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A SIMPLE SPECTROPHOTOMETRIC QUANTITATIVE DETERMINATION OF ACENOCOUMAROL IN BULK AND PHARMACEUTICAL DOSAGE FORMS USING DNPH REAGENT

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ABSTRACT

A simple, sensitive, cost effective spectrophotometric method developed and validated for the determination of Acenocoumarol, in bulk drug and its pharmaceutical formulations. The method is rely on the formation of a hydrazone with 2,4-dinitrophenylhydrazine (DNPH); the reaction of drug with reagent gives a bright yellow colour. The obtained coloured species absorbance was measured at its absorption maximum (λmax) 486 nm. The Beer's law has been obeyed in the concentration range 50-250 μg/ml. All the variables were examined to optimize the reaction conditions. There was no interference observed in the presence of common pharmaceutical excipients. The validity of the method was established by analyzing CIL in its pharmaceutical formulations and critically tested for its accuracy by statistical tests. Good recoveries were obtained by the developed method; the obtained results were critically analyzed and successfully employed for the determination of Acenocoumarol in its pharmaceutical dosage forms.

KEYWORDS: Acenocoumarol, DNPH, Spectrophotometry, antiplatelet- agregation, Vali.

MATERIALS AND METHOD

Instrument

All measurement were done on Milton Roy 1001spectrophotometer by using 10 mm matched quartz cuvettes. dation.

0.1786 M Alcoholic Potassium Hydroxide: 1.0019 g of Potassium Hydroxide (Fischer Scientific) dissolved in 20 ml of distilled water and diluted up to 100 ml with Methanol.

NN-Dimethyl Formamide: NN-Dimethyl Formamide (Fischer Scientific) is directly used.

Concentrated Hydrochloric acid: 36% Concentrated Hydrochloric acid (Fischer Scientific) is directly used.

2,4-Dinitrophenyl Hydrazene (2,4-DNP): 0.1 gram of 2,4-Dinitrophenyl Hydrazene (S.D. Fine Chem) is dissolved in 10 ml of Methanol and 0.5ml of Concentrated Hydrochloric Acid is added and make up the volume to 100 ml with Methanol to obtain 0.005Molar solution.

INTRODUCTION

Acenocoumarol is scientifically named 4-hydroxy-3-[1-(4-nitrophenyl)-3-oxobutyl]chromen-2-one. Acenocoumarol is a coumarin derivative used as an anticoagulant. Coumarin derivatives inhibit the reduction of vitamin K by vitamin K reductase. This prevents carboxylation of vitamin K-dependent clotting factors, II, VII, IX and X, and interferes with coagulation. Hematocrit, hemoglobin, international normalized ratio and liver panel should be monitored. Patients on acenocoumarol are prohibited from giving blood. Chemical Formula $C_{19}H_{15}NO_6$, molecular weight 353.3255. Acenocoumarol is having the following structure:

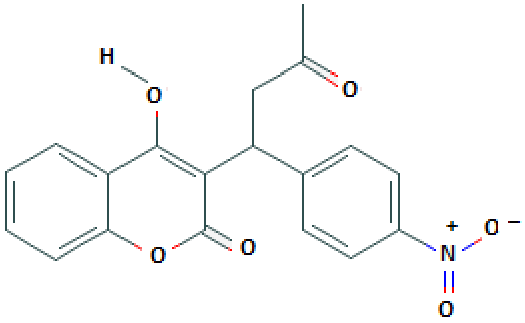

Fig. 1: Chemical Structure of Acenocoumarol.

MATERIALS AND METHOD

Instrument

All measurement were done on Milton Roy 1001 spectrophotometer by using 10 mm matched quartz cuvettes.

Materials and reagents

All the chemicals used were of analytical grade. All the solutions were prepared freshly and deionised water is used throughout the experiment. Acenocoumarol bulk drug obtained from Cipla Ltd. certified to contain 99.7% of active ingredient, which has been used as a reference substance, as received without further purifications. 2,4-dinitrophenylhydrazine (DNPH) was procured from Sd-Fine chemicals 99.5% purity and HCl procured from Sd-Fine chemicals 35% purity. Methanol AR grade procured from Sd-Fine chemicals.

