Research Article

DEVELOPMENT OF VALIDATED STABILITY INDICATING RP-HPLC METHOD FOR ESTIMATION OF ACENOCOUMAROL IN BULK AND TABLET DOSAGE FORM

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ABSTRACT

The present paper deals with development and validation of a stability indicating reverse phase HPLC method for the estimation of Acenocoumarol 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. Eluents were monitored by UV detector set at 283nm. The developed method was statistically validated for linearity and range, precision, limit of detection, limit of quantitation, accuracy, robustness and specificity. The specificity of the method was ascertained by forced degradation studies by acid hydrolysis, alkali hydrolysis, neutral hydrolysis, oxidative, thermal and photolytic degradation conditions. The degraded products were well resolved from the analyte peak.

Keywords: Acenocoumarol, Oral anticoagulant, stability indicating RP-HPLC method, Forced degradation, Validation.

INTRODUCTION

High Performance Liquid Chromatography (HPLC) is an integral analytical tool in assessing drug product stability. A stability-indicating method is "a validated quantitative analytical procedure that can detect the changes with time in the pertinent properties of the drug substance and drug product. A stability-indicating method accurately measures the active ingredients, without interference from degradation products, process impurities, excipients, or other potential impurities".[1] The International Conference on Harmonization (ICH) guideline Q_1A (R_2) entitled "Stability testing of new drug substances and products" requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance.[2] Forced degradation studies (chemical and physical stress testing) are essential to help develop and demonstrate the specificity of stability-indicating methods.

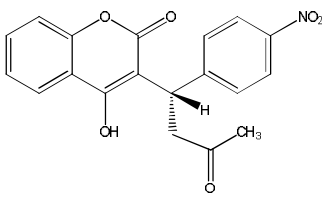

Figure 1: Structure of Acenocoumarol

Acenocoumarol[3] (previously known as nicoumalone) is an oral anticoagulant that functions as a vitamin K antagonist. Chemically it is 4-hydroxy-3-[1-(4-nitrophenyl)-3-oxobutyl]-Coumarin (Figure 1). It is administered in the management of thromboembolic disorders. Acenocoumarol is official in Indian[4] and British[5] pharmacopoeia. Both the pharmacopoeias explain the assay of acenocoumarol API by aqueous acid base titration while that of the tablet formulation by Spectrophotometric method (λmax at 306 nm and specific absorbance at 521).

Extensive literature survey revealed that a few analytical and bio-analytical methods have been reported for its quantitative estimation in pharmaceutical formulations and biological fluids, which include spectrophotometric[6-7], HPLC[8-9] and bioanalytical[10-16] methods. But to the best of our knowledge a rapid, precise and accurate stability indicating HPLC procedure is not yet reported which would serve as stability indicating assay method for the analysis of acenocoumarol. So the authors have made an attempt to develop and validate such a stability indicating HPLC method for the estimation of acenocoumarol.

MATERIALS AND METHODS

HPLC Instrumentation and conditions

The HPLC (Shimadzu, LC 2010 CHT, Kyoto, Japan) consisting of a quaternary system with automatic injection facility was used for analysis. The detector consisted of a UV-Vis model and operated at 283 nm. The capacity of loop was 20 µl. The software used was 'LC Solution'. Thermo BDS Hypersil C_{18} (250 mm X 4.6 mm, 5 µm) column was utilized for analysis. The mobile phase comprising of Acetonitrile: Ammonium acetate buffer 0.01M, pH 6 (adjusted using 0.1N NaOH) was delivered at a flow rate of 0.8 ml/min. The mobile phase and samples were filtered using 0.2µm membrane filter and degassed using an ultrasonicator (model UCB-40) before injecting in HPLC system. The separation was achieved by isocratic elution.

Chemicals and Reagents

Pharmaceutical grade acenocoumarol was kindly supplied as a gift sample by Century Pharmaceuticals, Vadodara, Gujarat, India. It was certified to contain 98.98% w/w of

acenocoumarol on dried basis and used as such without further purification. All chemicals and reagents used were of HPLC grade and were purchased from Loba Chemie Pvt. Ltd., Mumbai, India. Hydrogen peroxide I.P (3%) Solution and commercial formulation of Acenocoumarol [ACITROM, Abbott Healthcare Pvt. Ltd.] were purchased from local medicine store.

