Research Article

Development and Validation of Stability Indicating HPTLC Method for Estimation of Acenocoumarol in Tablet Dosage Form

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ABSTRACT

The project involves development and validation of stability-indicating HPTLC method for the estimation of acenocoumarol in the presence of its degradation products. Acenocoumarol was subjected to alkaline, acidic, oxidative, thermal (dry heat) and photo-degradation conditions. The drug was spotted on precoated silica gel G 60 F_{254} TLC plate using Toluene: Isopropyl Alcohol: Methanol (9.0: 1.0: 0.5) as a mobile phase. The drug was scanned at 290 nm. The R_f value of acenocoumarol was found to be 0.50 ± 0.03. The linearity was obtained in the range of 200 – 1000 ng/spot. The LOD and LOQ were found to be 26 ng/spot and 78.80 ng/spot respectively. The percentage recovery was found in the range of 98.36 – 101.28%. One degradation product was separated in acidic condition; two degradation products were separated in each oxidative and photolytic degradation conditions by HPTLC method. The drug was found to be stable when subjected to alkaline hydrolysis and under thermal (dry heat) conditions. The developed method was applied for quantitative analysis of acenocoumarol in marketed formulation.

Keywords: Acenocoumarol (ACC), HPTLC, Stability Indicating Method, Forced Degradation Study.

1. INTRODUCTION

Acenocoumarol (ACC) is a vitamin K antagonist. It is chemically 4-hydroxy-3-[1-(4-nitrophenyl)-3-oxobutyl]-2*H chromen*-2-one (Figure 1) [1]. It is orally used anticoagulant. It acts indirectly by inhibiting vitamin K reductase, results in depletion of vitamin K. It is also known as Nicoumalone. It is used in thromboembolic disorders.

Acenocoumarol API and tablet is official in IP and BP [2, 3]. Titrimetric and UV spectrophotometric method are official methods for estimation of Acenocoumarol in bulk and tablet dosage form. The literature review reveals that few analytical and bio-analytical methods have been reported for quantitative estimation in pharmaceutical formulations and biological fluids, which include spectrophotometric [5-7], stability indicating HPLC [8-10], HPTLC [11] and bio analytical methods [12, 13].

But stability indicating HPTLC method is not yet reported which would serve as stability indicating assay method for the analysis of Acenocoumarol. Therefore, it was thought of interest to develop and validate stability indicating HPTLC method for estimation of Acenocoumarol in tablet dosage

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form.

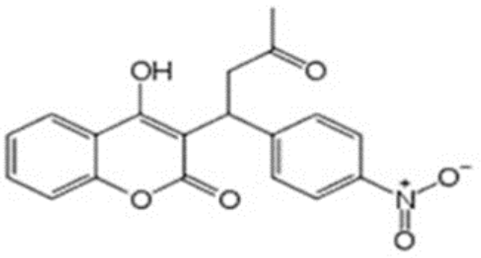


Figure 1: Structure of Acenocoumarol [13]

2. MATERIALS AND METHODS

2.1. Instruments

The HPTLC system (Camag Switzerland) consisting of Linomat V semiautomatic spotting device, TLC Scanner IV (Camag Muttenz, Switzerland), twin-trough developing chamber (10 x 10cm), UV cabinet with dual wavelength UV lamps, winCATS software, syringe (100 µl capacity, Hamilton) were used for chromatographic study. Electronic analytical balance (Shimadzu AUX-220) was used for all the weighings.

2.2. Reagents and Materials

Acenocoumarol (98.83%) was received as a Gift sample from Century Pharmaceuticals Ltd., Vadodara. Toluene, Isopropyl Alcohol, Methanol were purchased from s.d. Fine-Chem Limited, Mumbai, India. Acitrom tablets were purchased from local market.

