STABILITY INDICATING METHOD DEVELOPMENT AND VALIDATION, STRESS DEGRADATION STUDIES FOR DACARBAZINE BY USING RP-HPLC

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ABSTRACT

For the measurement and quantification of Dacarbazine, a rapid, simple, sensitive, and reliable RP-HPLC technique using a Waters HPLC System with PDA detection was designed and validated. In this work, Dacarbazine was subjected to different stress conditions mentioned by international conference on Harmonization. A stability indicating method was developed for the analysis of the drug in the presence of its degradation products. Chromatography was carried out on an Altimal ods 150 mmX4.6 mm 5 µm column with a rate of flow 1.0 ml/min and an effluent of 323 nm using filtered and mixed Degassed 0.1% OPA buffer: Acetonitrile (90:10) as a mobile phase, The buffer pH was maintained at 5 and the temperature was ambient i.e., 30^{0}c. Dacarbazine Retention time is 2.717 minutes. Even the percentage purity of the drug was observed at 99.99%. Later the system suitability criteria i.e., theoretical plates, tailing factor, and Asymmetry values for Dacarbazine was revealed at 4484, 1.10 and 1.101 respectively. Linearity range was observed well at 25-150 µg/mL concentration series, Linearity test Correlation Coefficient was found to be 0.999 for the compound. and then the %mean recovery for the drug at 50%, 100% and 150% were 100.16, 99.85 and 100.32 correspondingly, for %RSD precision that is intraday it was 0.53% and Inter-day it was 0.83%, The values of LOD and LOQ obtained from Dacarbazine was 0.17 µg/ml and 0.52µg/ml. The method was simple and economical that can be applied successfully for simultaneous estimation of Stability Studies of Dacarbazine.

Keywords: Method Development, Validation, Dacarbazine, Degradation, stability indicating, RP-HPLC.

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1. INTRODUCTION

Dacarbazine (DTIC), also considered as imidazole carboxamide. It is a chemotherapeutic drug used in the treatment of Malignant melanoma (MM), Hodgkin lymphoma (HL), as well as Soft Tissue Sarcoma. Its IUPAC designation is 5-(3,3-Dimethyl-1-triazenyl) imidazole-4-carboxamide. It belongs to the alkylating agent family, which acts on tumour cells and kills them by attaching an alkyl group onto their genome (Weber, G. F et al., 2014). DTIC may cause adverse effects related to the digestive system including nausea and vomiting. It may also cause anaemia and neutropenia by suppressing the synthesis of blood cells, as well as diarrhoea and a flu-like condition 1-2 weeks after taking injection (Crosby, T et al., 2000). Its significant anticancer potential was discovered and synthesized in 1962 for the first time (Iradyan, M.A et al., 2010). DTIC is a nonspecific antitumor drug and act as a genotoxic agent. For the treatment of Hodgkin's disease DTIC is prescribed in combination with

other agents like doxorubicin, bleomycin, and vinblastine (Sweetman, S.C et al., 2009).

