DESIGN AND VALIDATION OF AN IDEAL RP-HPLC METHOD FOR EVALUATION OF NICOUMALONE IN BULK DRUG AND TABLET FORMULATION

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ABSTRACT

The goal of this research aims to accomplish the efficacy of Nicoumalone through the utilization of an isocratic RP-HPLC (Reverse Phase High Performance Liquid Chromatography) method in both bulk and tablet formulation. Quantification was performed using a Phenomenex Luna C18, $100A^0$, 5µm, 250mm X4.6mm column. Various solvents were evaluated at varying proportions to determine the optimal outcome for the development of the system. The analysis revealed that Acetonitrile: Methanol (35:65 % V/V) is a suitable solvent for use as the mobile phase. The separation was conducted at room temperature using UV detectors to a specific value that has been established at 285 nm, at a flow rate of 1 millilitre per minute. The compound Nicoumalone exhibited a retention time of 4.07 minutes, which appears to be superior to that of other compounds. The recommended method is deemed validated in accordance with the regulations set forth by the International Conference of Harmonization (ICH). Several analytical parameters go through statistical validation, including precision, reliability, and limits of quantization, limit of detection, the concept of linearity and others. The proposed method is deemed useful for achieving optimal precision in the analysis of dosage forms of drugs and their formulations.

KEY WORDS: RP-HPLC, Nicoumalone, substantiation, ICH Guidelines, evaluation.

INTRODUCTION

An anticoagulant synthesized from the derivative of coumarin is nicoumalone. Vitamin K reductase cannot reduce vitamin K in the presence of coumarin derivatives. Vitamin K is counteracted by the

oral anticoagulant nicoumalone. It is 4-hydroxy-3-(1-(4-nitro phenyl)-3-oxobutyl)-Coumarin in its chemical structure (Figure-1)[1]. In the treatment of thromboembolic conditions, it is used. In more detail, it is suggested for the purpose of transient ischemic episodes, cerebral embolism, pulmonary embolism, and thromboembolism in infarction. It is used to treat myocardial infarction and deep vein thrombosis. Both the Indian Pharmacopoeia (also known as Acenocoumarol) and the British Pharmacopoeia recognise nicoumalone as an approved drug[2]. Both Pharmacopoeias described the aqueous acid base titration method as the assay method for Nicoumalone API, and the λmax at 306 nm and specific absorbance at 521 nm for the spectrophotometric analysis as the assay method for the tablet formulation[3]. The literature review conducted revealed that a limited number of spectroscopic, HPLC, and Biological analysis techniques have documented as a result of quantitative measurement of drugs and biological fluids[3]. The focus of current research is primarily on developing an isocratic RP-HPLC technique which can detect Nicoumalone and is rapid, accurate, precise, and repeatable. The method's specificity, linearity, precision, accuracy, range, limit of detection (LOD), limit of quantification (LOQ), and robustness have all been validated. The aforementioned technique has received clearance from the International Conference on Harmonization (ICH) [4].

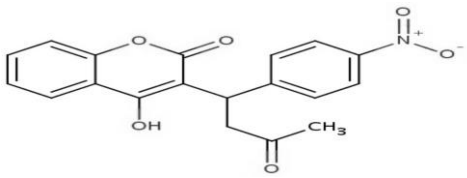

Figure: 1- Structure of Nicoumalone

MATERIALS & METHODS

CHEMICALS AND REAGENTS

A gift sample of Nicoumalone of pharmaceutical quality was acquired from Nicolas Piramal India, Pvt. Ltd. Reagents with a purity of 99.9% were utilized in the study, including Methanol (Loba Chem; Mumbai), Dipotassium hydrogen orthophosphate (Sd fine-Chem ltd; Mumbai), Acetonitrile (Loba Chem; Mumbai), Potassium dihydrogen orthophosphate (Sd fine-Chem ltd; Mumbai), and Orthophosphoric acid (Sd fine-Chem ltd; Mumbai). The chemicals and reagents employed in the experiment were of high-performance liquid chromatography (HPLC) grade. The Millipore purification technology was utilized to produce high-purity distilled water within the laboratory, which was subsequently utilized as a fresh source. A commercial formulation of Nicoumalone, specifically the 3mg tablet of Abbott Healthcare Pvt. Ltd. brand named ACITROM, was purchased from a local pharmacy.

