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HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF DAUNORUBICIN AND CYTARABINE IN RAT PLASMA

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ABSTRACT

Objective: A simple, accurate and precise reversed phase High Performance Liquid Chromatography (HPLC) method for rapid and simultaneous quantification of Daunorubicin and Cytarabine in rat plasma was developed and validated. **Methods:** The chromatographic separation was achieved on Symmetry C18 (150x4.6mm, 3.5µ) column. The Buffer was Hexane Sulphonic acid at pH 2.5 adjusted with Ortho Phosphoric acid (OPA) and the mobile phase was a mixture of buffer and Acetonitrile in the ratio of 65:35 v/v, flow rate 1.0ml/min and UV detection was carried out at 236nm. **Results:** The proposed method shows a good linearity in the concentration range of 1-15µg/ml for Daunorubicin and 2.27-34.05µg/ml for Cytarabine under optimised conditions. Precision and recovery study results are in range 98-102%. In the entire robustness conditions %RSD is below 2.0%. Degradation has very less effect in stress condition and solutions remained stable up to 24 hrs. This method is validated for different parameters like selectivity, sensitivity, matrix effect, linearity, precision and accuracy, recovery, reinjection, reproducibility, limit of detection (LOD), limit of quantification (LOQ) and stability were determined according to the ICH Q2B guidelines. All the parameters of validation were found to be in acceptance with ICH guidelines. **Conclusion:** The results obtained demonstrate that proposed strategy can be effortlessly and advantageously applied for routine examination of daunorubicin and cytarabine in rat plasma.

KEYWORDS

RP-HPLC, Daunorubicin, Cytarabine, HPLC, Validation and Rat plasma.

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INTRODUCTION

Daunorubicin also known as Daunomycin is a chemotherapy[1] medication used to treat cancer. Specifically it is used for acute myeloid leukaemia (AML)[2,3] acute lymphocytic leukaemia (ALL)[4,5] chronic myelogenous leukemia (CML)[6] and Kaposi's sarcoma[7]. It is used by injection into a vein[8]. A liposomal formulation also known as

liposomal Daunorubicin also exists. Common side effects include hair loss, vomiting, bone marrow suppression, and inflammation[9] of the inside of the mouth. The other severe side effects are heart disease and tissue death at the site of injection. Use in pregnancy may harm the baby. Daunorubicin is in the anthracycline[10,11] family of medication. It works in part by blocking the function of topoisomerase II[12]. It deteriorate the growth of cancer cells in the body. Treatment is usually performed together with other chemotherapy drugs (such as cytarabine) and its administration depends on the type of tumor and the degree of response. In addition to its major use in treating AML, daunorubicin is also used to treat neuroblastoma[13]. Daunorubicin is useful with other chemotherapy agents to treat the blastic phase of chronic myelogenous leukemia. Daunorubicin is also used as the Initiator for semi synthetic manufacturing of doxorubicin, epirubicin and idarubicin.

Cytarabine also known as cytosine arabinoside, is a chemotherapy drug used to treat acute myeloid leukemia, acute lymphocytic leukemia, chronic myelogenous leukemia, and non-Hodgkin's lymphoma[14]. It can be injected into a vein, under the skin, or into the cerebrospinal fluid[15]. There is a liposomal formulation for which there is tentative evidence of better outcomes in lymphoma involving the meninges.

Common side effects are bone marrow suppression, vomiting, diarrhea, liver problem, rash, ulcer formation in the mouth, and bleeding. Other serious side effects include loss of consciousness, lung disease and allergic reactions[16]. Use during pregnancy may harm the baby. Cytarabine is in the anti metabolite[17] and nucleoside analogy families of medication. It works by blocking the function of DNA polymerase. Cytarabine is mainly used in the treatment of acute myeloid leukemia, acute lymphocytic leukemia and lymphomas[18], where it is the backbone of induction chemotherapy. Cytarabine also possesses antiviral activity, and it has been used in the treatment of generalized herpes viral infection. However, cytarabine is not at all selective in this setting and may cause bone marrow suppression and other severe side effects. Therefore Ara-C is not used as antiviral agent in humans because of its toxic profile and it is used mainly for the chemotherapy of hematologic cancers. Cytarabine is also used in the study of the nervous system to control the proliferation of glial cells[19] in cultures. The amount of glial cells having an important impact on neurons[20].