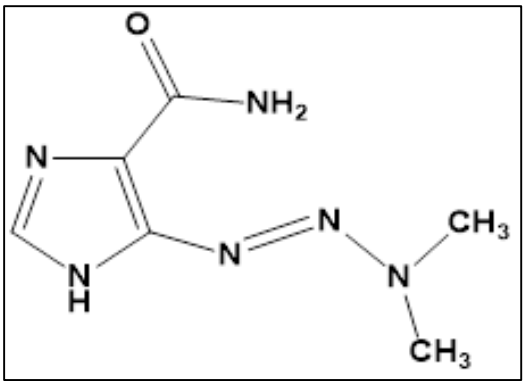

Figure 1. Dacarbazine Structure

The literature review was carried out to elucidate the reported analytical methods for the selected drug individually or in combination with other drugs such as HPLC, RP-HPLC, HPLC-MS/MS, UHPLC-MS/MS, RP-LC, LC-MS/MS, CAPILLARY ELECTROPHORESIS. RP-HPLC methods for Dacarbazine combination with Bleomycin, Vinblastine, Adriamycin (Malik, M.Z et al., 2013), HPLC methods are reported for the determination of Dacarbazine alone (Prasanth, M.L et al., 2014), for determination of Dacarbazine along with other 2 products in plasma and urine (Fiore, D et al., 1985), for determination of Dacarbazine combination with Ondansetron and Doxorubicin in 5% dextrose injection (King, D.T et al., 1993), LC-MS/MS method for determination of Dacarbazine and 4(5)-aminoimidazol-5(4)-carboxamide (AIC) in plasma (Liu, Y et al., 2008), Capillary Electrophoresis and HPLC methods are reported for determination of Dacarbazine and its associated contaminants (Haque, A et al., 1999), HPLC-MS/MS methods are reported for determination of Dacarbazine alone. UHPLC-MS/MS methods are reported for determination of 26 anticancer medicines, including Dacarbazine (Dugheri, S et al., 2021), for determination of Dacarbazine combination with Bleomycin (. Bragaru, A et al., 2011), for determination of Dacarbazine levels in urine of mouse (Yue, Y et al., 2020), and also for determination of primary degradation product in Dacarbazine (El Aatmani, M et al., 2002). and also, for determination of Dacarbazine as well as its metabolites (Safgren, S.L et al., 2001). RP-LC as well as Capillary Electrophoresis methods for determination of Dacarbazine alone (Abd El-Hady, D et al., 2015). As we all know that, thus far for Dacarbazine there is no RP-HPLC methods have been published. The present study is strived to construct, optimize and validate, for the stability determination of Dacarbazine, a simple, rapid RP-HPLC process.

2. MATERIALS AND INSTRUMENTS

2.1. Chemicals and reagents

Dacarbazine drug is bought from clintech labs, Dacatic TM tablets (merck & co., India), 0.1% Ortho phosphoric acid, Acetonitrile, Methanol (merck), water (HPLC grade).

2.2. Instruments Specification

Waters HPLC LC 2695 is used with Photo Diode Array Detector 2996 and a software application named Empower V.1.2.1.2. The analysis was performed with the column Altimal ods of dimensions 250mmx4.6mm,5µm and Automatic injector (Rheodyne) of 20µL.The mobile phase used is 0.1%OPA and Acetonitrile in 90:10 ratio with the Flow rate of 1.0ml/min at wavelength of 323nm.the retention time of dacarbazine is 2.7 minutes.

2.3. Preparation of Solutions

2.3.1. Preparation of Mobile Phase

90ml of OPA (0.1%) and 10ml of Acetonitrile, prior to use it was percolated using 0.45 membrane filter and any gas bubbles present were removed for 10minutes.

2.3.2. Preparation of Diluent

The acetonitrile: water in ratio of 1:1percent v/v ratio was preferred as diluent the optimal technique.

2.3.3. Preparation of Dacarbazine Standard Drug Solution

10mg of Dacarbazine is taken into a 10ml sterile, dried volumetric flask along with diluent(7ml), sonicated for 30mins, then diluents were added to make up the final quantity to get 1000 µg/ml concentration primary stock solution. The stock solution was placed correctly into volumetric flasks and adjusted to a concentration range of 25-150µg/ml.

2.3.4. Preparation of Dacarbazine Sample Solution

10mg of tablet (Dacatic TM) powder was weighed and taken into a 10 ml volumetric flask, the volume is made up to 10ml with diluent to get a concentration of 1000µg/ml concentration

primary stock solution, 1ml of the 1000 µg/ml solution was pipetted into a volumetric flask of 100ml, which was then diluted to the final volume using a solvent to get 10µg/ml concentration secondary stock solution. The resulting solution is filtered using a 0.45 syringe filter.

2.3.5. Preparation of Stock Solution for Stress Studies

Accurately weighed 10mg of each drug component was meticulously put into a volumetric flask, and the volume was increased to the specified value. Dilute the diluents to get 1000 µg/ml. 1ml of the 1000 µg/ml solution was pipetted into a volumetric flask of 10ml, which was then diluted to the final volume using a solvent to get 100 µg/ml. The same process was used to generate oxidation, acidic degradation, and basic degradation solutions by using 3% H_2O_2, 1N HCL, and 0.1N NaOH respectively.

2.4. Procedure for stress studies

2.4.1. Acid Degradation preparation

1ml of Dacarbazine stock solution was added to 1ml of 1N HCl and heated for 6 hours at 60°C. the resulting solution was again diluted to a concentration of 100µg/ml and 10µL solutions were injected into the HPLC system. Finally, chromatograms were plotted to determine the sample's stability.