METHOD DEVELOPMENT

The instrumentation employed in the study comprised of High-Performance Liquid Chromatography (HPLC) system equipped with Waters HPLC, Empower 2 Software, Isocratic mode, and a UV-Visible Detector. A 10 ml volumetric flask was utilized to dissolve 10 mg of Nicoumalone standard, which was subsequently made up to volume with mobile phase. One millilitre of the aforementioned solution is transferred to a ten ml volumetric flask, followed by the addition of mobile phase to achieve maximum volume, resulted in additional dilution. The conventional approach involves the utilization of UV spectrum scanning within the range of 200 to 400nm[5]. The objective of this study was to identify the maximum absorption wavelength of Nicoumalone, with the purpose of utilizing a consistent wavelength in an HPLC UV detector for the quantification of Nicoumalone. The maximum observed during the scanning of the Nicoumalone solution was found to be at 285nm. The ELICO SL-159 UV-Visible spectrophotometer model UV-2450 was utilized to obtain the ultraviolet spectrum. Table 1 displays the optimization of chromatography conditions through the utilization of various mobile phases and flow rates during sample preparation[6].

Table-1: Summary of Process Optimization

Column Used	Mobile Phase	Flow Rate	Wave length	Observation	Result
Phenomenex Luna C_{18}, 100A, 5µm, 250mmx4.6mm i.d.	Acetonitrile : Water = 70 :30	0.8 ml/min	285nm	Peak broken at the end	Method rejected
Phenomenex Luna C_{18}, 100A, 5µm, 250mmx4.6mm i.d.	Methanol : Water = 60 :40	1.0 ml/min	285nm	Tailing Peaks	Method rejected
Phenomenex Luna C_{18}, 100A, 5µm, 250mmx4.6mm i.d.	0.1%Orthophosphoric Acid: Methanol = 70:30	1.0 ml/ min	285nm	Tailing and Frontings	Method rejected
Phenomenex Luna C_{18}, 100A, 5µm, 250mmx4.6mm i.d.	0.1%Orthophosphoric Acid: Methanol 50:50	1.0 ml/ min	285nm	Tailing Peak	Method rejected
Phenomenex Luna C_{18}, 100A, 5µm, 250mmx4.6mm i.d.	Acetonitrile: Methanol = 20:80	1.0 ml/ min	285nm	Broad Peak	Method rejected
Phenomenex Luna C_{18}, 100A, 5µm, 250mmx4.6mm i.d.	Acetonitrile: Methanol = 35:65	1.0 ml/ min	285nm	Good Peak	Method Accepted

Summary of Optimized Chromatographic Conditions:

The Optimum conditions evolved from experiments can be summarized as below:

Table-2: Summary of Optimised Chromatographic Conditions

Mobile phase	Acetonitrile: Methanol = 35:65
Column (Stationary Phase)	Phenomenex Luna C_{18}, 100A, 5µm, 250mmx4.6mm i.d.
Column Temperature	Ambient
Detection Wavelength	285 nm
Flow rate	1.0 ml/ min.
Run time	09 min.
Temperature of Auto sampler	Ambient
Diluent	Mobile Phase
Injection Volume	20µl
Type of Elution	Isocratic
Retention time	4.078 minutes

MOBILE PHASE SET UP

The mobile phase employed in this study consisted of a 35:65% v/v combination of Acetonitrile and Methanol in a volume of up to 1000ml, with 350ml of Acetonitrile and 650ml of Methanol[7].

STOCK AND STANDARD SOLUTIONS SET UP

Accurately measure approximately 10 milligrams of Nicoumalone working standard and transfer it into a 10 milliliter volumetric flask that has been recently cleaned and dried. The initial stock solution was dispersed and volume-adjusted with the mobile phase to produce a solution of approximately 1000 mcg/ml or 1000ppm concentration, which was then determined[8]. A volume of 1 milliliter of the stock solution was taken and subsequently diluted with the mobile phase to a final volume of 100 milliliters, resulting in a final concentration of 10 micrograms per milliliter, which served as the standard solution[9].

