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A NOVEL STABILITY INDICATING RP-HPLC METHOD FOR ESTIMATION OF NICOUMALONE IN BULK AND TABLET DOSAGE FORM

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ABSTRACT

A simple and selective ,stability indicating LC method is described for the determination of Nicoumalone tablet dosage forms. The present paper deals with development and validation of a stability indicating reverse phase HPLC method for the estimation of Nicoumalone using Thermo BDS Hypersil column (250 mm X 4.6 mm, 5 µm). A mobile phase consisting of Acetonitrile: Ammonium Acetate buffer 0.01M, pH 6 adjusted using 0.1 N NaOH in 80:20 v/v ratio was employed in this study. The flow rate was set at 0.8 ml/min. Separation was performed at ambient temperature. Stability studies represented the degradation of drug in different condtions of acidic,alkali,oxidative and thermal. Linearity was observed in the range 48-112µg /ml for Nicoumalone (r^2 =0.9986) for the amount of drugs estimated by the proposed methods was in good agreement with the label claim.

Keywords *– Nicoumalone,HPLC, Stability.*

1. INTRODUCTION

Nicoumalone is a coumarin derivative used as an anticoagulant. Coumarin derivatives inhibit the reduction of vitamin K by vitamin K reductase. It has the IUPAC name of 4-hydroxy-3-[1-(4-nitrophenyl)-3-oxobutyl]-2H-chromen-2-one with the molecular formula of **$C_{19}H_{15}NO_6$**.

It inhibits vitamin K reductase, resulting in depletion of the reduced form of vitamin K (vitamin KH2). As vitamin K is a cofactor for the carboxylation of glutamate residues on the N-terminal regions of vitamin K-dependent clotting factors, this limits the gamma-carboxylation and subsequent activation of the vitamin K-dependent coagulant proteins. The synthesis of vitamin K-dependent coagulation factors II, VII, IX, and X and anticoagulant proteins C and S is inhibited resulting in decreased prothrombin levels and a decrease in the amount of thrombin generated and bound to fibrin. This reduces the thrombogenicity of clots.

It inhibits the reduction of vitamin K by vitamin K reductase. This prevents carboxylation of certain glutamic acid residues near the N-terminals of clotting factors II, VII, IX and X, the vitamin K-dependent clotting factors. Glutamic acid carboxylation is important for the interaction between these clotting factors and calcium. Without this interaction, clotting cannot occur. Both the extrinsic (via factors VII, X and II) and intrinsic (via factors IX, X and II)areaffectedbyacenocoumarol.Limited work was performed on Nicoumalone.Here an attempt was performed to get specific, precise method as per ICH guidelines.

2. EXPERIMENTAL

2.1 Materials ,Chemicals and Reagents

Nicoumalone standard was provided by Piramal health care, Hyderabad.Nocoulamine tablets containing 2 mg of Nocoulamine were procured from the market. Analytical grade dipotassium hydrogen phosphate,potassium dihydrogen phosphate,ammonium acetate,sodium hydroxide was purchased from S.S. fine chemicals , Hyderabad. HPLC grade methanol, acetonitrile and water were obtained from Merck,Mumbai.

2.2 Instrumentation

The chromatographic system used to perform development and validation of this assay method was comprised of a LC-10STvp binary pump, a SPD –M10 Avp photo diode array detector and a rheodyne manual injector model 7725i with 20µl loop connected to a multi – instrument data acquisition and data processing system.(Class-VP 6.13SP2, Shimadzu).

2.3 Mobile phase preparation

The mobile phase consisted of mixture of 30 volumes of Mixed Phosphate buffer(KH_2PO_4+K2HPO4) pH3.0 and 70volumes of Acetonitrile. . The solution was subjected to sonication.

2.4 Preparation of Mixed Phosphate buffer

1.625 gm of potassium di hydrogen phosphate (KH_2PO_4) and 0.3 g of Di potassium hydrogen phosphate was weighed and dissolved in 100ml of water and volume was made up to 550ml with water. Adjust the pH to 3.0 using ortho phosphoric acid. The buffer was filtered through 0.45µ filters to remove all fine particles and gases.

2.5 Diluent Preparation

Mobile phase was used as diluents.