Acenocoumarol tablets were purchased from pharmaceutical store with different contents and different makes such as Acitrom (4 mg Nicolas) and Nistrom(4 mg Neiss lab).

Preparation of 0.005M DNPH solution

Accurately weighed 0.099 mg of DNPH in a 100 ml volumetric flask, added 20 ml of methanol swirled to mix then the solution made up to the mark with methanol.

Preparation of standard stock solution

Accurately weighed (0.001g) 10 mg of the reference standard in 10 ml volumetric flask, added 5 ml methanol swirled to mix and brought to the mark with methanol. The apparent concentration reached to 1000 µg/ml. further dilutions made to obtain the working standard stock solution 100 µg/ml.

General procedure for the determination of Acenocoumarol

Variable aliquots of working standard solution containing 50-250 µg/ml of Acenocoumarol were transferred in to series 10 ml volumetric flasks. To each flask 2 ml of 0.005M DNPH and 1 drop of concentrated HCl was added, mixed the solution mechanically and then the flasks are heated on a water bath for 1hour. Then the contents were cooled for one hour. Then 3 ml of 0.1786 M KOH added. There is a formation of yellow coloured hydrazone, then the flasks are allowed to cool for 30 minutes to obtain room temperature and the solutions is mixed with 10 ml of NN-Dimethyl formamide. The coloured species absorbance was measured at 486 nm using reagent as a blank. The formation of hydrazone is shown in Scheme 1 The calibration graph was prepared by plotting absorbance versus concentration of drug and the concentration of unknown was read from the calibration graph or computed from the regression equation derived from the Beer's law data.

The same procedure was followed for the determination of acenocoumarol in the tablet formulations and the content of the tablets were calculated by using regression equation.
Scheme. 1: Possible reaction sequence.

Determination of absorption maxima (λmax)

To determine the λmax of the colored species, 1 ml of 100 μg/ml of the acenocoumarol was added to a 10 ml volumetric flask and 1 ml of concentrated HCl, mixed the contents mechanically then added 1 ml of 0.005M DNPH solution then the flasks are heated on a water bath for 10 min. there is a formation of yellow coloured hydrazone, then the flasks are allowed to cool to room temperature and the solutions made up to the mark with NN-Dimethyl formamide. The coloured species was measured against reagent blank in the range of 440 nm to 540 nm. The λmax of the complex was found to be 486 nm. Absorption spectrum of the proposed method was shown in Fig.2. Under the experimental conditions each reagent blank showed a negligible absorbance at the corresponding λmax.

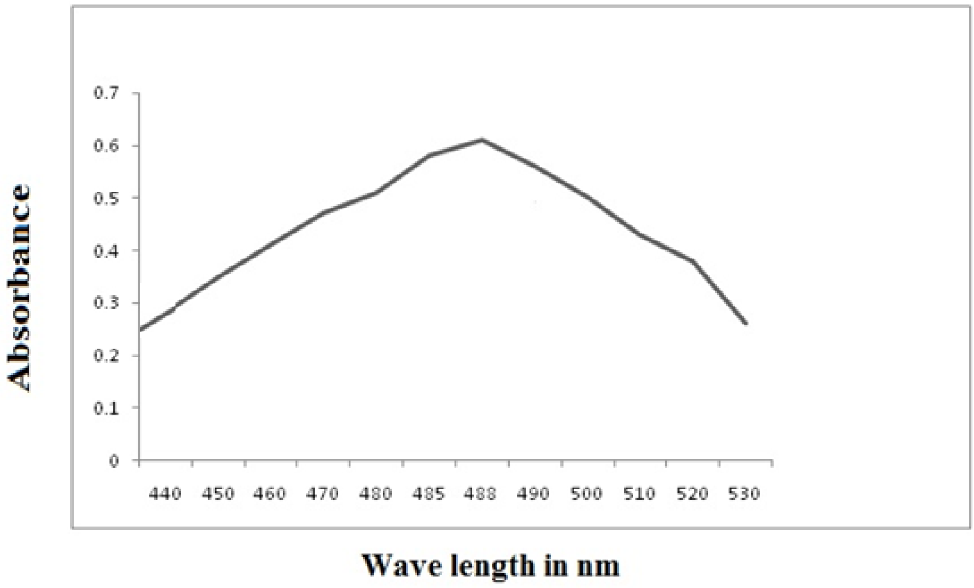

Fig. 2: Absorption spectrum of the coloured species.