PREPARATION OF SAMPLE SOLUTION FOR ASSAY

Twenty tablets are taken for the test. As per the composition given in the brand, 3mg of drug should present in each tablet. Individual tablet were weighed and calculate the average weight of tablet containing 1mg of drug. The weight of tablet containing 1mg of the drug is diluted up to 100ml of mobile phase to produce 10µg/ml of sample solution[10].

DEVELOPED METHOD VALIDATION

As per ICH criteria, the proposed methodology was validated for the following parameters.

ACCURACY

Recovery trials were performed to determine the correctness of the anticipated approach by adding completely various quantities (80%, 100%, and 120%) of pure Nicoumalone with varying concentrations of 12-18g/ml[11]. The percentage recovery numbers were determined, and the results are displayed in Table-3.

PRECISION

Repeatability

Using the peak areas and retention times obtained from six independent samples of a specific concentration of Nicoumalone, each methodology's precision was evaluated independently[12]. The percent relative standard deviations determined for the substance are displayed in Table-4.

Intra-Assay & Inter-Assay (Intermediate Precision)

The method was subjected to intra- and inter-day testing, and the results showed elevated mean assay readings and low standard deviation values, as well as a low percentage of relative standard deviation (% RSD < 2%) for Nicoumalone. These findings indicate that the proposed approach is precise and accurate in measuring the substance's fluctuations within a day and from day to day[13]. This is demonstrated in Table 5.

LINEARITY AND RANGE

In order to ascertain the linearity and range, it is necessary to measure approximately 10mg of Nicoumalone and transfer it into a 100 ml volumetric glass flask that has been recently cleaned and dried. The stock solution was dispersed and adjusted by volume using the mobile phase to produce a solution with a concentration of approximately 100 mcg/ml. Subsequent to the preparation of the stock solution, aliquots of 1ml, 2ml, 3ml, 4ml, and 5ml were extracted and subsequently solubilised with the mobile phase in a 10ml volumetric flask[14]. The mobile phase was introduced into the capacity, leading to the attainment of final concentrations of 10mcg/ml, 20mcg/ml, 30mcg/ml, 40mcg/ml, and 50mcg/ml, correspondingly. Subsequently, the aforementioned concentrations were subjected to high-performance liquid chromatography (HPLC) analysis employing a UV-visible spectrophotometer detector set at a wavelength of 285 nm. To ensure linearity, the resultant peak area shall be graphed against the concentration[15].

METHOD ROBUSTNESS

This study investigated the impact of slight modifications in the natural procedural parameters, including flow rate (±0.1ml/min), temperature (±2^0C), detection wavelength (±2nm), and content in mobile phase (±2%), on the efficacy of the methodology. The objective was to assess the suitability of the technique and to develop a suitable RP-HPLC protocol for the analysis of Nicoumalone (API)[16].

LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTITATION (LOQ)

The LOD and LOQ were calculated by the use of the equations

$$LOD = 3.3 \times \sigma / S$$

and

$$LOQ = 10 \times \sigma / S$$

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

SYSTEM SUITABILITY PARAMETERS

Several analytical approaches are related with system quality testing in nursing. The testing section aids in the development of instruments, physics, and materials to be analysed. It is an essential component of the system that will be assessed intrinsically. Table-7 shows the system quality parameters that were determined[17].

ASSAY OF NICOUMALONE IN TABLET FORMULATION

The ingestion of 20 pills was facilitated through the utilization of the I.P. method. The individual weight of each pill was measured and subsequently, the mean weight was calculated. The tablets underwent a comprehensive process of grinding and triturating. A specific amount of powder equivalent to 100 mg of medication was carefully transferred into a volumetric flask with a capacity of 100 ml[18]. Following the addition of 75 milliliters of mobile phase, the solution underwent sonication for a duration of 15 minutes. Subsequently, the volume was augmented to 100 milliliters utilizing the identical solvent. Subsequently, the solution was diluted by 10 ml in 100 ml of the mobile phase. The solution underwent filtration and sonication procedures utilizing a 0.45 micron membrane filter[19]. A 3.5 mL aliquot of the initial stock solution was distributed into five separate 10 mL volumetric flasks and subsequently adjusted to volume using the identical solvent. The resultant solutions were subjected to injection into the HPLC apparatus in five iterations, and subsequent observations

were recorded. Furthermore, a duplicate injection of a standardized solution was administered into the High-Performance Liquid Chromatography (HPLC) instrument, and the areas of the peaks were documented[20]. The results collected are presented in Table 8.

