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A Novel RP-UPLC Technique for Quantification of Daunorubicin and Cytarabine Simultaneous in Combined Tablet Dosage Forms - Its Application in Monitoring Forced Degradation Studies

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Abstract

To establish and evaluate new reverse-phase ultra-performance liquid chromatography (RP-UPLC) technique to measure daunorubicin and cytarabine at the same time in combined tablet forms and to use this technique to monitor forced degradation studies. Using standardized testing, the authors identified a technique, reverse-phase ultraperformance liquid chromatography (RP-UPLC), on a piriform C18 column, 50mmx2.1mm OD core particle sizes, 1.7 micron. This approach used a changing mix of acetonitrile and a phosphate buffer with a pH level of 3.0. The liquid moved through the system at a rate of 0.3mL per minute, and UVlight detected substances at a wavelength of 254 nm. For the robustness of the method to be ensured, the scientific (research) community has used the ICH guidelines, has estimated the following functionalities, i.e., repeatability, reproducibility, accuracy, robustness and reliability. Moreover, the investigators also proved the protocol's versatility in various stress environments (acidic and basic stress, oxidative stress and thermal stress, light exposure and radiation). The method exhibited excellent linearity for both daunorubicin and cytarabine of concentration of 0.5–50µg/mL (R^2 > 0.999). Forced degradation studies demonstrated efficient separation of the analytes and degradation productsof the corresponding analytes, confirming the stability-indicating capability of this technique. Validation results indicated high precision, accuracy, and robustness, making the method suitable for routine quality control.Thus, this newly established RP-UPLC techniqueis an efficient and reliable procedure for the quantification of both the drugs at the same timein combined formulation.It is also a strong stability-indicating method forforced degradation studies ensuring the quality and purity of the pharmaceutical formulation.

Keywords: RP-UPLC, Daunorubicin, Cytarabine, Simultaneous quantification, Forced degradation, Stability-indicating method.

Introduction

Daunorubicin and cytarabineare commonly used together for the treatment of acute myeloid leukaemia (AML) based on their synergistic therapeutic properties (1). Precise simultaneous determination of these drugs in combined formulations is important to assure dosing integrity and product purity. Ultra-performance liquid chromatography (UPLC) provides good resolution, fast analysis, and high sensitivity and is thus highly suitable for such determinations (2). Furthermore, forced degradation studies are required to evaluate

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the stability and degradation mechanism of these drugs, requiring a stability-indicating technique that is robust (3).

This research deals with the establishment and authentication of a new RP-UPLC method for quantitation of Daunorubicin and Cytarabine at the same time and its use in forced degradation studies for day-to-day quality control and stability evaluation (4).Daunorubicin was accepted by the FDA for treatment in the USAin 1979.It is mainly used for treating Kaposi's sarcoma, acute myeloid and lymphocyticleukaemia, and chronic myelogenous leukaemia. Daunorubicin is an anthracycline drug (5-8). In this manner, Daunorubicin interferes with the catalytic cycle of topoisomerase II by steadying the complex of DNA-topo II and indirectly through intercalation between DNA base pairs.Cytarabine is used in the treatment of majority of the leukaemia. Deoxycytidine kinase catalyses the transformation of the pyrimidine nucleoside cytarabine synthesized into its active form of cytarabine triphosphate which inhibits DNA synthesis by competing with deoxycytidine triphosphate for DNA polymerase (9,10).

Literature review revealed that there were numerous techniques for determining Daunorubicin and Cytarabine on their own, as well as certain techniques used in conjunction with other medications (Figures 1 and 2). Only a few number of HPLC methods, meanwhile, are documented for the measurement of Daunorubicin and Cytarabine in combination dose forms at the same time (11-15). An attempt was made in this study to create a UPLC approach for the parallel assessment of both the drugs.