2.4.2. Alkaline Degradation preparation

1ml of dacarbazine stock solution was added to 1ml of 0.1N NaOH and heated for 6 Hours at 60°C. The solution was again diluted to 100µg/ml and 10µL solution was injected into the HPLC system. Finally, chromatograms were plotted to determine the sample's stability.

2.4.3. Oxidative Degradation preparation

1ml of H_2O_2 was dissolved in 1ml of dacarbazine stock solution separately. This solution was then maintained at ambient temperature for 6 Hours. The resulting solution was diluted to get a concentration of 100µg/ml and 10µL solution was injected into the injector unit of the HPLC system. Finally, chromatograms were plotted to determine the sample's stability.

2.4.4. Solid State Stability preparation

It was conducted by putting the sample as a thin film which was preheated at 105°C. 10mg of heated samples were weighed, appropriately liquified, at different time intervals and adjusted to a concentration of 100µg/ml by using mobile phase, and injected into the system.

2.4.5. Photolytic Degradation preparation

The 100µg/ml, 10µg/ml, and 25µg/ml solutions were subjected to ultraviolet light for a week or placed in a photostability chamber at 200-watt hours/m^2. The resulting solution was adjusted to a concentration of 100µg/ml for HPLC analysis. 10µL solution was injected into HPLC system. Finally, chromatograms were plotted to determine the sample's stability.

2.4. 6.Neutral Condition preparation

The drug was treated with water for 7 hours at room temperature. The resulting solution was adjusted to a concentration of 100µg/ml and a 10µL solution was injected into the HPLC system. chromatograms were plotted to determine the sample's stability.

3. RESULTS AND DISCUSSION

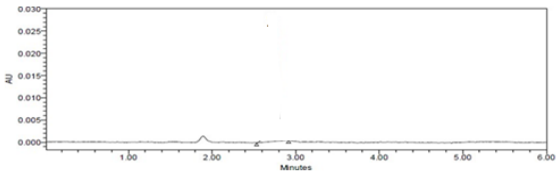

Figure 2. The chromatogram for Blank

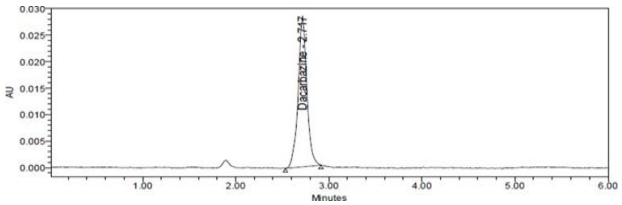

Figure 3. The chromatogram for Standard

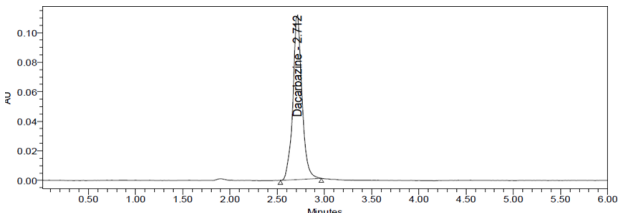

Figure 4. The chromatogram for the sample

3.1. Validation of the Analytical Method

The designed chromatographic approach was examined for system suitability, specificity, accuracy, precision, linearity, limit of detection

(LOD), limit of quantification (LOQ), robustness, and stress degradation studies based on ICH standards.

3.1.1. System Suitability

It is defined as the study used to determine whether a particular procedure produces results that are accurate and precise enough. The convenience of the system was determined followed by the completion of method development and validation (Sanjeev, A et al., 2020). The factors which are checked to know the appropriateness of the system are plate count, tailing factor, capacity factor, HETP (Height equivalent to theoretical plates), and asymmetry of samples. It can be performed by injecting five replicates of medication at a concentration of 100 µg/ml to determine the system's applicability as shown in table 1.

Table 1. System suitability parameters

S. No	Parameter	Obtained values	Acceptance criteria
1	Plate count	4484	>2000
2	Tailing Factor	1.10	≤2.0
3	Asymmetry (10%)	1.101	0.9-1.2
4	Capacity Factor	0.888	0.5-10

3.1.2. Specificity

The specificity is the ability to assess the analyte clearly in the presence of other components which may be expected to be present. A blank (only diluent without drug) was injected into the system. No peaks were observed. Chromatograms of Blank, standard, sample were shown in figure 2, figure 3, and figure 4.

