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Stability Indicating Reverse Phase-HPLC Method for the Estimation of Doxorubicin in Bulk and Pharmaceutical Dosage Forms

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Abstract: The current research goal was to develop a novel specific method for the quantitation of the Doxorubicin. The method development was done by using a High-Performance Liquid Chromatography (HPLC). The extensive method development was conducted to identify a right combination of chromatographic conditions and validated as per the regulatory guidelines. The current method overcome the disadvantages of complex sample preparation, long runtime and improved peak shape. The simple combination of organic modifier and buffer (40:60) used to elute the Doxorubicin in isocratic mode. The shorter run time of the method was achieved by optimizing the flow rate of 0.8mL per minute along with L1-octadecyl chemistry column. The doxorubicin peak was detected by using a sensitive Ultra violet detector at 254nm. The method was completely wetted with all the key validation parameters likewise suitability, precision, selectivity, linearity, robustness and recovery. The purity threshold of more than 990 proves that the method is free from any possible interferences and the linearity was established at 5 different levels with a correlation coefficient 0.999. The overall %RSD (relative standard deviation) of mean recovery was 1.1%. The marketed commercial formulation of Doxorubicin injection was tested on the developed method to confirm the suitability of the method for both bulk active substance and pharmaceutical formulations. Based on the experimental outcome method can be regarded as inventive, short run time, specific to quantitate Doxorubicin.

Keywords: Doxorubicin, Reverse Phase-HPLC, Estimation, Specific, Forced Degradation, Regulatory Guidelines, Validated.

INTRODUCTION

The drug product Doxorubicin commercialized and marketed under the name of Adriamycin. The drug is produced by bacterial species through semi synthetic chemical reactions. It is an anthracycline antitumor antibiotic, which is closely related to the natural product daunomycin and like all anthracyclines. It's mainly categorized to treat cancer and works by intercalating DNA (deoxyribonucleic acid), having a adverse effect heart injury. It is commonly used in the treatment of a wide range of cancers as a standalone drug and in combination. It's knowing to produce a synergistic effect in combination with other drugs. Its mainly used to treat many cancers like leukemia and tumors. [1-2].

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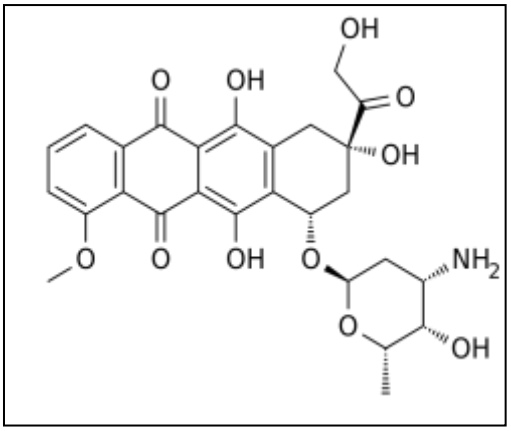

Fig. 1: Chemical structure of Doxorubicin

The key attributes of any analytical assay method developed must be stability indicating nature [3]. This key characteristic was achieved by performing many analytical experiments. The important activities that needs to be done has been explained by many of the scientific journals, review articles and drug specific articles. The major studies are forced degradation, injecting the blank, injecting the excipients and spiking known available impurities [4-5]. If the impurities not available while developing the methods, the best way to achieve the specificity is by performing the stress study. The purpose of this study is generating a potential degradant, which are expected to be occur during shelf life. There are several stability guidelines available to explain the quality attributes of the developed methods. Out of which, the international council for Harmonization (ICH) has well accepted guideline worldwide. This guideline is also named under trip late, which aligns the regulatory bodies of United States, Europe and Japan. These key team members contribute for the major technical and scientific discussion for Human consumption drug substances and products. The ICH has set if guidelines which must be followed while developing a drug substances and drug product to the respective regions. The Quality guideline Q1 plays a vital role stability testing of the New drug products and New drug substances. Which is further emphasize more on importance of photostability testing, evaluation of stability data to access the shelf life of the products. The crucial requirements of the method were defined by the ICH Q2 guideline. This guideline explains the mandatory parameters that need to be performed to show that the developed method can be used for the intended purpose. Also, the guideline explains the specification and acceptance criterial required for any method validation [6-11].

The various research articles demonstrate that analytical method such as Ultra Violet-Spectrophotometric [12] and Reverse Phase-Liquid chromatographic (RP-LC) methods for active and marketed dosage forms [13-16]. The mass spectroscopic [17-18] methods also available to determine the drug content in the plasma samples. The LC methods also been developed along with combination of drugs and same has been validated in many scientific literatures [19-20]. The efforts also made to determine the content of doxorubicin by using fluorescence derivatization in plasma samples [21].

The current research activity attempts, to advance a stability indicating and validated a novel, fast, precise, accurate, reliable, selective and specific reverse phase-LC method for quantitation of Doxorubicin in active substances and marketed commercial dosage form.

MATERIALS AND METHODS

Chemicals and Reagents: Doxorubicin was obtained from Shilpa Medicare Pvt. Ltd. and all the experimental reagents used were technical grade. The organic modifiers used for the study were Acetonitrile, HPLC grade supplied from Merck. The milli Q water was used for the buffer preparation.

Instrumentation: The development and method suitability for the intended purpose were executed by using Diode array detector (PDA)/Ultra violet (UV) Detector and the HPLC having a model number of Ultimate CAD 300 (Thermo scientific). The additional laboratory accessories used to prepare sample and mobile phase were pH meter, vortex mixer, ultrasonicator, water bath, micro and analytical weighing balance were used for the study.

Chromatographic mode: The data analysis software used was Chromeleon 7.2 version, RS4 with RP-HPLC.

Method development: The extensive method development was done to choose a suitable column chemistry. The stationary phase selection was done systematically by selecting a diverse chemistry

column like C8 and C18 with array of carbon loading. Also, the development was performed by using a range of different particle size like 3µ and 5 µ. The suitable column was opted finally due to its proficiency in separating compounds with better peak shape and ideal tailing. The column was supplied from Thermofischer. While developing the method the different range of pH like acidic, basic and neutral buffers with different pKa values were tried. The mobile phase with pH having 3.0 found to be suitable for better peaks shape and resolution of all potential degradants from forced degradation studies. The column temperature of 40°C found to elute a peak around 2.0min. Hence, the temperature was opted to be 25°C, which assisted in peak retention, better shape as well moderate system back pressure. The injection volume between 10µL to 50µL were tried, the higher injection volume having a peak broadening and poor peak shape. The injection volume 25µL found to be optimal and suitable. The final optimized method was considered for the validation as per the ICH regulatory triplite guideline.