A number of HPLC methods are developed and reported so far for estimation of Daunorubicin and cytarabine. But there is no single bioanalytical method reported for quantification of Daunorubicin and cytarabine. Hence we made a honest attempt and were successful in simultaneous estimation of Daunorubicin and Cytarabine in rat plasma.

MATERIAL AND METHODS

Chemicals and reagents

Acetonitrile, Ortho Phosphoric Acid (OPA) and water (HPLC grade), Hexane sulphonic acid (HPLC grade) were purchased from Merck (India) Limited. Worli, Mumbai, India. All API's of Daunorubicin and Cytarabine as reference standards were procured from Rakshit drugs Private, Limited, Hyderabad.

Instrumentation

Waters alliance-2695 chromatographic system consisting of quaternary pump, PDA detector-2996 and chromatographic software Empower-2.0 was used.

Chromatographic conditions

Chromatographic separation was carried out in isocratic mode at room temperature using Symmetry C18 (150x4.6mm, 3.5µ) column. The mixture of 0.1% Hexane sulphonic acid: Acetonitrile 65:35 v/v at a flow rate 1.0ml/min was used as a mobile phase. The injection volume was 10µl and eluents was monitored at 236nm using PDA detector. The run time was 10min.

Selection of wavelength

The absorption spectra of solution of each Daunorubicin and Cytarabine were scanned over the range 200-400nm by using PDA detector and the spectra were recorded. The spectrum was shown in Figure No.2.

Preparation of Standard stock solution

The stock solutions of Daunorubicin and Cytarabine were prepared separately for bulk spiking of calibration curve and quality control samples for the method validation exercise as well as subject sample analysis. The stock solutions of Daunorubicin and Cytarabine were prepared in acetonitrile at a free base concentration of 10µg/ml of Daunorubicin and 22.7µg/ml of Cytarabine. Primary dilutions and working standard solutions were prepared from stock solutions using mobile phase as diluent. These prepared working standard solutions were used to prepare the calibration curve and quality control samples. Blank rat plasma was screened prior to spiking to ensure it was free of endogenous interference at the retention time of Daunorubicin and Cytarabine. Eight point standard curve and four quality control samples were prepared by spiking the blank plasma with an appropriate amount of Daunorubicin and Cytarabine. Calibration samples were made at concentrations of 1.0, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0 and 20.0µg/ml of Daunorubicin and 2.28, 5.69, 11.38, 17.06, 22.75, 28.44, 34.13 and 45.5µg/ml of Cytarabine.

Preparation of Sample Solution

For sample preparation, 200µl of plasma sample, and quality control sample was added to 2ml ria vial tube add 300µl of acetonitrile and 500µl of internal standard (IS) and 500µl of standard stock and 500µl of diluent to precipitate all the proteins and mix in the vortex cyclo mixture. Centrifuge at 5000rpm for 20min. Collect the supernatant solution in HPLC vial and inject into the chromatogram.

BIOANALYTICAL METHOD VALIDATION OF DAUNORUBISINE AND CYTARABINE

A short pre -validation was performed before starting the final validation process to ensure that all procedures as set up yield the expected results.

System suitability

System suitability samples were included at the beginning, middle and end of each batch of samples. The final concentration of the system suitability samples was made up to contain 10µg/ml Daunorubisine, 22.7µg/ml Cytarabine and 25µg/ml IS in mobile phase. RSD % of peak area and retention time for Daunorubisine and Cytarabine and IS for 6 consecutive injections were checked to see whether they were below 2% and 5% respectively or not.