2.3. Preparation of standard solutions

2.3.1. Preparation of stock solution of standard ACC

Accurately weighed quantity of ACC (50 mg) was transferred into 10 ml volumetric flask, dissolved in 5 ml of methanol and diluted up to the mark with the same to obtain a stock solution having strength of 5,000 µg/ml.

2.3.2. Preparation of working standard solution

The stock solution (1 ml) was withdrawn and transferred into 10 ml volumetric flask and diluted up to the mark with methanol (500 µg/ml). The solution (1 ml) was transferred into 10 ml volumetric flask and diluted with methanol to obtain a solution having strength of 50 µg/ml.

2.4. Preparation of degradation solutions

ACC was degraded in acidic, alkaline, oxidative, photolytic and thermal (dry heat) stress conditions.

2.4.1. Acid Hydrolysis

The stock solution (1 ml) was transferred in 10 ml volumetric flask, diluted up to the mark with 1 N HCl (500 µg/ml). The solution was transferred into stoppered conical flask and heated for 5 hours at 80^0C. The solution (1 ml) was transferred in 10 ml volumetric flask, brought to RT and then neutralized with 1 N NaOH. Volume was made up to the mark with methanol.

2.4.2. Alkaline Hydrolysis

The stock solution (1 ml) was transferred in 10 ml volumetric flask, diluted up to the mark with 1 N NaOH (500 µg/ml). The solution was transferred into stoppered conical flask and heated for 5 hours at 80^0C.The solution (1 ml) was transferred in 10 ml volumetric flask, brought to RT and then neutralized with 1 N HCl. Volume was made up to the mark with methanol.

2.4.3. Oxidative Degradation

The stock solution (1 ml) was transferred in 10 ml volumetric flask, diluted up to the mark with 6% H_2O_2 (500 µg/ml). The solution was transferred into stoppered conical flask and heated for 4 hours at 80^0C. The solution (1 ml) was transferred in 10 ml volumetric flask and brought to RT and volume was made up to the mark with methanol.

2.4.4. Photolytic Degradation

The stock solution (1 ml) was transferred in 10 ml volumetric flask, diluted up to the mark with methanol (500 µg/ml). The solution was exposed to sun light for 10 hours. The solution (1 ml) was

transferred in 10 ml volumetric flask and volume was made up to the mark with methanol.

2.4.5. Thermal (Dry Heat) Degradation

Accurately weighed quantity of ACC (25 mg) was kept at 110° C for 5 hrs under dry heat. After bringing at room temperature, the powder was dissolved in 5 ml of methanol into 10 ml volumetric flask and diluted up to the mark with methanol (2500 µg/ml). The solution (2 ml) was transferred into 10 ml volumetric flask. Volume was made up to the mark with methanol (500 µg/ml).

The solution (1 ml) was transferred into 10 ml volumetric flask and volume was made up to the mark with methanol.

2.5. Chromatographic Conditions

Chromatographic separation was performed on 10 × 10 cm aluminium backed plates precoated with 250 µm layer of silica gel 60 F_{254} (E. Merck, Darmstadt, Germany). The TLC plates were prewashed with methanol and dried in oven at 60° C for 5 min. Samples were spotted on TLC plate 15 mm from the bottom edge by Linomat V semi-automatic spotter using following parameters: band width, 6 mm; track distance, 10 mm; application rate, 0.1µl/s. The TLC plate was developed in twin trough chamber using toluene: isopropyl alcohol: methanol (9:1:0.5, v/v/v) as mobile phase at temperature, 25 ± 2° C; chamber saturation time, 40 min; migration distance, 75 mm. The TLC plate was scanned and analysed by TLC Scanner IV and WinCATS software using following parameters: slit dimension, 4 × 0.30 mm; scanning speed, 20 mm/sec; detection wavelength, 290 nm.

2.6. Preparation of Calibration Curve

From working standard solution (50 µg/ml) aliquots of 4, 8, 12, 16 and 20 µl were spotted on the TLC plate and analysed as described under section 2.5. The calibration curve of peak area versus respective concentration was plotted and correlation coefficient and regression line equation were computed.

