Development and Validation of Stability Indicating RP-HPLC Method for the Simultaneous Estimation of Daunorubicin and Cytarabine in Bulk and Pharmaceutical Dosage Forms

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ABSTRACT:
A new, simple, precise, accurate and reproducible RP-HPLC method for Simultaneous estimation of Daunorubicin and Cytarabine bulk and pharmaceutical formulations. Separation of Daunorubicin and Cytarabine was successfully achieve Dona: Agilent150X4.6mm,5µm, C18 or equivalent in an isocratic mode utilizing Na_2HPO_4: Methanol (50:50) at a flow rate of1.0 mL/min and eluate were monitored at 228nm, with a retention time of 2.337 and 3.358 minutes for Daunorubicin and Cytarabine respectively. The method was validated and there response was found to be linear in the drug concentration range of 50µg/ml to150 µg/ml for Daunorubicin and 50µ g/ml to150 µg/ml for and Cytarabine. The values of the correlation coefficient were found to 1.000 for Daunorubicin and 0.999 for Cytarabine respectively. The LOD and LOQ for Daunorubicin were found to be 0.293 and 0.977 respectively. The LOD and LOQ for Cytarabine were found to be 0.239 and 0.795 respectively. This method was found to be good percentage recovery for Daunorubicin and Cytarabine were found to be 100 and 101 respectively indicates that the proposed method is highly accurate. The specificity of the method shows good correlation between retention times of standard with the sample so, the method specifically determines the analyte in the sample without interference from excipients of tablet dosage forms. The method was extensively validated according to ICH guidelines for Linearity, Accuracy, Precision, Specificity and Robustness.
Keywords: Daunorubicin, Cytarabine, RP-HPLC, ICH-guidelines, Method development andValidatio

I. INTRODUCTION

Daunorubicin is also known as Daunomycin, is a chemotherapy medication used to treat cancer (Fig.1). Specifically, it is used for acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL), chronic myelogenous leukemia (CML), and Kaposi's sarcoma. It is used by injection into a vein. Cytarabine (cytosine arabinoside, 1-b-D-arabinofuranosyl cytosine, area-C) is a pyrimidine nucleoside analog which is predominantly used against acute myelogenous leukemia and non-Hodgkin's lymphoma (Fig. 2)

Daunorubicin and Cytarabine (I.V injection) is a liposomal combination of that is FDA approved for the treatment of adults with newly-diagnosed therapy-related acute myeloid leukemia (t- AML) or AML with myelodysplasia-related changes (AML-MRC)[1-3].

Daunorubicin interacts with DNA by intercalation and inhibition of macromolecular biosynthesis. This inhibits the progression of the enzyme topoisomerase II, which relaxes supercoils in DNA for transcription. Daunorubicin stabilizes the topoisomerase II complex after it has broken the DNA chain for replication, preventing the DNA double helix from being resealed and thereby stopping the process of replication. Cytarabine is a cell cycle phase-specific anti-neoplastic agent, affecting cells only during the S-phase of cell division. Cytarabine acts primarily through inhibition of DNA polymerase.

Literature review reveals estimation of Daunorubicin by RP-HPLC4 and Cytarabine by RP- HPLC [4-6] and by Spectroscopy method [7] individually. In combination, Dounorubicin and Cytarabine only one method was published [8,] but yet there is a need to develop new stability indicating RP-HPLC method with more sensitivity, accuracy and precision.
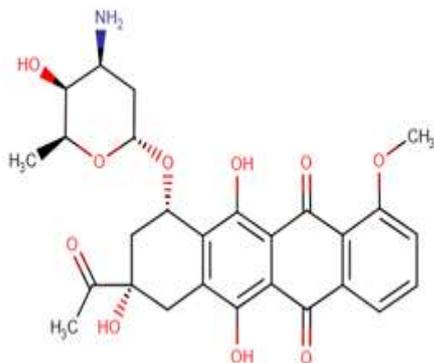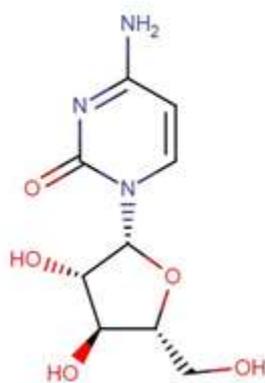
Fig: 1: structure of daunorubicin