Quality control (QC) standards

As the purpose of quality control standards (QC) is to assess the performance of the assay procedure; it must also cover the whole range of calibration line. The following QC samples were considered for HPLC method validation.

Low quality control (LQC), i.e. three times of LLOQ (lower limit of Quality control).

Mid quality control (MQC), i.e.100% or near about of highest calibration point.

High quality control (HQC), i.e.150% or near about of highest calibration point.

All the QC samples were selected such a way that those samples were not included into the calibration point.

Validation of developed bioanalytical HPLC method for Daunorubisine and Cytarabine

The HPLC method for Daunorubisine and Cytarabine was validated to meet the acceptance criteria of industrial guidance for the bio-analytical method validation (Food and Drug Administration of the United States, 2001).

Stability of stock solution

An aqueous stock solution containing 10µg/ml, 22.7µg/ml Daunorubisine and Cytarabine and 25µg/ml Alectinib was prepared in diluent. The solution was divided into three containers, the first one stored at room temperature, the second one stored at deep freezer and the last one stored at -20°C (assumed stable as a freshly prepared solution). The solutions of drug and IS from each storage conditions taken out at predetermined time intervals (0, 12, 24 hrs) and was injected onto the HPLC. The peak area from the chromatogram of each sample was compared with that of freshly prepared samples.

Specificity and selectivity

This test was performed by analyzing the blank plasma samples from six different sources to detect for any chromatographic interference at the

retention times of the Daunorubisine and Cytarabine and IS.

Calibration curve

An 8-point calibration curve was prepared by spiking appropriate amounts of working solution into the blank plasma to obtain final concentrations of 1.0, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0 and 20µg/ml for the Daunorubisine and 2.28, 5.69, 11.38, 17.06, 22.75, 28.44, 34.13 and 45.5µg/ml for the Cytarabine. The calibration curve was prepared by plotting the peak area ratio of the transition pair of Daunorubisine and Cytarabine to that of IS against the nominal concentration of calibration standards. The results were fitted to linear regression analysis. The acceptance criterion for each back-calculated standard concentration was ±15% deviation (SD) from the nominal value, except at LLOQ, which was set at ±20% (Food and Drug Administration of the United States, 2001).

Precision and accuracy

Intraday precision was determined by analyzing six replicates of LLOQ, LQC, MQC and HQC samples. Whereas reproducibility (day-to-day variation i.e. inter day precision) of the method was validated using six sets of LLOQ, LQC, MQC and HQC samples on three different days.

Intra and inter day assay precision were determined as % of co efficient of variance (CV %), i.e. the ratios of standard deviation (SD) to the mean and expressed as percentage.

Intra and inter assay accuracy was determined by analyzing six replicates at four QC levels (LQC, MQC, HQC including LLOQ) on the same day and on three different days respectively. Accuracy was determined by the ratio of determined concentration and actual concentration multiplied by 100%. The criteria for acceptability of the data included accuracy within ±15% deviation (SD) from the nominal values and a precision of within ±15% relative standard deviation (RSD), except for LLOQ, where it should not exceed ±20% of SD (Food and Drug Administration of the United States, 2001).

Stability experiments

Stability study was evaluated as part of the method validation. To assess the decomposition of the Daunorubisine and Cytarabine that may occur due to different reasons, the following stability test was prepared.

Freeze-thaw stability

Six replicates of each (LQC, MQC and HQC) that were stored at - 20ºC, were thawed completely thawing at room temperature and refrozen immediately to -20ºC. This cycle was repeated twice and the samples were extracted for injection in to HPLCB ench top stability.

For bench top stability experiment, stability of Daunorubisine and Cytarabine in the rat plasma after 8 h exposure on bench top was determined at three concentrations (LQC, MQC and HQC) in six replicates.

