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BIO ANALYTICAL METHOD DEVELOPMENT AND VALIADTION FOR THE QUANITIFICATION OF CYTARABINE IN RAT PLASMA BY USING HPTLC

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ABSTRACT

It is a simple, sensitive, rapid and economic chromatographic method has been developed for the determination of cytarabine in rat plasma using standard API. This analytical technique used for development was high performance thin layer chromatography. HPTLC aetron with already coated silica gel plates 60 F_{254} (10X10 cm) at 250 nm thickness was used as stationary phase. the mobile phase used consisted of 2-butanone :acetone :water (65:20:15 % v/v/v)[4] the plasma sample were extracted by protein precipitation with methanol concentration ranges of 1000,2000,3000,4000,5000 ng/ml respectively ,were used mixed plasma for the calibration curves .the stability of cytarabine in plasma were confirmed during three freeze-thaw cycles (-20 ºC) on a bench for 24 hrs and post preparattively for 48 hrs.this method was validated statistically and proved suitable for the determination of cytarabine in rat plasma.

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INTRODUCTION

Cytarabine or cytosine arabinoside is a chemotherapy agent used mainly in the treatment of cancers of white blood cells such as acute myeloid leukemia (AML) and non-Hodgkin lymphoma. It kills cancer cells by interfering with DNA synthesis. It is called cytosine arabinoside because it combines a cytosine base with an arabinose sugar. Cytosine normally combines with a different sugar, deoxyribose, to form deoxycytidine, a component of DNA. Certain sponges, where it was originally found, use arabinoside sugars to form a different compound (not part of DNA). Cytosine arabinoside is similar enough to human cytosine deoxyribose (deoxycytidine) to be incorporated into human DNA, but different enough that it kills the cell. This mechanism is used to kill cancer cells. Cytarabine is the first of a series of cancer drugs that altered the sugar component of nucleosides. Other cancer drugs modify the base .Cytarabine is rapidly delaminated in the body into the inactive uracil derivative and therefore is often given by continuous intravenous infusion.

Cytarabine is an antimetabolite antineoplastic agent that inhibits the synthesis of DNA. Its actions are specific for the S phase of the cell cycle to stop normal cell development and division. Cytosine arabinoside is an antimetabolic agent with the chemical name of 1 -arabinofuranosylcytosine.

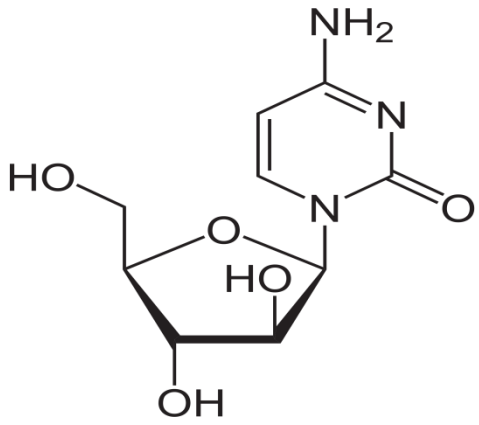


Figure.1 Structure of cytarabine.

Cytarabine (Cytosine Arabinoside-ARA-C) is one of the most effective agent in the treatment of nonlymphocytic leukemia in man. Mainly there are two main mechanism involved in its anti tumor activity. Firstly, cytarabine is transported across the cell membrane and activated to the form of 5'triphosphate, ara-triphosphate, which directly inhibits DNA polymerase by competing with the binding of 2-deoxycytidine 5'triphosphate(dCTP) to this enzyme. Secondly, incorporation of cytarabine into DNA, producing chain termination of polydeoxynucleotide elongation, leads to an inhibition of DNA synthesis. Cytarabine inhibit cytidylatedeoxycytidylated (Cyd- dCyd) deaminase and increase the activity of Cyd-dCyd kinase. Cytarabine is a S phase specific drug. Prolonged exposure of cells to cytotoxic concentration is critical to achieve maximum cytotoxic activity. Activity of cytarabine is decreased by its rapid deamination to the biologically inactive metabolite uracil arbinoside . Literature survey shows there are methods available for the quantization of cytarabine, it includes, High performance liquid chromatography alone. and liquid chromatography in combination with tandem mass spectrometry . Porous graphitic carbon chromatography/ tandem mass spectrometric was also used for the determination of cytarabine in mice plasma. Anion-pairing liquid chromatography/tandem mass spectrometric method is also reported for the analysis of cytarabine.

The present communication describes isocratic high performance liquid chromatographic method for estimation of Cytarabine, which would be a better choice for the quality control laboratory of the pharmaceutical industry. This study achieved satisfactory results in terms of formulation stability as well as behavior of cytarabine degradation under the influence of different stress conditions. It confirms the suitability of methods for the effectively separate drug from its degradation products. As indication of stability indicating assay methods. This method confirms suitability of selectivity, linearity, precision and accuracy under simple chromatographic condition. Adding to its advantage, the method is simple and time saving.

HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC)

High performance thin layer chromatography (HPTLC) is the most powerful advanced form of thin layer chromatography(TLC) and consists of chromatographic layers of utmost separation efficiency and the application of sophisticated all instrumentatial steps in the procedure include accurate sample application, standardized reproducible chromatogram development and software controlled evaluation. HPTLC is a concept that includes a widely standardized methodology based on scientific facts as well as the use of validated methods for qualitative and quantitative analysis. HPTLC meets all quality requirements for todays analytical labs, to increase the resolution and to allow more accurate quantitative measurements

Instrumentation

HPTLC method:

Stationary phase :

HPTLC is the most advanced from of modern TLC. HPTLC plates featuring small particles with a narrow size distribution which results in homogenous layers with a smooth surface to be obtained. HPTLC uses smaller plates (10 x10 or 10 x 20 cm).HPTLC plates provide improved insitu quantification and are used for industrial pharmaceutical densitometric quantitative analysis.normal phase adsorption TLC on silica gel with less with a less polar mobile phase, such as chloroform –methanol, has been used for more than 90% of reported analysis of pharmaceuticals and drugs.

MobilePhase:

The selection of mobile phase is based on adsorbent material used as stationary phase and physical and chemical properties of analyte. The mobile-phase systems are used based on their diverse selectivity properties..

Prewashing:

Plates are handled at the upper edge to avoid contamination. Plates are used without pretreatment unless chromatography produces impurity fronts due to contamination of the plate. For reproducibility and quantitative analysis, layers are often prewashed using 20 ml methanol.

Preparation of plate:

TLC plates can be made with the suitable apparatus. such layers do not adhere well to the glass support. Precoated plates use small quantities of very high molecular weight polymer as binder overcomes most limitations of a home- made layer.Precoated layers are reasonably abrasion resistant, very uniform in layer thickness, reproducible, preactivated, and ready to use. They are available with glass or aluminum or polyester support. Aluminum foil plates are the less expensive to buy, cheaper, can be cut, and therefore easy to carry around or transport or mail. Glass plates are the best for highest quality of results. Most often, layers containing a fluorescent indicator F 254 are used. This enables the visualization of samples in a UV cabinet very simply, instantly, and in a nondestructive manner.Commonly used for the size of plates in TLC is 20×20 cm and in HPTLC 20 × 10 cm or 10 × 10 cm is widespread.Steps involved in HPTLC procedure

- Sample Application
- Chromatogram Development
- Derivatization
- Evaluation: Detection
- Evaluation: Documentation

DRUG EXTRACTION FROM RAT BLOOD

Standard stock solution

Accurately weighed about quantities of 10 mg of cytarabine were transferred into 10 ml clean dry volumetric flask, and 3/4th volume of diluent was added, sonicated for 30 minutes , made up to the final volume with diluent.

↓

Take 15 ml 5 centrifuge tubes and 10,20,30,40,50 µl of stock solution in each test tube.

↓

Add drug free plasma to provide calibration standards of 1000,2000,3000,4000.5000 ng/ spot.

↓

For this carried out protein precipitation and liquid liquid extraction by using methanol : Acetonitrile (3.0:0.1 v/v).

↓

By vigorous vertex centrifugation can done speed 5000 rpm up to 10 minutes.[2]

↓

The organic phase was recover and evaporate to dry on hot plate.

↓

This residual mass was constituted with methanol and analyse on HPTLC .[2]

Method development

Method validation was performed as per the ICH guidelines. The developed method was validated for the following parameters.

- System suitability
- Linearity
- Accuracy
- Precision
- Robustness

SYSTEM SUITABILITY TESTING:

System suitability is a test should be carried out to verify the analytical system is working properly and can give accurate and precise results. Standard solutions were prepared as per the test method and injected into the chromatographic system.[1]

LINEARITY:

The linearity of analytical method was carried out to check 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.[1] Different levels of standard solutions were prepared and injected into the HPTLC and the chromatograms were recorded.

Procedure:

Preparation of standard stock solution (10mg cytarabine/10ml methanol)

Accurately weighed about quantities of 10 mg of cytarabine were transferred into 10 ml clean dry volumetric flask, and 3/4th volume of diluent was added, sonicated for 30 minutes and made up to the final volume with diluent.

Preparation of serial dilutions

Different volumes of solution is indication for different concentrations of solution like multilabile calibaration.

Acceptance criteria :

Correlation coefficient should not be less than 0.993.

ACCURACY:

The accuracy of an analytical method is the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

Accuracy was performed by following direct comparison method. The study was performed by giving same standard concentrations three times of known amounts of studied drugs. The accuracy of an analytical method should be established across its range. Finally, the final volume made up with diluent and mixed well. The resulting mixtures were analyzed by the proposed HPTLC method at 257 nm. The excellent mean recoveries and standard deviation suggested good accuracy results of the propose method.

Procedure:

Sample solutions prepared were injected three times into the chromatographic system and recorded the chromatograms.

Acceptance criteria:

The % RSD for the volume of three standard injections results should not be more than 2%.

PRECISION

The precision of an analytical method is a measure of the random error and is defined as the agreement between replicate measurements of the same sample. It is expressed as the percentage of relative standard deviation (%RSD) of the replicate measurements.

METHOD PRECISION:

i) Intradayprecision

Preparation of sample solution

Accurately weighed about quantities of 10 mg of cytarabine were transferred into 10 ml clean dry volumetric flask, and 3/4th volume of diluent was added, sonicated for 30 minutes and made up to the final volume with diluent.

Procedure

For precision studies 3 replicate injections of cytarabine standard were performed. %RSD was determined for volumes.

Acceptance criteria

The % RSD for the area of three standard injections results should not be more than 2%.

ii) Inter_day_precision

Preparation of sample solution

Accurately weighed about quantities of 10 mg of cytarabine were transferred into 10 ml clean dry volumetric flask, and 3/4th volume of diluent was added, sonicated for 30 minutes and made up to the final volume with diluent

Procedure

For inter day method precision studies 3 replicate injections of cytarabine sample were performed. %RSD was determined for volumes of cytarabine.

