DEVELOPMENT OF AN ANALYTICAL METHOD AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF CYTARABINE AND DAUNORUBICIN IN PURE AND PHARMACEUTICAL DOSAGE FORM

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Abstract:

The aim of the present work is to develop an analytical method and validation for Simultaneous estimation of Cytarabine and Daunorubicin in pure and pharmaceutical dosage form. From literature review and solubility analysis initial chromatographic conditions Mobile phase Phopshate buffer:Methanol 35:65 were set (Buffer P^H 2.5 adjusted with Triethylamine), Kromosil C 18 (250×4.6mm, 5µ) Column, Flow rate 1.0 ml/min and temperature was ambient, eluent was scanned with PDA detector in system and it showed maximum absorbance at 254 nm. As the methanol content was increased Cytarabine and Daunorubicin got eluted with good peak symmetric properties. The retention times for Cytarabine and Daunorubicin was found to be 2.589 min and 3.711 min respectively.System suitability parameters were studied by injecting the standard five times and results were well under the acceptance criteria. Linearity study was carried out between 50% to150 % levels, R^2 value was found to be as 0.999.

Key Words:

RPHPLC, Linearity, Precision, Suitability.

INTRODUCTION:

ANALYTICAL CHEMISTRY: [1]

Analytical chemistry is a branch of chemistry involved in separating, identifying and determining the relative amounts of the components making up a sample of matter.

It is mainly involved in the qualitative analysis or detection of compounds and quantitative analysis of the compounds. A qualitative method yields information about the identity of atomic or molecular species or functional groups in the sample. A quantitative method, in contrast provides numerical information as to the relative amount of one or more of these components.

CHROMATOGRAPHY [2] [5]

Chromatography is relatively a new technique which was first invented by M.Tswett, a botanist in 1906. Chromatography was derived from Greek words chroma and graphos meaning “colour” and “writing” respectively. It involves passing a mixture dissolved in a "mobile phase" through a stationary phase, which separates the analyte to be measured from other molecules in the mixture based on differential partitioning between the mobile and stationary phases. Differences in compounds partition coefficient results in differential retention on the stationary phase and thus changing the separation.

OBJECTIVE OF VALIDATION [5]

The primary objective of validation is to form a basis for written procedures for production and process control which are designed to assure that the drug products have the identity, strength, quality and purity they purport or are represented to process. Quality, safety and efficacy must be designed and built into the products. Each step of the manufacturing process must be controlled to maximize the probability that the finished product meets all quality and design specifications.

MATERIALS AND METHODS:

Cytarabine and Daunorubicin were purchased from Yarrow chem. products, Mumbai. Water Methanol and Acetonitrile for HPLC were purchased from Thermo Fisher Scientific India. Pvt. Ltd., Mumbai., All other chemicals and solvents were of analytical grade satisfying pharmacopeial specifications.

EXPERIMENTAL WORK AND RESULTS

Reference standards

1. Cytarabine and Daunorubicin- Yarrow Chem.

Marketed formulation

(Vyxeos) cytarabine/daunorubicin liposomal injection, lyophilized cake for reconstitution-100mg/44mg)/vial

METHOD DEVELOPMENT AND OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS

Selection of chromatographic condition

Proper selection of the method depends upon the nature of the sample, its molecular weight and solubility. The drugs selected in the present study are polar in nature and hence reversed phase or

ion-pair or ion exchange chromatography method may be used. The reversed phase HPLC was selected for the separation because of its simplicity and suitability.

Selection of detection wavelength:

The sensitivity of method that uses UV- Vis detector depends upon the proper selection of wavelength. An ideal wavelength is that gives maximum absorbance and good response for both the drugs to be detected.

Standard solutions of Cytarabine and Daunorubicin were scanned in the UV range (200-400nm) and the spectrums obtained were overlaid and the overlain spectrum was recorded. From the overlain spectrum, 254 nm was selected as the detection wavelength for the present study.