Stationary phase: The Column chemistry with C-18, 150 mm x 4.6mm, 5µm (Synchronis) was selected as a suitable column, upon method development.

Buffer Preparation: Weighed accurately 1.4g of sodium phosphate monobasic into 1Ltr of water, dissolved with the aid of sonication. Diluted orthophosphoric acid was used to adjust the pH to 3.0 (±0.05), Further buffer solution was degassed and filtered through suitable 0.45µm filter (Nylon).

Preparation of Mobile Phase composition: Prepared, filtered and degassed mixture of Acetonitrile and Buffer volumetrically in the ratio of 40:60.

Diluent solution: Use 100% Acetonitrile as a diluent.

Standard preparation: The 12.49mg of Doxorubicin accurately weighed and transferred into a 50.0 mL flask. Dissolved and diluted with the diluent. The above stock solution was diluted to 20mL with diluent.

Retention time (RT) of Doxorubicin: The RT of Doxorubicin were estimated by injecting 25µL of working standard solution at the flow rate of 1ml per minute into HPLC chromatograph having detection wavelength of 254 nm. The chromatography was finalized based on the extensive method development and system suitability parameters and taken forward for validation to confirm the further stability indicating nature.

ANALYTICAL PROCEDURE VALIDATION

In order to ensure the performance characteristics of the of developed RP-HPLC method the validation was performed using parameters as per the ICH guidelines [10].

System suitability: The developed method was determined by injecting 25 µL of working standard solution at 0.8 ml/min into HPLC chromatograph using UV detector at 254 nm. From the chromatograms obtained data such as tailing factor, theoretical plates and the % relative standard deviation of Doxorubicin were calculated from five standard injections.

Specificity: The study was conducted by injecting blank (1 injection), standard solution (5 injections) into the Chromatographic system. Recorded the RT (retention time) of Doxorubicin peak from standard solution. The specificity of developed RP-HPLC method was studied using by stressing the active drug substances under different conditions such as acid, alkali and peroxide.

Specificity and selectivity by degradation study of Doxorubicin was performed to achieve the stability inducting nature of the method. This was proved by injecting the highly degraded samples in to the chromatographic system. Specificity and selectivity ensure the peak of interest is free from any potential impurities.

Untreated sample (Control): Accurately weighed 20.0 mg of Doxorubicin active substance transferred in to a 50.0 mL volumetric flask, dissolved and diluted up to the mark with diluent. Further diluted the solution to achieve a concentration of 0.05mg/mL.

Acid treated sample: (0.1 N Hydro chloric acid): Accurately weighed 20.08 mg of Doxorubicin active substance in to a 50 mL flask, spiked 2ml of 0.1N Hydrochloric acid and placed on bench top at controlled room temperature for about 20 minutes. The acid treated sample was dissolved and diluted up to the mark with diluent. Further diluted the solution to achieve a concentration of about 0.05mg/mL. The samples were not neutralized to avoid the secondary degradation by the addition of acidic agent.

Alkali treated sample: (0.1 N Sodium Hydroxide): Accurately weighed 20.41 mg of Doxorubicin active substance in to a 50 mL flask, spiked 2ml of 0.1N sodium hydroxide and placed on bench top at controlled room temperature for about 20 minutes. The base treated sample was dissolved and diluted up

to the mark with diluent. Further diluted the solution to achieve a concentration of about 0.05mg/mL. The samples were not neutralized to avoid the secondary degradation by the addition of acidic agent.

Peroxide treated sample: (3.0% w/v Hydrogen Peroxide): Accurately weighed 20.36 mg of Doxorubicin active substance in to a 50.0 mL volumetric flask, added 2ml of 3.0% Hydrogen peroxide and placed on bench top at controlled room temperature for about 20 minutes. The oxidized sample was dissolved and diluted up to the mark with diluent. Further diluted the solution to achieve a concentration of about 0.05mg/mL.

Linearity: The study was established by preparing a series of dilutions in concentration range of 12-63 ppm of Doxorubicin, 25µl of each solution was injected into HPLC chromatograph. Calibration curve was constructed by plotting the peak area versus the drug concentration in ppm.

Precision: The method Precision attribute was established by preparing a active substance of a same batch at target working concentration (48 PPM).

Solution stability of the doxorubicin in selected diluent was achievd by injecting the standard solutions (0.05mg/mL) at 0 (initial), 6, 12 and 24 Hr. interval time.

Accuracy was performed to measure the closeness of the experimental values to the actual number of spiked samples. The recovery was established at ± 25% of the working concentration. The triplicate samples were prepared at 75%, 100% and 125% of the target concentrations.

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. In this study the robustness was performed by varying the flow rate by ±0.2mL, temperature by ±5.0°C and mobile phase composition by ±10%.

Assay of Doxorubicin in marketed Sample: The selected marketed formulation Doxorubicin hydrochloride injection-IP 50mg/25mL supplied from Fresenius Kabi was taken for the study. The marketed sample vial was reconstituted with 25mL of purified water. Further diluted accurately measured 5mL of the dissolved solution to 200mL. Diluted up to the mark with diluent, vortex to mix the solution Filtered through 0.45µm filter and vial for HPLC analysis.

RESULTS

An innovative stability indicating Reverse Phase-HPLC method for the estimation of Doxorubicin in active drug substance and pharmaceutical dosage forms was developed and validated.

System suitability: The conditions of developed method were given in Table 1 and chromatogram obtained were presented in Fig. (2). The requirements for SST (system suitability parameters) were met and result were presented in Table 2.

Specificity: Developed stability indicating RP-HPLC method was found to be selective and specific and results were tabilated in Table (3) and chromatogram of blank was presented in Fig. (3). The representative chromatograms of acid treated, base treated and Oxidative degradation samples were presented in Fig. (4), (5) and (6). Further the peak of interest (Doxorubicin) was proved through purity angle, which must be more than 990. All the major degradations were well separated from Doxorubicin peak.

Linearity: The method was found to be linear upon the concentration between of 12 to 63 ppm with regression coefficient 0.999 and data were presented in Table (4) and standard calibration graph was presented in Fig. (7).

