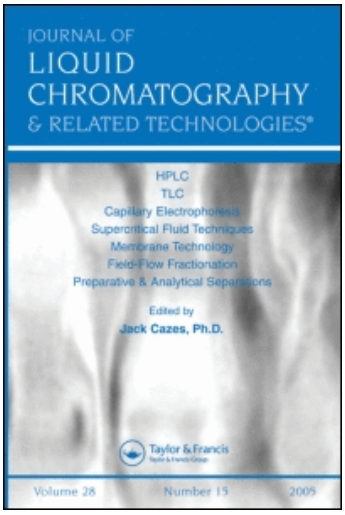

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HPLC Determination of Acenocoumarol and its Major Thermal Degradation Product

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Abstract: A High performance liquid chromatographic (HPLC) method is presented for the determination of acenocoumarol and its thermal degradation product using a reverse phase C_{18} column at ambient temperature with the mobile phase consisting of water, and the pH adjusted to 3.0 using phosphoric acid:acetonitrile (50:50). Quantitation was achieved with UV detection at 280 nm based on peak area. The method was developed and validated for the determination of acenocoumarol in tablets. The proposed method was validated for selectivity, linearity, accuracy, and robustness. The method was found to be suitable for the quality control of acenocoumarol in tablets as well as the stability-indicating studies.

Keywords: Acenocoumarol, HPLC, Assay, Stability-indicating method

INTRODUCTION

Acenocoumarol or Nicoumalone, 4-hydroxy-3-[1-(4-nitrophenyl)-3-oxobutyl]-coumarin, is an orally administered coumarin anticoagulant with actions similar to those of warfarin sodium (Figure 1). It is used in the management of thrombo-embolic disorders. The usual dose on the first day is 8 to 12 mg, on the second day 4 to 8 mg; subsequent maintenance doses range from 1 to 8 mg depending on the response.[1]

The method was validated by following the analytical performance parameters suggested by the International Conference on Harmonization (ICH).[2]

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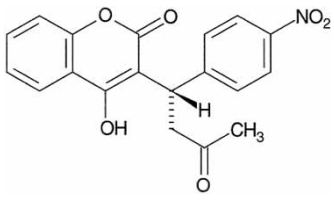

Figure 1. Acenocoumarol.

Most of the analytical techniques for acenocoumarol that are described in the literature are based on the determination of this drug in human plasma.[3–12] An analytical technique for acenocoumarol described in the literature is based on the liquid chromatographic determination of this drug in pharmaceutical formulations and raw materials with warfarin.[13] An UV quantitation of acenocoumarol in tablets is described in British Pharmacopoeia.[14]

The aim of our investigation was to develop and validate a liquid chromatographic method for the simultaneous determination of acenocoumarol in the presence of its degradation products in pharmaceutical dosage forms.

EXPERIMENTAL

Chemicals and Reagents

The acenocoumarol working standard was provided by Anzen Exports (Mumbai, India) 99.10%, calculated with reference to the dried substance.

A commercial tablet formulation was studied. Its composition was acenocoumarol 4 mg, in a matrix of hydroxypropyl methylcellulose, lactose, starch, magnesium stearate, talc, silicon dioxide, and sodium lauryl sulphate.

Acetonitrile used was HPLC grade, J. T. Baker, (Estado de Mexico, México). Phosphoric acid, 85%, was AR grade (Mallinckrodt Baker Inc., Phillipsburg, New Jersey, USA). Distilled water was passed through a 0.45 μm membrane filter.

Equipment

The HPLC system consisted of a dual piston reciprocating Spectra Physics pump (Irvine, CA, United States, Model ISO Chrom. LC pump), a UV-Vis Hewlett Packard detector (Model 1050), a Hewlett Packard integrator (Loveland, CO, United States, Series 3395), and a Rheodyne injector (Model 7125).

Chromatographic Conditions

The analytical column was a reversed phase C_{18} column (Ace, Aberdeen, Scotland) (250 × 4.6 mm, 5 μm). The separation was carried out under

isocratic elution with water, pH adjusted to 3.0 using phosphoric acid: acetonitrile (50:50) (v/v). The flow rate was 1.5 mL/min. The wavelength was monitored at 280 nm, and the injection volume was 20 μL. The HPLC was operated at ambient temperature. Under these conditions, the retention time (t_R) of acenocoumarol was approximately 7 min.

Preparation of Standard Solutions

A standard stock solution of acenocoumarol was prepared at a concentration of 0.8 mg/mL by dissolving the appropriated amount of standard in acetonitrile. The standard solution was obtained by diluting the standard stock solution with mobile phase to obtain a solution containing acenocoumarol at 48 μg/mL.

Sample Preparation

Twenty tablets were weighed, finely powered, and an accurately weighed powered sample equivalent to one tablet was transferred to a 25 mL volumetric flask; 20 mL acetonitrile was added, and the flask was kept in an ultrasonic bath for 5 min. The contents of the flask were then diluted to 25 mL with acetonitrile and thoroughly mixed. A 3 mL aliquot of the solution was transferred to a 10 mL volumetric flask. The sample was diluted to volume with mobile phase and filtered through a 0.2 μm nylon membrane (25 mm disposable filter; Cat. N° Y02025WPH μicroclar, Buenos Aires, Argentina).