Materials and Methods

Instrumentation

The chromatographic system consists of an SPD-20A photo diode array detector, LC-20AD pumps, and a Waters Acquity H-Class UPLC (Model 2695) chromatograph fitted using an SB C18 100x 2.1mm; 1.8m column. With a 2µL loop and a Rheodyne 7725 injector valve, samples were

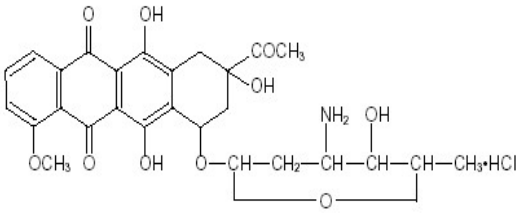

Figure 1: Structure of Daunorubicin

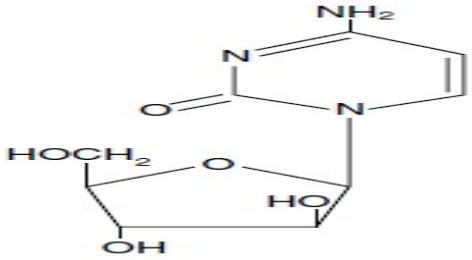

Figure 2: Structure of Cytarabine

injected into the system. Empower-2 software integrated and monitored the output signal. Sonication on an ultrasonicator (PCI Analytics PCI81) enhanced the compound's solubility. In trials, a Sartorius balance (model CPA225D) was used for all weighing. Merck Millipore provided the PVDF membrane filters that were utilized for the filtration.

Drugs and chemicals

Cytarabine and Daunorubicin reference standard samples were obtained from Suven Life Sciences Ltd. Potassium dihydrogen orthophosphate sodium hydroxide and hydrogen peroxide were of GR quality (Merck Ltd. Mumbai, India), while acetonitrile and orthophosphoric acid were of HPLC grade. Throughout the whole analysis, Milli-Q water was used.

Methodology

Preparation of diluted Orthophosphoric acid

1 mL of Orthophosphoric acid was transferred in to 100 mL flask and volume was makeup with water and mixed well.

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Preparation of pH 4.0 buffer

Correctly weighed 1.36 g of Potassium dihydrogen orthophosphate and added it to 1000 mL volumetric flask. Add approximately 900 mL of Milli-Q water, degassed using sonicator, and then brought up to the mark with water. Finally, adjust the pH to 4.0 using a diluted orthophosphoric acid solution.

Preparation of the mobile phase

To degas, acetonitrile and pH 4.0 buffer were combined in a ratio of 60:40 v/v and put it in sonicator for degassing.

Preparation of diluent

Water was combined with acetonitrile in the ratio of 50:50 v/v and used as diluent for preparing drug solutions.

Preparation of the mixed working standard solution of Daunorubicin and Cytarabine

22 mg of Daunorubicin and 50mg of Cytarabine working Standards was accurately weighed and transferred into a 50mL volumetric flask, 3/4th volume of diluents was added, sonicated for 5 mins and made up to the final volume with diluents. (440 ppm of Daunorubicin and 1000 ppm of Cytarabine).

1 mL from the above two stock solutions were taken in a 10mL volumetric flask and made up to 10 mL (44ppm of Daunorubicin and 100ppm of Cytarabine).

Assay of daunorubicin and cytarabine from tablet dosage forms

Ten Jazz Pharmaceuticals "VyxeosTM" tablets were weighed and coarsely pulverized. A 100 mL volumetric flask was filled with powder equal to one tablet's weight. Fifty milliliters of diluent were then added, and the mixture was sonicated for half an hour. Following diluent adjustment, the volume was filtered. After removing the first few milliliters of filtrate, a fraction of the solution was run via a 0.22µm membrane filter. A solution comprising 44 µg/mL of Daunorubicin and 100 µg/mL of Cytarabine was obtained by transferring 5 mL of the filtered solution into a 50 mL volumetric flask and diluting it with diluent to 50 mL. This solution (2 µL) was chromatographed 6 times. The mean peak areas of both drugs were estimated, and their constituents in the formulation was determined. Typical chromatogram from the analysis of 'Vyxeos™' tablets are shown in Figure 3.

Results and Discussion

Optimization of Chromatographic Conditions and Method Development

The retention times acquired under the previously mentioned ideal circumstances for Daunorubicin and Cytarabine were 1.009 mins and 1.284 mins each (Table 1).