Precision: The %RSD values of 6 preparation at 100% working concentration were found to be less than 2.0%. Which indicates the method is more precise and the results were presented in Table (5). The demonstrative chromatogram was presented in Fig. (8).

Solution stability Data: The solution stability up to 5°C was established up to 24 Hr. The solution stability graph was present in Fig. (9). The Doxorubicin satisfactory a good solution stability data up to 24 Hr. with the % RSD of less than 2.0.

Recovery: The method was found to be accurate and results were presented in Table 6. The over all recovery of the method found to be 101.1% and the % RSD of 1.1.

Robustness: The data obtained from the robustness study by varying flow rate, temperature and mobile phase composition was presented in the Table 7.The illustrative chromatogram of the study was presented in the Fig. (10), (11), (12) and (13) respectively.

Marketed sample Testing: The developed methods was used to test the marketed sample of Doxorubicin Injection IP, sample purchased from Fresenius KABI. The assay of Doxorubicin was found to be 98.8% and results were presented in Table 8. The chromatogram of Doxorubicin in marketed sample were presented in Fig. (14).

Table 1: Developed method specification

Column	: Synchronis C18, 150mm x 4.6mm, 5µm.
Flow rate (ml/min)	: 0.8
Wavelength (nm)	: 254
Column Temperature (°C)	: 25
Auto Sampler Temperature	: 5
Injection Volume (µL)	: 25
Runtime (minutes)	: 6
Needle wash	: Acetonitrile: water
Elution	: Isocratic mode

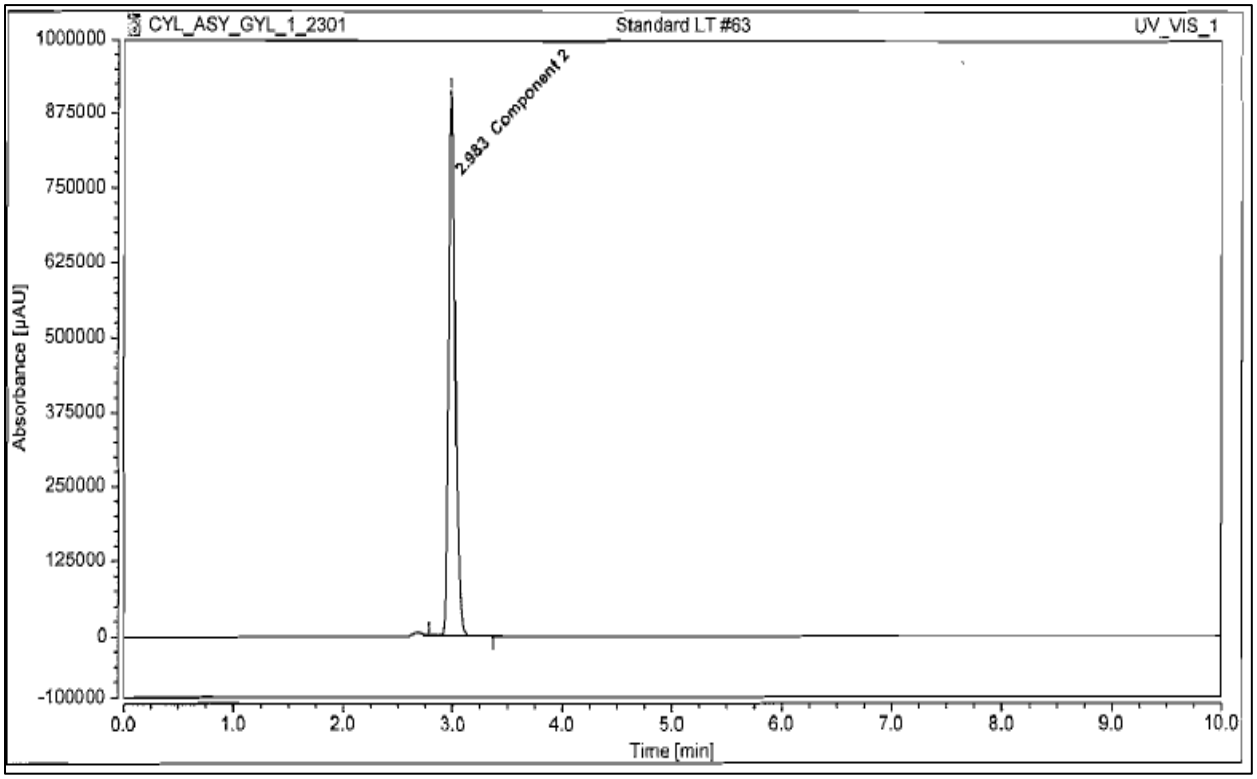

Fig. 2: Representative chromatogram of system suitability

Table 2: System Suitability Test (SST) data

Set No.	System Precision	
1	3779401.491	Acceptance criteria
2	3783104.267	
3	3783127.710	
4	3783140.757	
5	3769209.218	
Mean	3779597	
% RSD	0.16	Not More Than 2.0
Tailing	1.50	Not More Than 2.0
Theoretical Plate	12924	Not More Less Than 2000

