Original Article

UPLC Method Development And Validation For The Simultaneous Estimation Of Cytarabine And Daunorubicin In Pharmaceutical Dosage Form

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Abstract

Objective: A modest, specific and reliable method UPLC method was developed for the simultaneous estimation of Cytarabine and Daunorubicin in bulk and Pharmaceutical dosage form.
Method: An ultra-performance liquid chromatographic technique has been developed to separate Cytarabine and Daunorubicin. This can be achieved by Leapsil C 18 column (Dikma) (4.6 x 50 mm, 2.7µm) using mobile phase of 0.05M Phosphate Buffer pH 3: Methanol: Acetonitrile; 40:30:30 v/v. The detector wavelength was set at 240nm. The flow rate was maintained at 0.4 ml/min, with 4 µL injection volume.
Results: The optimum concentrations of Cytarabine and Daunorubicin were found to be 37.5µg/mL and 16.5µg/mL. The mean retention time was found to be 0.842 and 0.492 min correspondingly. Linearity studies were carried out and the linear response (r2 = 0.999) was observed in the range of 12.5 - 62.5 µg/mL for Cytarabine and 5.5 – 27.5 µg/mL for Daunorubicin. The % RSD values of method precision were found to be 0.10163 and 0.10168 respectively. The accuracy of the method was performed by recovery studies. The percentage recovery was found to be in the range of 99.95–100.466 % for Cytarabine and 99.89 - 100.206 % for Daunorubicin respectively.
Conclusion: The proposed method was validated in terms of Linearity, Range, Precision, Accuracy, Specificity, LOD, LOQ and Robustness. The method was successfully applied to the estimation of Cytarabine and Daunorubicin Pharmaceutical dosage form.

Keywords: UPLC, ICH guidelines, Validation, Cytarabine and Daunorubicin.

1. INTRODUCTION

Cytarabine and Daunorubicin is a novel fixed-dose combination medication with multiple actions used in treatment of several forms of leukemia. It also has antiviral and immunosuppressant properties [1].
Cytarabine [Fig.1 (a)] is a pyrimidine nucleoside analog used mainly in the treatment of leukemia, especially acute non-lymphoblastic leukemia, acute myelogenous leukemia, and meningeal leukemia. Cytarabine is chemically 4-amino-1-[(2R,3S,4S,5R)-3, 4-dihydroxy-5-(hydroxymethyl) oxolan- 2-yl] pyrimidin-2-one (Fig.1). It has moderate emetogenicity which has been managed with antiemetic drugs [2].
Daunorubicin [Fig.1 (b)] is an anthracycline antibiotic that has antineoplastic activity and is used for the treatment of acute leukemia and acquired immune deficiency syndrome related Kaposi sarcoma. Chemically it is (1S,3S)-3-Acetyl-3 5,12-trihydroxy-10-methoxy-6,11-dioxo-1,2,3,4,6,11-hexahydro-1-tetracenyl3-amino-2,3,6-trideoxy-α-L-lyxo hexopyranoside. It is one among six Topo II inhibitors is prescribed as highly antineoplastic drug in clinical use [3].
Along with these activities, a well documented literature reports that there are few analytical methods like HPLC [4, 6], LC-MS/MS [7, 8] are available for quantitative estimation and therapeutic effectiveness of Cytarabine and Daunorubicin in bulk as well as formulation. To the best of our knowledge, there was no testified simultaneous estimation of Cytarabine and Daunorubicin by UPLC. Application of UPLC technology has been proposed to get out of the drawbacks like reducing the time of analysis and accordingly decreasing the environmental impact by reducing solvent consumption.

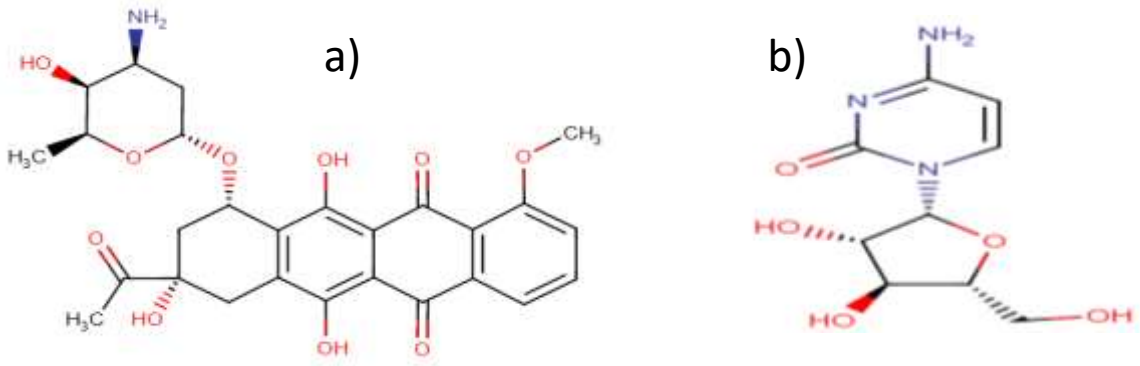

Fig 1. Chemical structure of Cytarabine (a) and Daunorubicin (b)

2. MATERIAL AND METHOD:

2.1 Materials:

The Cytarabine and Daunorubicin reference standards with purity greater than 98% were gratis from Inception Source, Hyderabad, India. The reagents and solvents used (Acetonitrile, Methanol, potassium dihydrogen orthophasphate, Orthophosphoric acid were of AR grade obtained from Merck Chemicals, Mumbai, India. An ultrasonic device, a sensitive balance, Sartorius analytic balance and a pH meter, glass electrode, were used for the preparation of solutions.