RESULTS AND DISCUSSIONS

The most reliable, precise, and accurate analytical method for quantifying nicoumalone from bulk drug, in addition to the pharmaceutical formulations and dossage form, is the current RP-HPLC method. For calculation of tailing factor, Plate count, and % RSD of Peak Area in analysing Nicoumalone, six replicate injections of the Standard Solution was taken.

Accuracy

The usual addition method was used to calculate the method of recovery. The average recovery of total 09 samples is coming around 100.0819% having average % RSD value 0.5722(< 2%) ,which is accurate. The accuracy of the procedure is demonstrated by the values of % recovery & % RSD in Table -3.

Table-3-: Accuracy Readings

Sample ID	Concentration (µg/ml)			% Recovery of Pure drug	Statistical Analysis
	Conc. Injected	Conc. Recovered	Peak Area		
S_1 : 80 %	12	11.87153	158252	98.92942	Mean= 99.44 S.D. = 0.503409 % R.S.D.= 0.506447
S_2 : 80 %	12	11.99225	159861	99.93544	
S_3 : 80 %	12	11.93606	159112	99.46713	
S_4 : 100 %	15	14.97777	199652	99.85182	Mean= 100.0949 S.D. = 0.24745 % R.S.D.= 0.024721
S_5 : 100 %	15	15.01296	200121	100.0864	
S_6 : 100 %	15	15.05198	200641	100.3465	
S_7 : 120 %	18	18.17923	242321	100.9957	Mean= 100.711 S.D. = 1.048512 % R.S.D.= 1.0411200
S_8 : 120 %	18	18.28585	243742	101.588	
S_9 : 120 %	18	17.91895	238852	99.54972	

Precision

In Repeatability, A relative standard deviation was visible after six replicate injections of a standard solution mixture at working concentration. The mean % RSD of six replicates has came as 0.393811 (which is < 2%) about the drug's peak regions, indicating adequate reproducibility and, as a result, the system's accuracy. Table 4 presents the results regarding system precision repeatability.

Table-4: Repeatability Results of Precision

HPLC Injection Replicates of Nicoumalone	**Retention Time (Minutes)**	**Peak Area**
Replicate – 1	4.078	324295
Replicate – 2	4.077	324262
Replicate – 3	4.078	327007
Replicate – 4	4.076	324815
Replicate – 5	4.075	323124
Replicate -6	4.077	324726
Average		**324704.8**
Standard Deviation		**1278.722**
% RSD		**0.393811**

The approach was tested intra- and inter-day, and the elevated levels of mean assay along with low values of the standard deviation and% RSD (% RSD < 2%) within a day and day to day fluctuations for Nicoumalone demonstrated that the suggested method is exact.

Table – 5: Results of Intra-assay & Inter-assay

Conc. Of Nicoumalone (API) (µg/ml)	Observed Conc. Of Nicoumalone (µg/ml) by the proposed method			
	Intra Day		Inter Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
8	7.96	1.09	8.06	1.06
10	10.09	0.95	9.86	0.92
12	12.03	0.96	11.96	0.99

Linearity and range

The linearity of the calibration curve for Nicoumalone (API) was found to be satisfactory within the range of 0-50g/ml, as evidenced by a high correlation coefficient (r2) of 0.999 (Fig-2). A regression equation of y = 13228x - 2825 is commonly utilized for the calibration graph of Nicoumalone. The prerequisite for acceptance is that the correlation coefficient should exceed 0.990.

