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Research Article

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Development and validation of stability indicating method for the quantitative determination of doxorubicin hydrochloride and its related impurities in pharmaceutical dosage forms using RP-HPLC

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ABSTRACT

A simple, linear, rapid, precise and stability-indicating analytical method was developed for the estimation of related substances and degradants of Doxorubicin hydrochloride API and tablets. The chromatographic separations were achieved using high performance liquid chromatograph (X-Terra C18, 100mm X 4.6mm, ID with 3.5 microns particles column) employing mixture of 0.05mM ammonium acetate, methanol and Acetonitrile (500:250 :250v/v) as mobile phase in an isocratic elution mode with a 0.5 mL/min flow rate was chosen. Two impurities were eluted within ten minutes of run time. The column temperature was maintained at 25ºC and a detector wavelength of 254 nm was employed. Doxorubicin HCl was exposed to acid, base thermal, photolytic, and oxidative stress conditions. The stressed samples were analyzed by the proposed method. The degradation of Doxorubicin hydrochloride was observed under Acid hydrolysis, base hydrolysis and oxidative hydrolysis. The drug was found to be stable in all other stress conditions applied. Successful separation of the drug from organic impurities and degradation products formed under forced degradation was achieved. The developed HPLC method to determine the related substances and assay of Doxorubicin HCl can be used to evaluate the quality of regular production samples. It can be also used to test the stability samples of Doxorubicin HCl.

Key words: Reverse Phase Liquid Chromatography, Doxorubicin hydrochloride, Validation, Stress conditions, Degradation Products.

INTRODUCTION

Doxorubicin hydrochloride/Adriamycin HCl ((7S,9S)-7-[(2R,4S,5S,6S)-4-amino-5-hydroxy-6-methyloxan-2-yl]oxy-6,9,11-trihydroxy-9-(2-hydroxyacetyl)-4-methoxy-8,10-dihydro-7H-tetracene-5,12- dione; hydrochloride) is antineoplastic agent, also called as anticancerous drug. Doxorubicin (HCl) is the prototype agent of anthracycline antibiotic, isolated from Streptomyces peucetius var caesius. It contains an amino sugar and an anthracycline ring. Doxorubicin (HCl) is among the most useful cytotoxic anticancerous drugs. It has a broad spectrum of potent activity against many different types of cancers especially haematologic malagnancies. Doxorubicin (HCl) is used in combination with different anticancerous drugs to obtain best therapeutic effects and to reduce the side effects or toxicities.

Doxorubicin (HCl) is of Semi Synthetic origin and belongs to Anthracycyline. It belongs to intercalation (DNA acting) pharmacological group on the basis of mechanism of action and also classified in Antineoplastic Agent, Antibiotic pharmacological group.

DXRH (doxorubicin HCl liposome injection) is indicated for the treatment of patients with ovarian cancer whose disease has progressed or recurred after platinum-based chemotherapy. DXRH is indicated for the treatment of AIDS-related Kaposi's sarcoma in patients after failure of prior systemic chemotherapy or intolerance to such therapy.

The HPLC system will significantly decrease the time and cost per sample in the analytical process while improving the quality of the results.

Liquid chromatographic methods using different detection techniques are described in the literature for the determination of Doxorubicin HCl in rat plasma. Additionally, UPLC method also available for the determination of irinotecan and doxorubicin extracted from murine plasma, along with their major metabolites SN-38 and doxorubicinol respectively. And spectrophotometry methods are available for determination of assay in doxorubicin HCl. Few analytical methods using HPLC are available for determination of assay in doxorubicin HCl. These method require longer runtime and do not represent degradants studied in this paper. To the best of our knowledge, no method available in the literature can separate impurities Methyl paraben and Epirubicin using only a 10-minute run time. The method was validated as per ICH guidelines.

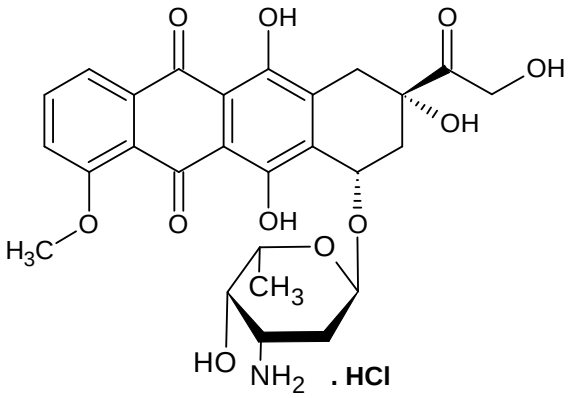
The chemical structures and names of Doxorubicin HCl and its impurities Methyl paraben and Epirubicin are depicted in Figure 1. Although two impurities are considered for method development and selectivity purposes, these impurities are not detected in the samples of Doxorubicin HCl.