Investigation of assay parameters

Optimum reagent concentrations required for the formation of sensitive and quantitative coloured products were determined by varying one reagent concentration and fixing the concentrations of other reagents and its effect on absorbance was measured at 486 nm. **(b)**

Parameter fixation

(i) Effect of amount of concentrated hydrochloric acid required for the absorbance of method

To determine the amount of concentrated hydrochloric **required for the method**, a series of a 25 ml volumetric flasks are taken, 1 ml of 100 µg/ml of the acenocoumarol was added. To each flask 2 ml of 0.005M DNPH and varying amounts ranging from 1to 4 drops of concentrated HCl was added to the series of flasks, mixed the solutions mechanically and then the flasks are heated on a water bath for 1hour. Then the contents were cooled for one hour. Then 3 ml of 0.1786 M KOH added. There is a formation of yellow coloured hydrazone, then the flasks are allowed to cool for 30 minutes to obtain room temperature and the solutions is mixed with 10 ml of NN-Dimethyl formamide. The coloured species absorbance was measured at 486 nm using reagent as a blank. Under the experimental conditions all the ingridients uniformly added excepting noof drops of concentrated hydrochloric acid. Absorbance verses noof drops of concentrated hydrochloric acid noted in the Table. 6.1.2.

Table 6.1.2: Effect of amount of concentrated hydrochloric acid required for the absorbance of method.

Number of drops of Hydrochloric acid added	Absorbance at 486 nm.
1	0.372
2	0.372
3	0.371
4	0.371

The data in table 7.1.1 indicates that 1drop concentrated hydrochloric acid is sufficient for attaining maximum absorbance. Hence it is maintained throughout the experimental studies.

(ii) Effect of amount of 0.005M DNPH required for the absorbance of method

To determine the amount of 0.005M DNPH required for the method, a series of a 25 ml volumetric flasks are taken, 1 ml of 100 µg/ml of the acenocoumarol was added. To each flask, varying amounts ranging from 1 to 4 ml of 0.005M DNPH and 1 drop of concentrated HCl was added to the series of flasks, mixed the solutions mechanically and then the flasks are heated on a water bath for 1hour. Then the contents were cooled for one hour. Then 3 ml of 0.1786 M KOH added. There is a formation of yellow coloured hydrazone, then the flasks are allowed to cool for 30 minutes to obtain room temperature and the solutions is mixed with

10 ml of NN-Dimethyl formamide. The coloured species absorbance was measured at 486 nm using reagent as a blank. Under the experimental conditions all the ingridents uniformly added excepting amount of 0.005M DNPH. Absorbance verses ml of 0.005M DNPH is noted in the Table. 6.1.2

Table 6.1.2: Effect of amount of 0.005M DNPH required for the absorbance of method.

Amount of 0.005M DNPH taken in ml	Absorbance at 486 nm.
1	0.262
2	0.374
3	0.374
4	0.374

The data in table 7.1.1 indicates that 2 ml **of 0.005M DNPH** is required for attaining maximum absorbance. Hence it is maintained throughout the experimental studies.

(iii) Effect of amount of 0.1786 M KOH required for the absorbance of method

To determine the amount of 0.1786 M KOH required for the method, a series of a 25 ml volumetric flasks are taken, 1 ml of 100 µg/ml of the acenocoumarol was added. To each flask, 2 ml of 0.005M DNPH and 1 drop of concentrated HCl was added to the series of flasks, mixed the solutions mechanically and then the flasks are heated on a water bath for 1hour. Then the contents were cooled for one hour. To each flask, varying amounts ranging from 1 to 4 ml of 0.1786 M KOH added. There is a formation of yellow coloured hydrazone, then the flasks are allowed to cool for 30 minutes to obtain room temperature and the solutions is mixed with 10 ml of NN-Dimethyl formamide. The coloured species absorbance was measured at 486 nm using reagent as a blank. Under the experimental conditions all the ingridents uniformly added excepting amount of 0.1786 M KOH. To each flask, varying amounts ranging from 1 to 4 ml of 0.1786 M KOH. Absorbance verses ml of 0.1786 M KOH is noted in the Table. 6.1.2.