Selection of mobile phase:

Initially the mobile phase tried was methanol and water, methanol and Methanol, buffer and water in various proportions. Finally, the mobile phase was optimized to Buffer: Methanol in proportion 65:35 v/v respectively.

Optimization of flow rate

The method was performed with flow rates 0.8ml, 1.5ml and 1ml/min. Flow rate of 1ml/min was found to be ideal as it gave sharp peak.

Based on the above study, the following chromatographic conditions were selected for the simultaneous estimation of drugs in multi component dosage forms

OPTIMIZED METHOD

Preparation of Buffer:
About 7.0g of potassium dihydrogen orthophosphate was dissolved in 1000ml of HPLC grade water and pH 2.5 was adjusted with orthophosphoric acid. It was filtered through 0.45µm nylon membrane filter and degassed with sonicator. It was used as a diluent for the preparation of sample and standard solution.
Preparation of mobile phase:
Mobile phase consist of buffer: Methanol of P^H 2.5 (35:65) was taken sonicated and degassed for 10min and filtered through 0.45 µm nylon membrane filter

Standard Preparation:
Weigh accurately 100 mg Cytarabine Working Reference Standard and 50 mg of Daunorubicin Working Reference Standard is taken in to 100ml volumetric flask and then it was dissolved and diluted to volume with mobile phase up to the mark. (Stock solution)
Further pipette 0.4 ml of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluent.
Assay

Preparation of samples for Assay

Standard preparation:

Weigh accurately 100 mg Cytarabine Working Reference Standard and 50 mg of Daunorubicin Working Reference Standard is taken in to 100ml volumetric flask and then it was dissolved and diluted to volume with mobile phase up to the mark. (Stock solution)

Further pipette 0.4 ml of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluent.

Sample preparation:

An Equivalent amount of Lyophilized injection powder was transferred into a 100ml standard flask. A volume of 70ml of mobile phase was added and sonicate for 30min.Then the solution was cooled and diluted to volume with mobile phase and filtered through 0.45µm membrane filter. (Stock solution)

Further pipette 0.4 ml of Cytarabine and Daunorubicin of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluent.

Assay procedure

20µL of the standard and sample solutions of Cytarabine and Daunorubicin were injected into the HPLC system and the chromatograms were recorded. Amount of drug present in the capsules were calculated using the peak areas.

Amount of drug in tablet was calculated using following formula :

$$\% \text{ Label claim} = \frac{Asp}{Ast} \times \frac{Dst}{Dsp} \times \frac{A}{Lc} \times P$$

VALIDATION:

Validation of an analytical method is the process to establish by laboratory studies that the performance characteristic of the method meets the requirements for the intended analytical application. Performance characteristics were expressed in terms of analytical parameters. After development of RP-HPLC method for estimation of Cytarabine and Daunorubicin, validation of the method was carried out according to ICH guidelines

The developed method was validated for the following parameters.

A. System suitability
B. Linearity
C. Specificity
D. Precision
E. Accuracy

F. LOD & LOQ

G. Robustness

SYSTEM SUITABILITY:

A Standard solution of Cytarabine and Daunorubicin working standard was prepared as per procedure and was injected five times into the HPLC system. The system suitability parameters were evaluated from standard Chromatograms obtained by calculating the % RSD of retention times, tailing factor, theoretical plates and peak areas from five replicate injections.

LINEARITY:

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range.

Serial dilutions of Cytarabine and Daunorubicin (20-60µg/ml and 10-30 µg/ml) were injected into the column and detected at a wavelength set at 254 nm. The calibration curve was obtained by plotting the concentration vs. peak area.

SPECIFICITY:

ICH defines specificity as "the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically this might include impurities, degradants, matrix, etc.

PRECISION:

The precision of the method was demonstrated by intra-day and inter-day precision studies. Intra-day studies were performed by injecting three (3) repeated injections within a day. Peak area and %RSD were calculated and reported.