Preparation of stock and standard solutions

100 mg of pure drug was weighed accurately and dissolved in 100 ml of ethanol, with the help of sonication, to prepare the stock solution of 1000 µg/ml concentration. This solution was further diluted with the same solvent to obtain concentrations in the range of 25-150 µg/ml. These solutions were used for analysis.

Preparation of sample solution for assay

20 tablets containing label claim of 4 mg were weighed accurately and average weight of tablet was determined. A quantity of the powder equivalent to 50 mg of acenocoumarol was dissolved in 100 ml of ethanol with the help of sonication to prepare a solution of concentration 500 µg/ml. Appropriate aliquot was further diluted to get a working standard of 100 µg/ml concentration.

Forced Degradation Studies

In order to determine the specificity of developed method and to observe the rate and extent of degradation that is likely to occur in the course of storage and/or after administration to body, the drug was subjected to stress conditions. The various degradation pathways studied are acidic hydrolysis, Basic hydrolysis, neutral hydrolysis, thermal degradation, oxidative degradation and photolytic degradation.

Acidic and Basic Hydrolysis

To 10 ml of stock solution of Acenocoumarol API, 10 ml each of 1M HCl and 1M NaOH was added. The resultant solution was refluxed at 70^0 C for a period of 6 hrs. Samples were withdrawn at every 2 hrs. From the resultant solutions 2 ml were diluted with the mobile phase to obtain the solution of 100 µg/ml concentration and 20 µl were injected into the system.

Neutral Hydrolysis

To 10 ml of stock solution of Acenocoumarol API, 10 ml of HPLC grade water was added and the mixture was refluxed for 6 hrs at 70^0C to study the degradation under neutral conditions.

Oxidative degradation

Hot condition: To 10 ml of stock solution of 1000 µg/ml pure acenocoumarol, 10 ml of 3 % Hydrogen peroxide solution was added. The solution was refluxed at 70°C for 6 hrs. Samples were withdrawn at every 2 hrs. 2 ml from the resultant solutions were diluted with mobile phase to obtain concentration of 100 µg/ml and 20 µl was injected into the system. Cold condition: To 10 ml of stock solution of 1000 µg/ml pure acenocoumarol, 10 ml of 3% hydrogen peroxide solution was added. The solution was kept at room temperature in dark for 6 hrs. Samples were withdrawn at every 2 hrs. 2 ml from the resultant solutions were diluted with mobile phase to obtain concentration of 100 µg/ml and 20 µl was injected into the system.

Thermal degradation

Acenocoumarol API was placed in oven at 100°C for 6 hrs. After 6 hrs, 5 mg of the drug was taken and dissolved in 10 ml of ethanol to obtain a solution of concentration 500 µg/ml. Final dilution was made in mobile phase to obtain a solution of 100 µg/ml concentration to study the effect of dry heat degradation.

Photolytic degradation

The photochemical stability of the drug was studied by exposing the stock solution of 1000 µg/ml acenocoumarol API to direct sunlight for 6 hrs, by keeping it on terrace in the month of May. The resultant solution was diluted to obtain concentration of 100 µg/ml and 20 µl was injected into the system under optimized conditions.

Validation of the developed method

Linearity and range

The stock solution 1000 µg/ml of acenocoumarol was prepared in ethanol and this stock solution was diluted with mobile phase to obtain final concentrations 25-150 µg/ml. A 20 µl volume of each concentration was injected into LC, six times, under the optimized conditions described above.

Precision

Intermediate precision studies (intra-day and inter-day) were performed by assay of acenocoumarol tablet at concentration of 100 µg/ml on the same day and on three consecutive days respectively.

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

For Acenocoumarol API, LOD and LOQ were separately determined based on the standard deviation of response of the respective calibration curves.

Accuracy

To ascertain the accuracy of proposed method, recovery studies were carried out by standard addition method, as per ICH guidelines. Recovery studies were carried out at a concentration of 100 µg/ml, by applying the method to drug sample to which known amount of Acenocoumarol corresponding to 80, 100, 120% of label claim was added. Results are expressed as % recovery.

Robustness

To study the robustness of the developed method, small changes in system parameters were done and its effect on resultant retention time (t_R) was assessed. Variations in mobile phase composition (± 2%), oven temperature (±

2°C) and flow rate (± 2ml/min) were done and the results obtained were compared with the expected results.