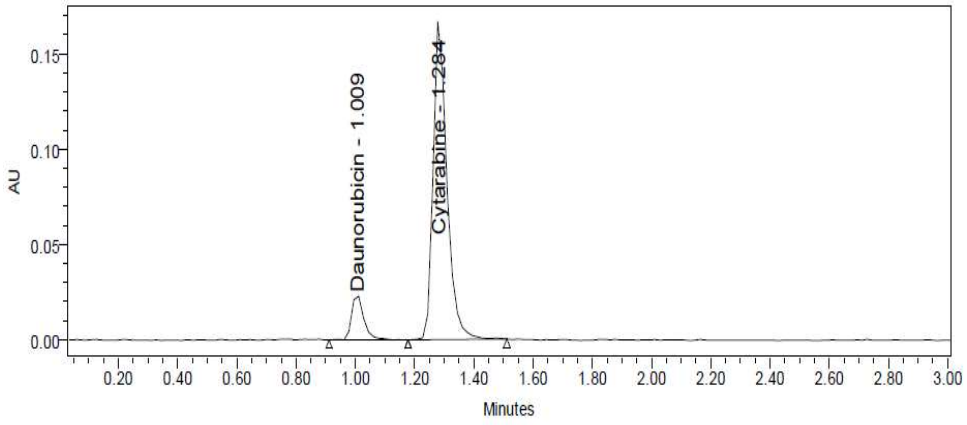

Figure 3: Chromatogram of the mixed standard solution Daunorubicin and Cytarabine

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Table 1: Optimized chromatographic conditions of this method		
S. No.	Parameters	Values
1.	Stationary phase	SB C18 (100x2.1mm;1.8µ)
2.	Mobile phase	Buffer: Acetonitrile (50:50 %)
3.	Flow rate	0.3 mL/min
4.	Column temperature	30ºC
5.	Injection Volume	2 µL
6.	Detection wavelength (λmax)	240 nm

Table 2: System suitability data for this method			
S. No.	Parameters	Daunorubicin	Cytarabine
1.	Retention time (min)	1.004	1.284
2.	Peak area	58341	450734
3.	Resolution	-	3.4
4.	Theoretical Plates	3311	2630
5.	Tailing Factor	1.20	1.28

Validation of the Developed Technique

In compliance with the ICH requirements for system suitability, linearity, accuracy, precision, robustness, limit of detection, and limit of quantification, the established method was validated with the following methodologies (16-20).

System suitability

The mixed standard drug solution (44 ppm of Daunorubicin and 100 ppm of Cytarabine) and the chromatographic parameters, assisted in a determination of system suitability were analyzed (Table 2).

Specificity

Interference wasn't found from excipients present in the formulation; thus, UPLC chromatograms of the drug matrix (drug and excipients mixture) did not record overlapping peaks within the retention time ranges. Figures 4 and 5 show chromatograms

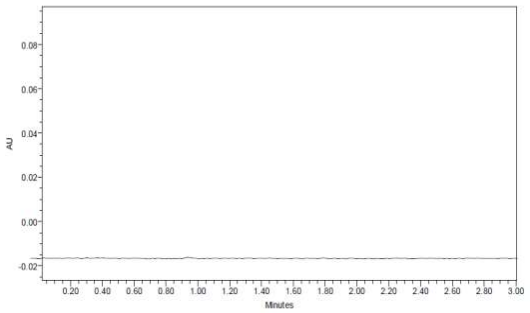

Figure 4: A Chromatogram of Placebo

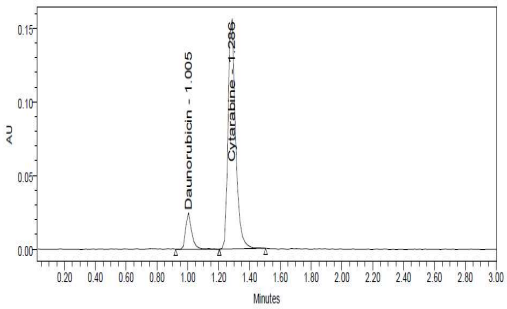

Figure 5: Chromatogram of Daunorubicin and Cytarabine (mixed standard solution)

for the standard and for the formulation, respectively, in which the separation of drugs is easily recognized, thus proving the selectivity of the proposed UPLC method.