Wet Extract stability

Freezer stability of Daunorubisine and Cytarabine in plasma was assessed by analyzing LQC, MQC and HQC samples in six replicates stored at -20°C for 24 Hours for stability study. All sample compared with the fresh prepare samples of three different QC in six replicates. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (i.e. ±15% SD) and precision (i.e. ±15% RSD; Food and Drug Administration of the United States, 2001).

Auto sampler stability

Samples of Daunorubisine and Cytarabine in plasma was assessed by analyzing LQC, MQC and HQC samples are injected every 1 hour upto 24 Hours for stability study. All sample compared with the fresh prepare samples of 0Hr of different QC in six replicates. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (i.e. ±15% SD) and precision (i.e. ±15% RSD; Food and Drug Administration of the United States, 2001).

Recovery

Extraction recovery of the analytes were determined by comparing the analytical results for extracted samples at three different concentrations (LQC, MQC, HQC) in six replicates compared with extracted standards (unprocessed) which represent 100 % recovery. Whereas recovery experiment was carried out for IS at a single concentration of 25µg/mL.

RESULTS AND DISCUSSION

In the present HPLC work Hexane sulphonic acid and Acetonitrile (65:35) is used as mobile phase. The column used is Symmetry C_{18} with a flow rate of 1ml/min at 236nm. By using this chromatographic conditions the peaks were eluted at 3.029, 7.782 and 9.311 respectively.

By using the above chromatographic conditions the tailing factor, plate count for Daunorubicin and Cytarabine were 1.09, 3940 and 1.09, 12957 respectively and the %CV for peak area <5%. The results Shown in Table No.1 suggest that the method was suitable for simultaneous detection of Daunorubicin and Cytarabine using Alectinib as internal standard. The %RSD for Daunorubisine and Cytarabine and istd area ratio was found to be 0.24 and 1.07%. Hence it passed the system suitability. In selectivity and Sensitivity representative chromatograms are obtained from blank plasma and plasma spiked with Daunorubicin and Cytarabine.

In matrix effect, the %CV of Daunorubicin and Cytarabine at HQC and LQC levels was found to be 0.05%, 0.52% and 2.20%, 1.41% indicating that the matrix effect of analyte is within the acceptable range under these conditions.

Linearity of the method was found to be in the concentration range of 1-20µg/ml of Daunorubicin and 2.28-45.5µg/ml of Cytarabine. The linearity graph was plotted between concentrations vs. area. The results of linearity were shown in Table No.2 and Table No.3.

The linear regression equation for Daunorubicin is $y=0.362577x + 0.00088$ with $r^2 = 0.99927$. The linear regression equation for Cytarabine is $y = 0.03644x + 0.00361$ with $r^2 = 0.99941$. Precision and accuracy were determined by %CV.

The inter-run and precision were determined by pooling all individual assay results of replicate quality control. The %CV and accuracy results were found to be within the acceptable limits. The results were shown in Table No.4 and Table No.5. In recovery, six aqueous at low, medium and high concentration levels for Daunorubicin and Cytarabine were prepared for recovery determination, and the areas obtained for extracted samples of the same concentration levels from a precision and accuracy batch run with in the same day. The mean recovery for daunorubicin and cytarabine was 99.20% with a precision of 0.93% and 97.96% with a precision of 0.9% for cytarabine.

In Stability, the predicted concentrations for Daunorubicin (10µg/ml) and Cytarabine (22.7µg/ml) were deviated within ±15% of fresh sample concentration in a battery of stability tests namely freeze thaw, bench top, long term stabilities. The results were shown in Table No.6 and Table No.7.