Acceptance criteria

The % RSD for the area of three standard injections results should not be more than 2%**.**

ROBUSTNESS:

The robustness of an analytical method 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.

The robustness of the proposed method was determined by analysis of aliquots from homogenous lots by differing physical parameters like volume, mobile phase volumes. The standard solution and sample solution were injected into the chromatograph at varied conditions of flow ± 5 ml/min, mobile phase volumes ±5ml. .

OPTIMIZED CHROMATOGRAPHIC CONDITIONS:

Chromatographic mode	Optimized conditions
HPTLC system	HPTLC-Aetron
Stationary phase	Precoated silica plates (10×10cm)
Mobile phase	2-butanone: acetone: water(65:20:15)
Detection wavelength	257 nm
Injection volume	20 µl

METHOD VALIDATION

System suitability:

Parameters	Cytarabine
Linearity range[a]	1000 -5000
r^2	0.998
Slope	0.013
Intercept	36.39

Data interpretation

From the results tabulated above, it is observed that the system suitability parameters are within the acceptable limit and meet the requirements of method validation**.**

Linearity:

Acceptance criteria: Correlation coefficient should be NLT 0.993.

Table 1 : linearity of cytarabine.

s.no	Concentration(ng/ml)	volume
1	1000	49.63
2	2000	62.18
3	3000	76.82
4	4000	90.20
5	5000	101.85

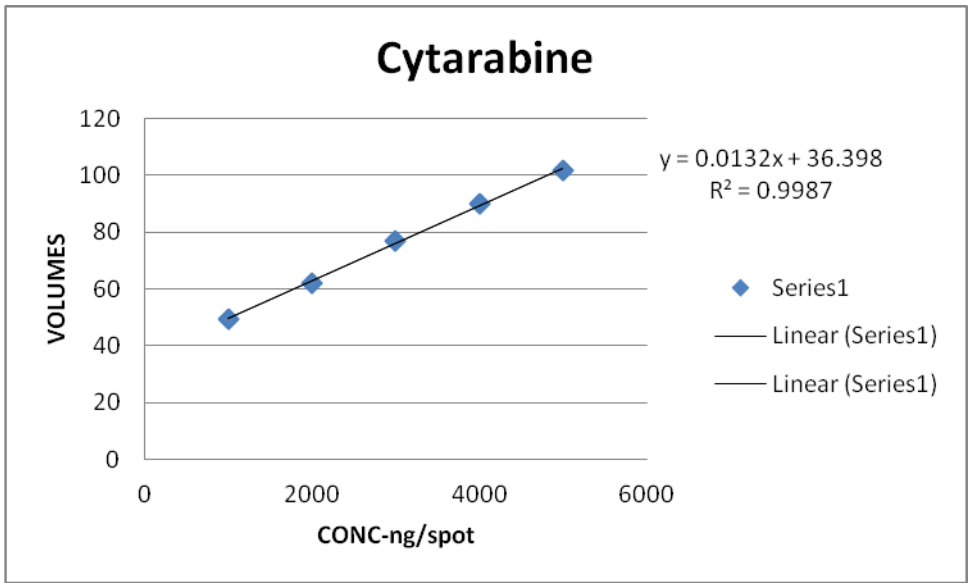


Figure.5 linearity of cytarabine.

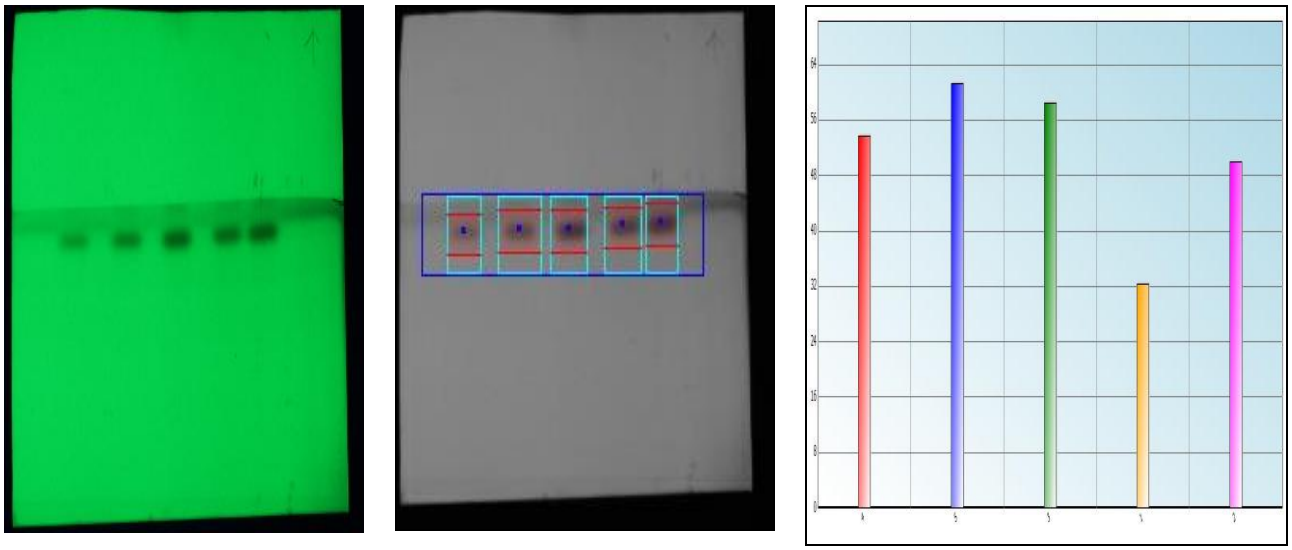


Figure.6 :TLC plate for cytarabine linearity.