3.1.3. Accuracy

The accuracy of the method is determined with the help of recovery studies. Recovery studies are conducted by taking 3 replicates of 3 different concentrations, namely 50%, 100%, 150% of the target concentration level (100µg/ml Dacarbazine) in tablets. Then the individual percentage recovery of items and mean recovery values were determined shown in table 2.

Table 2. Accuracy report of tablet dosage

Drug	Amount	Recovery level	Amount recovered(µg/ml)	% Recover Y(n=3)	Acceptance criteria
DACA	50	50%	50.06	100.16	98-102%
	100	100 %	99.82	99.85	98-102%
	150	150 %	150.09	100.32	98-102%

3.1.4. Precision

The system's precision was determined, by measuring the six replicate injections of Dacarbazine concentration (100µg/ml). Inject six replicate injections and note down the area and then calculate the %RSD for the area of six injections were shown in table3,4,5.

Table 3. Precision: Repeatability (system precision)

Injection No.	Concentration(µg/ml)	Area(n=6)	RT(n=6)
1	100	789369	2.703
2	100	783318	2.708
3	100	786363	2.711
4	100	785071	2.716
5	100	794991	2.722
6	100	789928	2.718
MEAN		788173	
SD		4182.5	
%RSD		0.53	

Table 4. Precision: Reproducibility (Method precision)

Injection no.	Concentration (µg/ml)	Area(n=6)	RT(n=6)
1	100	796225	2.717
2	100	782220	2.720
3	100	790135	2.716
4	100	777540	2.718
5	100	790679	2.706

6	100	793785	2.711
Mean		788430	
SD		7134.7	
%RSD		0.9	

Table 5. Summarizes the intra-day and inter-day precision results

Drug	Amount	Intraday(n=3)		Inter day (n=3)	
		Amount found	%RSD	Amount found	%RSD
Dacarbazine	100	99.25	0.53	99.87	0.83

3.1.5. Linearity

The linearity of calibration curves was carried out using pure solution of Dacarbazine concentrations ranging from 25µg/ml to 150µg/ml. The total time required for eluting doesn't take more than 30 minutes. With the help of regression analysis, the regression line of standard drug levels was determined. Under the examined range, the calibration curves were linear, and the regression equation Y=7133x+368.9, R^2=0.999 were obtained. The mean and standard deviation (SD) of the slope, correlation coefficient of three standard curves (n=3) and intercept were determined and shown in table 6 and figure 5.

Table 6. Linearity Range of Dacarbazine

Sr. No.	Concentration(µg/ml)	Peak area
1	0	0
2	25	183678
3	50	346942
4	75	526895
5	100	734196
6	125	894385
7	150	1061610

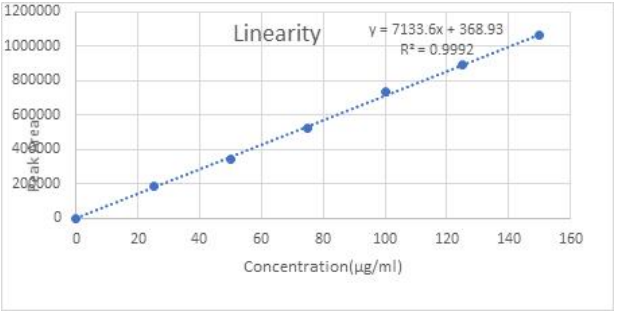

Figure 5. linearity range graph of Dacarbazine

3.1.6. LOD and LOQ

The LOD and LOQ procedures were examined using the samples having very low concentration of analyte in conformity with ICH recommendations. Limit of detection is typically defined as the lowest amount of analyte present in the sample that can be reliably distinguished but not necessarily quantified, as well as Limit of quantification is defined as the lowest amount of analyte present in the sample that can be quantitatively determined with suitable accuracy and precision. The values were shown in table 7.

$$LOD=3.3\sigma/S, \text{ and } LOQ=10\sigma/S$$

Where,

σ = Standard deviation of y-intercept of regression line.

S = Slope of the calibration curve.