Table No.1: System suitability results of Daunorubicin and Cytarabine

S.No	Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
1	Daunorubicin	3.029	70264		1.09	3940
2	Cytarabine	7.782	164257	20.21	1.09	12957
3	Alectinib	9.311	201632	5.06	1.1	14535

Table No.2: Linearity data of Daunorubicin

S.No	Linearity	Daunorubicin conc. (µg/ml)	Daunorubicin response	IS response	Area ratio
1	Linearity-1	1.0	7139	201365	0.0355
2	Linearity-2	2.5	18066	201975	0.0894
3	Linearity-3	5.0	36132	203679	0.1774
4	Linearity-4	7.5	57199	203462	0.2811
5	Linearity-5	10.0	70863	201576	0.3515
6	Linearity-6	12.5	92359	204531	0.4516
7	Linearity-7	15.0	109455	201379	0.5435
8	Linearity-8	20.0	147548	201596	0.7319
9	Slope	0.362577			
10	Intercept	0.00088			
11	CC	0.99927			

Table No.3: Linearity data of Cytarabine

S.No	Linearity	Cytarabine Conc (µg/ml)	Cytarabine response	IS response	Area ratio
1	Linearity-1	2.28	17139	201365	0.0851
2	Linearity-2	5.69	45224	201975	0.2239
3	Linearity-3	11.38	80219	203679	0.3939
4	Linearity-4	17.06	125421	203462	0.6164
5	Linearity-5	22.75	167236	201576	0.8296
6	Linearity-6	28.44	212359	204531	1.0383
7	Linearity-7	34.13	253455	201379	1.2586
8	Linearity-8	45.5	339548	201596	1.6843
9	Slope	0.036344			
10	Intercept	0.00361			
11	CC	0.99941			

Table No.4: Within run and between run precision and accuracy of Daunorubicin

S.No	Nominal conc (µg/ml)	Within run			Between run		
		Mean (µg/ml)	Precision (%CV)	Accuracy	Mean (µg/ml)	Precision (%CV)	Accuracy
1	0.5	0.525	0.76	97.32	0.542	0.74	97.85
2	5	5.165	0.1	99.38	5.236	0.15	99.68
3	10	10.26	0.05	100.14	10.34	0.07	99.64
4	15	15.33	0.25	98.89	15.45	0.31	97.35

Table No.5: Within run and between run precision and accuracy of Cytarabine

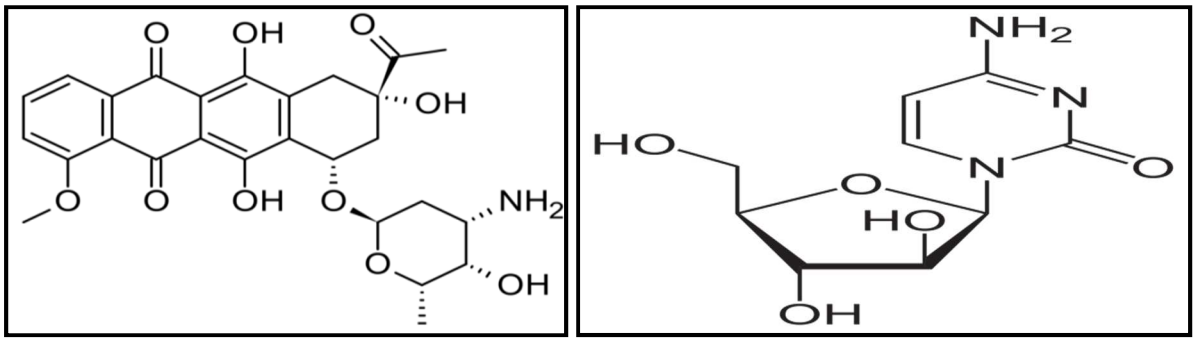
S.No	Nominal Conc (µg/ml)	Within run			Between run		
		Mean (µg/ml)	Precision (%CV)	Accuracy	Mean (µg/ml)	Precision (%CV)	Accuracy
1	1.13	1.14	0.29	96.39	1.129	0.35	95.85
2	11.35	11.37	0.40	99.67	11.45	0.44	99.32
3	22.75	22.78	0.19	99.87	22.86	0.23	99.17
4	34.05	34.08	0.13	98.69	34.22	0.18	99.46