Inference:

Calibration curve was plotted by taking concentration on x-axis and volumes on y-axis. Cytarabine shows linearity in the range of 1000 to 5000 ng/ml with a regression coefficient of 0.998

Accuracy:

Acceptance criteria:

The % RSD for each level should be NMT 2 .

Accuracy

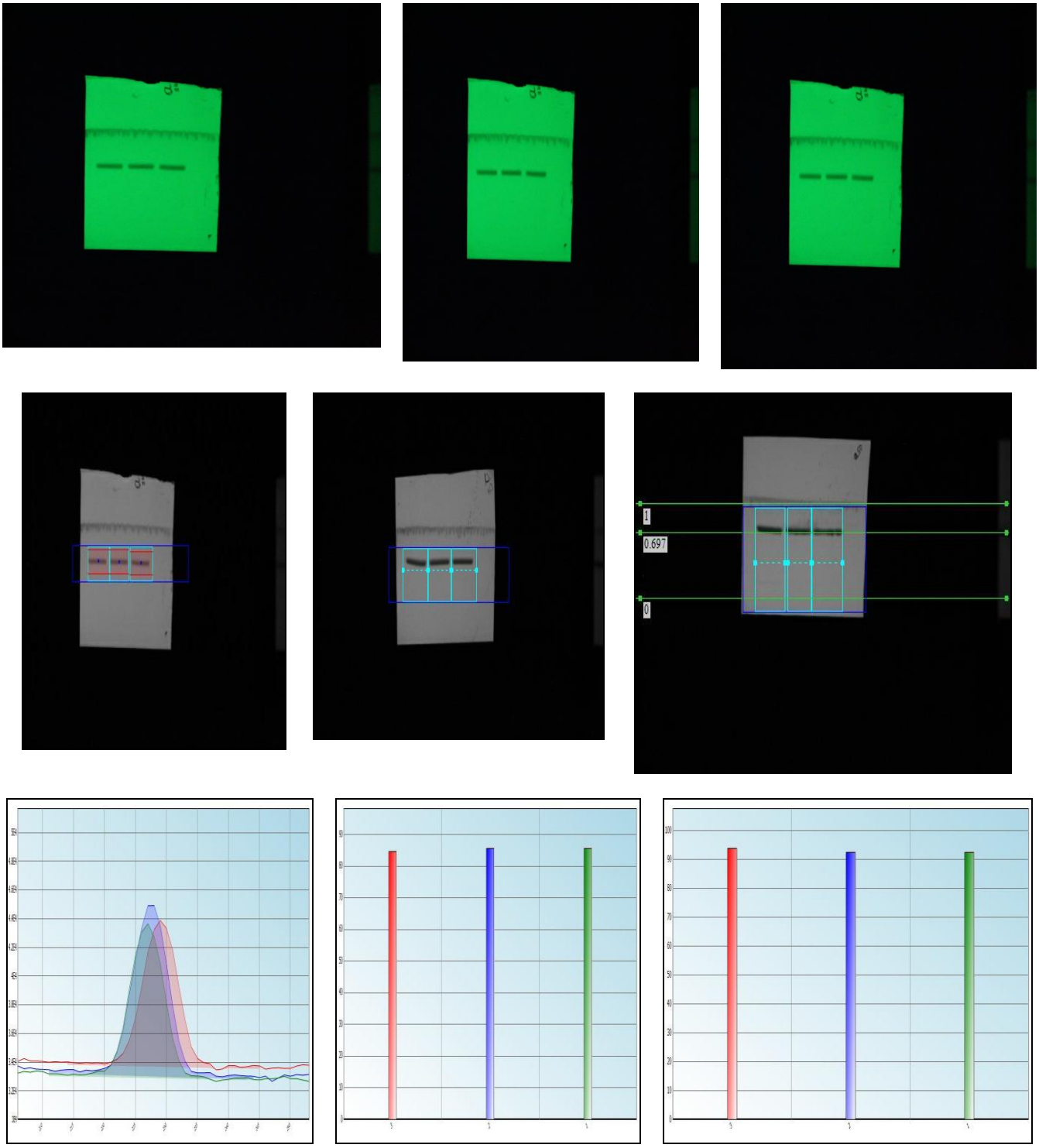


Figure.7 :TLC plate for cytarabine accuracy.