Fig:2: Structure of cytarabine

II.   MATERIALS AND METHODS:

Chemicals and Reagents: Both Daunorubicin and Cytarabine (API) were obtained as a gift sample from Hetro Pvt. Ltd., Hyderabad, India. The marketed formulation in the brand name Vyoxeos (Dauno-44 mg & Cyta-100 mg) procured from the local pharmacy. All the chemicals and reagents used in this work were HPLC grade water, acetonitrile, methanol, potassium dihydrogen Phosphate ,Sodium dihydrogen phosphate was obtained from Merck.

Instrumentation: A HPLC system with waters 2695 separation module provided with an UV detector, autosampler injection with Empower-2 software. Electronic balance, ultrasonicator, hot air oven and pH meter were used.
Chromatographic Conditions: The chromatographic separations achieved on a Xbridge C18 column (150 × 4.6 mm, 5 µm particle size) as a stationary phase. The mobile phase was composed of 50:50 v/v of 0.1% Octane sulphonic acid pH 3 and

acetonitrile at a flow rate of 1.0 ml/min and injection volume is 20 µl. The column oven temperature was maintained at 30 ℃, and the drugs were detected at 238 nm.

Preparation of mobile phase:
Transfer 1000ml of HPLC water into 1000ml of beaker and Na_2HPO_4 adjust pH 4.6

Transfer the above solution 500ml Na2HPO4 of, 500ml of Methanol is used as mobile phase. They are mixed and sonicate for 20min

Diluent Preparation:
The Mobile phase was used as a diluent.

Mixed Standard Solution:
Accurately weighed quantity of 44mg Daunorubicin and 100mg Cytarabine was taken in a 100 ml volumetric flask and 50 ml of mobile phase was added. The mixture was subjected to sonicate for 20 min with intermediate shaking for complete extraction of drugs. Filtered and cooled to room temperature and solution was made up to mark with mobile phase. From the above solution 1 ml is taken and further diluted in 10 ml volumetric flasks with mobile phase. To acquire a concentration of 44mg Daunorubicin and 100mg Cytarabine.

Sample Solution:
10 tablets were weighed and crushed, from the powdered tablets, weighed accurately about 144mg (44mg Daunorubicin and 100mg Cytarabine) into a 100 ml volumetric flask and 50 ml of mobile phase was added. The mixture was subjected to sonication for 20 min with intermediate shaking for complete extraction of drugs. Filtered and cooled to room temperature and solution was made up to mark with mobile phase. From the above solution 1 ml is taken and further diluted in 10 ml volumetric flasks with mobile phase. To acquire a concentration of 44mg Daunorubicin and 100 mg Cytarabine.

Procedure:
10 □ l of the standard, sample were injected into the chromatographic system and measured the areas for Cytarabine and Daunorubicin peaks and calculate the % assay by using the formulae.

Validation of the RP-HPLC Method: The proposed RP-HPLC method was validated as per ICH guidelines [9-12].

System Suitability Parameters: The system suitability parameters were determined by preparing standard solutions of Daunorubicin (50 µg/mL) and Cytarabine (22 µg/mL), and the solutions were injected six times and the parameters like retention time, peak tailing, resolution and USP plate count were determined.

Specificity: As per ICH guidelines "Specificity" can be defined as the ability of the method to specifically separate the particular API or analyte in the presence of other components.

Linearity: The stock solution of Daunorubicin and Cytarabine was prepared by using diluent From that, various working standard solutions were prepared in the range of 22µg/ml to 110µg/ml, 50µg/ml to 250µg/ml and injected into the HPLC system. The calibration plot (peak area vs. concentration) was generated by replicate analysis (n=5) at all concentration levels. The linear relationship was evaluated using the least square method within Microsoft excel program.