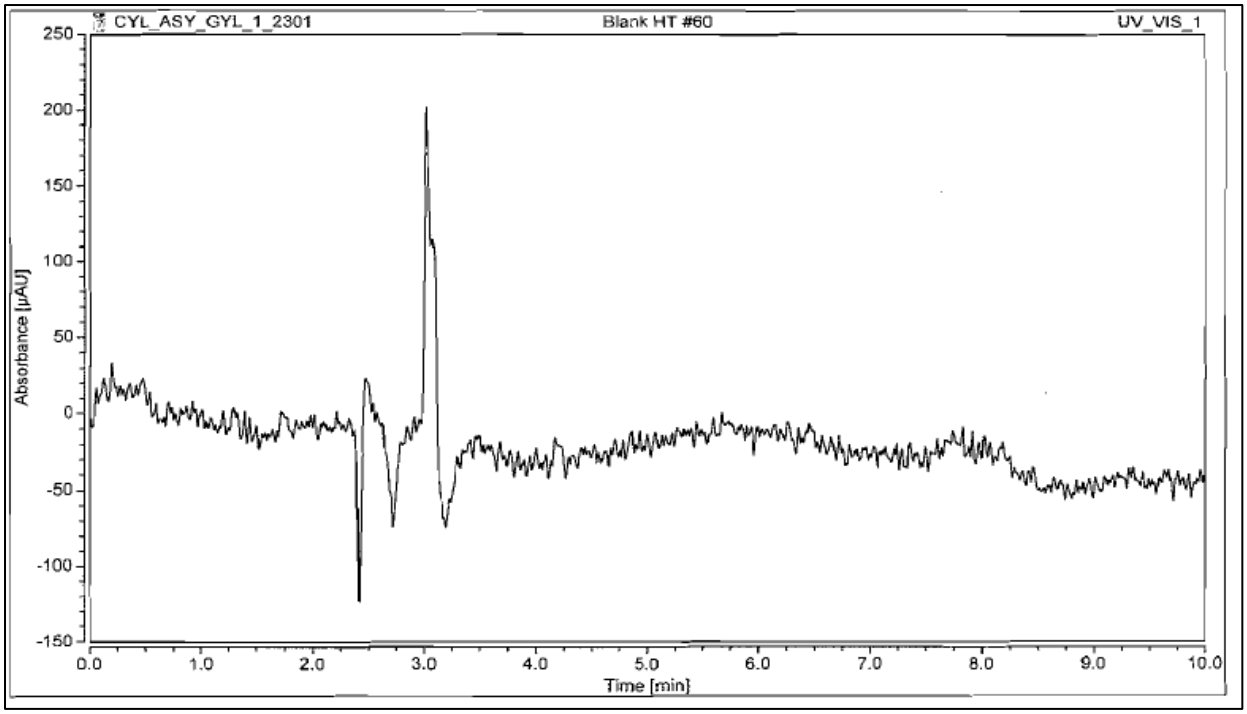

Fig. 3: Representative Chromatogram of Blank

Table 3: Specificity studied data

Stress Condition	Peak Purity**	Purity Match	% of Assay
Control sample (Untreated)	P	1000	100.0%
0.1 N Hydro chloric acid treated sample	P	1000	69.3%
0.1 N Sodium Hydroxide treated sample	P	999	47.4%
3.0%w/v Peroxide treated sample	P	1000	64.1%

Peak Purity **: 'P' indicates Doxorubicin Peak is free from any interference. The purity match factor above 990 is indicator of peak purity.