Linearity

To establish linearity, a stock solution containing 44 µg/mL of Daunorubicin and 100 µg/mL of Cytarabinewere prepared using diluent and additionally diluted to yield solutions in the range 11-66µg/mL of Daunorubicin and 25-150 µg/mL Cytarabine. Three separate tests of the solutions were carried out then the experiment was analysed by injecting two microliters into a UPLC. Data for Daunorubicin and Cytarabineare given in the Tables 3 and 4 respectively. Linearity plots for Daunorubicin and Cytarabine are depicted in Figures 6 and 7 respectively.

Accuracy

Accuracy for both drugs was evaluated by spiking the placebo powder with

Table 3: Linearity of Daunorubicin

Conc. of Daunorubicin (µg/mL)	Peak Area	Mean Area	RSD
11	19279	19958	0.03
	20176		
	20418		
22	38136	38323	0.03
	39514		
	37319		
33	55693	55338	0.01
	55063		
	55259		
44	72452	74542	0.03
	75039		
	76135		
55	90654	93850	0.03
	94831		
	96066		
66	112515	112081	0.01
	110526		
	113202		

Table 4: Linearity data of Cytarabine

Conc. of Cytarabine (µg/mL)	Peak Area	Mean Area	RSD
25	147566	148804	0.03
	153275		
	145572		
50	280584	280688	0.00
	280738		
	280741		
75	429322	429281	0.00
	429256		
	429264		
100	561251	565121	0.01
	567938		
	566174		
125	700825	703835	0.00
	705046		
	705635		
150	848053	845678	0.01
	840706		
	848274		

the drug at 3 different level concentrations (50%, 100%, and 150%), with each tested thrice. The mean %recovery and %RSD values were estimated, and the % recovery

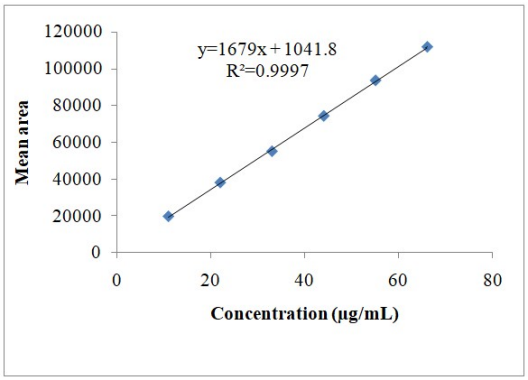

Figure 6: Linearity plot of Daunorubicin

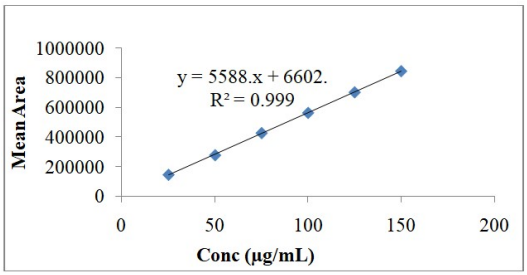

Figure 7: Linearity plot of Cytarabine

ranged from 98.0% to 102.0% (21, 22). Tables 5 and 6 represent the derived values.

Precision

Suitability tests were done to check the method efficacy of the standard stock solution which was prepared freshly. The optimized chromatographic system was filled with 2µL of this solution. The working standard samples were injected for system suitability in six duplicates, and shares with the peak response for the sample wasderived, the derived datais presented in Table 7. On the two separate days, a different instrument was used to determine the intermediate precision. The results are represented in Table 8. Both the parameters were calculated with the formula. (Table 9).

Limit of detection (LOD) and Limit of Quantification (LOQ)

The results are tabulated in Table 9.