Table 2 : Accuracy of cytarabine.

concentration	Rf value	Area	Volume	Average	SD	%RSD
1000	0.571	1888	49.03			
1000	0.551	1716	49.48			
1000	0.510	1891	49.14	49.216	0.23459	0.476651
3000	0.733	1364	85.41			
3000	0.733	1176	85.41			
3000	0.747	1220	84.41	85.0766	0.57735	0.67862
5000	0.722	1368	95.23			
5000	0.722	1125	95.42			
5000	0.705	1357	95.54	95.396	0.156312	0.163

Inference:

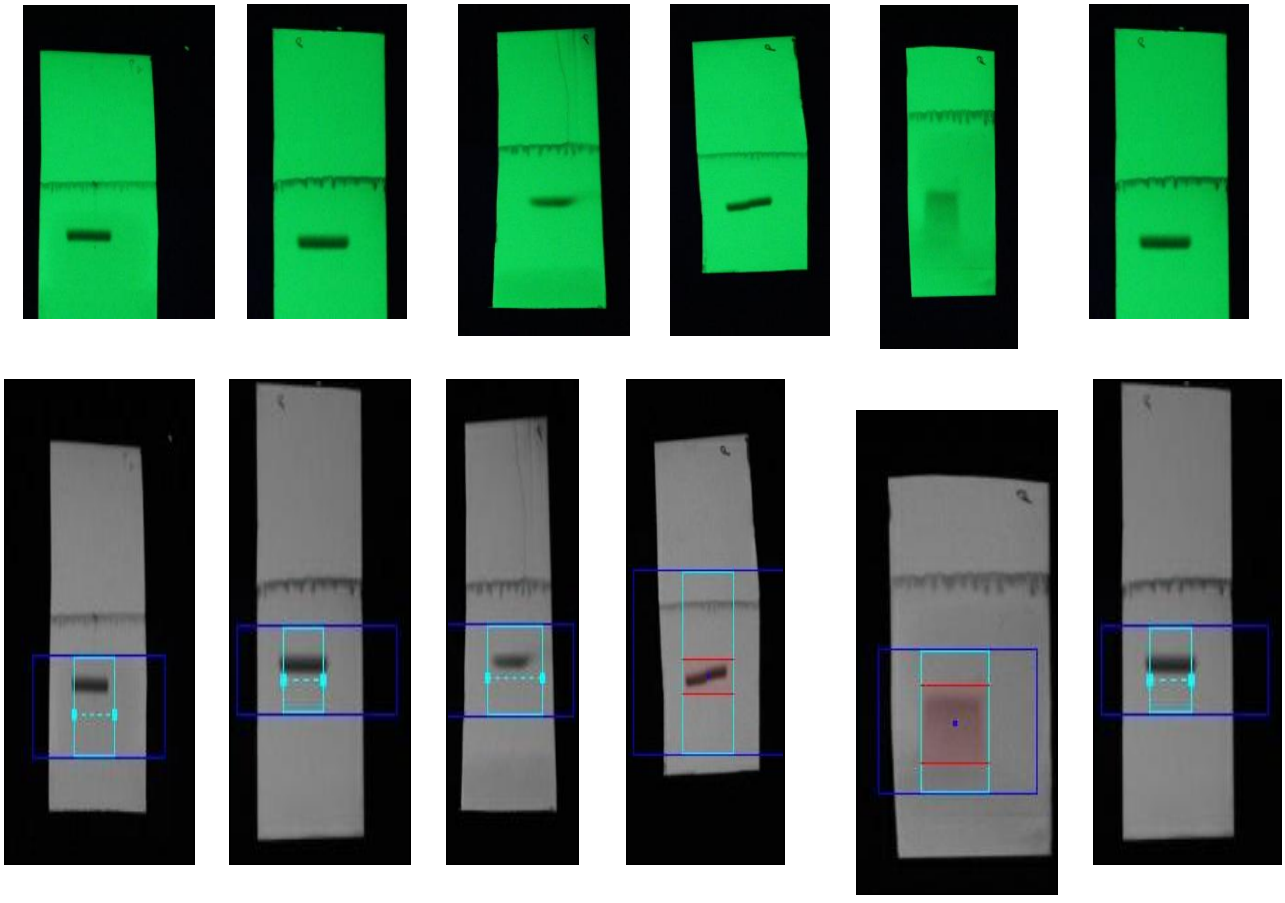
The accuracy of the method was determined by measuring drug recoveries. The mean recovery was found to be 96.50 –98.53 % which is within acceptable range. The excellent mean recoveries and standard deviation suggested that the good accuracy of the proposed method.

Method Precision:

Acceptance Criteria: The RSD should be NMT 2.0 %.

Data interpretation:

The volumes for cytarabine obtained from six replicate injections are consistent as evidenced by the values of relative standard deviation. Hence it can be concluded that the system precision parameter meets the requirement of method validation.



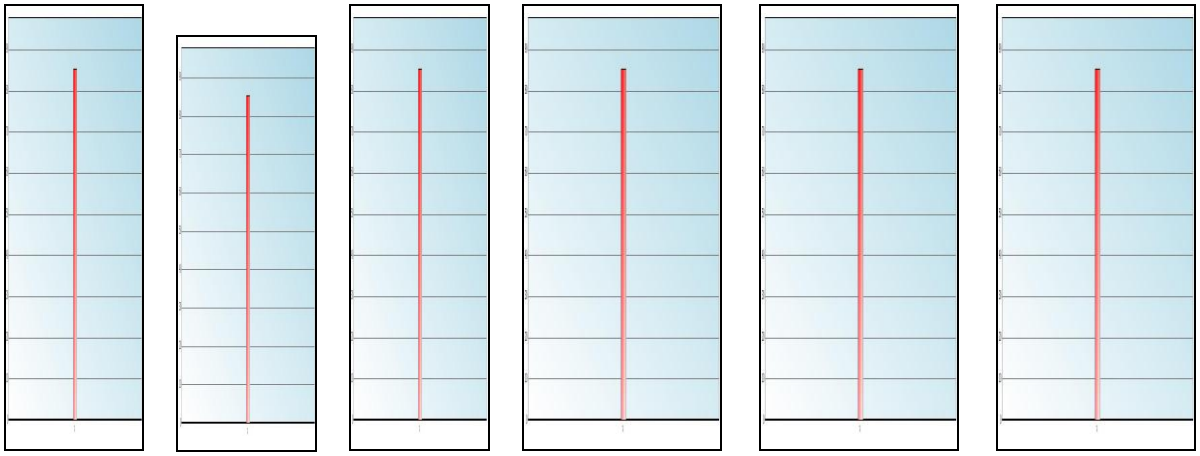


Figure.7:TLC plate for cytarabine precision.