2.6 Wave length Detection

The wavelength of maximum absorption (λ_{max}) of the drug, 10 µg/ml solution of the drugs in methanol were scanned using UV-Visible spectrophotometer within the wavelength region of 200–400 nm against methanol as blank. The resulting spectra of absorption curve shows characteristic absorption maxima at 291 nm for nicoulamine.

2.7 Chromatographic Conditions

Chromatographic analysis was performed on a Inertsil ODS C18,250x 4.6mm ,5µm. column. The mobile consisted of 30:70 volumes of mixed phosphate buffer and acetonitril.. The flow rate of the mobile phase was adjusted to 1.0 ml/min and the injection volume was 20 µl.Detection was performed at 291nm.

3. METHOD DEVELOPMENT.

3.1 Development and Optimization of the HPLC method

3.1.1 Standard Preparation

Weigh accurately 100 mg of Nicoumalone, in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 100 µg/ml of Nicoumalone,is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

3.1.2 Test Preparation

Twenty tablets (each Tab contains 100 mg of Nicoumalone,**)** were weighed and taken into a mortar uniformly mixed. Test stock solutions of Nicoumalone,E (100µg/ml) and was prepared. by dissolving weight equivalent to 100 mg of Nicoumalone, and dissolved in sufficient mobile phase. After that filtered the solution using 0.45-micron syringe filter and Sonicated for 5 min and dilute to 100ml with mobile phase. Further dilutions are prepared in 5 replicates of 100 µg/ml of Nicoumalone, was made by adding 1 ml of stock solution to 10 ml of mobile phase.

3.2 Forced degradation studies

It is carried out under the following stress condition

3.2.1 Photo stability (By UV)

The photochemical stability of the drug was studied by exposing the stock solution of 800 µg/ml Nicoumalone API to UV light for 6 hrs, by keeping it on terrace in UV chamber. The resultant solution was diluted to obtain concentration of 80 µg/ml and 20 µl was injected into the system under optimized conditions

3.2.2 Basic Hydrolysis (BY 1NaOH):

To 10 ml of stock solution of 800 µg/ml Nicoumalone API, 10 ml each of 1M NaOH. The resultant solution was refluxed at 70^0 C for a period of 4 hrs From the resultant solutions 1 ml were diluted with the mobile phase to obtain the solution of 80 µg/ml concentration and 20 µl were injected into the system.

3.2.3 Acidic hydrolysis (1M HCl) :

To 10 ml of stock solution of 800 µg/ml Nicoumalone API, 10 ml each of 1M HCl. The resultant solution was refluxed at 70^0 C for a period of 4 hrs. From the resultant solutions 1 ml were diluted with the mobile phase to obtain the solution of 80 µg/ml concentration and 20 µl were injected into the system.

3.2.4 Oxidative degradation(6%w/v):

To 10 ml of stock solution of 800 µg/ml pure Nicoumalone, 10 ml of 6% hydrogen peroxide solution was added. The solution was kept at room temperature in dark for 4 hrs. 1 ml from the resultant solutions were diluted with mobile phase to obtain concentration of 80 µg/ml and 20 µl was injected into the system.

4. RESULTS AND DISCUSSIONS

4.1 System Suitability

Standard solutions were prepared as per the test method and injected into the chromatographic system. The system suitability parameters like theoretical plates, resolution and asymmetric factor were evaluated.

4.2 Specificity by Direct comparison method

There is no interference of mobile phase, solvent and placebo with the analyte peak and also the peak purity of analyte peak which indicate that the method is specific for the analysis of analytes in their dosage form.

4.3 Standard sample

Weigh accurately 80 mg of N Nicoumalone, in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 80 µg/ml of Nicoumalone, is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

4.4 Tablet sample

Twenty tablets (each Tablet contains 2 mg of Nicoumalone,) were weighed and taken into a mortar uniformly mixed. Test stock solutions of Nicoumalone,800µg/ml) and was prepared by dissolving weight equivalent to 80mg of Nicoumalone, and dissolved in sufficient mobile phase. After that filtered the solution using 0.45-micron syringe filter and Sonicated for 5 min and dilute to 100ml with mobile phase. Further dilutions are prepared in 5 replicates of 80µg/ml of Nicoumalone, was made by adding 1 ml of stock solution to 10 ml of mobile phase.