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Table 5: Recovery of Daunorubicin

Accuracy Level	Peak area differences	Added quantity (µg/mL)	Amount Found (µg/mL)	%Recovery	Mean %Assay
50%	37406	22	21.95	99.77	99.4
	37229	22	21.85	99.30	
	37174	22	21.81	99.15	
100%	74684	44	44.02	100.05	100.23
	75251	44	44.36	100.1	
	74517	44	43.92	99.82	
150%	111348	66	65.73	99.59	99.81
	111091	66	65.58	99.36	
	112334	66	66.31	100.47	

Table 6: Recovery of Cytarabine

Accuracy Level	Peak area differences	Added quantity (µg/mL)	Amount Found (µg/mL)	%Recovery	Mean %Assay
50%	280488	50	49.94	99.88	99.96
	280874	50	50.01	100.02	
	280748	50	49.99	99.98	
100%	556176	100	99.03	99.03	99.67
	564563	100	100.52	100.52	
	558476	100	99.44	99.44	
150%	841275	150	149.80	99.86	99.95
	844009	150	150.28	100.19	
	840697	150	149.69	99.80	

Table 7: Intra-day precision data

S. No.	Injection	Daunorubicin	Cytarabine
1	Injection-1	58151	449062
2	Injection-2	58603	451480
3	Injection-3	58481	450890
4	Injection-4	58294	451312
5	Injection-5	58629	450059
6	Injection-6	57885	451602
Mean		58341	450734
SD		288.9	991.9
%RSD		0.5	0.2

Monitoring Forced Degradation Studies

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Table 8: Inter-dayprecision data

S. No.	Injections	1st Day		2nd Day	
		Daunorubicin	Cytarabine	Daunorubicin	Cytarabine
1	Injection-1	58151	449062	52587	404369
2	Injection-2	58603	451480	52172	408091
3	Injection-3	58481	450890	52615	408363
4	Injection-4	58294	451312	52643	402199
5	Injection-5	58629	450059	52092	404929
6	Injection-6	57885	451602	52975	408859
Mean		58341	450734	52514	406135
SD		288.9	991.9	328.6	2693.5
% RSD		0.5	0.2	0.6	0.7

Table 9: LOD and LOQ data of this method

S. No	Parameters	Daunorubicin	Cytarabine
1	Limit of detection	0.98	1.38
2	Limit of Quantification	2.96	4.18

Table 10: Robustness data of Daunorubicin

Chromatographic conditions	Daunorubicin			
	%Assay	Theoretical Plates	Asymmetry	Retention time(rt)
Water:Acetonitrile (60:40% v/v)	99.81	3104	1.49	0.986
Water: Acetonitrile (40:60% v/v)	99.53	3078	1.17	1.023
0.2mL/min	99.12	2539	1.29	1.039
0.4mL/min	99.98	3114	1.18	0.972
28°C	100.02	3014	1.47	1.007
32°C	99.87	3039	1.21	0.995

Robustness

Using small but purposeful variations in chromatographic settings and assessing system suitability of the two drugs, a robustness study was conducted. Among the parameters tested were the flow rate, temperature of column and composition of mobile phase. This method used a mixed standard solution containing 100µg/mL of Cytarabine and 44µg/mL of Daunorubicin. Outcomes were not affected by a small change in these conditions (Tables 10 and 11).

Assay of Tablet Dosage Forms

The results are captured in the shown in Table 12 and Figures 8 and 9.

Forced Degration Studies

Preparation of Standard stock solution of Daunorubicin and Cytarabine

22 mg of Daunorubicin and 50mg of Cytarabine working standards were weighed and shifted into a dry 50 mL volumetric flask. Approximately three-quarters of the flask's volume was filled with diluent, degassed for 5 mins, andthen the final volume was adjusted using diluent to get a solution containing 440 ppm of Daunorubicin and 1000 ppm of Cytarabine(23-25).

In the acid-hydrolytic degradation, to 5 mL of the standard stock solution ofboth the drugs, there was added 5 mL of 1 N HCl

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Table 11: Robustness data of Cytarabine

Chromatographic conditions	Cytarabine				
	%Assay	Theoretical Plates	Asymmetry	Resolution	Retention time(rt)
Water:Methanol (60:40% v/v)	99.07	2135	1.220	3.5	1.262
Water: Methanol (40:60% v/v)	98.99	2282	1.25	3.3	1.319
0.2 mL/min	101.0	2604	1.27	3.5	1.331
0.4 mL/min	99.7	2283	1.26	3.1	1.219
28°C	99.2	2060	1.25	3.6	1.302
32°C	100.1	2292	1.26	3.5	1.277