2.2. Chromatographic conditions:

Waters Acquity UPLC system (Waters, Milford, MA, USA) equipped with a quaternary gradient pump, auto sampler, column oven, and photodiode array detector and empower 2 software connected with a Leapsil C18 (Dikma) (4.6 x 50 mm, 2.7 µm)

2.3 Mobile phase:

Mix a mixture of 400 ml phosphate buffer (40%) and 300ml of Methanol (30%) and 300 ml of Acetonitrile (30%) degas in ultrasonic water bath for 5 minutes. Filter through 0.45 µ filter under vacuum filtration. The detector wavelength was set at 240 nm. The flow rate is maintained at 0.4 ml/min, at an ambient column temperature with 4 µL injection volume.

2.4 Preparation of stock solution and standard solution

Accurately weigh and transfer 50 & 22mg of Cytarabine & Daunorubicin working standards into a 100 mL clean dry volumetric flask add Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Pipette 0.75 ml of Cytarabine & Daunorubicin from the stock solution and transferred in to a 10ml volumetric flask and dilute up to the mark with diluent.

2.5 Preparation of sample Solution:

Accurately weigh and transfer the lyophilized powder equivalent to 50 & 22 mg (162mg) of Cytarabine & Daunorubicin sample into a 100 ml clean dry volumetric flask add about 7mL of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further dilute 0.75 ml of Cytarabine & Daunorubicin solution into a 10ml volumetric flask.

3. RESULTS AND DISCUSSION:

3.1. Method development and Optimized Chromatographic Conditions

Cytarabine and Daunorubicin is a novel combination with multiple actions used in treatment of several forms of leukemia. It also has antiviral and immunosuppressant properties. To achieve good separation among the two components, different buffer pH conditions and different proportions of mobile phase and different column are used [9, 10].

A RP-UPLC method was developed and validated for the quantitative determination of Cytarabine and Daunorubicin in powder for injection dosage form. The optimum concentrations of Cytarabine and Daunorubicin were found to be 37.5µg/mL & 16.5µg/m. The chromatographic separation was achieved by Leapsil C 18(Dikma) (4.6 x 50 mm, 2.7 µm) using 0.05M Phosphate Buffer pH 3: Methanol: Acetonitrile in the ratio of 40:30:30 v/v selected as the mobile phase and the pH was adjusted by adding diluted orthophosphoric acid up to 3.0. A sharp and symmetrical peak with a retention value of 0.492 and 0.842 for Daunorubicin and Cytarabine at 240nm. The flow rate was maintained all over analysis at 0.4 ml/min at ambient column temperature with the injection volume was 4 µL (Fig. 2, 3). The system suitability results were shown in Table 1.