Table No.6: Stability data of Daunorubicin

S.No	Stability experiments		Spiked plasma concentration	Concentration measured	%CV
1	Bench top stability	LQC	5	5.556	0.98
		HQC	15	15.608	0.34
2	Auto sampler stability	LQC	5	5.477	0.91
		HQC	15	15.453	0.83
3	Long term stability	LQC	5	5.144	1.71
		HQC	15	15.361	0.59
4	Freeze thaw stability	LQC	5	5.187	2.43
		HQC	15	15.215	0.31

Table No.7: Stability data of Cytarabine

S.No	Stability experiments		Spiked plasma concentration	Concentration measured	%CV
1	Bench top stability	LQC	11.35	11.354	0.12
		HQC	34.01	34.11	0.17
2	Auto sampler stability	LQC	11.35	11.501	0.14
		HQC	34.01	34.502	0.07
3	Long term stability	LQC	11.35	11.513	0.08
		HQC	34.01	34.512	0.02
4	Freeze thaw stability	LQC	11.35	11.348	0.35
		HQC	34.01	34.524	0.03



(A) **(B)**

Figure No.1: Chemical structure: (A) Daunorubicin(B) Cytarabine

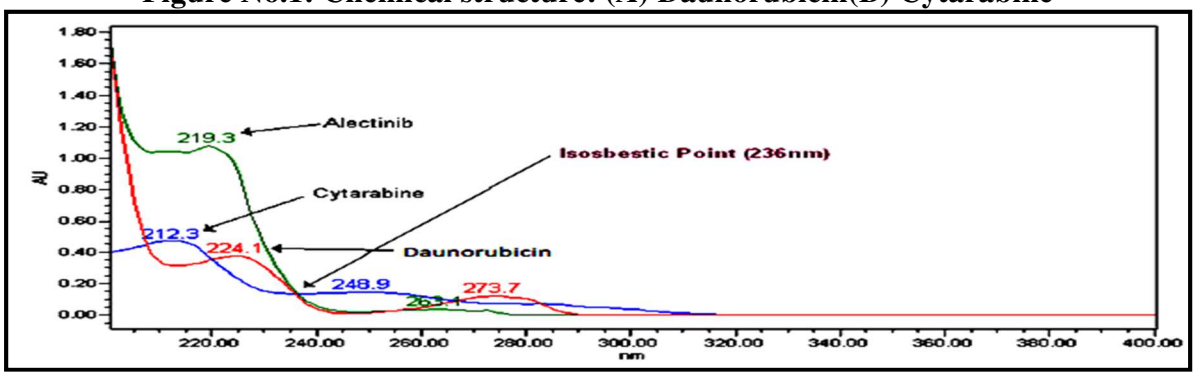


Figure No.2: PDA Spectrum for Daunorubicin and Cytarabine

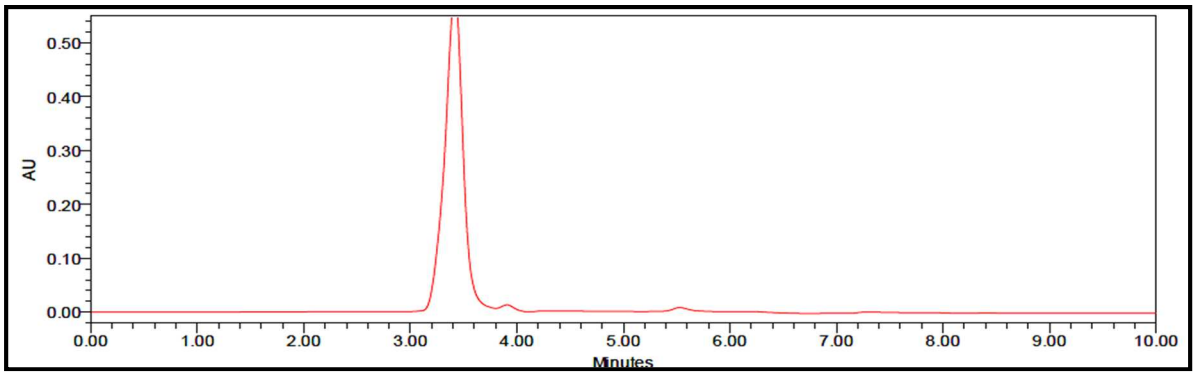


Figure No.3: Blank plasma chromatogram for Daunorubicin and Cytarabine in rat plasma