Table 12: Method precision results of Daunorubicin and Cytarabine intablets

S. No.	% Assay	
	Daunorubicin	Cytarabine
1	99.97	99.90
2	100.74	100.43
3	100.53	100.30
4	100.21	100.40
5	100.79	100.12
6	99.51	100.46
Mean	100.29	100.27
SD	0.50	0.2
%RSD	0.5	0.2

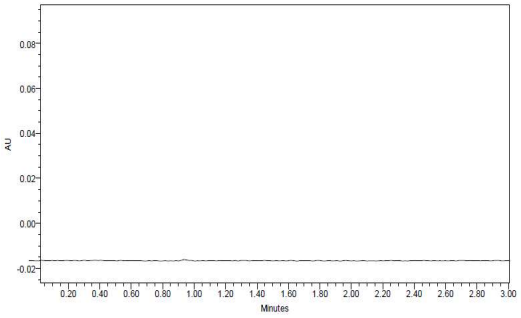

Figure 8: A Chromatogram of Placebo

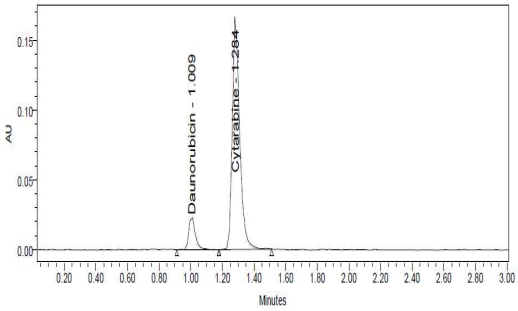

Figure 9: A Chromatogram of formulation Vyxeos™

and refluxed for 30 mins at 60O and then it was neutralized with 5mL of 1N sodium hydroxide solution. Dilution of final solution was done to get 44 µg/mL of Daunorubicin and 100µg/mL of Cytarabine (desired concentration); 2µL solutions were injected into the UPLC system, and the chromatograms (a) were notedfor the stability check. For alkaline hydrolysis degradation study, 5 mL of Daunorubicin and 5 mL of 1N NaOH solution were mixed and refluxed for 30 minutes at 60 degrees Celsius. The blend was neutralized using 5 mL of 1N hydrochloric acid.44 µg/mL concentration of Daunorubicin and 100 µg/mL of Cytarabine was prepared by dilution. 2 µL of that solution was injected into the UPLC system, and the chromatograms (b) were obtained for sample stability and degradation samples evaluation. In degradation induced hydrogen peroxide study, a 10% (H2O2) solution (5mL) was mixed with 5mL of Daunorubicin and Cytarabine stock solution. The solution was incubated on Bench for 30min. The final solution set to be examined by UPLC was then diluted to achieve final concentrations as mentioned above for both the drugs, and 2µL volume was injected into the UPLC system for the recording of chromatograms (c) to determine the stability of the drugs. In thermal degradation, 5mL of the stock solution of Daunorubicin and Cytarabine standard was kept in the oven at 105°C for

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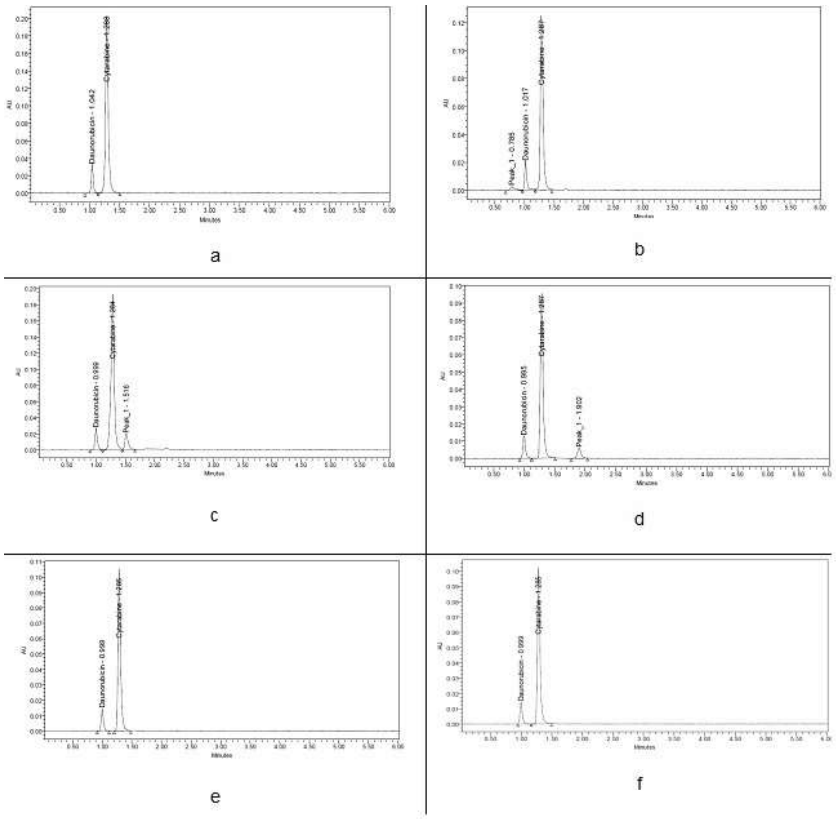