4.5 Linearity

The linearity plot was prepared with five concentration levels (48, 64,80,96 and 112µg/ml of Nicoumalone,). These concentration levels were respectively corresponding to 60,80,100,120,140% of standard solution concentration. The peak area versus concentration data were evaluated by linear regression analysis.80 µg/ml of standard stock solution of Nicoumalone, was prepared and further diluted to attain concentration of about 48,64,80,96 and 112µg/ml of standard solution concentration. From standard stock solution of 80 µg/ml accurately pipette out exact 0.6,0.8,1.0,1.2 and 1.4 ml and dilute it up to 10 ml each with diluents to achieve 48-112 µg/ml concentration range. Correlation coefficient of the linearity study was found to R^2=0.999 with linear regression equation Y=53.71x+88.54, which proves the method is highly linear over the working range.

4.6 LOD and LOQ Study

LOD is the lowest amount of the drug content which can be detected by the proposed method while LOQ is the lowest amount which can be quantified by the method.The guideline suggest minimum signal to noise ratio (S/N) more than 3.3 for LOD and more than 10 for LOQ.On the basis of linearity data theoretically it can also be calculated by the given formula.

$$\mathbf{LOD = 3.3\sigma/S} \qquad \mathbf{LOQ = 10\,\sigma/S}$$

Where σ=residual standard deviation of regression line

S=slope of regression line.

4.7 Precision Study

Precision study was established by evaluating method precision and system precision study. Method precision of the analytical method was determined by analyzing six sets of the sample preparation. Assay of all six replicate sample preparation was determined and mean % assay value, standard deviation and% RSD for the same was calculated. System precision of the analytical method was carried out to ensure that the analytical system was working properly. Standard solution was injected six times in to system and chromatograms were recorded.

4.8 System Precision

Standard Solution Preparation

Weigh accurately 100 mg of Nicoumalone, in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 100 µg/ml of Nicoumalone,is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

4.9 Accuracy Study

This experiment can be performed by the recovery test. Recovery of the method was evaluated at three different concentration levels by addition of known amounts of standard placebo preparation. For each concentration level three sets were prepared and injected as duplicate. Accuracy of the method was determined by Recovery studies. To the formulation (pre analyzed sample), the reference standards of the drugs were added at the level of 80%, 100%, 120%. The recovery studies were carried out three times and the percentage recovery and percentage mean recovery were calculated for drug is shown in table. To check the accuracy of the method, recovery studies were carried out by addition of standard drug solution to pre-analyzed sample solution at three different levels 80%, 100%, 120%.

4.10 Robustness study

Robustness of the method was evaluated by assaying test solution under slight but deliberate changes in analytical conditions such as change in flow rate, change in wave length, change in proportions of mobile phase.

4.10.1 Flow rate change

In this experiment the test samples were analyzed at the flow rate of 0.8 ml/min and 1.4 ml/min each and the results were observed in terms of assay value and chromatographic compatability. Blank, standard and sample solutions were prepared as per the assay procedure . The result shown that during all variance conditions assay value of the test preparation solution was not affected and it was the accordance with that of actual .System suitability parameters were also found satisfactory. Hence the analytical method would be concluded as robust.

4.10.2 Wave length change

In this experiment the test samples were analyzed at the wave length of 290 and 294 nm each and the results were observed in terms of assay value and chromatographic compatability. Blank, standard and sample solutions were prepared as per the assay procedure .

4.11 Ruggedness study

The ruggedness of the method was studied by the determining instrument to instrument variation and the analyst to analyst variation by performing the Assay by two different analysts.

Table 1: Optimized Chromatographic Conditions

Mobile phase	(KH_2PO_4+K_2HPO_4)+ACN
pH	3.0
Column	INERTSIL ODS 3V column,C18 (250x4.6mm ID)
Flow rate	1.0 ml/min
Column temperature	Room temperature(20-25^oC)
Sample temperature	Room temperature(20-25^oC)
Wavelength	291 nm
Injection volume	20 µl
Run time	5 min
Retention time	4.377 min for Nicoumalone,

Table 2: **Assay Results Of Nicoumalone**

Injection no.	**Standard Area**	**Sample Area**
Injection-1	4236.32	4260.493
Injection-2	4256.047	4236.32
Injection-3	4250.49	4256.047
Injection-4	4236.32	4262.647
Injection-5	4260.403	4252.55
Average Area	4247.916	4253.611
Tablet average weight	81 mg	
Standard weight	20 mg	
Sample weight	800 mg	
Label amount	2 mg	
std. purity	99.87 %	
Amount found in mg	**2.03**	
Assay (%purity)	**101.25**	