Table 7. LOD and LOQ

Sr. NO	LOD	LOQ
1	**0.17 µg/ml**	**0.52 µg/ml**

3.1.7. Robustness

Robustness measures the ability of the method to remain unaffected by deliberate changes in flow rate, the organic solvent concentration, and pH within a realistic range and the quantitative influence of the variables is determined. It is a measure of reliability of a method. Not a single change results in a significant alteration in the RSD of the peak area, the USP tailing factor, or the theoretical plates. Although the differences in retention times were greater, measurement remained accomplishable. The values are shown in table 8.

Table 8. Robustness Studies

Parameter	Condition	Retention time	Area(n=3)	%RSD
Optimized	Flow rate:1ml/min, Mobile Phase: 9:10, pH 5.0	2.717	397058	0.47
Flow rate	0.8	3.03	432719	0.3
(ml/min)	1.1	2.70	412678	0.5
Mobile phase	89:11	2.71	387589	0.2
	91.09	2.79	418975	0.7

3.2. Stress degradation studies

The therapeutically active substance Dacarbazine was subjected to accelerated degradation under acidic, alkaline, neutral, heat treatment, oxidation, as well as photolytic stress conditions. Under stress investigation, the portions of the stressed sample were diluted using the mobile phase to obtain a concentration of 100µg/ml and then injected under optimal conditions with an appropriate blank.

3.2.1. Acid degradation studies

Acid degradation studies are conducted for 6 hours, at 60ºC temperature in 1N HCL. The Dacarbazine was found to be prone to acid hydrolysis and the degradation was found to be 7.08%. The chromatogram was shown in figure 6.

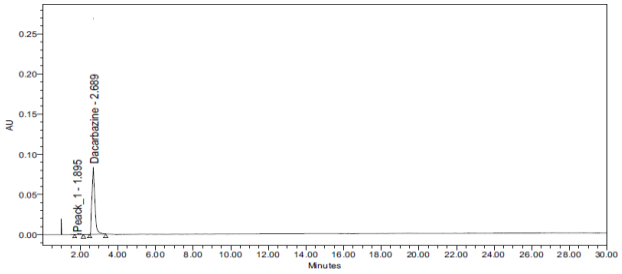

Figure 6. Acid degradation in 1N HCl at 60°C

Table 9. Acid degradation of Dacarbazine using 1N HCl

Sr. No.	Time (Hrs)	Peak Area Mean± SD(n=3)	%Degradation
1	6	719467	7.08

3.2.2. Alkaline degradation studies

Alkaline degradation studies are conducted for 6 hours, at 60ºC temperature in 1N NaOH. Dacarbazine was found to be prone to base hydrolysis and the degradation was found to be 6.38%. The chromatogram was shown in figure 7.

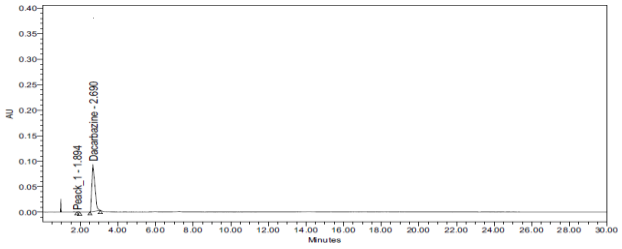

Figure 7. Alkaline Degradation in 0.1N NaOH at room temperature

Table 10. Alkaline degradation report of Dacarbazine in 0.1N NaOH

S. No	Time (Hrs)	Peak Area Mean ± S.D (n=3)	%Degradation
1	6	724898	6.38

3.2.3. Thermal degradation studies

Thermal degradation studies are conducted to know the solid-state stability of the drug at 105ºC. The given drug in solid form was found to be susceptible to heat above 105ºC temperature and the degradation was found to be 4.70%. The chromatogram was shown in figure 8.

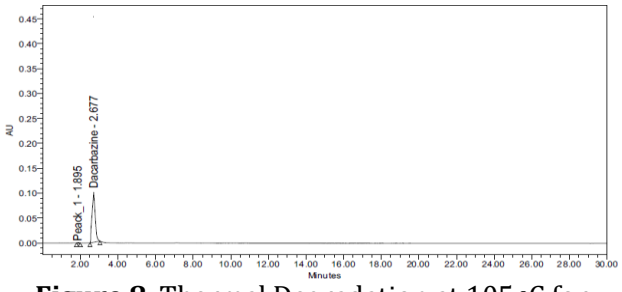

Figure 8. Thermal Degradation at 105 °C for 6hrs

Table 11. Thermal degradation (105 °C) report

S. No	Time (Hrs)	Peak area Mean ± S.D (n=3)	%Degradation
1	6	737862	4.70

3.2.4. Oxidative degradation studies

At ambient temperature, using 3%H_2O_2 the oxidative degradation experiments were conducted. The given drug was found to be prone to oxidative stress and the degradation was found to be 5.42%. The chromatogram was shown in figure 9.