Specificity

The specificity of the HPLC method was determined by complete separation of acenocoumarol from its degradation products.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

The HPLC procedure was optimized with a view to develop stability-indicating assay method. The method was optimized on Thermo BDS Hypersil column (250 mm X 4.6 mm, 5 µm). Initially different combinations of mobile phases such as methanol: water 80:20 v/v, acetonitrile (ACN): water 80: 20 v/v, 0.01M ammonium acetate buffer: ACN 40:60 v/v were tried. Using the optimized mobile phase best results was obtained in terms of peak symmetry, selectivity and analysis time for the selected tablet formulation. Results are summarized in table 1.

Assay of Acenocoumarol in tablet formulation

Assay was performed by using the regression equation (y=45359x+8959, r^2 =0.997) obtained from the standard calibration curve of Acenocoumarol API. Results are given in table 2. The percentage purity was found to be 99.51% as per the method.

Table 1: Optimization of chromatographic conditions

Column	Mobile phase composition	Flow rate ml/min.	Retention time	Tailing Factor	Theoretical Plates	Peak Description	Result
Thermo BDS Hypersil C_{18} (250 mm X 4.6 mm, 5 µm)	Methanol : Water 80 : 20(v/v)	0.8	2.449	1.2	783	Theoretical plates were less	Rejected
	Acetonitrile : Water 80 : 20(v/v)	0.8	2.339	0.9	641	Theoretical plates were less	Rejected
	Acetonitrile : Ammonium acetate buffer 60 : 40(v/v)	0.8	5.59	0.98	1113	Theoretical plates were less	Rejected
	Acetonitrile : Ammonium acetate buffer 80 : 20(v/v)	0.8	2.746	2.03	4081	Slightly tailing peak	Rejected
	Acetonitrile : Ammonium acetate buffer pH 6 80 : 20(v/v)	0.8	2.675	1.2	4470	Good peak shape, Symmetrical peak, Good Theoretical plates	Accepted

Table 2: Result for analysis of marketed Formulations

Marketed Formulation	Label claim (mg/tab)	Drug content (%) ± SD	%RSD*
Acitrom tablet	4	99.51% ± 0.26	1.28

*n= 6 *determinations*

Table 3: Results of forced degradation studies of Acenocoumarol

Stress condition	Amount of Acenocoumarol degraded (%)	Amount of Acenocoumarol recovered (%)	Retention time (t_R) of degraded products (min)
1M HCl (2hrs at 70°C)	98.45	1.55	2.9,3.5
1M NaOH (4hrs at 70°C)	1.85	98.15	-----
Neutral (4hrs at 70°C)	1.31	98.69	-----
H_2O_2 (3hrs at 70°C)	19.81	80.19	3.2,4.1
H_2O_2 (3hrs at RT)	9.32	90.68	3.2,4.1
Dry heat (4hrs at 100°C)	3.20	96.80	-----
Sunlight (4hrs)	31.88	68.11	-----

Forced degradation studies

The chromatogram of pure Acenocoumarol API is shown in figure 2. The results of the forced degradation studies are given in table 3 and figure 3-7. Significant degradation was observed under acidic (98.45%), photolytic (31.88%) and oxidative (19.81%) conditions. Additional peaks of degradation products were observed for acidic (at retention time 2.9 and 3.5 min.) and oxidative (at retention time 3.2 and 4.1 min.) conditions. No additional peak was observed for photolytic degradation but significant reduction in peak area was found. Basic, neutral and thermal degradation was not considerable as neither any additional peaks nor significant reduction in peak area was seen. Well separation of degradation products from parent peak shows that the method is stability indicating.