Accuracy: The accuracy method was carried out using one set of different standard addition methods at different concentration levels 50%, 100% and 150% and then comparing the theoretical value and found value.

Precision: The precision of the method was ascertained from the peak area obtained by actual determination of six replicates of a fixed amount of the drug (50 µg/mL Daunorubicine, 22 µg/mL Cytarabine). The precision of the assay also determined in terms of intraday and interday variation in the peak area of a set of drug solutions on three different days. The peak area of a set of drug solutions was calculated in terms of relative standard deviation (RSD).

Detection Limit and Quantification Limit: Detection limit and quantification limit established based on the calibration curve parameters, according to the following formulas.
LOD = 3.3 SD/slope and LOQ = 10 SD/slopeor
Detection limit = $3.3\sigma/s$, Quantification limit = $10\sigma/s$,
Where σ is the standard deviation of Y-intercept of the regression line and S is the slope of the curve.

Robustness: The Robustness of the proposed method carried out by small but deliberate changes in method parameters such as flow rate (±0.1), column temperature (±5), mobile phase ratio (±5%). The percentage recovery and RSD of peak area were evaluated.

Forced Degradation Tests: The stability indicating of the method can be demonstrated by applying stress conditions [13,14] using acid, alkaline, peroxide, thermal, UV, water degradations. The sample was exposed to these conditions the main peak of the both drugs were studied for peak purity, that indicating the method effectively separated the degradation products from the pure active ingredient.

Acid Degradation Studies: To 1 ml of Daunorubicin and Cytarabine stock, 1 ml of 2N HCl was added and refluxed for 30 min at 60 ℃. The resultant solution was neutralized with 1 ml 2N NaOH and makeup to final volume to obtain (50 µg/mL and 22 µg/mL) solution. Cool the solution to room temperature and filtered with 0.22 µm membrane filter. A sample of 10 µl was injected into the HPLC system, and the chromatograms were recorded to assess the stability of the sample.

Alkali Degradation Studies: To 1 ml of stock solution of Daunorubicin and Cytarabine 1 ml of 2N sodium hydroxide was added and refluxed for 30 min at 60 ℃. The resultant solution was neutralized with 1 ml 2N HCl and makeup to final volume to obtain (50 µg/mL and 22 µg/mL) solution. Cool the solution to room temperature and filtered with 0.22 µm membrane filter. The sample of 10 µl was injected into the system, and the chromatograms were recorded to an assessment of sample stability.

Peroxide Degradation Studies: To 1 ml of stock solution of Daunorubicin and Cytarabine 1 ml of 20% hydrogen peroxide (H_2O_2) was added separately. The solution was kept for 30 min at 60℃.
For HPLC study, the resultant solution was diluted to obtain (50 µg/mL and 22 µg/mL) solution. Cool the solution to room temperature and filtered with 0.22 µm membrane filter. A sample of 10 µl solution was injected into the system, and the chromatograms were recorded to assess the stability of the sample.

Thermal Degradation Studies: The 1 ml of standard drug solution was placed in the oven at 105 ℃ for 6 h to study dry heat degradation. For HPLC study, the resultant solution was makeup to

final volume to obtain (50 μg/mL and 22 μg/mL) solution.

Cool the solution to room temperature and filtered through a 0.22 μm membrane filter. A sample of 10 μl solution was injected into the system, and the chromatograms were recorded for the assessment of sample stability.

Photo Degradation Studies: The photostability of the drug was studied by exposing the stock solution to UV light for 7 days or 200 Watt-hours/m^2 in photostability chamber. For HPLC study, the resultant solution was diluted to obtain (50 μg/mL and 22 μg/mL) solution and filtered with 0.22 μm membrane filter. A sample of 10 μl solution was injected into the system, and the chromatograms were recorded for the assessment of sample stability.

III. RESULTS AND DISCUSSION:

Method validation was performed according to ICH Q2 guidelines. In the blank chromatogram, there were no peaks observed at the retention times of Daunorubicine and Cytarabine.