Figure 10: Chromatograms of Forced Degradation studies

6 hrs. In the UPLC method, the resulting solution was diluted to desired concentration for both drugs. 2 ml of the solutions were injected into the UPLC system. Proper chromatograms were taken to evaluate sample stability. For neutral degradation, 5 mL of the Daunorubicin standard stock solution and 5 mL of Cytarabine standard stock solution were combined and refluxed at 80°C for 6 hours with water. The formed solution was then diluted desired concentrations as above .2 µL aliquot of the solution was introduced into the UPLC system, and chromatograms (e) and monitored for any further. 5 mL aliquots of standard stock solution of both the drugs were exposed to UV radiation. The photochemical stability tested was 1 hour in a UV chamber or for 200Watt-hours/m² in a photostability chamber. For the UPLC study, the formulation solution with Daunorubicin and Cytarabine was diluted to obtain 44 µg/mL and 100 µg/mL, respectively. The solution was injected in a 2µL quantity into the UPLC system, and chromatograms were studied for the formation of extra peaks to detect degradation of the formulation. Both the drugs were both placed under stress conditions to facilitate the degradation of the sample and subsequently injected into a UPLC system with a photodiode array detector. All samples were able to successfully separate the distinct degradant peaks from Daunorubicin and Cytarabine peaks. The forced degradation sample chromatograms are presented in Figure 10

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Table 13: Forced degradation study data

Sample	Daunorubicin		Cytarabine	
	% Assay	% Degradation	% Assay	% Degradation
Control sample	100.29	-	100.27	-
Acid sample	93.5	6.79	93.64	6.63
Base sample	95.40	4.89	95.45	4.82
Peroxide sample	96.3	3.99	95.44	4.83
Thermal sample	98.51	1.78	98.14	2.13
UV sample	98.13	2.16	98.47	1.8
Water sample	99.09	1.2	99.43	0.84

and Table 13. Degradants were noted to have a presence of 5% in alkaline conditions and 6% in acidic conditions. A little bit of degradation of the sample was noted under oxidation and alkaline conditions. In neutral or photolytic conditions, no notable degradation was noted along with a little degradation under neutral and photolytic conditions (26-29).

Conclusion

An analytical technique was established by studying various parameters. The SB C18 100 X 2. 1mm 1. 8µ column was used as it produced good peak shapes and separation. The ideal wavelength, λ max, was determined to be at 240 nm, and an injection volume of 2µL was chosen for optimal peak area. A continuous flow rate of 0.3mL/min provided suitable retention times. A buffer and acetonitrile mixture at 50:50% v/v yielded good drug resolution. Daunorubicin and Cytarabine peaks appeared at approximately 1.009 and 1.284 ±0. 02 mins each, with a run time of 3 mins. The percent recovery ranged from 98. 0 to 102.0%. The method showed linearity for Daunorubicin (11-66µg/mL) and Cytarabine (25-150µg/mL). It passed robustness and ruggedness tests, with a relative standard deviation below 2.0. The technique is simple, sensitive, precise, and accurate for measuring both drugs in tablet forms, with no interference from excipients.

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Conflict of Interest

The authors report that there are no competing interests to declare.

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