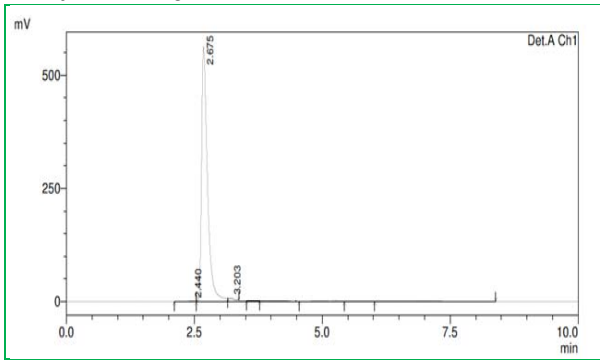

Figure 2: Chromatogram of Acenocoumarol API in optimized mobile phase

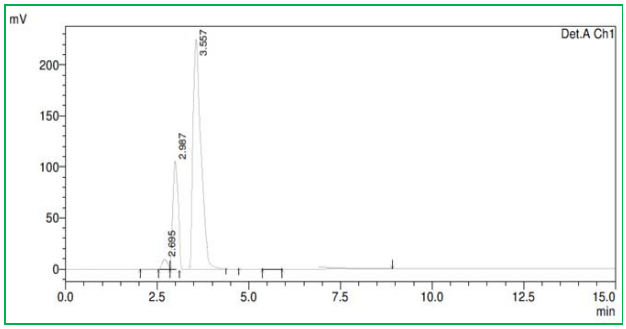

Figure 3: Chromatogram of acid (1M HCl, 2hrs at 70°C) treated Acenocoumarol

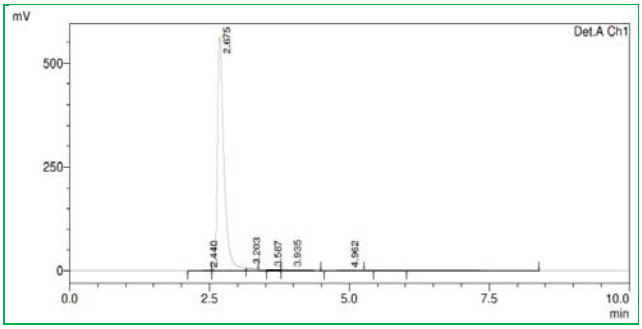

Figure 4: Chromatogram of base (1M NaOH, 4hrs at 70°C) treated Acenocoumarol

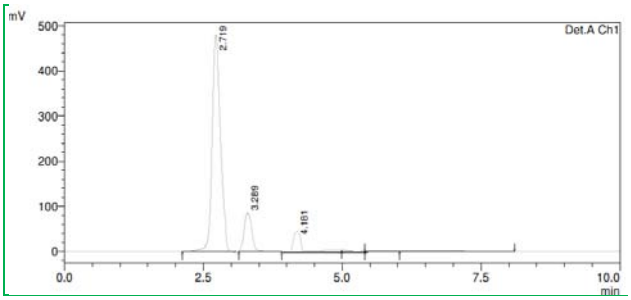

Figure 5: Chromatogram of H_2O_2 (3 hrs at 70°C) treated Acenocoumarol

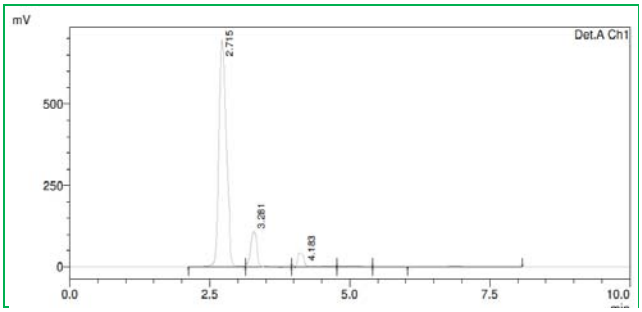

Figure 6: Chromatogram of H_2O_2 (3 hrs at RT) treated Acenocoumarol

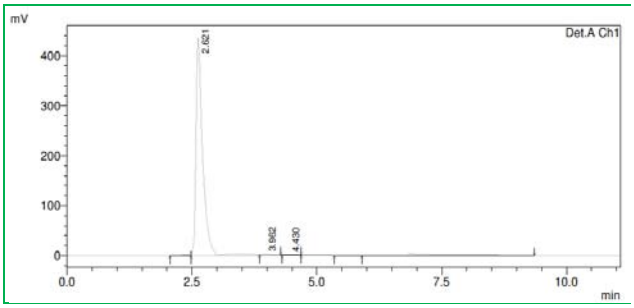

Figure 7: Chromatogram of photochemically (sunlight, 4 hrs) treated Acenocoumarol

Method validation

Linearity and range

The calibration curve showed good linearity in the range of 25-150 µg/ml for Acenocoumarol API with correlation coefficient (r^2) of 0.997 (figure 8). A typical calibration curve has the regression equation y=45359x+8959 for Acenocoumarol as shown in table 4.