System Suitability: System suitability was performed to evaluate the parameters like tailing factor, theoretical plates, resolution and % RSD for replicate injections. The results were within limits and were given in Table 1 and shown in Fig. 3.

Specificity: Retention times of Daunorubicin and Cytarabine were 4.144 min and 2.461 min for standard and 4.144 min and 2.461 min for sample respectively. Which were shown in Fig. 4, 5, 6. We did not find any interfering peaks in blank at retention times of these drugs in this method. So, this method was said to be specific.
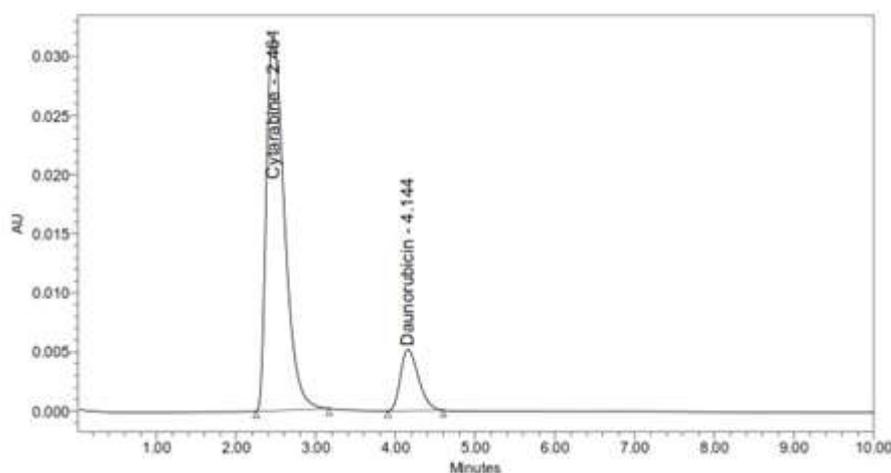

Figure 3: Chromatogram for system suitability

Table 1: Results of system suitability parameters

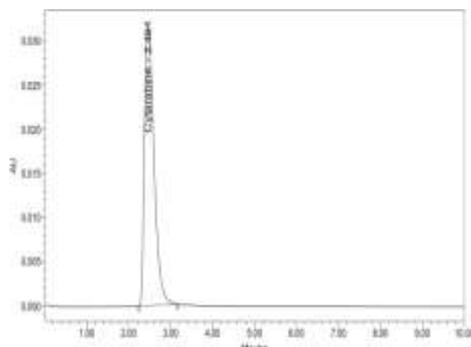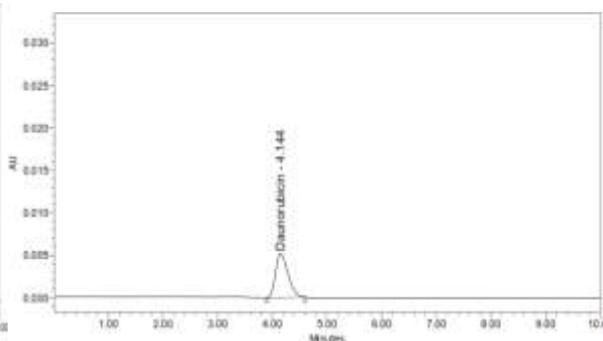| Parameter	Daunorubicin	Cytarabine	AcceptanceCriteria
Retention time	2.337	3.358	+-10
Theoretical plates	8358	6826	>2500
Tailing factor	1.54	1.41	<2.00
% RSD	0.2	0.3	<2.00