EXPERIMENTAL SECTION

2.1 Chemicals:

Samples of Doxorubicin hydrochloride and its two impurities namely Epi rubicin and Methyl paraben (Fig.1) was received from Naprod Life sciences Pvt. Ltd, Mumbai. Merck gradeMethanol, Acetonitrile & Ammonium Acetate was purchased from Merck, Darmstadt, Germany. High purity water was prepared by using a Millipore Milli Q plus purification system.

Doxorubicin HCl: Structure and its imp – Methyl paraben & Epi rubicin



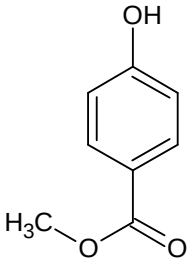
Molecular Formula = $C_{27}H_{30}ClNO_{11}$

Formula Weight = 579.9802

Chemical Name: (7S, 9S)-7-[(2R, 4S, 5S, 6S)-4-amino-5-hydroxy-6-methyloxan-2-yl]oxy-6,9,11-trihydroxy-9-(2-hydroxyacetyl)-4-methoxy-8,10-dihydro-7H-tetracene-5,12- dione; hydrochloride

Figure 1: Structure of doxorubicin hydrochloride

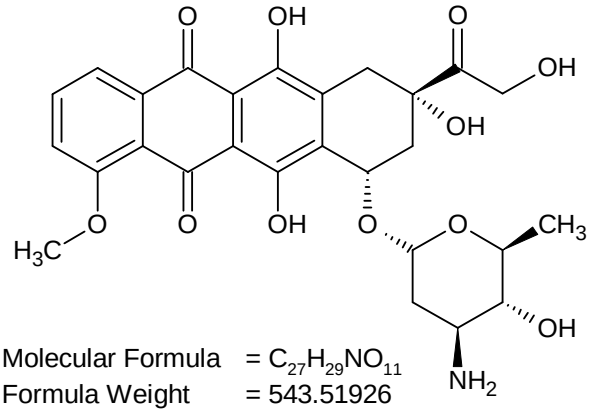
Structure of Methyl paraben



Molecular Formula = $C_8H_8O_3$
Formula Weight = 152.14732

Chemical Name: Methyl 4-hydroxybenzoate

Structure of Epi rubicin



Molecular Formula = $C_{27}H_{29}NO_{11}$
Formula Weight = 543.51926

Chemical Name : 7S,9S)-7-[(2R,4S,5R,6S)-4-amino-5-hydroxy-6-methyloxan-2-yl]oxy-6,9,11-trihydroxy-9-(2-hydroxyacetyl)-4-methoxy-8,10-dihydro-7H-tetracene-5,12-dione

2.2 Equipment:

The HPLC system used for the method development and validation consisted of gradient pumps from Agilent 1260 Technologies, Ultra violet detector from Agilent Technologies. USA, with autosampler and auto injector. The HPLC system was equipped with data acquision and processing software "EZ Chrome software" Agilent Technologies. USA.

2.3 Preparation of Standard solutions:

A stock solution of Doxorubicin Hydrochloride was prepared by dissolving appropriate amount of substance in water and Acetonitrile (1:1). Working solutions of 100µg /mL were prepared from the above stock solutions for the determination of related substance and assay. Stock solutions of impurities(mixture of Epi rubicin and Methyl paraben) at 0.1 mg/mL were also prepared in water and Acetonitrile (1:1).

2.4 Chromatographic Conditions:

The Chromatographic separation was achieved on an X-Terra C18, 100mm X 4.6mm, ID with 3.5 microns particles column. Aqueous Ammonium Acetate (0.05mM) Acetonitrile and Methanol (500:250:250v/v) used as a mobile phase. The mobile phase was filtered through nylon membrane (pore size 0.45 µm) and degassed by using vaccum pump and sonicate for 15 minutes prior to use. The flow rate of mobile phase was 0.5 mL/min. The column temperature was maintained at 25°C and wave length was monitored at 254 nm. The injection volume was 10µL. The standard and the test dilutions were prepared in water and Acetonitrile (1:1).