The chromatograms of intra-day precision studies were shown. Inter-day precision studies, was done by injecting three (3) repeated injections for three consecutive days. Peak area and %RSD were calculated and reported.

METHOD PRECISION:

Method precision also called as repeatability/Intra-day precision indicates whether a method gives consistent results for a single batch. Method precision was demonstrated by preparing six test solutions at 100% concentration as per the test procedure & recording the chromatograms of six test solutions.

The % RSD of peak areas of six samples was calculated. The method precision was performed on Cytarabine and Daunorubicin formulation. The % RSD of the assay value for six determinations should not be more than 2.0%.

INTERMEDIATE PRECISION:

Intermediate precision of the analytical method was determined by performing method precision on another day by different analysts under same experimental condition. Assay of all six replicate sample preparations was determined and mean %assay value, standard deviation & %RSD was calculated.

SYSTEM PRECISION:

System precision was established to ensure that the optimized analytical method is precise. System precision was performed by injecting six replicate injections of standard solution at 100% concentration and the chromatograms were reviewed for the %RSD of peak areas. % RSD of the assay value for six determinations should not be more than 2.0%.

ACCURACY:

Accuracy of the method was determined by recovery experiments. There are mainly 2types of recovery studies are there.

a) Standard addition method: To the formulation, the reference standard of the respective drug of known concentration was added, analyzed by HPLC and compared with the standard drug concentration.

b) Percentage method: For these assay method samples are prepared in three concentrations of 50%, 100%, and 150% respectively.

LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION:

The Sensitivity of measurement of Cytarabine and Daunorubicin by use of the proposed method was estimated in terms of the Limit of Detection (LOD) and the Limit of Quantitation (LOQ). The LOD and LOQ were calculated by the use of the equations:

$$\text{LOD} = 3.3 \times \frac{\sigma}{S}$$

$$\text{LOQ} = 10 \times \frac{\sigma}{S}$$

Where, σ is the standard deviation of intercept of calibration plot and S is the average of the slope of the corresponding calibration plot.

ROBUSTNESS:

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. For the determination of a method's robustness, deliberate change in the Flow rate was made to evaluate the impact on the method.

Effect of variation in flow rate:

A study was conducted to determine the effect of variation in flow rate. Standard and Test solutions of 100% concentration was prepared & injected into the HPLC system by keeping flow rates 0.8 ml/min& 1.2 ml/min. The effect of variation of flow rate was evaluated.

Effect of variation in mobile phase composition: A study was conducted to determine the effect of variation in mobile phase ratio by changing the ratio of organic solvent i.e., Buffer: Methanol by ±2ml. Standard & test solutions of 100% concentration were prepared and injected into the HPLC system and the chromatograms were recorded. The retention times, tailing factors & %RSD values were calculated.

RESULTS AND DISCUSSION:

Selection of detection wavelength:

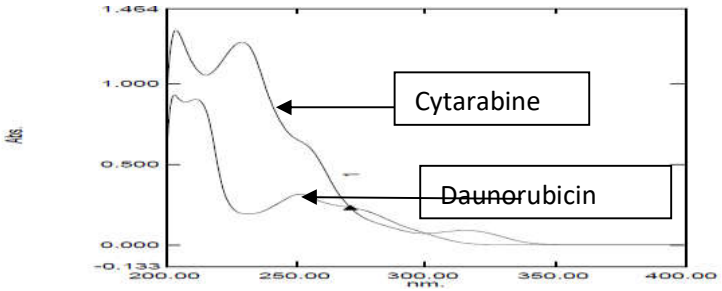


Fig 1. Over line Spectrum of Cytarabine and Daunorubicin

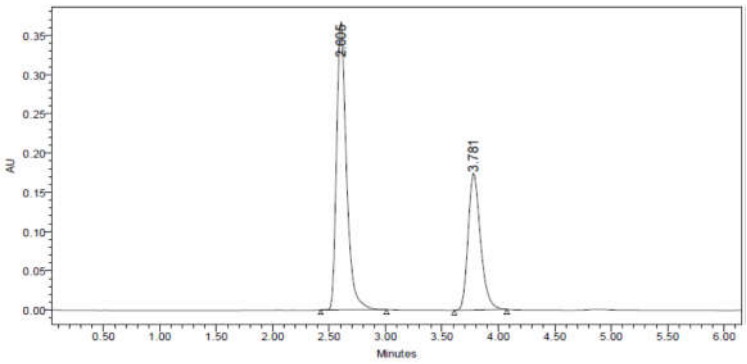
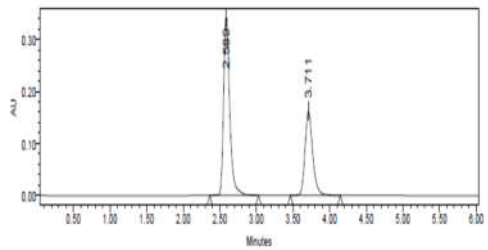


Fig 2.Chromatogram of Optimized Trail

Observation: The separation of two analytical peaks was good. The plate count also above 2000, tailing factor below 2, and the resolution is above 2. The condition is taken as optimized method.

Assay:



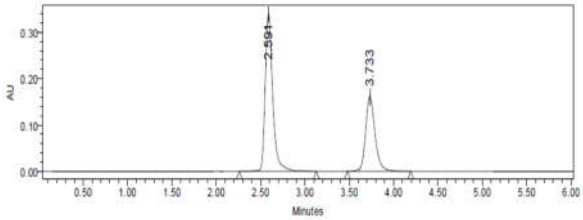


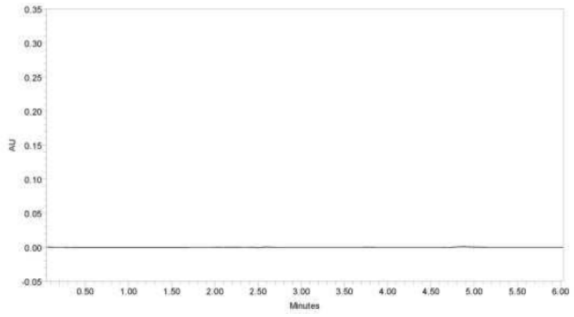
Fig 3. Chromatogram of standard **Fig 4. Chromatogram of Test**

Table 1: Results of Assay

Parameters	Cytarabine	Daunorubicin
Standard peak area	2008408	1185786
Test peak area (mean)	2005829	1189695
Average Weight	694.2mg	694.2mg
Label claim	100 mg	44 mg
% Purity of Standard	99.50	99.58
Amt obtained	399.88 mg	150.10 mg
% Assay	99.77%	100.12%

The % assays of Cytarabine and Daunorubicin were found to be 99.77% and 100.12% respectively. Thus, % Assay results were found to be within the limits i.e., 98-102% for both the drugs. Hence the developed method can be routinely used for the simultaneous estimation of Cytarabine and Daunorubicin in the marketed formulations.

System Suitability:



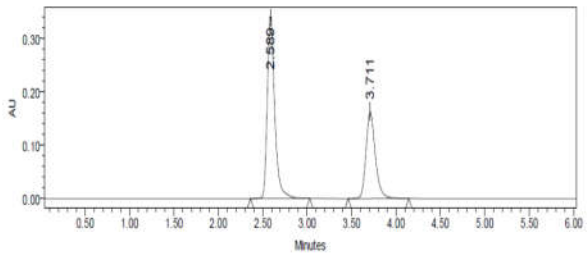


Figure 4: Chromatogram of Blank **Figure 5: Chromatogram of system suitability**

Table 2. Results of System suitability Test for DAUNORUBICIN

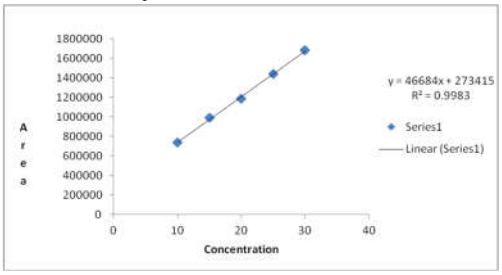
Injection	Retention time (t_R)	Peak Area	Plate count	Tailing factor
1	3.711	1185786	6389	1.3
2	3.702	1184759	6455	1.3
3	3.698	1187496	6234	1.6
4	3.708	1190478	6478	1.3
5	3.715	1183897	6502	1.30
6	3.714	1184759	6384	1.2
Mean	-	1186196	-	-
SD	-	2433.47	-	-
% RSD	-	0.20	-	-

Table 3. Results of System suitability Test for CYTARABINE

Injection	Retention time (t_R)	Peak Area	Plate count	Tailing Factor
1	2.589	2008408	5752	1.4
2	2.570	2008412	5758	1.3
3	2.572	2008357	5672	1.2
4	2.578	2007478	5674	1.4
5	2.582	2008475	5749	1.3
6	2.584	2008364	5843	1.4
Mean	-	2008249	-	-
SD	-	380.0	-	-
% RSD	-	0.01	-	-

Report: All the System suitability parameters were satisfied, thus the method passed the System suitability test.

Linearity:



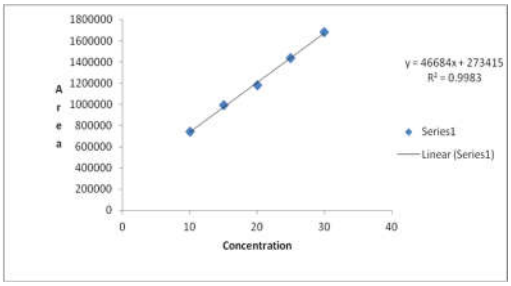


Figure 6: Linearity Graph of DAUNORUBICIN Figure 7. Linearity Graph of CYTARABINE

Precision:
Method precision:

The % RSD of peak areas of six samples was calculated. The method precision was performed on Cytarabine and Daunorubicin formulation. The % RSD of the assay value for six determinations should not be more than 2.0%.

Table 4. Method Precision data for Cytarabine & Daunorubicin

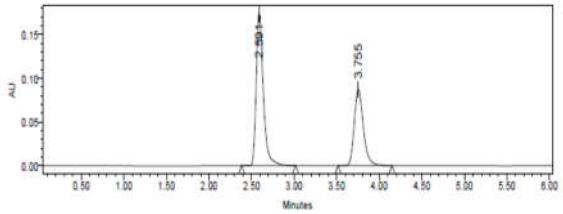
S.No.	Concentration (µg/ml)	Cytarabine		Daunorubicin	
		Retention time(Rt)	Peak Area	Retention time(Rt)	Peak Area
1	40 & 20	2.586	2010800	3.713	1184689
2	40 & 20	2.588	2002956	3.714	1188199
3	40 & 20	2.590	2012800	3.734	1195842
4	40 & 20	2.590	2005243	3.737	1184210
5	40 & 20	2.591	2011092	3.741	1198327
6	40 & 20	2.589	2011098	3.740	1198320
Avg			2008998		1191598
SD			3920.9		6668.5
%RSD			**0.19**		**0.55**

System precision:

Table 5. System Precision data for Cytarabine & Daunorubicin

S.No.	Cytarabine		Daunorubicin	
	Retention time(Rt)	Area	Retention time(Rt)	Area
1	2.592	2025051	3.743	1175422
2	2.594	2026574	3.734	1175841
3	2.594	2026471	3.736	1175234
4	2.576	2026489	3.724	1174894
5	2.585	2026523	3.723	1175023
6	2.592	2026471	3.735	1175236
Avg		2026263		1175275
SD		595.1		332.8
%RSD		**0.02**		**0.02**