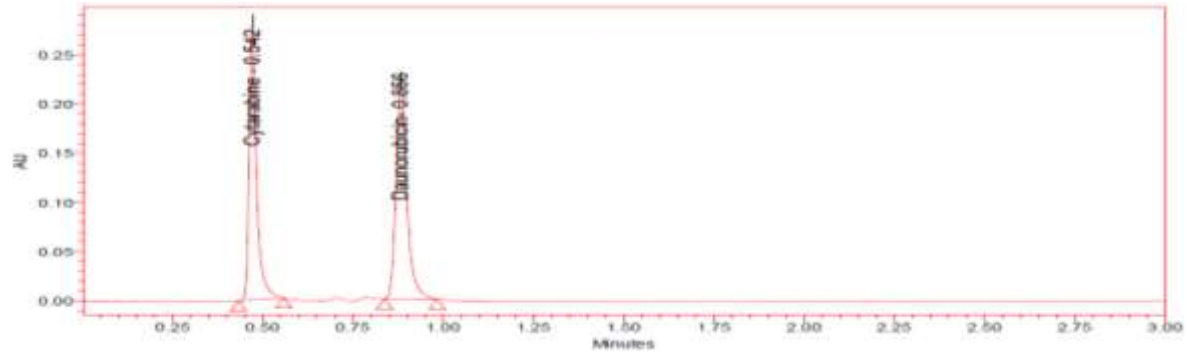

Fig 2. Chromatograms of Cytarabine and Daunorubicin standard solution

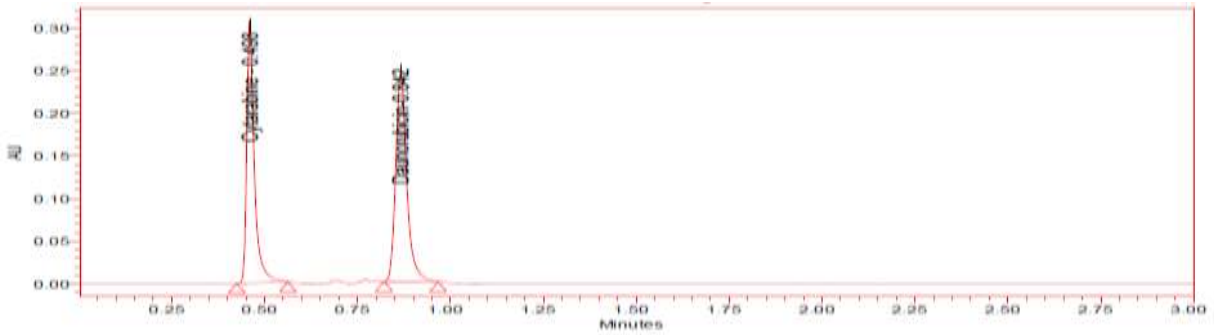

Fig 3. Chromatograms of Cytarabine and Daunorubicin sample solution

Table 1: System Suitability data of Cytarabine & Daunorubicin

S. No	Cytarabine		Daunorubicin	
	RT	Peak Area	RT	Peak Area
1	0.462	488408	0.864	215103
2	0.494	485617	0.884	216003
3	0.486	482556	0.864	210386
4	0.476	487966	0.882	216088
5	0.502	480038	0.874	219300
6	0.504	489801	0.884	215551
Average		485731		215405
SD		3774.2		2874.4
%RSD		0.8		1.3

4. METHOD VALIDATION

The results of the analysis were validated statistically as per the ICH guidelines in terms of its linearity, method precision, accuracy, specificity, LOD and limit LOQ and robustness [11-16].

4.1. Linearity (Calibration Curve)

The linearity was assessed by measuring dissimilar aliquots of 12.5, 25, 37.5, 50, and 62.5 µg/ml of Cytarabine and 5.5, 11, 16.5, 22 and 27.5 µg/ml of Daunorubicin (Fig. 4) from standard stock solutions. The calibration curve was linear with an average correlation coefficient of r^2 0.999 (Fig. 5& 6). The summary of the results shown in Table 2.