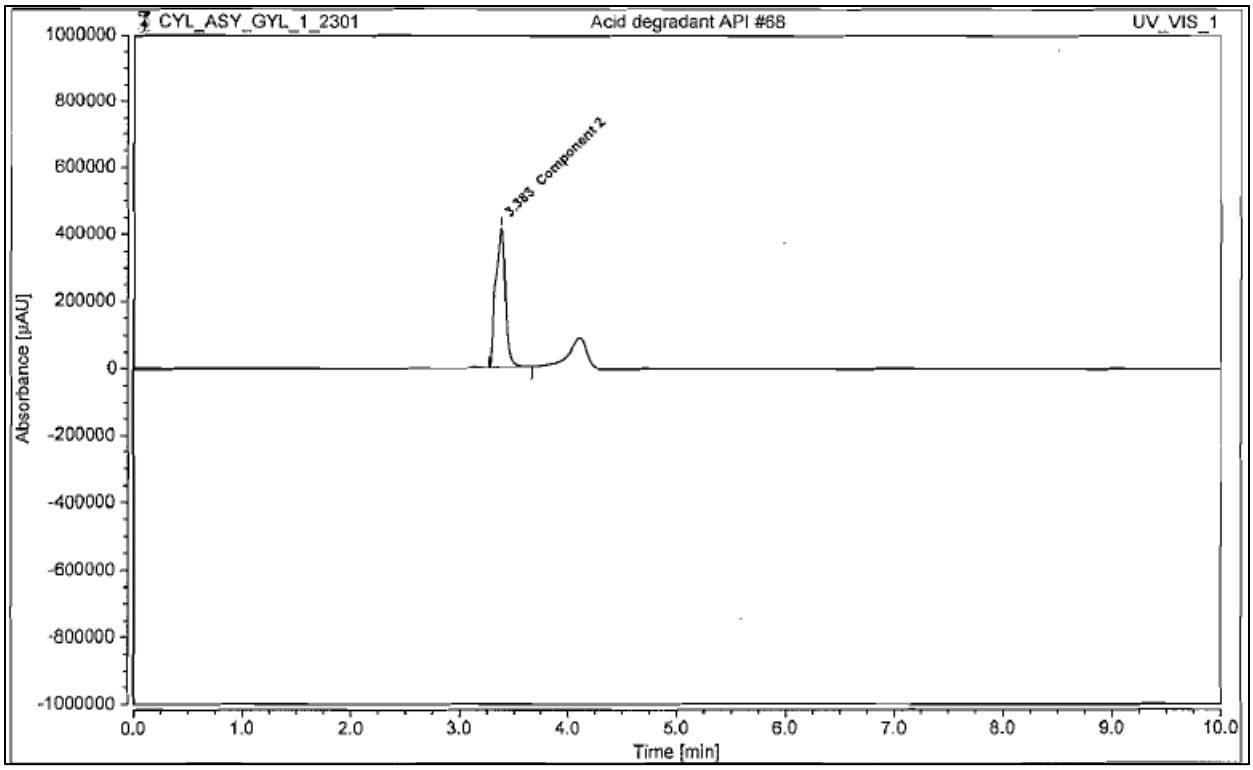

Fig. 4: Representative chromatogram of Acid Forced degradation

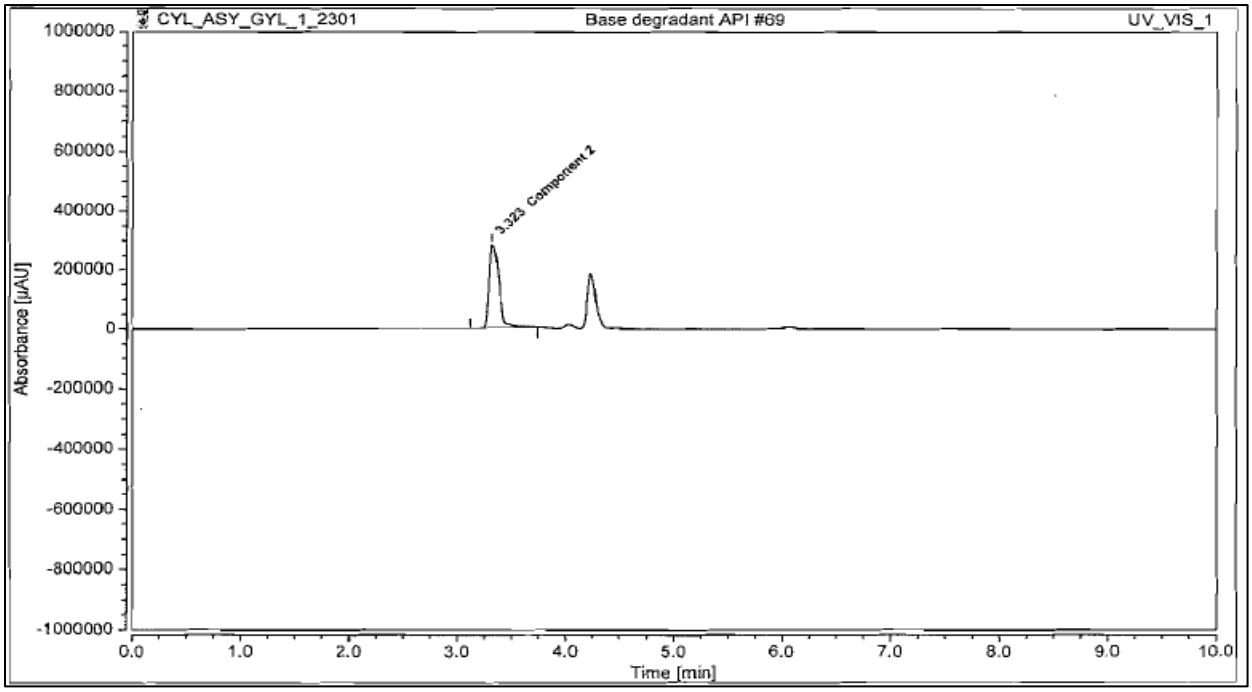

Fig. 5: Representative chromatogram of Base Forced degradation

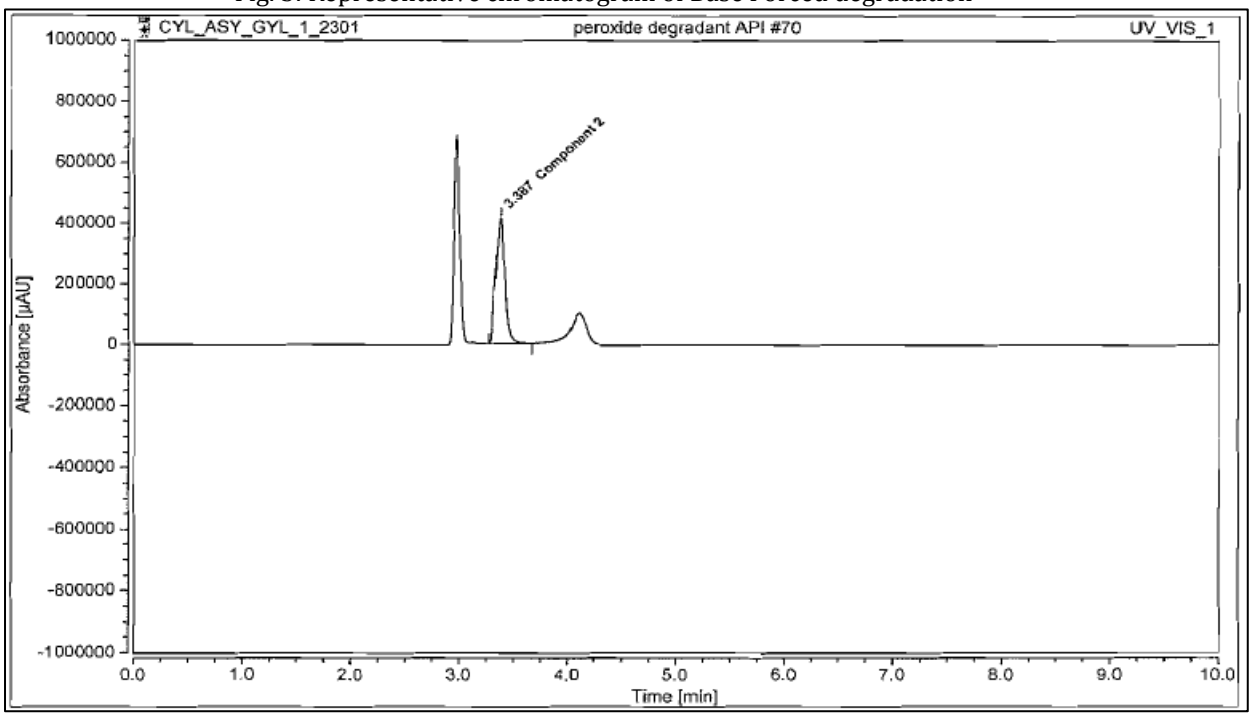

Fig. 6: Representative chromatogram of Peroxide Forced degradation

Table 4: Linearity data

Sr. No.	Conc. in ppm	Area Response
1	13	977495
2	25	1896265
3	38	2818321
4	50	3790509
5	63	4776349
Correlation Coefficient		0.9997
Regression Coefficient		0.9999
Criteria		0.998