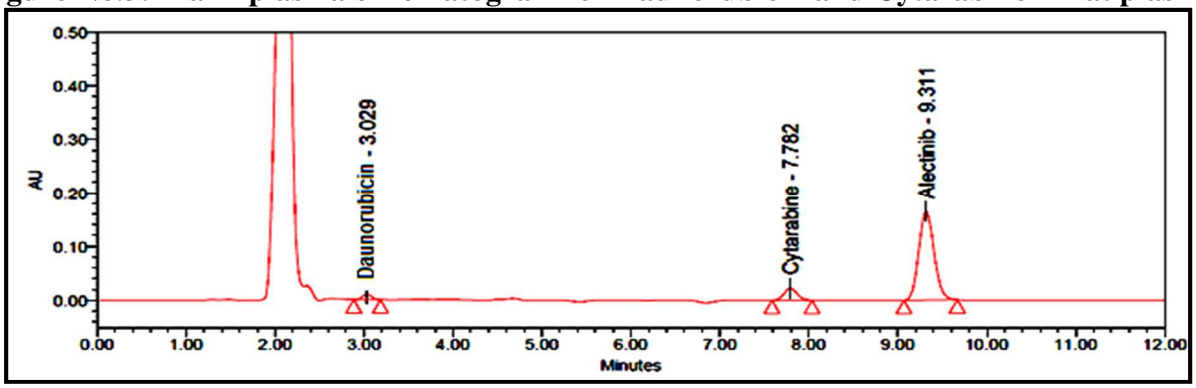
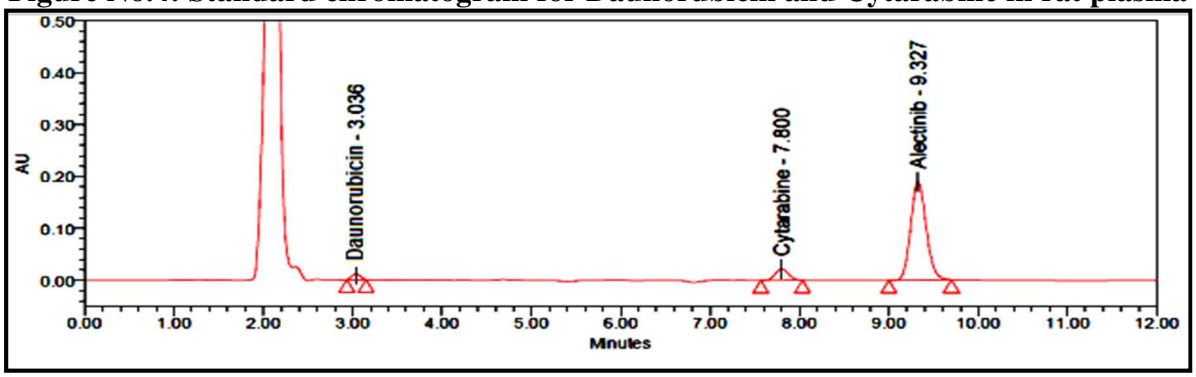
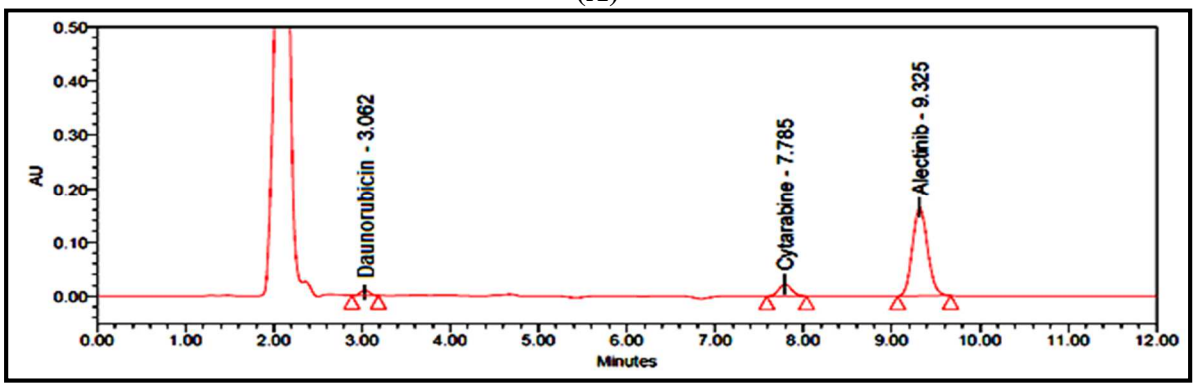