Figure 4: Chromatogram for Cytarabine   Figure 5: Chromatogram for Daunorubicin
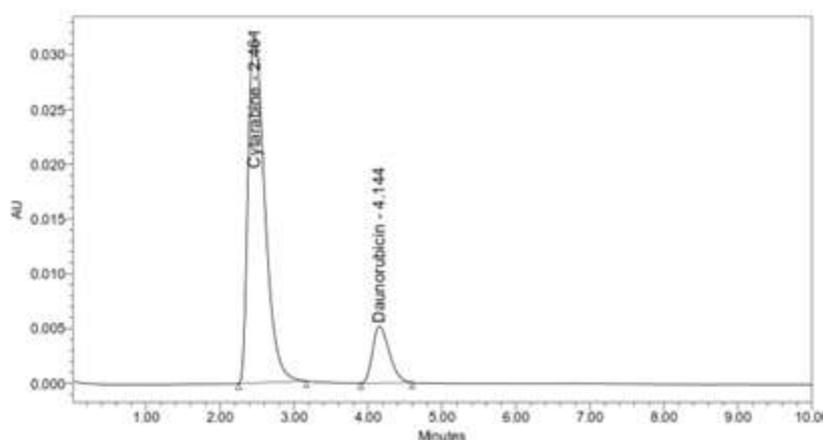

Figure 6: Chromatogram for Cytarabine and Daunorubicin

Linearity: The linearity of the measurement was evaluated by analyzing different concentrations (50% to 250%) of the standard solutions of Daunorubicin and Cytarabine. The calibration curve was constructed by plotting concentration against mean peak area, and the regression equation was computed. The coefficient of correlation (R^2) for Daunorubicin and Cytarabine were 0.999. The summary of the parameters is given in Table 2 and shown in Fig. 7, 8.

Table 2: Area of different concentration of Cytarabine and Daunorubicin

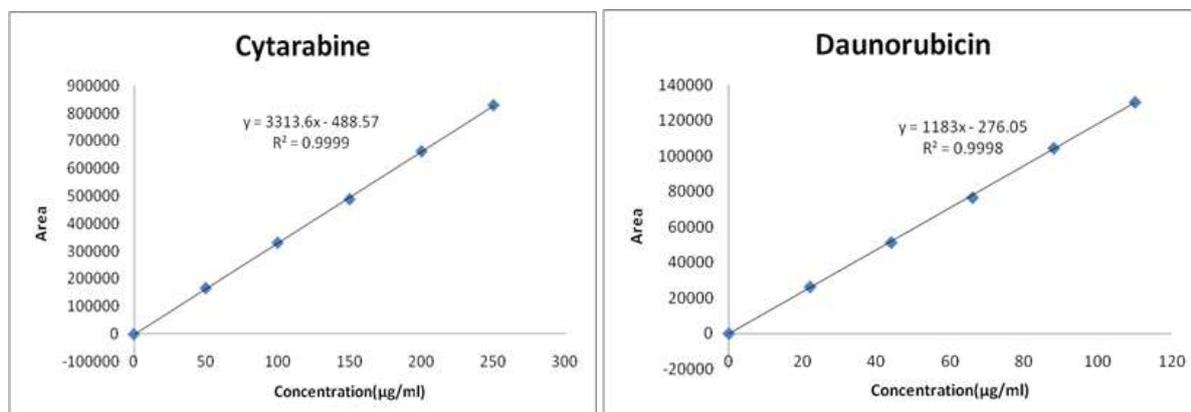| S. No.	Cytarabine		Daunorubicin	
	Concentration (µg/ml)	Area	Concentration (µg/ml)	Area
1	50	570005	50	117168
2	75	857388	75	176713
3	100	1129222	100	236953
4	125	1412940	125	295699
5	150	1696240	150	355161

Figure 7: Calibration graph for Cytarabine Figure 8: Calibration graph for Daunorubicin

Accuracy: To determine the accuracy of the proposed method, recovery studies were conducted at three different levels, 50, 100 and 150% and were calculated. Accuracy was calculated as the percentage of recovery, and the results were shown in Table 3,4.