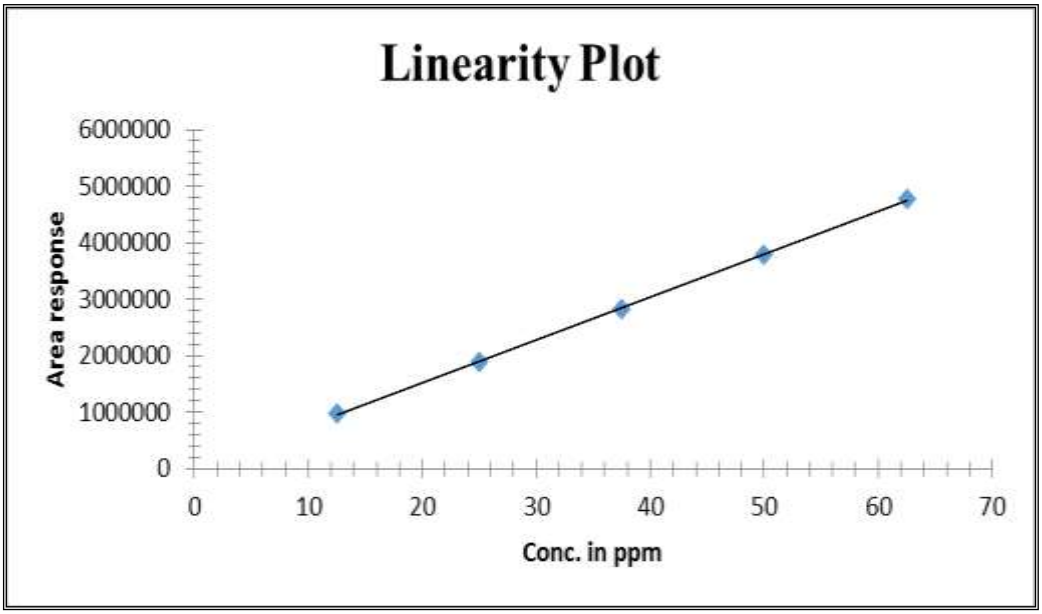

Fig. 7: Standard calibration curve

Table 5: Precision study data

Set No.	Method Precision
1	102.1
2	99.5
3	99.4
4	101.2
5	101.5
6	99.2
Mean	100.5
% RSD	1.25
Acceptance criteria	NMT 2.0%

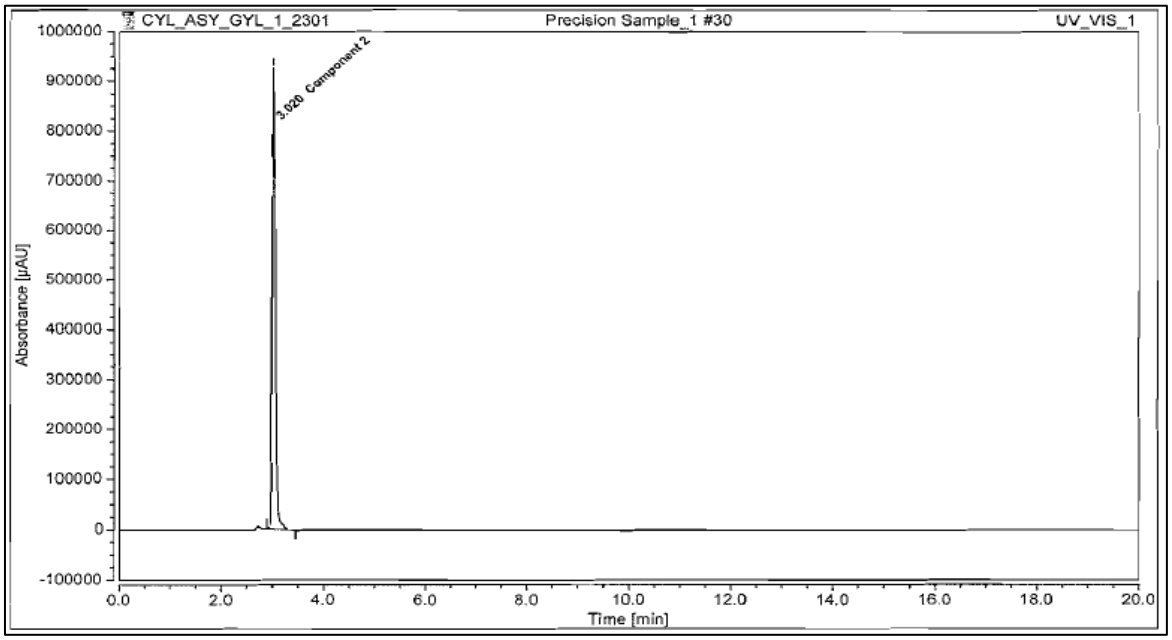

Fig. 8: Representative chromatogram of Precision sample

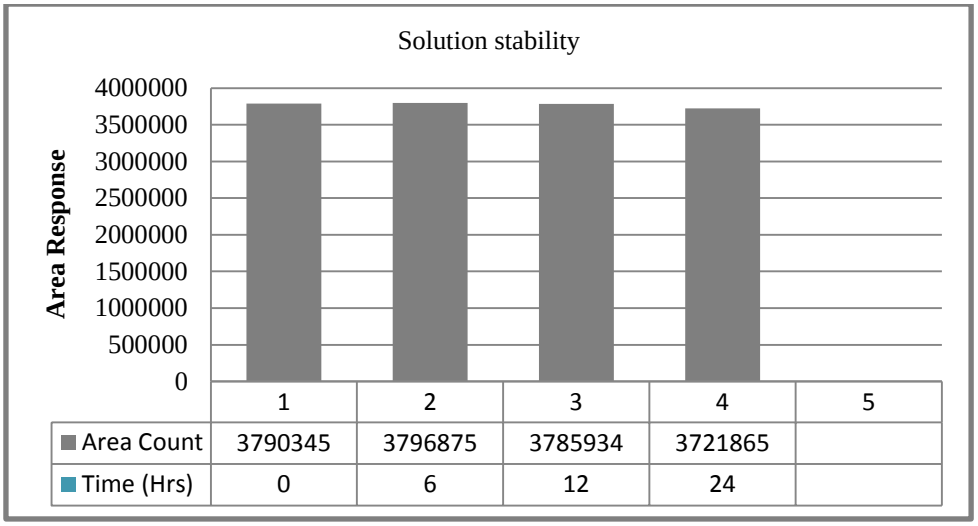

Fig. 9: Solution stability study data

Table 6: Accuracy study data

SL. No	% Recovery	Peak Response	*Sample added (mg)	*Sample Recovered (mg)	% Recovered	Average % Value	% RSD
1	75 %	2821121	15.39	15.53	100.9	101.1 %	0.6%
2		2794397	15.12	15.38	101.7		
3		2815575	15.41	15.50	100.6		
1	100 %	3770390	20.87	20.75	99.4	100.3%	1.3%
2		3811599	21.07	20.98	99.6		
3		3757642	20.31	20.68	101.8		
1	125 %	4720220	25.63	25.98	101.4	102.0%	0.6%
2		4761887	25.56	26.21	102.5		
3		4788982	25.81	26.36	102.1		
Over all Mean Recovery					101.1%		
Over all % RSD					1.1%		
Acceptance Criteria					RSD NMT 2.0%		

Table 7: Robustness study data

Robustness Parameter	RT (min)	Plate count	Tailing
Increase in Flow(-0.2ml/min)	2.760	9268	1.16
Decrease in Flow(+0.2ml/min)	3.373	7730	0.95
Increase in Temperature(-5°C)	2.983	12503	1.48
Decrease in Temperature(+5°C)	3.033	12205	1.58
Increase in Organic modifier -10% in Mobile Phase-B	2.427	9662	1.43
Decrease in Organic modifier 10%) in Mobile Phase-A	4.027	15758	1.45