2.5 Validation of the method:

2.5.1 Specificity:

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. The specificity of the developed HPLC method for Doxorubicin HCl was carried out in the presence of its impurities

namely Epi rubicin and Methyl paraben. Stress studies were performed for Doxorubicin HCl bulk drug to provide an indication of the stability indicating property and specificity of the proposed method. Intentional degradation was attempted to stress conditions of UV light (254nm), heat (70 °C), acid (0.1N HCl), base (0.1N NaOH),and Oxidation (3.0 % H2O2) to evaluate the ability of the proposed method to separate Doxorubicin hydrochloride from its degradation products. For all degradation studies, period was 24 hours. Assay and related substance studies were carried out for stress samples against qualified Doxorubicin hydrochloride reference standard. Assay was also calculated for Doxorubicin hydrochloride samples by spikin gall for impurities at the specification level (i.e., 0.1%).

2.5.2 Precision:
The precision of the assay method was evaluated by carrying out six independent assays of Doxorubicin hydrochloride test samples against a qualified reference standard and calculate the %RSD of assay. The precision of the related substances method was checked by injecting sixi ndividual preparations of Doxorubicin hydrochloride (0.1mg/ mL) spiked with 0.1 % of Epi rubicin and Methyl paraben with respect to Doxorubicin hydrochloride analyte concentration. %RSD of area for each impurity of Epi rubicin and Methyl paraben was calculated. The intermediate precision of the method was also evaluated using different analyst and different instrument in the same laboratory.

2.5.3 Limit of detection (LOD) and Limit of Quantification (LOQ):
The limit of detection and limit of quantification were determined at a signal to noise of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations. Precision study was also carried out at the LOQ level by injecting five individual preparations of imp Epi rubicin and Methyl paraben and then calculated the %RSD of the peak area.

2.5.4 Linearity
Linearity test solutions for the assay method were prepared from Doxorubicin Hydrochloride stock solutions at five concentration levels from 50% to 250% of assay analyte concentration (50%, 100%, 150%, 200%, and 250%). The peak area verses concentration data was treated by least squares linear regression analysis.

Linearity test solutions for the related substance method were prepared by dilution of stock solution to the required concentrations. The solutions were prepared at five concentration levels from LOQ to 150% (100 µg/mL) of specification level of impurities namely imp - Epi rubicin and Methyl paraben (LOQ, 25%,50% 75%, 100%, and 150%). Above test were carried out of 3consecutives days in the same concentration range for both assay and related substances method.

The % RSD value for the Slope and Y-intercept of the calibration curve was calculated.

2.5.5 Accuracy:
The accuracy of the assay method was evaluated in triplicate at three concentration levels50%,100% and 150 % of test concentration (0.1 mg/mL).The percentage of recoveries were Calculated. The accuracy study of impurities was carried out in triplicate at50%, 100%, & 150% of specification level (0.1%) to the Doxorubicin Hydrochloride analyte concentration (100 µg /mL).The percentages of recoveries for impurities were calculated from the slope and Y- Intercept of the calibration curve.

2.5.6 Robustness:
To determine the robustness of the developed method, experimental conditions were deliberately altered and the resolution between Doxorubicin Hydrochloride, imp - Epi rubicin and Methyl paraben was recorded. The effect of the methanol ratio in mobile phase preparation studied on resolution by varying by-5 to + 5 %, while other mobile phase components were held constant as stated in Chromatographic conditions. The column temperature was varied by -5 to + 5°C and flow rate of the mobile phase varied from – 0.1 to +0.1 mL/min.

2.5.7 Solution stability and Mobile phase stability:
The solution stability of Doxorubicin Hydrochloride in the assay method was carried out by leaving both the test solutions of sample and reference standard in tightly capped volumetric flasks at room temperature for 24 hrs. The same sample solutions were assayed for 6 hrs. interval up to the study period. The mobile phase stability was also carried out by assaying the freshly prepared sample solution against freshly prepared reference standard solution for 6 hrs. interval up to 48 hrs. Mobile phase prepared was kept constant during the study period. The % RSD for the

assay of Doxorubicin Hydrochloride was calculated during mobile phase and solution stability experiment. The solution stability of Doxorubicin Hydrochloride and its impurities in the related substance method was carried out by leaving spiked sample solution in tightly capped volumetric flasks at room temperature for 24 hrs. Content of imp - Epi rubicin and Methyl paraben were checked in the test solutions.