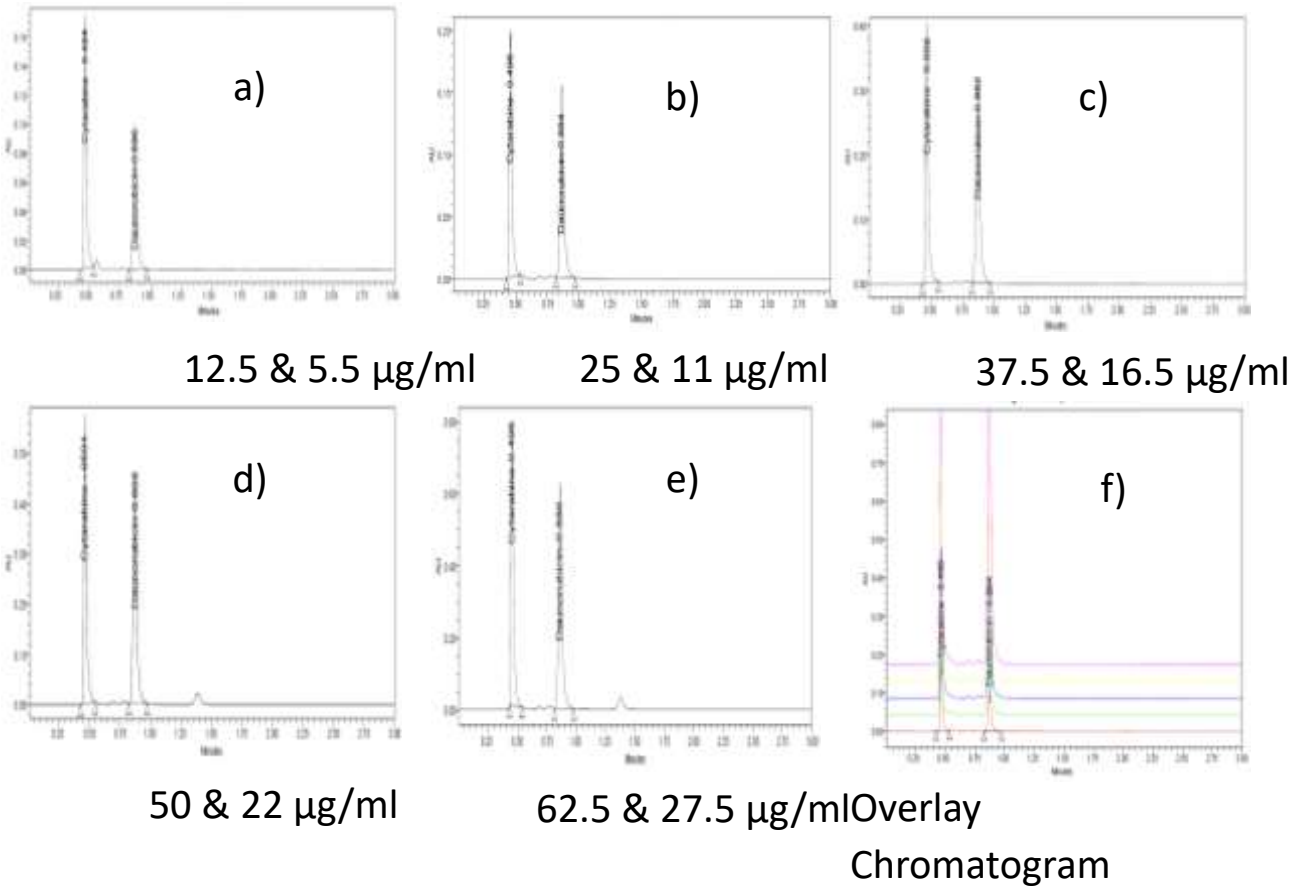

Fig 4. Linearity chromatograms of Cytarabine and Daunorubicin; 12.5 & 5.5 µg/ml (a), 25 & 11 µg/ml (b), 37.5 & l6.5 µg/ml (c), 50 & 22 µg/ml (d), 62.5 & 27.5 µg/ml (e) Overlay Chromatogram (f).

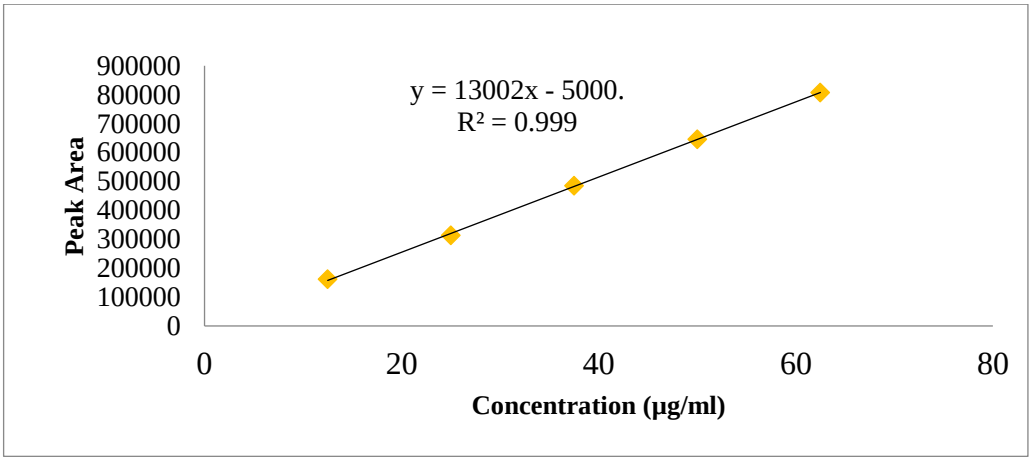

Fig 5. Cytarabine Calibration curve

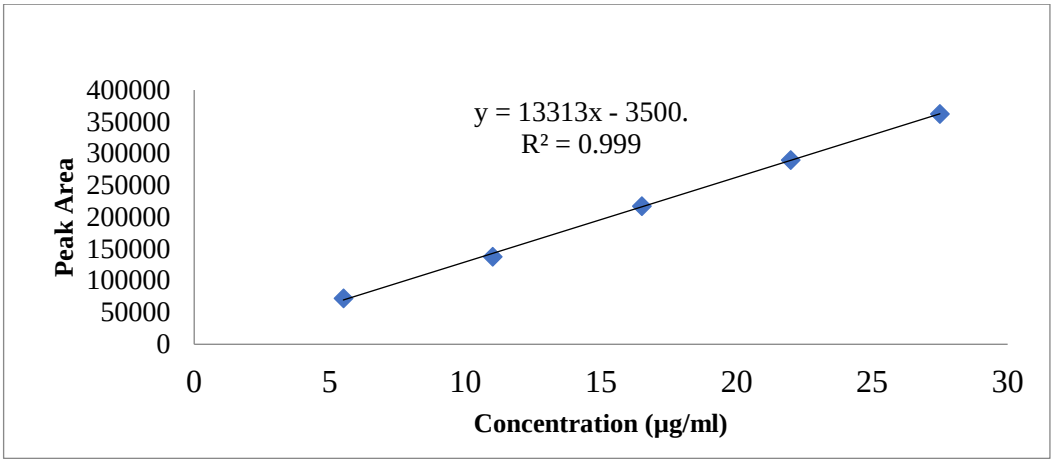

Fig 6. Daunorubicin Calibration curve

Table 2: Linearity data of Cytarabine & Daunorubicin

Cytarabine		Daunorubicin	
Concentration (µg/ml)	**Peak Area**	**Concentration (µg/ml)**	**Peak Area**
12.5	161521	5.5	72522
25.0	313042	11.0	138044
37.5	484564	16.5	217567
50.0	646085	22.0	290089
62.5	807606	27.5	362611

4.2. Precision

The precision was evaluated at three levels, repeatability, reproducibility and intermediate precision. Each level of precision was investigated by six replicate injections of concentrations 37.5µg/mL & 16.5µg/mL Cytarabine & Daunorubicin (Fig.7) respectively. The result of precision was expressed as % of RSD and was tabulated in Table 3 and 4.