Resolution Solution

Forty milligrams of acenocoumarol was accurately weighed, transferred to a 50 mL volumetric flask, and dissolved in acetonitrile. Ten milliliters of this solution were placed in an open container in an oven at 110°C for 24 h. The solution was diluted to a concentration of 48 μg/mL.

Method Validation

System Suitability

Relative standard deviations (RSD) values of the peak area, tailing factor, and retention time were the chromatographic parameters selected for the system suitability test.[15]

Specificity

Forced degradation studies were performed to evaluate the specificity of the method. Degraded samples were prepared by refluxing 0.8 mg/mL acenocoumarol working standard with acid (1 N hydrochloric acid), base (1 N NaOH), water, 30% hydrogen peroxide, and refluxing for at least 30 min. The drug was subjected to thermal degradation (either in the solid state or in solution in an open container in an oven at 110°C for 24 h) and photochemical degradation. The solution was transferred to a container and exposed to daylight for 24 h. After degradation treatment, samples were allowed to cool at room temperature and diluted, if necessary, to the same concentration as that of the standard solution, after being neutralized. After degradation, samples were analyzed using the methodology and the chromatographic conditions described.

Linearity

The linearity of the HPLC detector response for determination of acenocoumarol was evaluated by analyzing a series of different concentrations of the compound. In this study six concentrations were chosen, ranging between 18.7 and 93.4 μg/mL. Each concentration was repeated three times, this approach will provide information on the variation in peak area between samples of the same concentration.

Precision

System precision: Six replicates of a standard solution were analyzed to assess system precision.
Method precision (repeatability) and intermediate precision: Six replicates ($n = 6$) of sample solutions were analyzed on the same day to determine method precision, and on two different days by two different analysts to evaluate intermediate precision.

Accuracy

The accuracy was evaluated by the recovery studies at concentration levels of 80, 100, and 120% (3 samples each). Twenty tablets from the same lot of a commercial formulation were emptied into a mortar. The amount of acenocoumarol recovered in relation to the results obtained in the intermediate precision study was calculated.

Robustness

Variation of the organic strength of the mobile phase or pH have a significant effect on chromatographic resolution of acenocoumarol and its major thermal degradation product.

RESULTS AND DISCUSSION

The described reversed phase liquid chromatography method was developed to provide a rapid quality control determination of acenocoumarol in tablets. Validation of the method was performed according to ICH. This method uses a simple mobile phase. All samples were analyzed using the assay chromatographic conditions described.

Selectivity

The stability indicating capability of the assay was examined by accelerated stress testing. Standard and samples were subjected to degradation under alkaline, acid, oxidative, hydrolysis, thermal, and photochemical conditions. Degradation was indicated in the stressed sample by a decrease in the expected concentration of the drug and increased levels of degradation products. Acenocoumarol was degraded to numerous products under the thermal condition. The retention times of degradation products were shorter and larger than that of ACN, indicating that the degradation products were more or less polar than their parent drug (Table 1).

Regarding the thermal condition (solution), acenocoumarol was found to decompose significantly. The major degradant peak was eluted at 10.96 min (Figure 2). Selectivity was demonstrated showing that acenocoumarol was free of interference from degradation products, and that no interference from the sample excipients was observed at the detection wavelength; thus, the proposed method can be used in a stability assay.

Table 1. Selectivity: degradation conditions of acenocoumarol

Condition	Time (h)	ACN (%)	RRT of degradation products[a]
Acid (1 N HCl, reflux)	0.5	101.0	Non detected
Base (1 N NaOH, reflux)	0.5	96.7	Non detected
Hydrogen peroxide 30% (reflux)	0.5	99.9	Non detected
Water (reflux)	0.5	98.0	Non detected
Heat dry, 110°C (solution)	24	45.9	0.34, 0.68, 0.82, 1.54, 3.67, 4.03
Heat dry, 110°C (solid)	24	92.6	3.25
Daylight exposure	24	99.1	Non detected

[a]RRT, relative retention time.

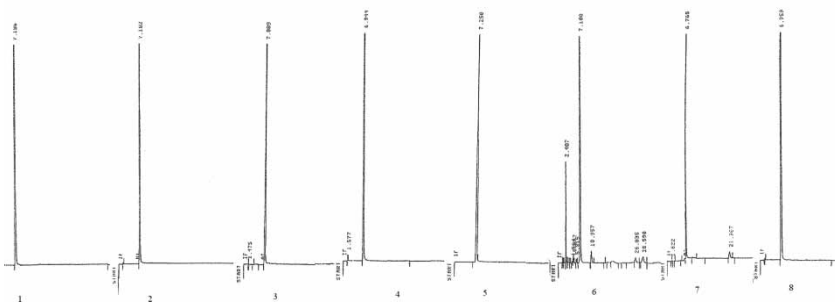

Figure 2. Chromatograms of acenocoumarol: 1- Standard; 2- Acid hydrolysis (1 N HCl, reflux, 0.5 h); 3- Alkaline hydrolysis (1 N NaOH, reflux, 0.5 h); 4- Oxidation (Hydrogen peroxide 30%, reflux, 0.5 h); 5- Hydrolysis (Water, reflux, 0.5 h); 6- Heat dry, 110°C (solution, 24 h); 7- Heat dry, 110°C (solid, 24 h); 8- Daylight exposure (24 h).