Accuracy:



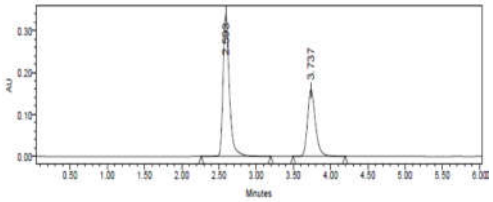


Figure 8 Chromatogram of Accuracy 50% **Figure 9 Chromatogram of Accuracy 100%**

Table 6. Accuracy Study of Cytarabine

Sample Id	Conc found (µg/ml)	Concn Obtained (µg/ml)	%Recovery	Mean recovery	Statistical Analysis
50%	5	5.01	100.2		
50%	5	4.96	99.2	99.73	
50%	5	4.99	99.8		**%RSD= 0.505**
100%	10	9.95	99.5		
100%	10	9.87	98.7	98.8	
100%	10	9.82	98.2		**%RSD=0.66**
150%	15	14.64	97.6		
150%	15	14.76	98.4	98.8	
150%	15	15.06	100.4		**%RSD=1.45**

Table 7. Accuracy Study of Daunorubicin

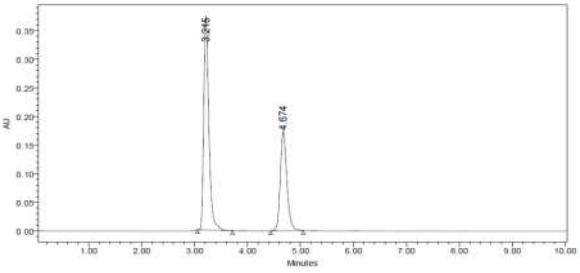
Conc (µg/ml)	Concn Obtained(µg/ml)	%Recovery of drug	Mean accuracy	%RSD
5	4.92	98.0		
5	4.96	99.2		
5	5.02	100.4	**99.2**	**1.2**
10	9.95	99.5		
10	9.94	99.4		
10	9.98	99.8	**99.5**	**0.2**
15	14.78	98.6		
15	14.94	99.6	**99.0**	**0.530**
15	14.83	98.8		

LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION:

Table 8. LOD and LOQ Data of Cytarabine and Daunorubicin

Cytarabine			Daunorubicin		
Conc.(x) (µg/ml)	**Peak Areas (y)**	**Statistical Analysis**	**Conc.(x) (µg/ml)**	**Peak Areas (y)**	**Statistical Analysis**
40	2004682	S = 39092 c = 618048 LOD: 0.001µg/ml LOQ: 0.004µg/ml	20	1184227	S = 39092 c =369381 LOD:0.005 µg/ml LOQ: 0.015µg/ml
40	2004587		20	1186425	

Robustness:



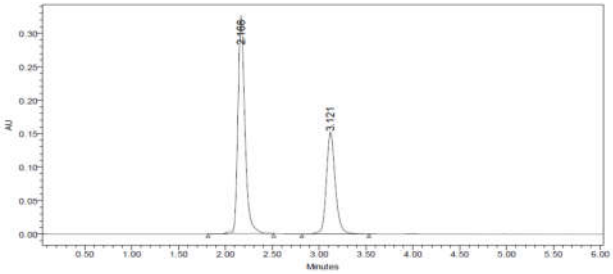


Figure.9 Representative Chromatogram at Flow rate of 0.8 ml/min **Figure.9 Chromatogram at Flow rate of 1.2 ml/min**