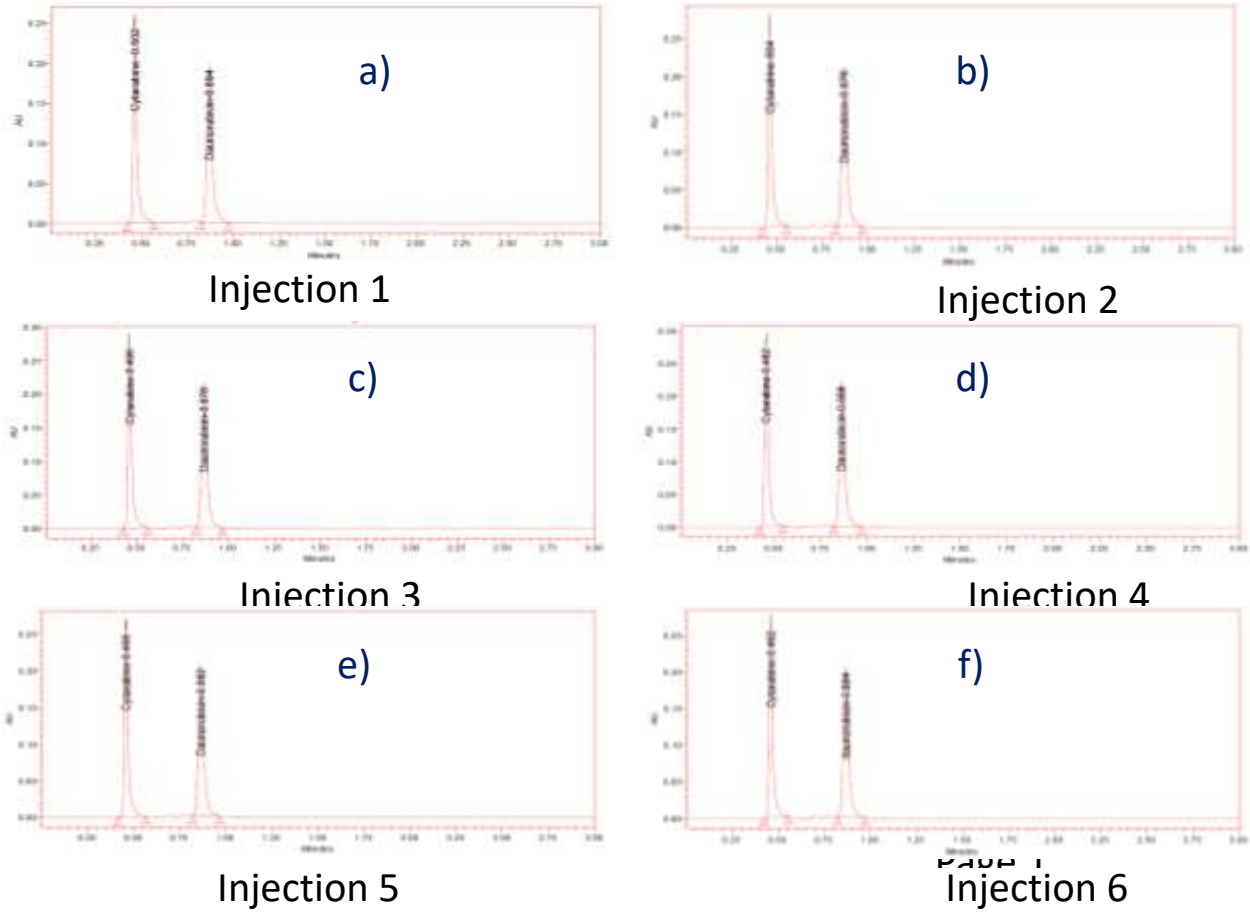

Fig 7. Method Precision chromatograms of Cytarabine and Daunorubicin; Injection 1-6 (a -f).

Table 3: Method precision data of Cytarabine & Daunorubicin

	Cytarabine			Daunorubicin		
S. No	**RT**	**Peak Area**	**% Assay**	**RT**	**Peak Area**	**% Assay**
1	0.498	480038	100.01	0.864	215103	99.99
2	0.496	485617	100.14	0.884	219300	100.19
3	0.494	488408	100.20	0.864	216003	100.05
4	0.502	489801	100.23	0.882	216088	100.07
5	0.498	487966	100.07	0.874	210386	99.88
6	0.496	482556	99.98	0.884	215551	100.02
Average			100.105			100.033
SD			0.10173			0.10171
%RSD			0.10163			0.10168

Table 4: Intermediate precision data of Cytarabine & Daunorubicin

	Cytarabine			Daunorubicin		
S. No	**RT**	**Peak Area**	**% Assay**	**RT**	**Peak Area**	**% Assay**
1	0.492	481038	99.99	0.864	215103	99.99
2	0.496	483617	100.11	0.894	219300	100.37
3	0.494	481408	100.08	0.874	216003	100.06
4	0.502	489801	100.13	0.882	216088	100.07
5	0.498	484966	100.04	0.867	210386	99.92
6	0.496	482556	100.01	0.884	215551	100.02
Average			100.082			100.071
SD			0.05585			0.15587
%RSD			0.05582			0.15578

4.3. Accuracy

The accuracy of the method was performed by recovery studies. Acknowledged amount of pure drug concentrations were spiked in placebo at three different levels, i.e., 50%, 100% and 150% was calculated (Fig. 8). Results of recovery data were shown in Table 5 and 6.