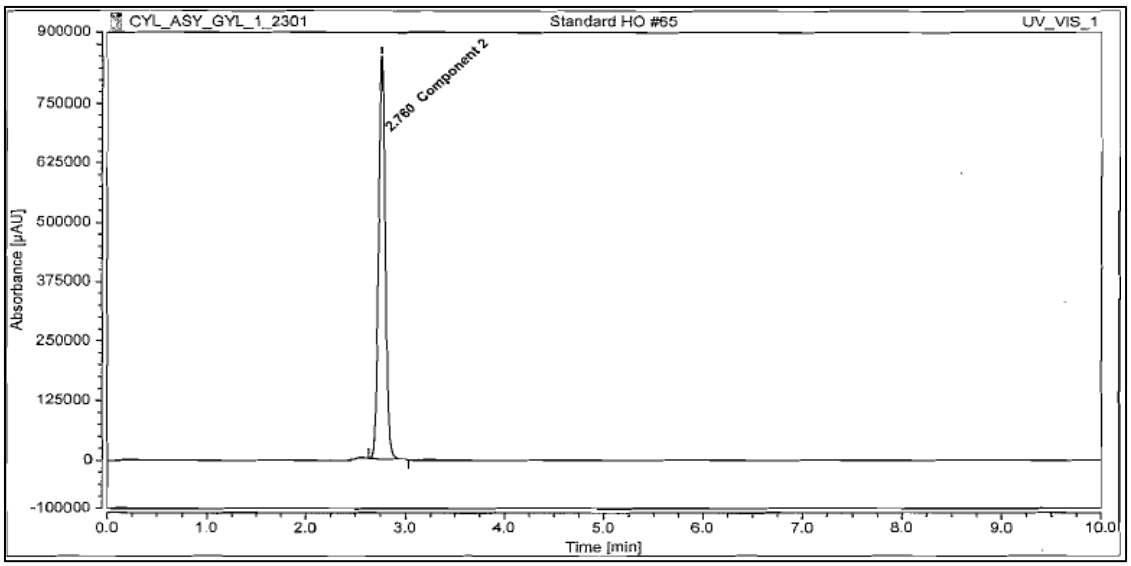

Fig. 10: Representative chromatogram of Robustness by increase in flow

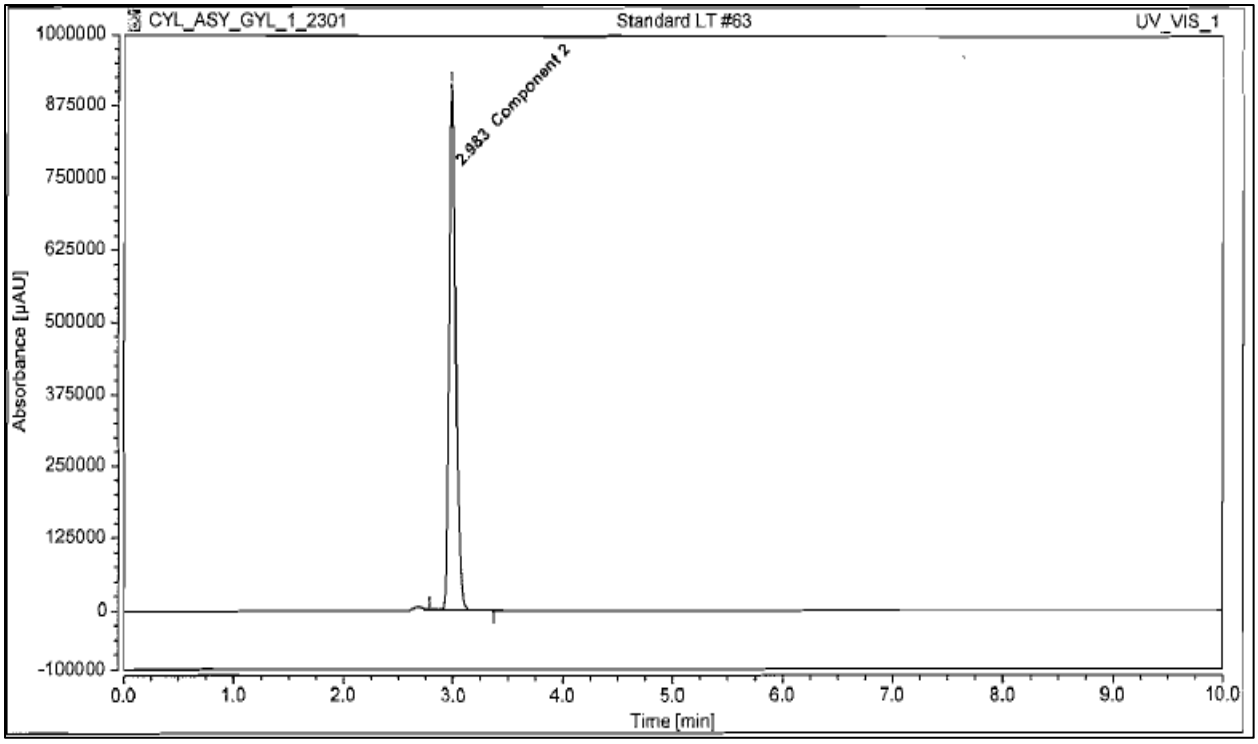

Fig. 11: Representative chromatogram of Robustness by increase in Temperature

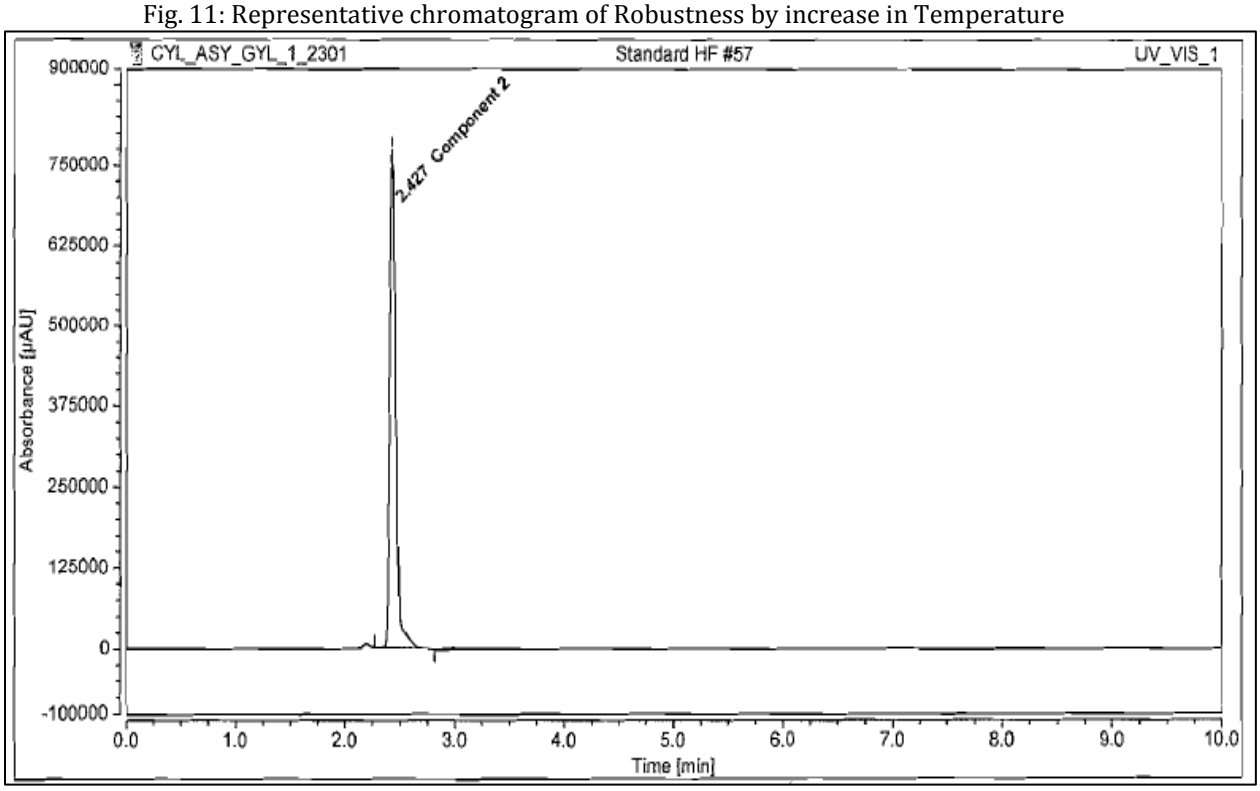

Fig. 12: Representative chromatogram of Robustness by increase in Mobile phase ratio

Table 8: Assay data of Doxorubicin in marketed formulation

Sample No.	% Assay	Average
Sample 1	99.5%	98.8%
Sample 2	98.1%	

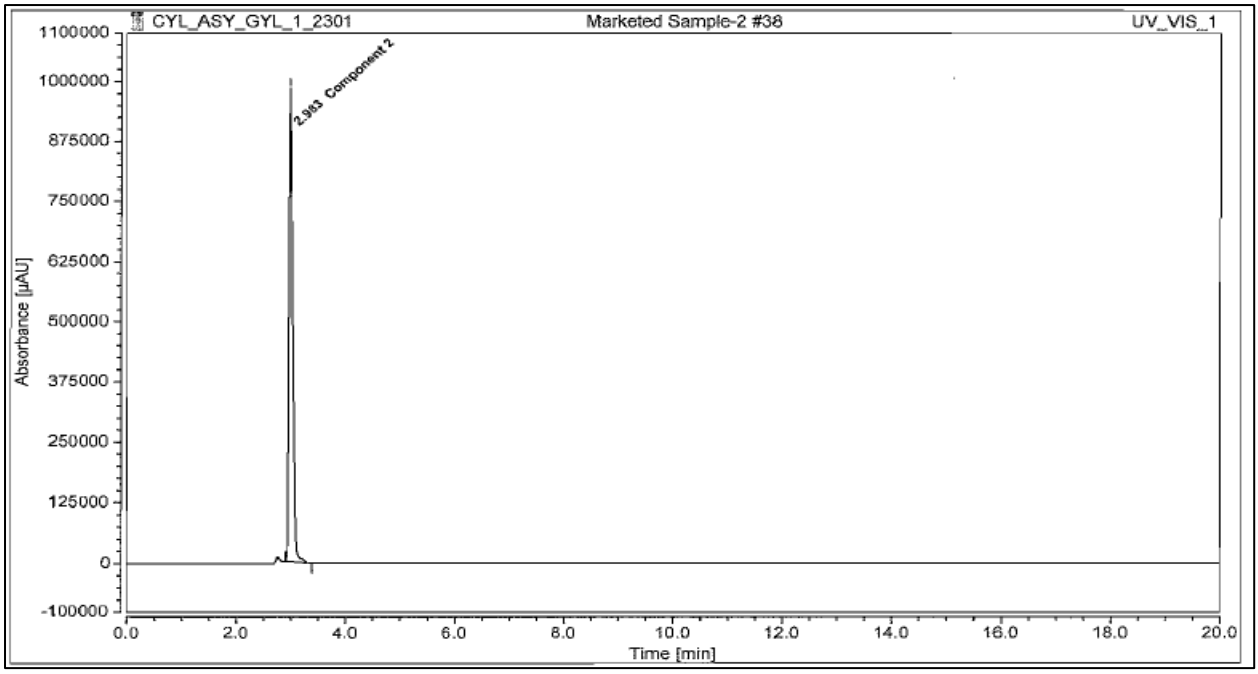

Fig. 13: Representative Chromatogram of marketed sample

DISCUSSION

The extensive method development was done to identify the suitable chromatographic conditions. Further, the method suitability was proved by performing the key forced degradation study. The presented method is specific, and peak of interest was very well separated from all potential degradants and the method was validated as per ICH guidelines. The derived Statistical data shows that the method validated concluded as accurate, precise and repeatable. The validated results indicated the suitability of the method to study shelf life of Doxorubicin in Active Pharmaceutical ingredients (API) as well as in commercial formulations. The developed method may be employed for analysis of routine, development and stability samples of Doxorubicin in bulk drug substances as well as pharmaceutical formulation.

APPLICATIONS

The developed method can be used for the analysis of development samples, routine quality check, exhibit batch stability testing of Doxorubicin in Pharmaceutical formulations and bulk active ingredients. Also, these methods can be further explored as cleaning methods at Pharmaceutical GMP manufacturing facilities to identify the trace amount of Doxorubicin present in machines after batch manufacture.

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