Table.9. Robustness data for Cytarabine

Std. Replicate	Variation in flow rate		Variation in Mobile phase composition	
	Flow Rate 0.8ml/min	**Flow Rate 1.2ml/min**	**Buffer: Methanol (40:60)**	**Buffer: Methanol (30:70)**
1	2492492	1676589	1951632	1979168
2	2495874	1675428	1954783	1967452
Mean	2494183	1676009	1953208.0	1973310
SD	2391.4	820.9	2228.0	8284.46
%RSD	0.09	0.04	0.11	0.4
Retention time	3.150	2.168	2.618	2.572
Tailing factor	1.4	1.3	1.3	1.3
Theoretical plates	5752	4207	4577	4476

Table.10. Robustness data for Daunorubicin

Parameter	Variation in flow rate		Variation in Mobile phase composition	
Standard	**Flow Rate 0.8ml/min**	**Flow Rate 1.2ml/min**	**Buffer: Methanol (40:60)**	**Buffer: Methanol (30:70)**
1	1500192	100524	1196996	1153397
2	1500426	100468	1198547	1154782
Mean	1500309	100496	1197772	1154090
SD	165.5	39.59	1096.2	979.34
%RSD	0.01	0.03	0.09	0.08
Retention time	4.674	3.121	4.394	3.331
Tailing factor	1.2	1.2	1.2	1.2
Theoretical plates	7187	5412	6498	6471

Report:

Cytarabine & Daunorubicin peaks in the chromatogram passed the system suitability criteria. %RSD of peak areas of Cytarabine & Daunorubicin was not more than 2.0% for variation in mobile phase composition. From the above data, it was concluded that the method was robust

In RP-HPLC method, the conditions were optimized to obtain an adequate separation of eluted compounds. Initially, various mobile phase compositions were tried, to separate title ingredients. Mobile phase and flow rate selection was based on peak parameters (height, tailing, theoretical plates, capacity or symmetry factor), run time and resolution. The mobile phase containing mixture of Phosphatebuffer solution: Methanol (35:65v/v, pH 2.5) with a flow rate of 1.0 ml/min is quite robust.

The optimum wavelength for detection was 254 nm at which better detector response for both the drugs was obtained. The retention times for Cytarabine and Daunorubicin was found to be 2.589 ± 0.004 min and 3.711 ± 0.005 min, respectively. To ascertain its effectiveness, system suitability tests were carried out on freshly prepared stock solutions. The calibration was linear in concentration range of 20 to 60 µg/ ml and 10 to 30 µg/ml, with regression 0.9979 and 0.9999, Cytarabine and Daunorubicin respectively. The low values of % R.S.D indicate the method is precise and accurate. The mean recoveries were found above 99.3 % for both the drugs.

Robustness of the proposed method was determined by varying various parameters, the %RSD reported was found to be less than 2 %. The proposed method was validated in accordance with ICH parameters and the applied for analysis of the same in marketed formulations.

CONCLUSION:

The proposed HPLC method was found to be simple, specific, precise, accurate, rapid and economical for simultaneous estimation of Cytarabine and Daunorubicin in Injection dosage form. The developed method was validated in terms of accuracy, precision, linearity, robustness and ruggedness, and results will be validated statistically according to ICH guidelines. The Sample recoveries in all formulations were in good agreement with their respective label claims.

From literature review and solubility analysis initial chromatographic conditions Mobile phase Phopshate buffer:Methanol 35:65 were set (Buffer P^H 2.5 adjusted with Triethylamine), Kromosil C 18 (250×4.6mm, 5µ) Column, Flow rate 1.0 ml/min and temperature was ambient,

eluent was scanned with PDA detector in system and it showed maximum absorbance at 254 nm. As the methanol content was increased Cytarabine and Daunorubicin got eluted with good peak symmetric properties. The retention times for Cytarabine and Daunorubicin was found to be 2.589 min and 3.711 min respectively.

System suitability parameters were studied by injecting the standard five times and results were well under the acceptance criteria.

Linearity study was carried out between 50% to150 % levels, R^2 value was found to be as 0.999.

By using above method assay of marketed formulation was carried out, 100.7% was present.

Full length method was not performed; if it is done this method can be used for routine analysis of Cytarabine and Daunorubicin.

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