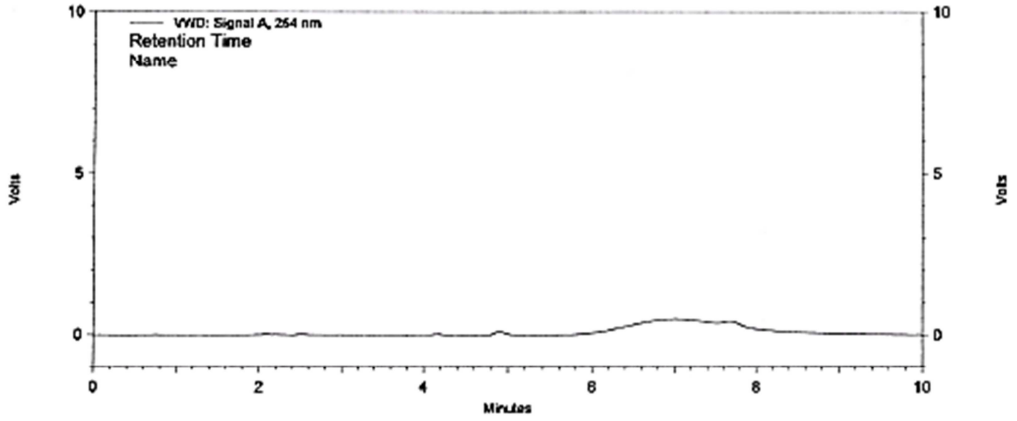
RESULTS AND DISCUSSION

3.1 Optimization of Chromatographic conditions

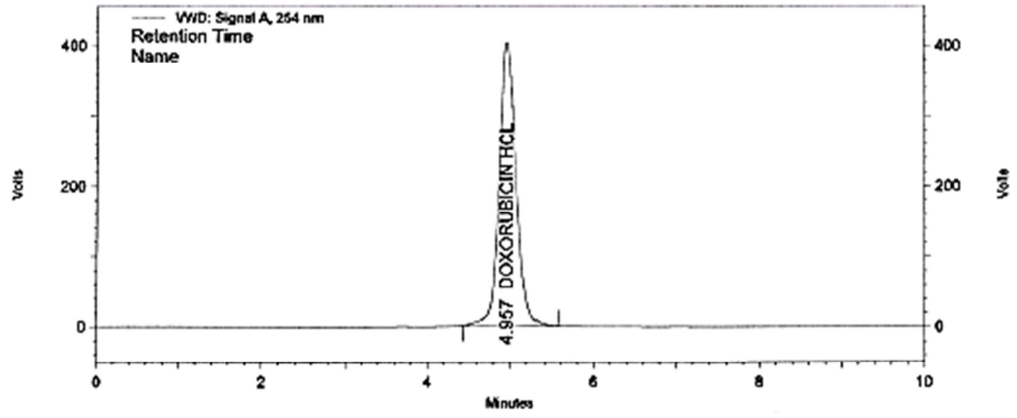
The main objective of Chromatographic method is to separate Doxorubicin Hydrochloride from Its impurities- Epi rubicin and Methyl paraben. Impurities were co-eluted using different stationary phases such as Symmetry C18, 150mm X 4.6, 5µm ,Symmetry C18, 250mm X 4.6, 5µm and Zorabax C8, 150mm X 4.6, 5µm as well as different mobile phases. The Chromatographic separation was achieved on aX-Terra C18, 100mm X 4.6mm, ID with 3.5 microns particles column using mixture of 0.05mM Ammonium acetate, Acetonitrile and methanol(500:250 :250v/v) as a mobile phase. The flow rate of the mobile phase was 0.5 mL / min, at 25 °C column temperature, the peak shape of the Doxorubicin Hydrochloride was found to be symmetrical. In optimized chromatographic conditions of Doxorubicin Hydrochloride, Imp - Epi rubicin and Methyl paraben were separated with resolution greater than 2, typical relative retention times were about 0.83, 1.17, respectively (**Fig 2**).The system suitability results are given in **Table-2**and developed HPLC method was found to specific for Doxorubicin Hydrochloride and its two impurities namely Imp -Epi rubicin and Methyl paraben (**Fig 1**).