Table 3: Degradation study of Nicoumalone

Stress Condition	Acidic	Alkali	Oxidative	Photolytic
% Degradation	4.1%	7.3%	3.4%	6.3%

Table 4: Results for system suitability of Nicoumalone,

Injection	Retention time (min)	Peak area
1	4.389	4256.908
2	4.387	4254.980
3	4.387	4250.876
4	4.387	4256.980
5	4.433	4256.083
6	4.438	4250.980
Mean	4.4035	4254.468
SD	0.0248	2.836
%RSD	**0.14**	**0.27**

Table 5: Results for recovery of Nicoumalone

Recovery level	**Accuracy of Nicoumalone,**					Average % Recovery
	Amount taken(mcg/ml)	Area	Average area	Amount recovered(mcg/ml)	%Recovery	
80%	80	4266.47	4255.722	81.27	101.59	100.45%
	80	4240.819				
	80	4259.877				
100%	96	5166.633	5091.955	96.82	100.86	
	96	5054.273				
	96	5054.958				
120%	112	5833.427	5838.054	110.78	98.91	
	112	5822.224				
	112	5858.512				

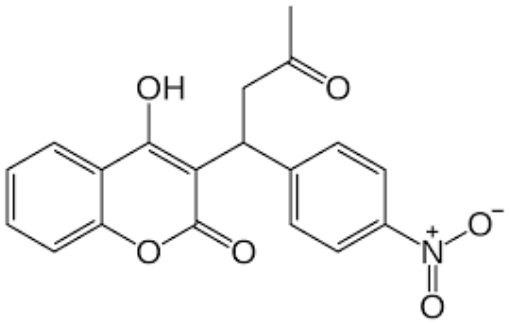

Figure 1: Structure of Nicoumalone

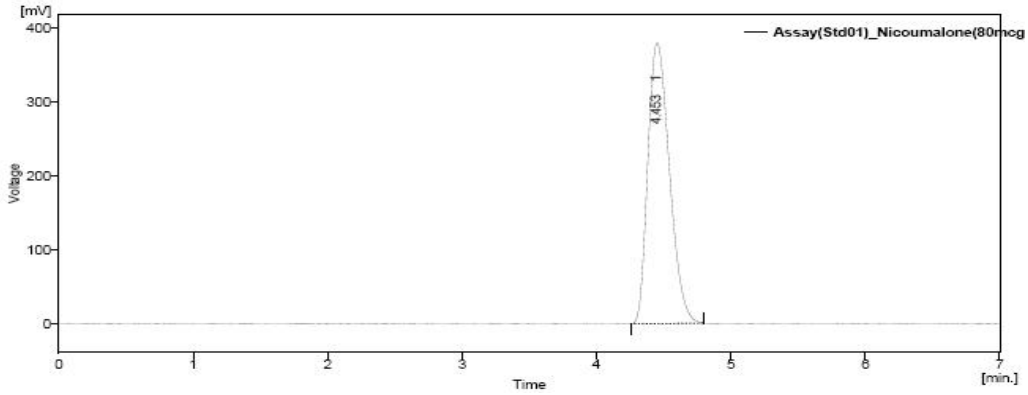

Figure 2: Assay Standard Preparation of Nicoumalone

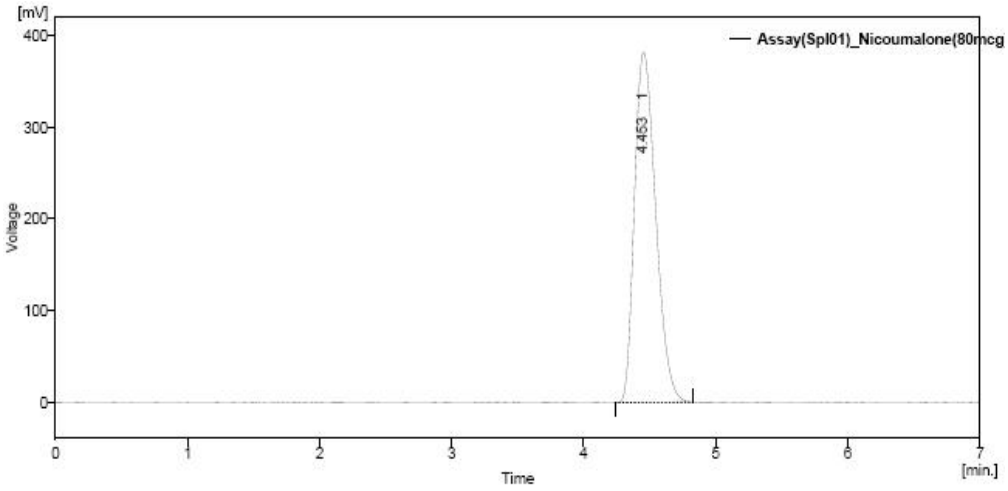

Figure3: Assay Sample Preparation of Nicoumalone

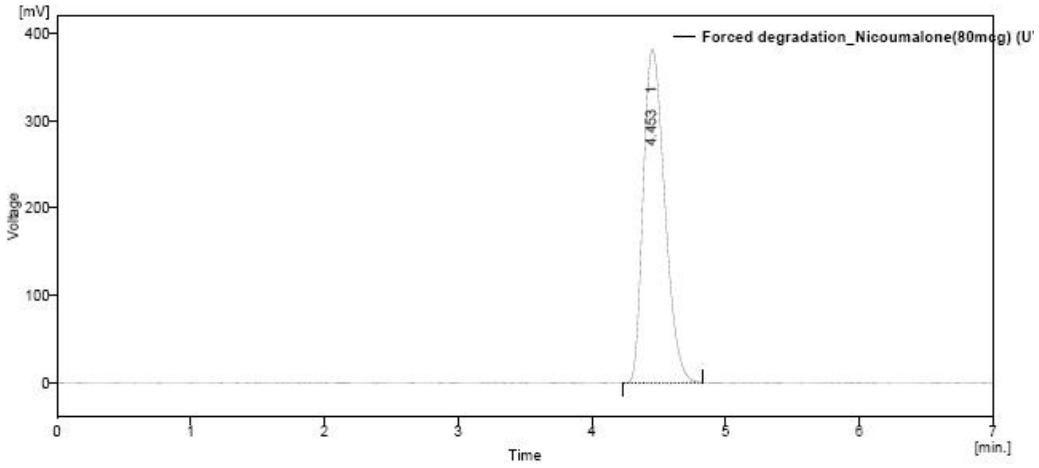

Figure4 :A typical chromatogram of UV exposure solution

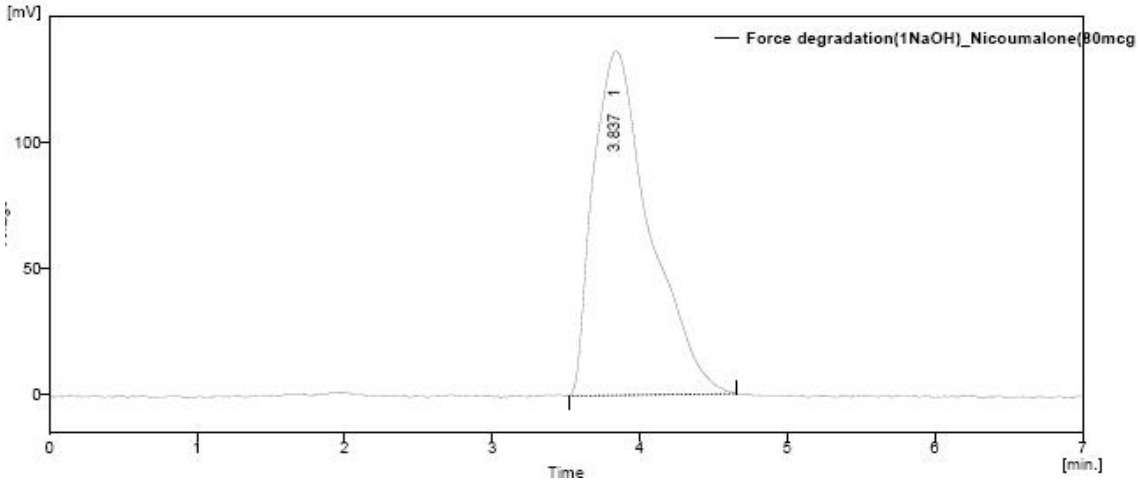