Table 3: Accuracy (recovery) data for Cytarabine

%Concentration (at specification Level)	Area	Concentration of pre analyzedsample (µg)	Amount Added (µg)	Amount Found (µg)	% Recovery	Mean Recovery
50%	248273.7	100	50	50.13	100.25	100.42
100%	497256.3	100	100	100.40	100.40	
150%	747586.3	100	150	150.94	100.63	

Table 4: Accuracy (recovery) data for Daunorubicin

% Concentration (at specification Level)	Concentration of pre analysed sample (µg)	Area	AmountAdded (µg)	AmountFound (µg)	% Recovery	Mean Recovery
50%	44	72.00	49.000	49.70	101	101
100%	44	144.00	98.000	97.81	100	
150%	44	216.00	147.000	148.33	101	

Precision: Precision was carried out in terms of system precision, repeatability, and intermediate accuracy. These are assessed by using six replicates at a concentration of 50 µg/mL of Daunorubicin and 22 µg/mL of Cytarabine. The data was given in Table 5& 6. The % RSD was found to be <2, indicating the repeatability of the method.

Table 5: Results of Precision for Cytarabine & Daunorubicin

Injection	Peak Area for Cytarabine	Peak Area for Daunorubicin
Injection-1	1133202	236238
Injection-2	1122993	236467
Injection-3	1131651	237514
Injection-4	1128831	237564
Injection-5	1123120	236994
Injection-6	1129733	236535
Average	98	99
Standard Deviation	0.37	0.24
%RSD	0.38	0.24

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LOD and LOQ: Estimation of the limit of detection (LOD) and limit of quantification (LOQ) considered the acceptable signal-to-noise ratios 3: 1 and 10: 1, respectively. LOD and LOQ of Daunorubicin and Cytarabine were determined 2.465 µg/mL, 4.156 µg/mL and 2.461 µg/mL, 4.144 µg/mL respectively. Which were given in Table 7, 8 and shown in Fig. 9 and 10.

Table 7: Results of LOD

S. No	Sample name	RT	Area
1	Daunorubicin	2.294	762
2	Cytarabine	3.306	3839

Table 8: Results of LOQ

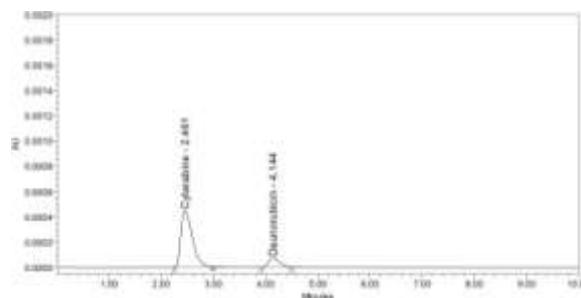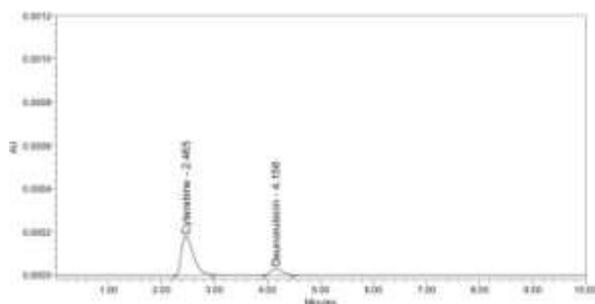| S.no	Sample name	RT	Area
1	Daunorubicin	2.294	3994
2	Cytarabine	3.310	15826

Figure 9: Chromatogram showing LOD **Figure 10: Chromatogram of showing LOQ**

Degradation Studies:

Since no interference of blank and degradants, the HPLC results showed that the three active ingredients Daunorubicin and Cytarabine purity angle was less than the purity threshold and hence the proposed method was the specific and revealed its stability-indicating power. The results were summarized in Table 14.

The drug Daunorubicin and Cytarabine were found to be more degraded when exposed to peroxide and acidic conditions and least degraded when exposed to necessary, thermal and photolysis degradation.

Table 14: Results for Stability of Cytarabine and Daunorubicin

Condition	Percent assay		Percent degradation	
	Daunorubicin	Cytarabine	Daunorubicin	Cytarabine
0.1 N HCl	90.33	88.68	9.67	11.32
0.1N NaOH	91.89	92.58	8.11	7.42
30% H2O2	94.31	95.71	5.69	4.29
105°C	89.31	90.45	10.69	9.55
Sunlight	92.30	93.51	7.70	6.49
Water	99.15	98.78	0.85	1.22

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