Fig.2: Chromatogram of (a) Blank (b) Doxorubicin Hydrochloride sample (c) Doxorubicin Hydrochloride sample spiked with impurities

(a) Blank



(b) Doxorubicin Hydrochloride sample



(c) Doxorubicin Hydrochloride sample and spiked with impurities

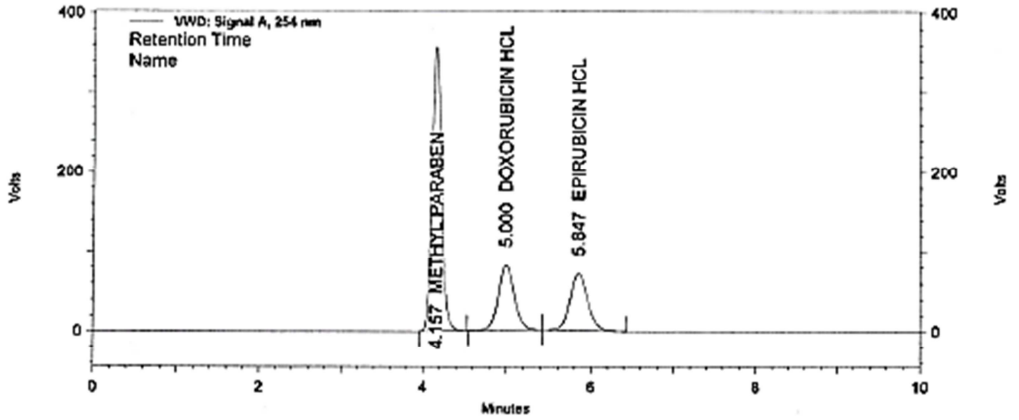


Table-1: System suitability data

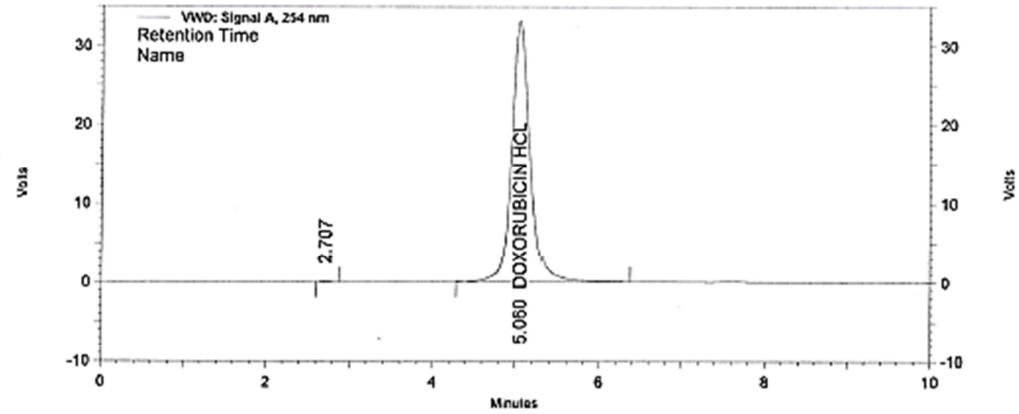
System suitability	Methyl paraben	Doxorubicin HCl	Epirubicin
Rt	4.16	5.0	5.85
RRt	0.83	1	1.17
Rs	-	3.03	2.28
T	1.2	1.1	1.1
N	6513	3258	3581