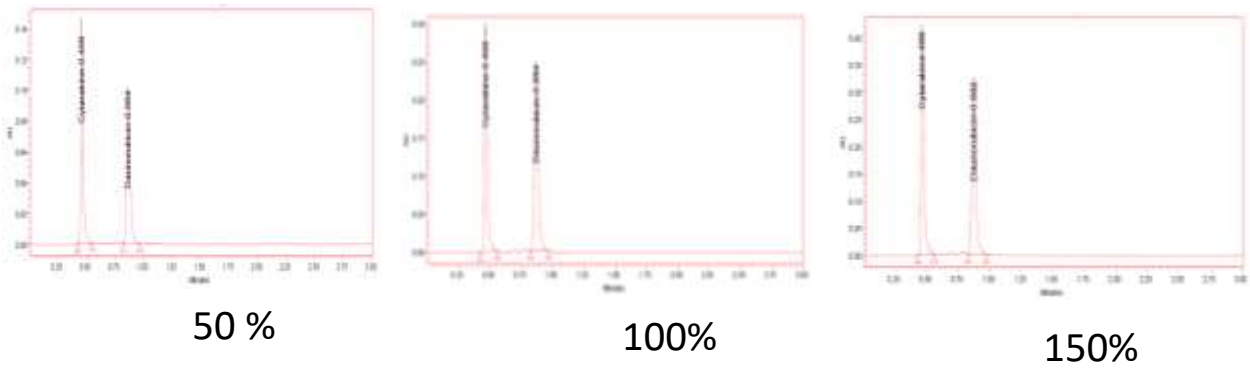

Fig 8. Accuracy chromatograms of Cytarabine and Daunorubicin; 50% (a), 100% (b), 150% (c).

Table 5: Accuracy data of Cytarabine

Sample No.	Spiked Level	Sample Area	µg/ml added	µg/ml found	% Recovery	% Mean Recovery
1	50%	243576	25.07	25.09	100.08	100.466
2		244447	25.03	25.34	101.23	
3		240458	25.01	25.03	100.07	
1	100%	483010	50.03	50.01	99.98	100.033
2		480082	50.05	50.02	99.94	
3		485883	50.13	50.22	100.18	
1	150%	720586	75.03	74.96	99.90	99.95
2		727596	75.10	75.04	100.01	
3		724823	75.08	75.04	99.94	

Table 6: Accuracy data of Daunorubicin

Sample No.	Spiked Level	Sample Area	µg/ml added	µg/ml found	% Recovery	% Mean Recovery
1	50%	110245	11.07	11.09	100.18	100.206
2		109783	11.03	11.09	100.54	
3		109630	11.01	11.00	99.90	
1	100%	211009	22.03	22.05	100.09	99.89
2		212674	22.05	22.02	100.04	
3		214032	22.13	22.22	99.62	
1	150%	332737	33.03	32.96	99.78	100.023
2		335561	33.10	33.14	100.12	
3		337204	33.08	33.19	100.33	

4.4. Specificity

In the placebo chromatogram there were no peaks observed at the retention times of Cytarabine & Daunorubicin indicate the method is specific (Fig. 9).

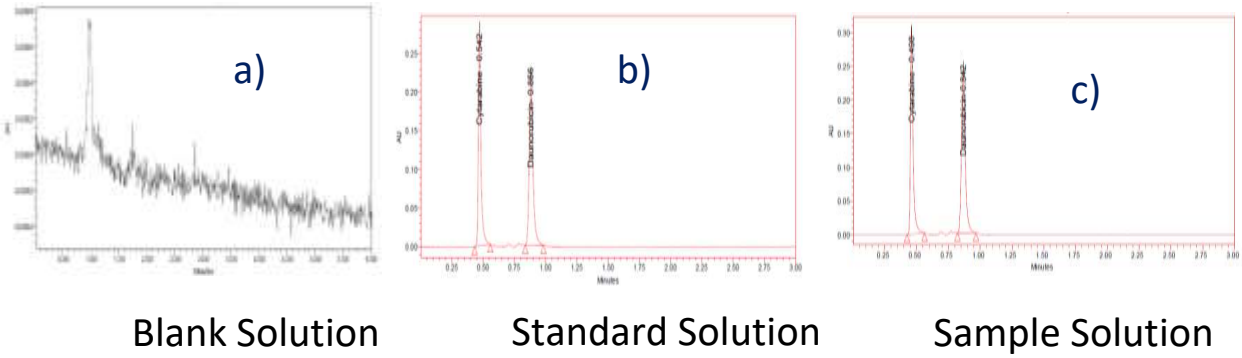

Fig 9. Specificity Chromatograms of Blank solution (a), Standard solution (b), Sample solution (c).