Table 3 : Precision of cytarabine.

s.no	concentration	volume	Average	SD	%RSD
Interday	3000	85.07			
1					
2	3000	86.99			
3	3000	85.44	85.8333	1.6186	1.1867
Intraday					
1	3000	85.66			
2	3000	86.02			
3	3000	85..36	85.68	0.33045	0.385684

Robustness:
Chromatogram of change in spray volume of sample 25 ml

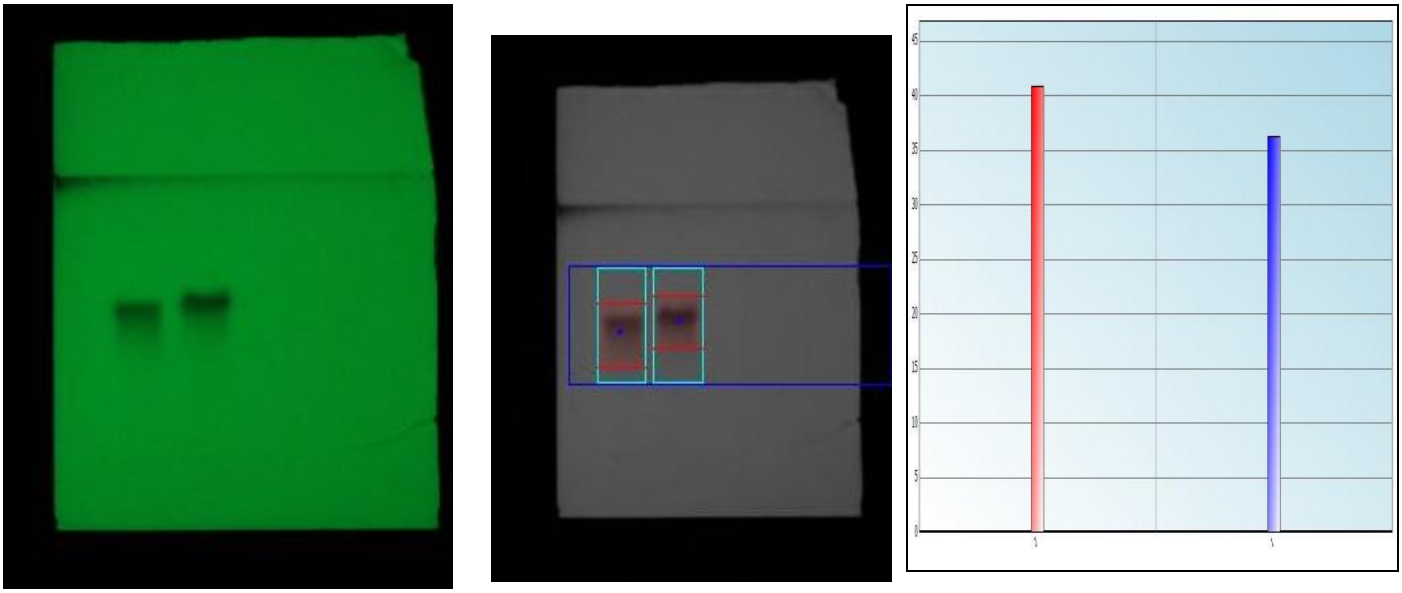


Figure.8 :TLC plate for cytarabine robustness.

Table 4 : Robustness of cytarabine volume changed.

Concentration	Area	RF value	volume	Average	SD	%RSD
2500	1139	0.439	36.35			
2500	1116	0.439	36.22	36.285	0.091924	0.283339

