide degradation	-1.5
		UV degradation	-0.3
		Thermal degradation	-0.5

Validation Parameters	**Acceptance Criteria**	**Results**	
Linearity	a. Correlation coefficient should not be less than 0.999 b. Report the slope of regression line c. Report the Y-intercept of regression line d. Y-intercept at 100% level should be between ± 5.0%	**Clofarabine**	
		Correlation coefficient	1.000
		slope of regression line	72366.0
		Y-intercept of regression line	26262.5
		Y-intercept bias at 100% level	0.6

Validation Parameters	**Acceptance Criteria**	**Results**
Intermediate Precision	The cumulative %RSD of method precision and intermediate precision results obtained from twelve sample preparations should not be more than 2.0%.	0.42%

Validation Parameters	**Acceptance Criteria**	**Results**		
Accuracy	% Recovery at each level and overall % recovery should be between 95.0% and 105.0% for Clofarabine. The %RSD at each level and overall %RSD of %recovery should not be more than 3.0%.	**Accuracy Level**	**Average % Recovery**	**%RSD**
		50 %	100.1	0.2
		100 %	100.0	0.1
		150 %	98.8	0.1
		Overall % Recovery	99.6	
		Overall % RSD	0.6	

Validation Parameters	**Acceptance Criteria**	**Results**	
Robustnes	System suitability criteria defined in test	**Condition**	**Clofarabine**

s	procedure should meet in each condition. 1. The Tailing factor for Clofarabine should be NMT 2.0 2. The relative standard deviation for Clofarabine peak from five replicate injections of standard solution should be NMT 2.0 %. 3. The Theoretical plates for Clofarabine peak in standard in standard solution should be not less than 3000.		**% RSD**	**Tailing factor**	**Theoretical plates**
		Flow rate:0.8 mL/min	0.05	1.1	6243
		Flow rate:1.2 mL/min	0.02	1.0	3940
		Column oven temperature: 38°C	0.03	1.1	4914
		Column oven temperature: 40°C	0.08	1.1	4875
		Low organic composition (142.5 mL)	0.02	1.1	5489
		High organic composition (157.5 mL)	0.04	1.1	4545

Validation Parameters	**Acceptance Criteria**	**Results**		
Stability of analyte in solution	% Difference of Clofarabine peak area obtained from standard solution at each time point should not be more than ±2.0 from the initial area.	**% Difference of area**		
		Time Interval	**Standard Solution**	
			RT	2-8°c
		Initial	0.0	0.0
		24 hrs.	0.37	0.24
		48 hrs.	0.37	0.42
	% Difference of Clofarabine peak area obtained from sample solution at each time point should not be more than ±2.0 from the initial area.	**% Difference of area**		
		Time Interval	**Sample Solution**	
			RT	2-8°c
		Initial	0.0	0.0
		24 hrs.	0.25	0.38
		48 hrs.	0.30	0.06
		Standard solution is stable up to 48Hours and sample solution is stable up to 48Hours at room temperature and 2-8°C for Clofarabine peak.		

Validation Parameters	**Acceptance Criteria**	**Results**			
System suitability	System suitability criteria should meet during overall validation studies, otherwise needs to be justified. Report	**System suitability criteria**	**Minimum**	**Maximum**	**Average**

Validation Parameters	Acceptance Criteria	Results			
	minimum, maximum and average values of system suitability parameters. ➢ The Tailing factor for Clofarabine should be NMT 2.0 ➢ The relative standard deviation for Clofarabine peak from five replicate injections of standard solution should be NMT 2.0 %. ➢ The Theoretical plates for Clofarabine peak in standard in standard solution should be not less than 3000.	**% RSD**	0.02	0.08	0.03
		Tailing factor	1.0	1.1	1.1
		Theoretical plates	3940	6243	5078

Final Conclusion:

A simple isocratic HPLC method is developed for the determination of Busulfan, Bendamustine Hydrochloride and Clofarabine in pharmaceutical formulations. The result meets the acceptance criteria and found comparable, indicates that the method is precision (System precision and Method precision), (Interference, Linearity), Stability of Analyte in solution, Filter compatibility and System Suitability with respect to analyst, day to day, column to column and equipment to equipment for its intended use. Therefore the method can be used for routine analysis in quality control. The analytical test attributes and evaluated as per the guidelines of ICH Q2 (R1).

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