Table 4: Linear regression data for calibration curves

Parameters	Result
Linearity range (µg/ml)	25-150
r^2 ± SD	0.997 ± 0.0006
Slope ± SD	45332 ± 0.14
Intercept ± SD	8959 ± 105

*n= 6 *determinations*

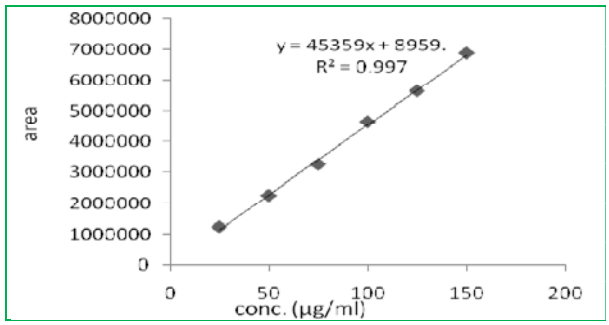

Figure 8: Calibration graph of Acenocoumarol

Precision

The intra and inter day assay for the method was done and high values of mean assay (99.51%) and low values of % RSD (0.02 and 0.01) for Acenocoumarol revealed that the proposed method is precise (table 5).

Table 5: Results for intra and inter day precision

Intra-day precision	**Acenocoumarol**
Drug content ± SD	99.51 ± 936
% RSD	0.02
Inter-day precision	**Acenocoumarol**
Drug content ± SD	99.51 ± 462
% RSD	0.01

*n= 6 *determinations*

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

The minimum concentration level at which the analyte can be reliably detected (LOD) and quantified (LOQ) were found to be 0.0076 and 0.0231 µg/ml respectively as shown in table 6.

Table 6: LOD and LOQ of Acenocoumarol

Parameters	Acenocoumarol
LOD (µg/ml)	0.0076
LOQ (µg/ml)	0.0231

*n= 6 *determinations*

Accuracy

The mean recovery of the method, determined by standard addition method, was found to be 96.33%. The values of % recovery and % RSD, listed in table 7 indicate that the method is accurate.

Table 7: Recovery data of Acenocoumarol

Level	Theoretical Amount (µg/ml)	Amount Found (µg/ml)	% Recovery ± SD	% RSD*
80	180	172.26	95.70 ± 0.03	0.03
100	200	190.27	95.48 % ± 0.52	0.55
120	220	215.25	97.83 % ± 0.71	0.73

*n= 6 *determinations*

Table 8: Result of Robustness evaluation of RP-HPLC method

Factor	Retention time (t_R) (Mean ± S.D)	% RSD
Flow rate (ml/min)	6.77 ± 0.01	0.26
Percentage of acetonitrile in mobile phase (v/v)	6.71 ± 0.03	0.52
Column temperature	6.72 ± 0.02	0.31

*n= 6 *determinations*

Robustness

Influence of small changes in chromatographic conditions such as change in flow rate (±0.2ml/min), percentage of acetonitrile in mobile phase (±2%) and column temperature (±2°C), studied to determine the robustness of the method, were also in favor of (Table 8, %RSD<2%) the developed RP-HPLC method for the analysis of Acenocoumarol API.

Specificity

The International Conference on Harmonization (ICH) guidelines define specificity as the ability of method to assess unequivocally the analyte of interest in the presence of potential interferences.[17] The results of specificity indicated that the peak was pure in presence of degraded sample.

The results of linearity, intra and inter day precision, LOD, LOQ, accuracy, robustness and specificity in analytical solution established the validation of the developed RP-HPLC method for the analysis of Acenocoumarol.

CONCLUSION

An accurate, precise and specific stability indicating RP-HPLC method has been developed and validated for the estimation of Acenocoumarol. The method is rapid owing to its shorter run time. Absence of co-eluting peaks along with the main peak indicated that the developed RP-HPLC method is specific for Acenocoumarol in presence of degradation products. Even though no attempt has been made to identify the degraded products, proposed method can be used as a stability indicating method for assay of Acenocoumarol in commercial tablet formulation.

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