35 ml

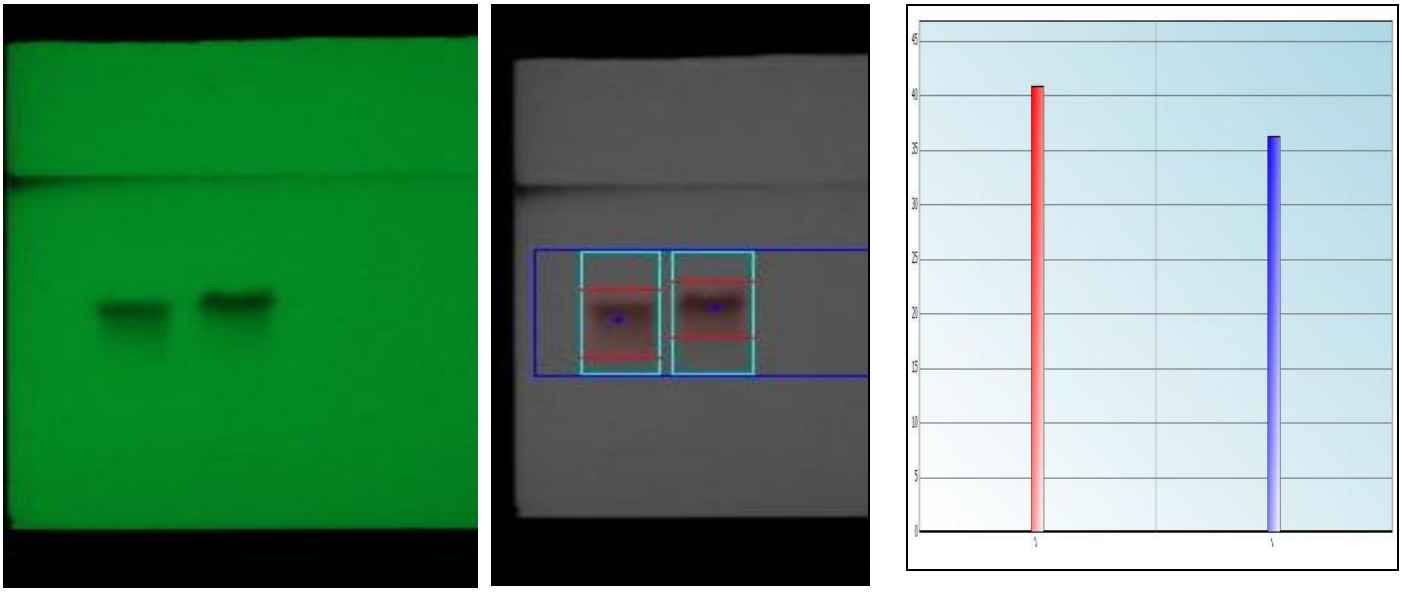


Figure.9 :TLC plate for cytarabine robustness.

Table 5 : Robustness of cytarabine volume changed.

Concentration	Area	RF value	volume	Average	SD	%RSD
3500	960	0.526	40.72			
3500	967	0.531	40.68	40.65	0.098995	0.24353

Chromatogram of change in Mobile Phase ratio

Mobile phase ratios

2-butanone : acetone : water (35:5:10)

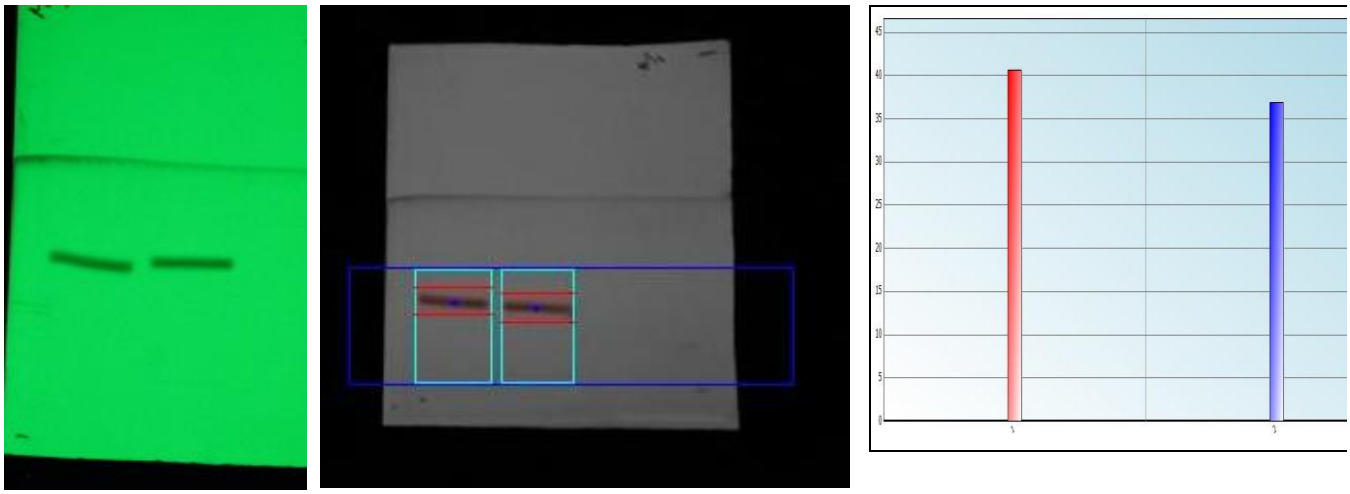


Figure.10 :TLC plate for cytarabine robustness.

Table 6 : Robustness of cytarabine mobile phase ratios changed.

Concentration	Area	RF value	Volume	Average	SD	%RSD
3000	960	0.659	36.52			
3000	950	0.707	36.09	36.26	0.367696	1.014053

2-butanone : acetone : water (25:10:15)