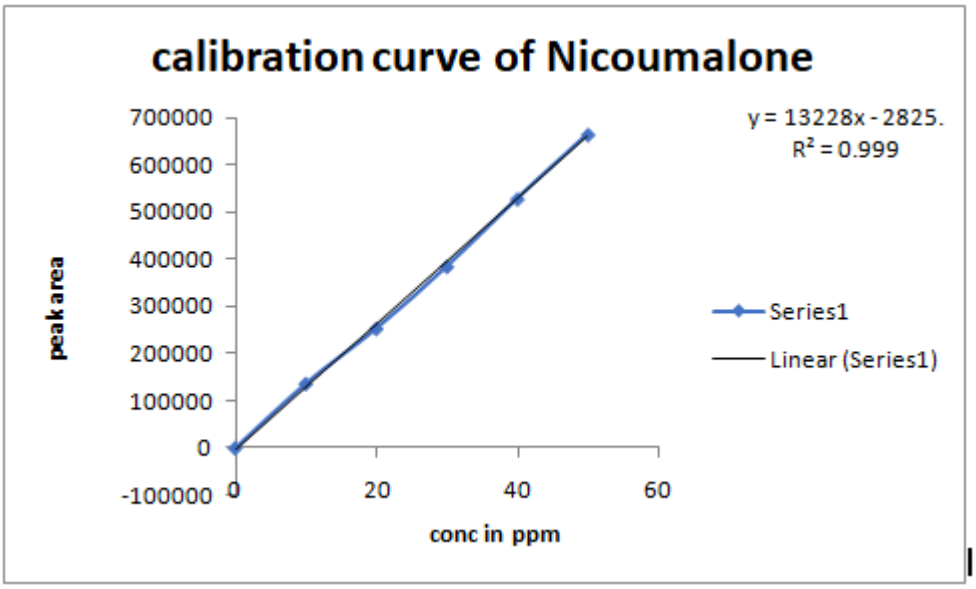

Fig-2: Calibration curve of Nicoumalone (API).

Table-6: Linearity results of Nicoumalone

CONC.	AUC (n=6)
0	0
10	136465
20	253214
30	384782
40	528192
50	664624

Robustness

When tested for robustness, the proposed RP-HPLC technique for the analysis of Nicoumalone (API) performed well with minor changes to a chromatographic conditions, such as a change in flow rate (± 0.1ml/min), change in column temperature (± 2°C), change in maximum wave length(± 2nm) which can be shown in Table-6.

Table – 6: Result of Method Robustness Test

Change in parameter	% RSD
Flow (1.1 ml/min)	0.09
Flow (0.9 ml/min)	0.07
Temperature (27^0C)	0.96
Temperature (23^0C)	0.89
Wavelength of Detection (285 nm)	0.06
Wavelength of detection (289 nm)	0.08

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

Both the Limit of Detection (LOD) and Limit of Quantification (LOQ) represent the minimum concentration at which the analyte can be deemed reliable for the completion of the experiment. The dependability of the system is evidenced by the determination of the detected (LOD) and quantified (LOQ) concentrations, which were found to be 0.09 µg/ml and 0.29 µg/ml, respectively.

System Suitability Parameter

The results demonstrate the Resolution (Rs) parameter value is 8.64(should be more than 2.0), Asymmetry value is 0.87(should be less than equal to 2.0),Theoretical Plate count is 4689(should be more than 2000) and USP Tailing factor of 1.29 (which should be less than 2.0). So all system suitability parameters are found to be within the range, hence the proposed research approves the system suitability which can be shown in the Table-7.

Table-7: Data of System Suitability Parameter

S.No.	Parameter	Limit	Result
1	Resolution	Rs > 2	8.64
2	Asymmetry	T ≤ 2	Nicoumalone =0.87
3	Theoretical plate	N > 2000	Nicoumalone =4689
4	Tailing Factor	T<2	Nicoumalone =1.29

ASSAY OF NICOUMALONE IN TABLET FORMULATION

The % Purity of ACETROM tablets containing NICOUMALONE was found to be 99.66% with RSD of 0.48%(should be less than 2%)which has been shown in the Table-8

Table-8: Assay of Nicoumalone Tablets

Brand name of Tablets/Capsules	Labelled amount of Drug (mg)	Mean (±SD) amount (mg) found by the proposed method (n=6)	Mean (± SD) Assay (n = 6)
ACITROM(ABBOTT)	3	2.99 (±0.09)	99.66(±0.48)

CONCLUSION

The suggested RP-HPLC technique produces results with high sensitivity, accuracy and consistency. In the written word, there was no clear, time-saving analytical approach for Nicoumalone. The devised method was approved in compliance with ICH guidelines. Every validation parameter was verified to meet the acceptance standards. We conclude that the technique was precise, reproducible, linear, and accurate. These results suggest that the approach might be utilised in quality control labs to examine the medicine in tablet dosage forms. The results suggest that the new approach is yet another acceptable method for assay, purity, and analysis of Nicoumalone in various formulations.

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