Table 6.1.2: Effect of amount of 0.1786 M KOH required for the absorbance of method.

Amount of 0.1786 M KOH taken in ml	Absorbance at 486 nm.
1	0.224
2	0.294
3	0.378
4	0.378

The data in table 7.1.1 indicates that 3 ml of 0.1786 M KOH is required for attaining maximum absorbance. Hence it is maintained throughout the experimental studies.

(iii) Effect of heating time absorbance of method

To determine the effect of heating time for the method, a series of a 25 ml volumetric flasks are taken, 1 ml of 100 μg/ml of the acenocoumarol was added. To each flask, 2 ml of 0.005M DNPH and 1 drop of concentrated HCl was added to the series of flasks, mixed the solutions mechanically and then the flasks are heated on a water bath ranging from 30,45,60 and 75 minutes. Then the contents were cooled for one hour. To each flask, 3 ml of 0.1786 M KOH added. There is a formation of yellow coloured hydrazone, then the flasks are allowed to cool for 30 minutes to obtain room temperature and the solutions is mixed with 10 ml of NN-Dimethyl formamide. The coloured species absorbance was measured at 486 nm using reagent as a blank. Under the experimental conditions all the ingridents uniformly added excepting the heating time. To each flask, varying heating time ranging from 30,45,60 and 75 minutes areheated. Absorbance verses varying heating time ranging from 30,45,60 and 75 minutes is noted in the Table. 6.1.2.

Table 6.1.2: Effect of heating time ranging from 30,45,60 and 75 minutes required for the absorbance of method.

Heating time in minutes	Absorbance at 486 nm.
30	0.224
45	0.294
60	0.378
75	0.378

The data in table 7.1.1 indicates that 60minutes of heating is required for attaining maximum absorbance. Hence it is maintained throughout the experimental studies.

Construction of Calibration Curve

To study the effect of drug concentration on the absorbance of the reaction under optimal conditions is studied by the following method to know the suitability of the method for the assay of acenocoumarol.

Various aliquots of the standard acenocoumarol solution (100 μg/ml) ranging from 0.2-1.0 ml are transferred into a series of 25 ml volumetric flasks. To each flask, 2 ml of 0.005M DNPH and 1 drop of concentrated HCl was added to the series of flasks, mixed the solutions mechanically and then the flasks are heated on a water bath 60 minutes. Then the contents were cooled for one hour. To each flask, 3 ml of 0.1786 M KOH added. There is a formation of yellow coloured hydrazone, then the flasks are allowed to cool for 30 minutes to obtain room temperature and the solutions is mixed with 10 ml of NN-Dimethyl formamide. The coloured species absorbance was measured at 486 nm using reagent as a blank. Calibration graph is obtained by plotting absorbance values against the concentration of acenocoumarol solution. The calibration curve is found to be linear over a concentration range of 20 to 100 μg of acenocoumarol. The amount of acenocoumarol present in the sample is estimated from the calibration graph. The results are presented in fig.6.1.2.