4.5. Limit of Detection (LOD) and Limit of Quantitation (LOQ)

Estimation of LOD and LOQ considered the acceptable signal-to noise ratios 3:1 and 10:1 respectively. The limit of detection and quantitation to be determined. The LOQ's were found to be 9.983 for and 9.965 for Daunorubicin and Cytarabine respectively (Fig. 10, 11).

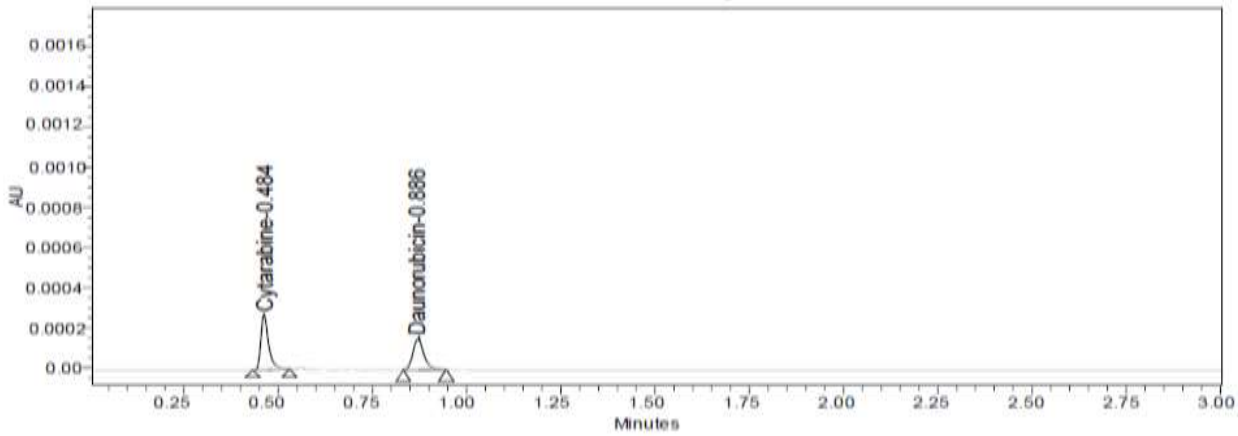

Fig 10. LOD chromatogram of Cytarabine and Daunorubicin

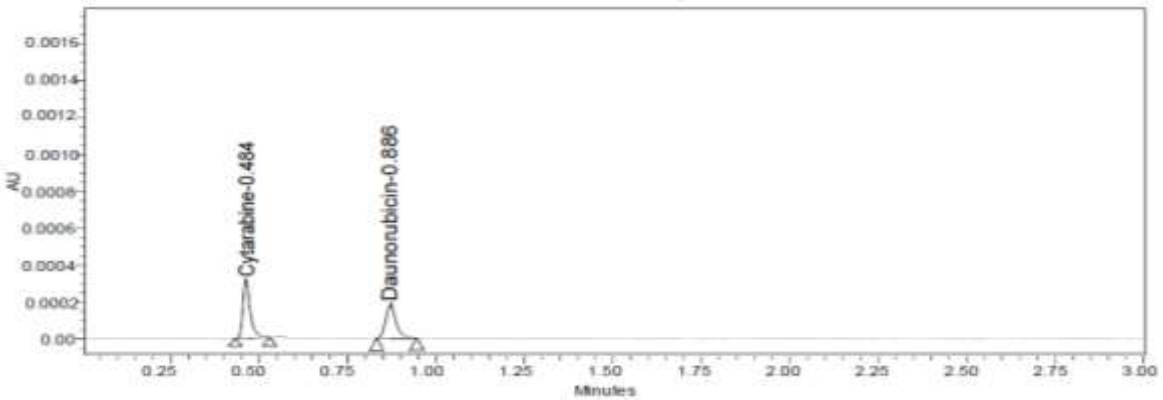

Fig 11. LOQ chromatogram of Cytarabine and Daunorubicin

The LOD's were found to be 2.983 and 2.965 for Cytarabine and Daunorubicin. The LOQ's were found to be 9.965 and 9.983 for Cytarabine and Daunorubicin respectively.

4.6. Robustness

The robustness of the method was genuine when small, deliberate changes like flow rate, mobile phase composition were performed at 100% test concentration (Fig. 12). Results are shown in Table 7. It can be concluded that the variation in flow rate and mobile phase not affected the method significantly.