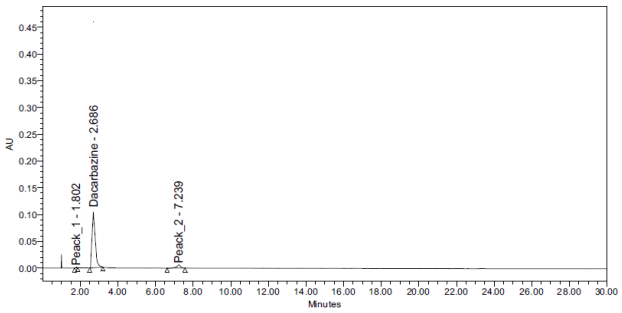

Figure 9. Degradation in 3% H_2O_2 at Room temperature

Table 12. Oxidative degradation report in 3% H_2O_2

S. No	Time 1(Hrs)	Peak Area Mean ± S.D(n=3)	%Degradation
1	6	732273	5.42

3.2.5. Photolytic degradation studies

Photolytic degradation studies are conducted by exposing the sample to sunlight, for about 7 days. Dacarbazine was found to be susceptible to photo degradation in sunlight and the degradation was found to be 1.99%. The chromatogram was shown in figure 10.

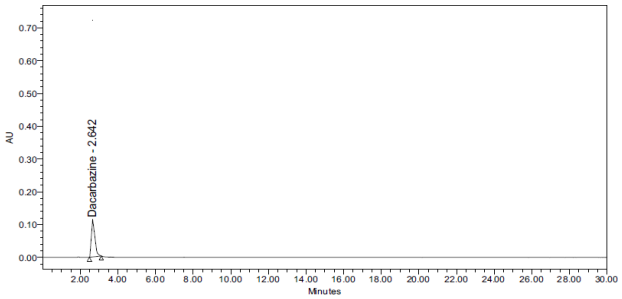

Figure 10. Degradation in UV chamber for 7 days

Table 13. Photo Degradation Report

S. No	Time (Hrs)	Peak area Mean± S.D(n=3)	% Degradation
1	7 days	758900	1.99

3.2.6. Neutral Degradation

The drug showed liability to Neutral condition. Degradation was done for 7 days and degradation was found to be 0.39%. The chromatogram was shown in figure 11.

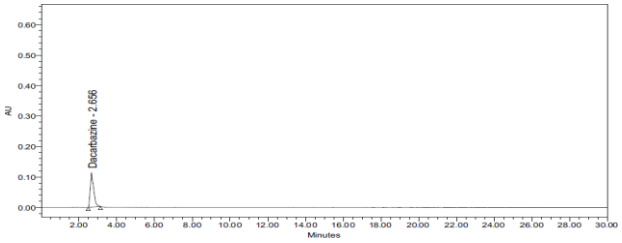

Figure 11. Degradation in neutral condition

Table 14. Neutral condition report in drug solution

S. No.	Time (days)	Peak area Mean ± S.D. (n=3)	% Degradation
1	**7**	**77**1792	0.39

3.3. Assay Report

The recommended method was efficiently verified, and also tested for tablet dosage form of Dacarbazine, assay results were shown in table 15.

Table 15. Assay report of tablet dosage form of Dacarbazine

Formulation	Drugs	Labelled claim (in mg)	Amount Found (µg)	Assay (in %)
Dacatic	Dacarbazine	100	99.99	99.99

4. CONCLUSION

The RP-HPLC technique recommended for the measurement and quantification of Dacarbazine, this method was considered as a simple, economical, rapid, sensitive and reliable, precise, accurate, linear, robust, specific, technique in pharmaceutical dose types. In this work, Dacarbazine was subjected to different stress conditions mentioned by international conference on Harmonization. It can be applicable successfully for estimation of Dacarbazine bulk substances and pharmaceutical dosage forms and also applicable for routine analytical purpose in industries and labs.

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