Linearity

The linearity of the method was determined by analysis of three replicates of six concentrations of standard solutions (range from 18.7 and 93.4 μg/mL). The calibration curve showed good linearity over the concentration range. The regression line was $y = 8158395.79x + 191157.22$, with a correlation coefficient (r^2) of 0.99997. The linearity of the calibration graphs was validated by the high value of the correlation coefficient and the intercept value that was not statistically ($p = 0.05$) different from zero (Table 2).

Precision

The precision is usually expressed as the RSD of a series of measurements. The RSD of peak area response and retention time showed the satisfactory

Table 2. Linearity data of acenocoumarol

% of Nominal value	Injected (μg)	Average peak area response	RSD
33	0.3736	3281952.33	0.77
50	0.5604	4705163.00	0.86
67	0.7472	6308180.33	0.85
100	1.1208	9329086.00	0.54
133	1.4944	12369957.33	0.11
166	1.8680	15447482.70	0.20
Slope[a]	8158395.79 ± 30945185.60		
Intercept[b]	191157.22 ± 39122542.60		

[a]Confidence limits of the slope ($p = 0.05$).
[b]Confidence limits of the intercept ($p = 0.05$).

Table 3. Precision of the assay method for acenocoumarol

Analyst 1 sample N°	mg per tablet	RSD (%)	Analyst 2 sample N°	mg per tablet	RSD (%)
1	4.31	0.60	1	4.25	0.83
2	4.32	0.60	2	4.22	0.83
3	4.42	0.60	3	4.27	0.83
4	4.43	0.60	4	4.35	0.83
5	4.27	0.60	5	4.29	0.83
6	4.39	0.60	6	4.41	0.83
Mean	4.36	1.47	Mean	4.30	1.64

repeatability of the system (<1%). The intra-day precision was performed by assaying the samples on two different days by two different analysts. The results were given both individually and as the average. For each precision assay, the results were as follows: mean values 4.36 and 4.30 mg per tablet, RSD 1.47% and 1.64%. Test "*t*", comparing two samples with 95% confidence for 10 degrees of freedom, disclosed that both results were not significantly different *inter se* ($t_{n-2,\ \alpha:0.05}$) = 2.23 (Table 3).

Accuracy

The results obtained in the accuracy study (recovery test) with 9 samples of one commercial formulation studied (n = 3 for 80%, 100%, and 120%) indicated that the mean recovery was 100.33%. The RSD was 1.69. Also studied was the experimental *t* of the recovery percentage of which the

Table 4. Recovery analysis of acenocoumarol

% of Nominal value	Added amount (mg)	Found amount (mg)	Recovery (%)	Average recovery (n = 3)	RSD (%)
80	3.568	3.660	102.58	100.56	2.92
	3.508	3.575	101.91		
	3.516	3.417	97.18		
100	4.647	4.719	101.55	100.14	1.57
	4.348	4.315	99.24		
	4.332	4.298	99.22		
120	5.236	5.263	100.52	100.29	0.45
	5.214	5.244	100.57		
	5.219	5.207	99.77		
Mean (n = 9)				100.33	1.69

Table 5. Robustness of acenocoumarol method

Mobile phase	RT acenocoumarol (min)	RRT R	R
Acetonitrile:water pH 3.0 (50:50)	7.1	1.54	9.6
Acetonitrile:water pH 3.5 (50:50)	7.0	1.15	2.2
Acetonitrile:water pH 4.0 (50:50)	6.8	1.06	1.0
Acetonitrile:water pH 3.0 (60:40)	3.6	1.21	3.1
Acetonitrile:water pH 3.0 (40:60)	16.5	2.12	14.3

value was 0.803, it being far below the 2.306 established in the tabulated *t* (95% level of probability, 8 d.f) (Table 4).

Robustness

The chromatographic conditions were optimized with respect to specificity, resolution, and time of analysis. The specificity of the method was established through the study of resolution factor of acenocoumarol from the nearest resolving peak, its thermal degradation product. Effects of pH and mobile phase proportion on resolution and retention time could be seen in Table 5. It was found, that retention time of acenocoumarol was significantly affected by changes of mobile phase proportion but not by different pHs. The major thermal degradation product was significantly affected by both changes.

CONCLUSIONS

The proposed RP-LC method provides simple, accurate, and reproducible quantitative analysis for the determination of acenocoumarol and its thermal degradation product. All validation parameters were within the acceptance range. The advantages of the proposed method in comparison to the compendial method are shorter analysis time, less toxic organic solvent used, and no sample extraction required. The developed method is considered to be reliable and suitable for the routine quality control and stability indicating studies of acenocoumarol.

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