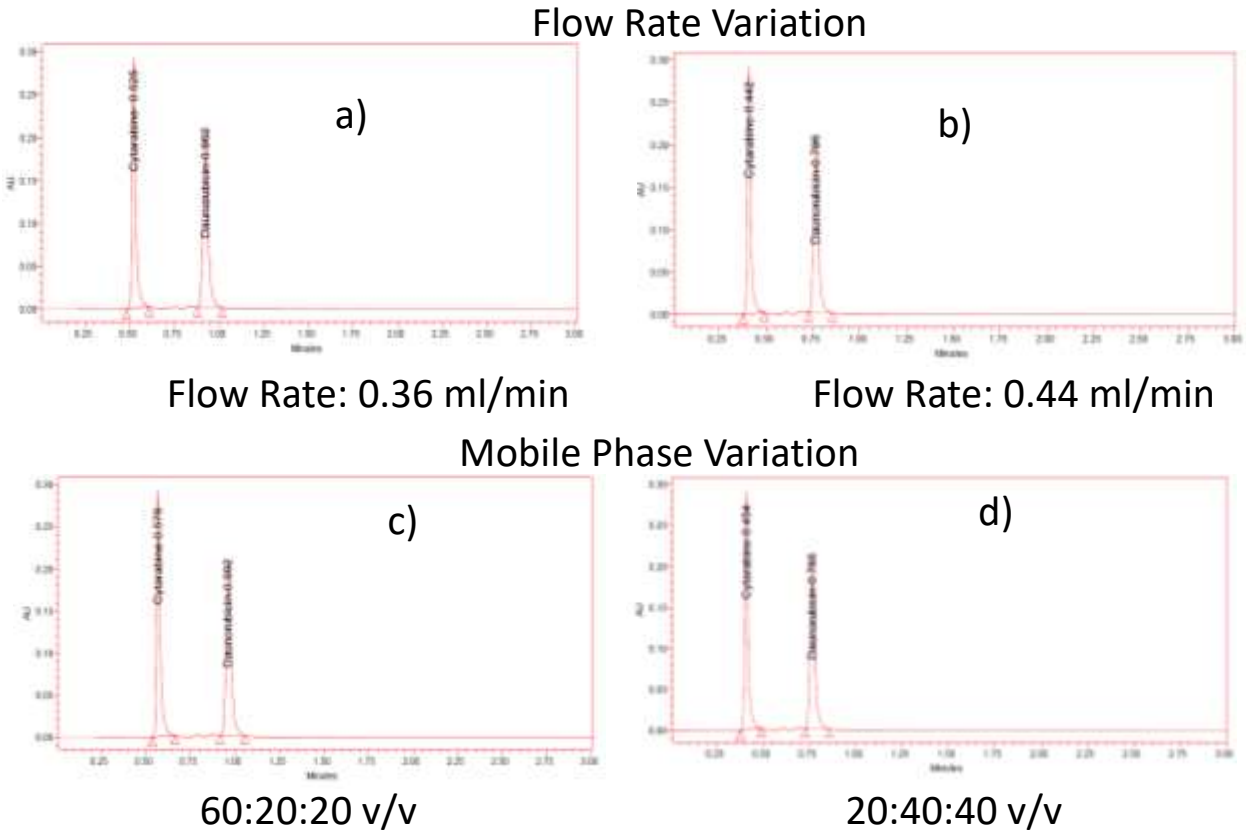

Fig 12. Robustness chromatograms of Flow rate variation and Mobile phase variation;
Flow rate- 0.36ml/min (a), Flow rate 0.44ml/min (b), Mobile phase variation- 60:20:20 v/v (c), Mobile phase variation- 20:40:40 v/v (d).

Table 7: Robustness data Cytarabine & Daunorubicin

S. No	Parameter	Cytarabine		Daunorubicin	
		Change	USP Plate Count	Change	USP Plate Count
1	Flow Rate (ml/min)	0.36	9564	0.36	7478
2		0.40	9732	0.40	7496
3		0.44	9678	0.44	7423
4	Mobile phase composition	60:20:20	9647	60:20:20	7459
5		40:30:30	9789	40:30:30	7469
6		20:40:40	9697	20:40:40	7421

The proposed method met the accepted criteria's and hence it was successfully applied to a routine analysis of bulk and powder for injection dosage form. Analytical performance summary data was shown in Table 8.

Table 8: Analytical performance summary data of Cytarabine and Daunorubicin

Validation Parameter	Results		Acceptance criteria
	Cytarabine	Daunorubicin	
Accuracy (%Recovery) (n=9)	Mean Recovery 100.15 %	Mean Recovery 100.04 %	Mean assay – 98% - 102% % RSD should be < 2
Precision (n=6)	Mean assay – 100.105 % % RSD – 0.10163	Mean assay – 100.033% % RSD – 0.10168	
Linearity	y = 13002x - 5000. R^2 = 0.999	y = 13313x - 3500. R^2 = 0.999	R^2 = 0.999
LOD - S / N Ratio	2.983	2.965	3

LOQ - S / N Ratio	9.965	9.983	10

5. CONCLUSION

A modest, specific and reliable method UPLC method was developed for the simultaneous estimation of Cytarabine and Daunorubicin in bulk and Pharmaceutical dosage form. The projected method was successfully separated the Cytarabine and Daunorubicin compounds with proper resolution, able to quantify Cytarabine and Daunorubicin with precision, accuracy. The results demonstrated a highly sensitive, specific and robust method, without any interference from the matrices. Hence the technologically advanced method can be adapted to regular quality control analysis.

6. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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