3.2 Results of forced degradation studies:

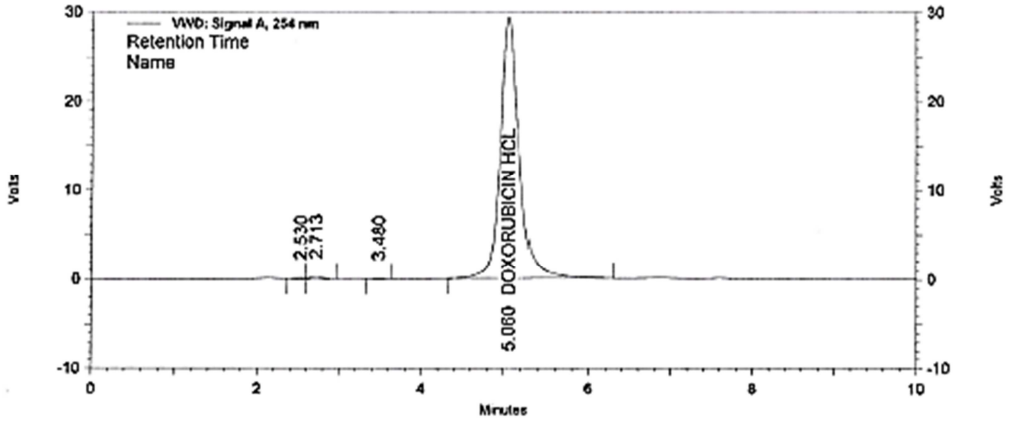
Degradation was not observed in Doxorubicin HCl sample when subjected to stress conditions like light, heat. Doxorubicin HCl was degraded to Impurities Methyl paraben and Epi rubicin under Oxidative hydrolysis, in base hydrolysis and acid hydrolysis. Doxorubicin HCl was degraded under Oxidative hydrolysis **(Fig.3).**Peak purity test results confirmed that the Doxorubicin HCl Peak is homogenous and pure in all the analyzed stress samples. The assay of Doxorubicin e HCl is unaffected in the presence of impurities and its degradation products confirms the stability indicating power of the method. The summary of forced degradation studies is given in Table 2.

Fig.3: Stress study Chromatogram of Doxorubicin HCl a) in Photo degradation b) in base c) in acid d) thermal e) peroxideblank f) peroxide degradation

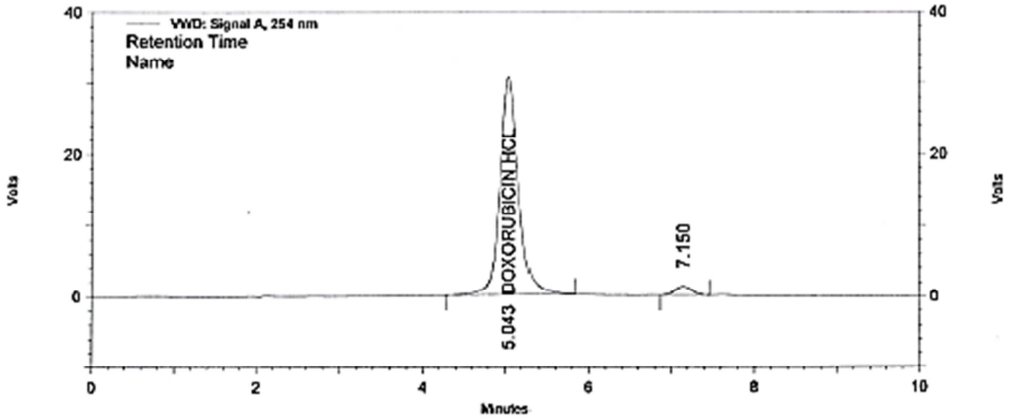
(a) Stress study Chromatogram of Doxorubicin HCl Photo degradation



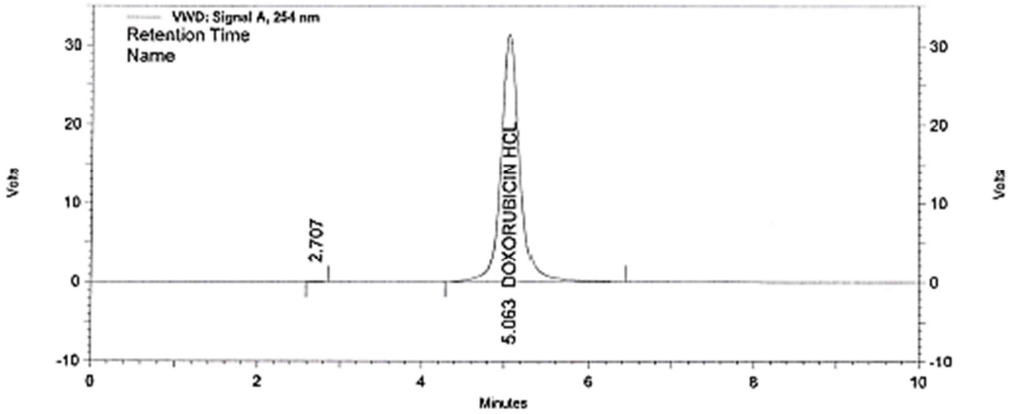
(b) Stress study Chromatogram of Doxorubicin HCl Base degradation