Figure 5: A typical chromatogram of Base hydrolysis solution

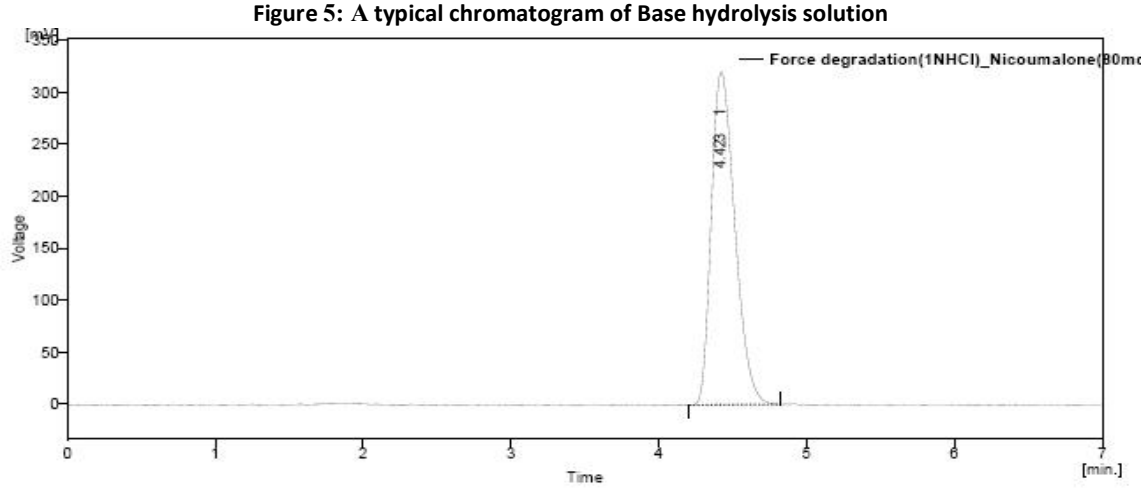

Figure 6: A typical chromatogram of Acid hydrolysis solution

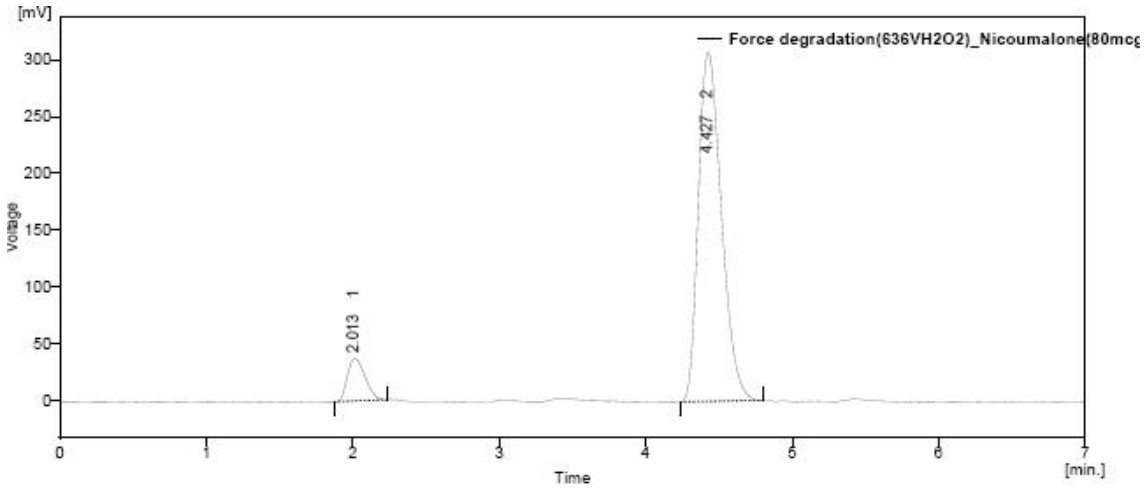

Figure7: A typical chromatogram of oxidation solution

5. CONCLUSION

A validated RP-HPLC method has been developed for determination of Nicoumalone in the bulk and tablet dosage forms. The results show that the method was found to be specific, simple, accurate, precise and sensitive. The method was successfully applied for the determination of Nicoumalone in quality control laboratories.

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