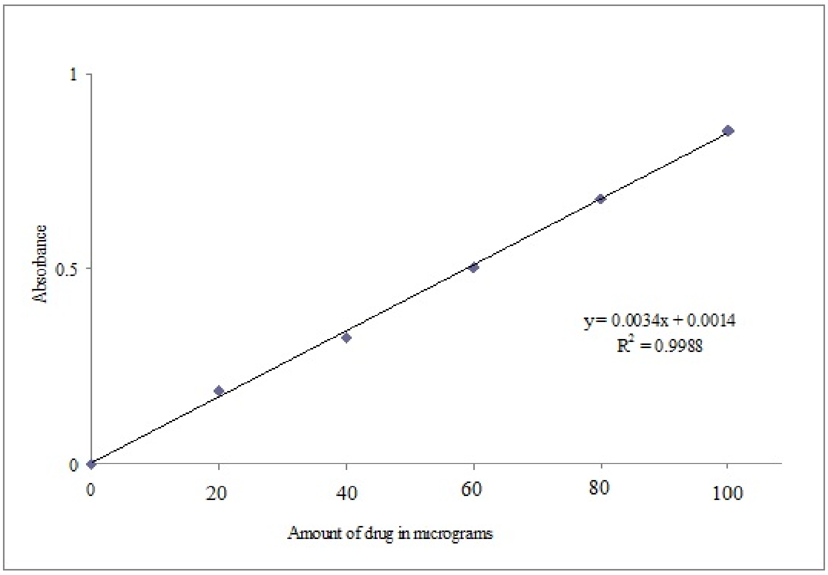

Fig. 3: Beer's law calibration curve of acenocoumarol.

Assay of acenocoumarol in pharmaceutical formulations

The proposed procedure for the assay of acenocoumarol is applied for its determination in commercial tablets.

Preparation of the sample solution

Powdered tablet equivalent to 50 mg of the drug is weighed accurately and transferred into a 50 ml beaker and mixed well with 30 ml of methanol. The solution is filtered and transferred into a 50 ml volumetric flask and the volume is made up to 50 ml with methanol. The concentration of the drug solutions is now 1mg/ml. This stock solution is further diluted to obtain the working concentration.

The pharmaceutical preparation as prepared above is analysed by the following procedure.

Assay Procedure

The amount of acenocoumarol in the pharmaceutical formulation is evaluated from the predetermined calibration plot.

RESULTS AND DISCUSSION

Acenocoumarol form hydrazone when treated with 24DNPH and hydrochloric acid. This orange red colour product shows maximum absorbance at 486 nm. The colour of the product is stable for more than 24 hours. The calibration curve (concentration vs. absorbance) is linear over the range of 20-100 µg of acenocoumarol. The optical characteristics of the proposed method such as absorption maxima, Beer´s law limits, molar absorptivity and Sandell´s sensitivity are presented in Table 6.1.4. The molar absorptivity and Sandell´s sensitivity values shows sensitivity of the method. The regression analysis using method of least squares was made for the slope (b), intercept (a) and correlation (r) obtained from different concentrations and results are summarized in the Table 6.1.4. The value of correlation coefficient was 0.999, which indicated the good linearity of calibration lines. The values of standard deviation are low, indicates high accuracy and reproducibility of the method. The 't' calculated values are compares well with the theoretical value of 2.78 there by indicating that the precision of the method is good. There no effect of additives and excipients such starch, calcium lactose and glucose in the concentrations those present in general pharmaceutical preparations.

The proposed method is found to be simple, precise, accurate and time saving, reproducible and can be conveniently adopted for routine analysis of estimation of acenocoumarol in bulk drugs samples and pharmaceutical formulations.

Table 6.1.4: Optical and Regression characteristics, precision and accuracy of the proposed method.

Parameters	**Proposed method**
λmax (nm)	318
Beer's law limit (µg/ml)	20-100
Molar absorptivity (l mole-^{1} cm-^{1})	2.034x10^4
Sandell's sensitivity(µg cm-2 / 0.001 absorbance unit)	0.0203
Regression equation (Y = a + bC)	Y=0.0034X+0.0014
Slope (b)	0.0034
Intercept (a)	0.0014
correlation coefficient (r)	0.9998

*Y = a+bx, where Y is the absorbance and x concentration in µg/ml

Table No 2: Assay of acenocoumarol in tablet formulations.

Tablets	Labeled amount (mg)	*Amount found (mg)±S.D*	% label claim	*t value
Tablet 1	4	3.99±0.46	99.75	0.0971
Tablet 2	4	4,02±0.69	100.5	0.0644

*Average of five determination based on label claim

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