Figure No.4: Standard chromatogram for Daunorubicin and Cytarabine in rat plasma

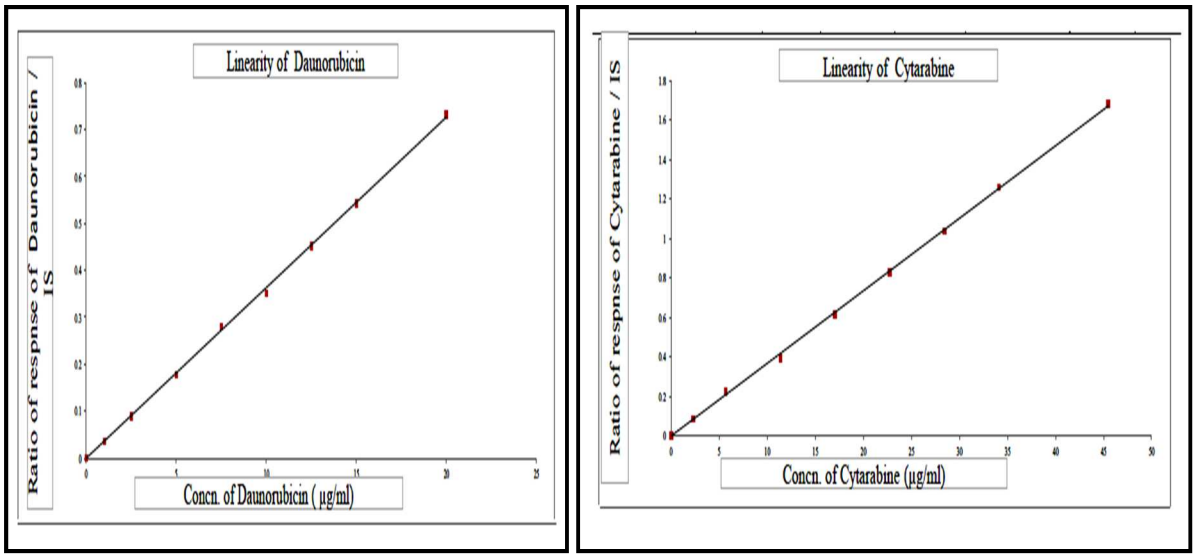


(A)



(B)

Figure No.5: Sensitivity: (A) LOD (B) LOQ



(A) **(B)**

Figure No.6: Linearity plot: (A) Daunorubicin (B) Cytarabine

CONCLUSION

In summary a highly sensitive, specific, reproducible and rapid and high through put HPLC assay was developed and validated to quantify Daunorubicin and Cytarabine in rat plasma as per the regulatory guidelines. Acquired results demonstrate that proposed strategy can be effortlessly and advantageously applied for routine examination of daunorubicin and cytarabine in rat plasma. Hence, the combination was taken up for developing a bio analytical method development and validation so that further it would be useful for performing pharmacokinetic study.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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