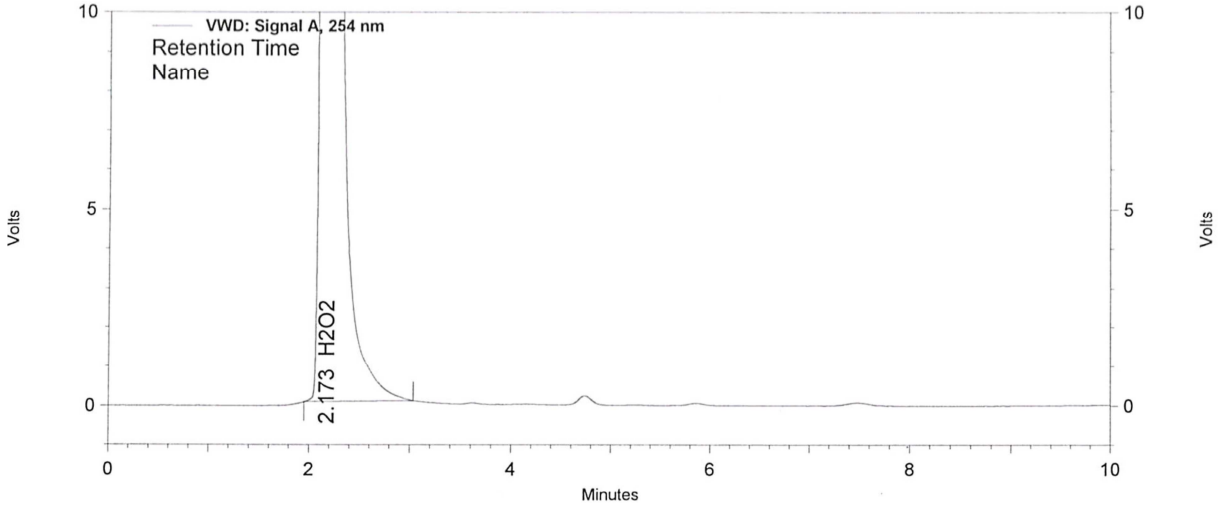
(c) Stress study Chromatogram of Doxorubicin HCl Acid degradation



(d) Stress study Chromatogram of Doxorubicin HCl Thermal degradation



(e) Stress study Chromatogram of Blank for Doxorubicin HCl Peroxide degradation



(f) Stress study Chromatogram of Doxorubicin HCl Peroxide degradation

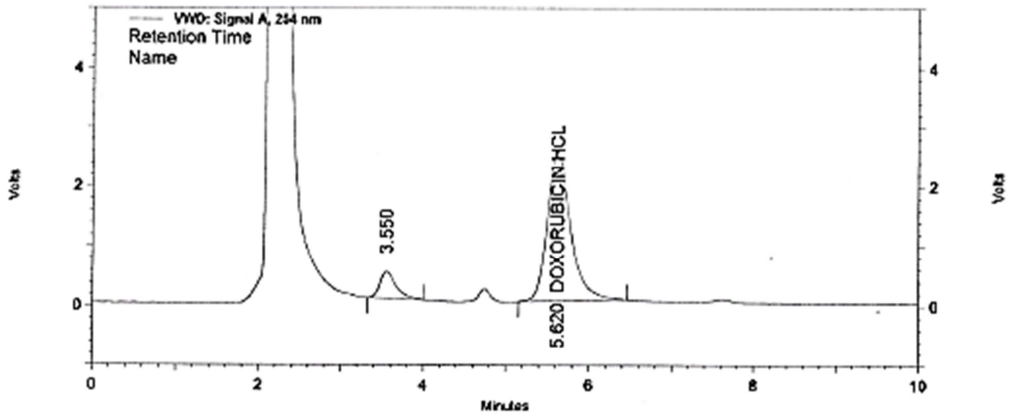


Table-2: Summary of forced degradation

Stress conditions	Time (hours)	Assay of Active substance (%)	Total impurities (%)	Mass balance (Assay + impurities (%))
Normal	24	99.90	0.10	100
Acid hydrolysis	24	96.50	3.50	100
Base hydrolysis	24	99.30	0.49	99.79
Oxidation(3%H2O2)	24	88.99	11.01	100
Thermal at 70 oC	24	99.87	0.13	100
UV light	24	99.90	0.10	100