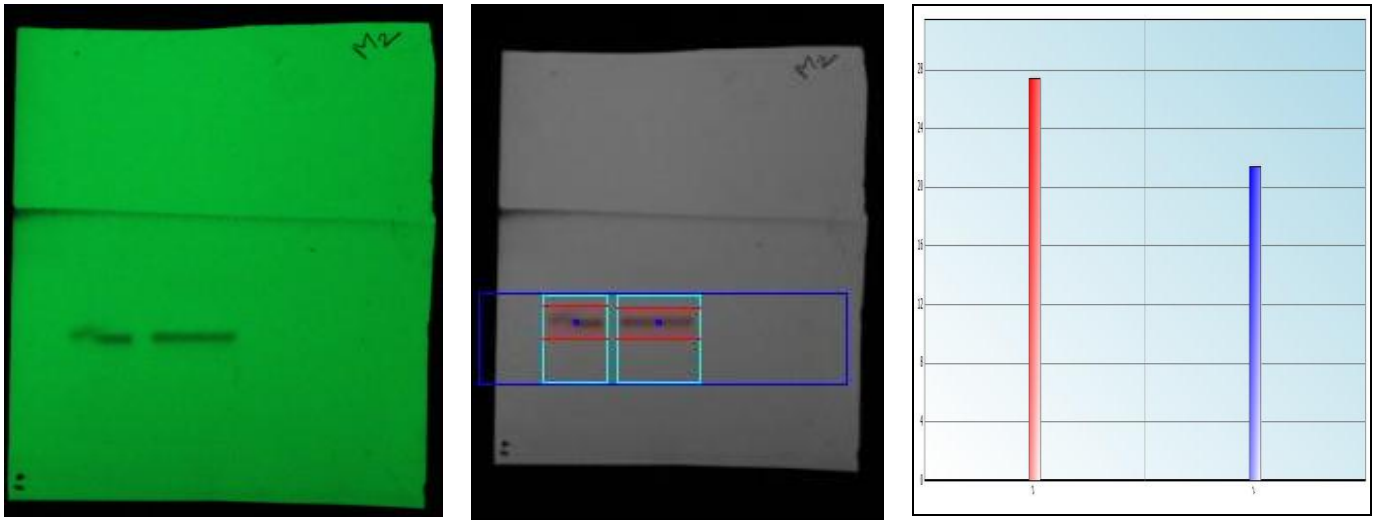


Figure.11 :TLC plate for cytarabine robustness.

Table 6 : Robustness of cytarabine mobile phase ratios changed.

Concentration	Area	RF value	Volume	average	SD	%RSD
3000	924	0.667	22.76			
3000	1080	0.667	22.40	22.58	0.254558	1.127362

2-butanone : acetone : water (30:12:8)

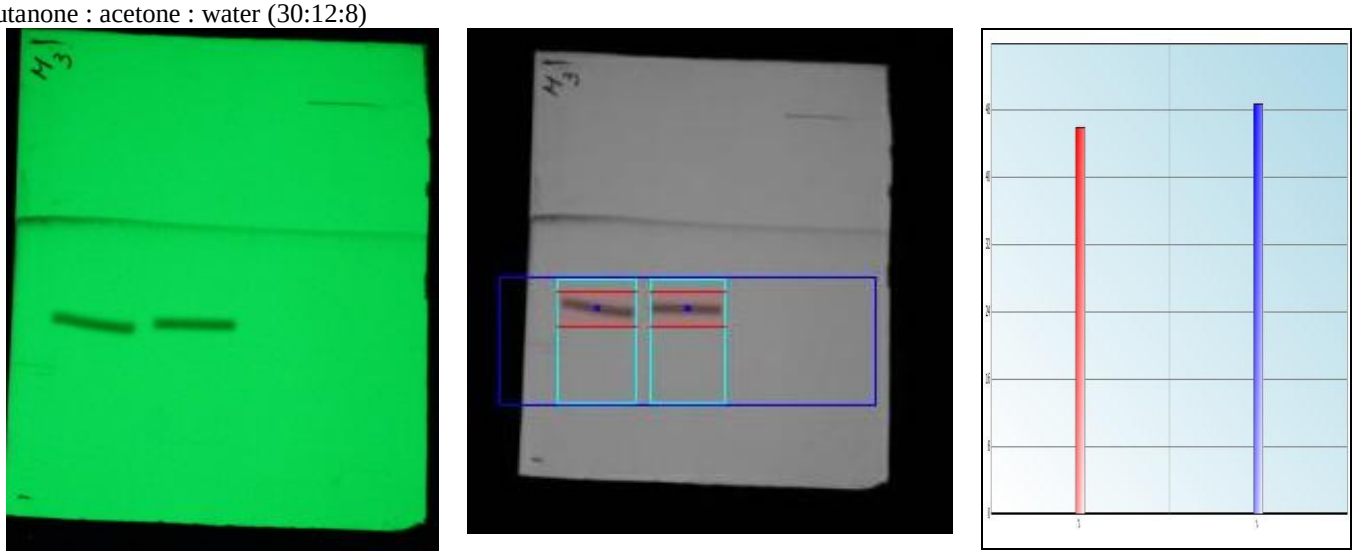


Figure.12 :TLC plate for cytarabine robustness.

Table 6 : Robustness of cytarabine mobile phase ratios changed.

concentration	Area	RF value	Volume	Average	SD	%RSD
3000	1144	0.75	48.59			
3000	1056	0.75	48.06	48.325	0.374767	0.775513

RESULTS AND DISCUSSION

A simple high-performance thin layer chromatographic method was developed for the quantification of cytarabine. using silica as stationary phase (10x10cm) equilibrated with mobile phase containing combination of 2-butanone: acetone:water in ratio of 65:20:15 v/v/v .

❖The Rf values was found to be 0.495, 0.563,0.733,0.662,0.705 respectively .the plate was scanned quantified at 254 nm. .

❖Calibration curve for each drug was plotted using to parameters concentration v/s peak height. The linearity range of cytarabine were 1000,2000,3000,4000,5000 ng/spot.The validation of the developed method was performed in accordance with ICH guidelines (Q_2B validation of analytical procedure methodology)

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