3.3 Precision

The %RSD of assay of Doxorubicin Hydrochloride during the assay method precision study was within 0.62% and the %RSD for the area of Imp –Methyl paraben and Epi rubicin in related substances method precision study was within0.31 % & 2.19%. The %RSD of the assay results obtained inthe intermediate precision study was within 1.5 % ,%RSD for the area of Imp - Methyl paraben and Epi rubicin were well within 2.5%& 2.78 conforming good precision of the method.

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

The limit of detection of all impurities namely Imp - Methyl paraben and Epi rubicin were achieved0.32, and 0.32 µg/mL respectively for 10 µL injection volume. The limit of quantification of impurities namely Imp - Methyl

paraben and Epi rubicin are 0.96, & 0.97, µg/mL for 10µL injection volume. The precision at the LOQ concentrations for Imp - Methyl paraben and Epi rubicin were below 7.5%.

Table-3 : Limit of Detection and Limit of Quantification

Name	Methyl paraben	Epirubicin
LOD	0.32	0.32
LOQ	0.96	0.97

3.5 Linearity:

The linearity calibration plot for the assay method was obtained over the calibration ranges tested, i.e. 50 to 150 µg/ mL and correlation coefficient obtained was greater than 0.999.Linearitywas checked for assay method over same concentration range for 3 consecutives days. The %RSD value of the Slope and Y-Intercept of calibration curve were 1.4and 2.5 respectively. The result shows that an excellent correlation existed between the peak area and concentration of the analysis. Linear calibration plot for the related substances method was obtained over the calibration ranges tested i.e. LOQ to 150 % for Imp - Methyl paraben and Epi rubicin. The correlation coefficient obtained greater than 0.999. Linearity was checked for the related substances method over the same concentration ranges for 3 consecutives days. The %RSD values of the Slope and Y-intercept of calibration curve were 3.2 and 2.8 respectively. The above results shows that an excellent correlation existed between the peaks area and the concentrations of Imp - Methyl paraben and Epi rubicin.

Table-4: Linearity data

Name	Doxorubicin HCl	Methyl paraben	Epirubicin
Linearity (n=3)			
Intercept	-2761031.93	904.29	-1696.56
Slope	70017316.40	1203891.53	422054.32
r	0.99974	0.99991	0.99991

n, number of determinations

3.6 Accuracy

The percentage recovery of Doxorubicin hydrochloride in bulk drug samples was ranged from 99.5 to 100.2 % (Table-5).The percentage recoveries of all two impurities in Doxorubicin Hydrochloride samples varied from 98.5-101.5%

Table-5: Accuracy data

Name	Doxorubicin HCl	Methyl paraben	Epirubicin
Accuracy % Recovery	99.5-100.2	98.5-101.5	99.1-100.5

3.7 Robustness

In all the deliberate varied chromatographic conditions (flow rate, composition of organic solvent & column temperature) the resolution between critical pair, i.e. Doxorubicin HCl and imp- Methyl paraben, Epirubicin was greater than 2.0, illustrating the robustness of the method.

3.8 Solution stability and Mobile phase stability

The % RSD of assay of Doxorubicin Hydrochloride during solution stability experiments were within 0.1% .No significant change were observed in the content of impurities namely imp –Methyl paraben and Epi rubicin during the solution stability and mobile phase stability experiments when performed using the related substance method. The solution stability and mobile phase stability experiment data confirms that the sample solution and mobile phase used during the assay and the related substance determination were stable for 48 hrs.

CONCLUSION

The RP HPLC method developed for quantitative and related substance determination of Doxorubicin hydrochloride is linear, accurate, precise, rugged, rapid and specific. The method was fully validated showing satisfactory data for all method validation parameters tested. The developed method is stability indicating and can be conveniently used by quality control department to determine the related substance and assay in regular Doxorubicin Hydrochloride production samples and also stability samples. In the RP HPLC technique all the impurities were separated within a short time.

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