2.7. Validation of Developed HPTLC Method

2.7.1. Linearity

The linearity response for ACC was determined by analyzing five independent levels of calibration curves in the range of 200 – 1000 ng/spot.

2.7.2. Precision

2.7.2.1. Repeatability of measurement of peak area

Working standard solution (12 µl) was spotted on a TLC plate and analysed as described under section 2.5. The separated spot of ACC was scanned for seven times without changing plate position and RSD for measurement of peak area was computed.

2.7.2.2. Repeatability of sample application

Working standard solution (12 µl) was spotted on a TLC plate seven times. The areas of seven spots were measured and the RSD of peak area was calculated.

2.7.2.3. Intermediate Precision

Intraday Precision: It was determined by applying freshly prepared solution of ACC (200-1000 ng/spot) three times on same day. RSD of peak area at each level was calculated.

Inter-day Precision: It was determined by applying freshly prepared solution of ACC (200-1000 ng/spot) three times on different days. RSD of peak area at each level was calculated.

2.7.3. Specificity

The specificity of the method was ascertained by analysing standard drug and sample. The band in sample was confirmed by comparing the R_f value and UV spectra of the band with that obtained from the standard.

The peak purity was assessed by comparing the spectra acquired at three different positions on the band, i.e. peak start (s), peak apex (m) and peak end (e).

Table 1: Preparation of solutions for accuracy study.

ACC from tablet (mg)	Std. ACC spiked (mg)	Concentration of ACC in final solution (µg/ml)	Volume of solution spotted	Total ACC (ng/spot)
10	0	50	6 µl	300
10	8	90	6 µl	540
10	10	100	6 µl	600
10	12	110	6 µl	660

Table 2: Recovery data of ACC from formulation (Acitrom Tablet)

Amount of ACC in tablet(mg)	Amount of standard ACC added (mg)	Total amount of ACC (mg)	Total peak area	Recovered amount of ACC (mg)	% Recovery ± S.D (n=3)
10	00	10	2796.27	-	-
10	08	18	4323.73	8.12	101.28 ± 1.44
10	10	20	4620.34	9.83	98.36 ± 1.52
10	12	22	5064.00	12.06	100.23 ± 1.87

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

The limits of detection and quantification of the developed methods were calculated from the standard deviation of the intercepts and mean slope of the calibration curves of ACC using the formula as given below.

$$LOD = 3.3\ \alpha/S$$

$$LOQ = 10\ \alpha/S$$

Where, α is the standard deviation of the intercepts of the five calibration curves and S is the mean slope of the five calibration curves.

2.7.4. Accuracy

Accuracy was determined in terms of percent recovery. The proposed method was applied to determine ACC in pharmaceutical dosage form. The recovery experiment was carried out in triplicate by spiking previously analyzed samples with three different concentrations of standards at 80%, 100% and 120%. Preparation of solutions for accuracy study is given in Table 1.

2.8. Analysis of Marketed Formulation

Twenty tablets were individually weighed and powdered. Powder equivalent to 10 mg of ACC was accurately weighed and transferred into 10 ml volumetric flask and 5 ml methanol was added. This solution was sonicated for 2 min and volume was made up to 10 ml with methanol.

The solution was filtered. After filtration, the solution (1 ml) was transferred in 10 ml volumetric flask and diluted up to the mark with methanol.

The solution (6 µl) was applied in triplicate on TLC plate and percentage of ACC was calculated from the calibration curve.

3. RESULTS AND DISCUSSION

3.1. Optimisation of mobile phase

Number of trials were taken by changing the ratio of mobile phase with respect to achieve good resolution between ACC and degradation products obtained in different degradation conditions with minimum tailing. The mobile phase Toluene: Isopropyl Alcohol: Methanol (9:1:0.5, v/v/v) gave good resolution with Rf values of 0.50 ± 0.03. Chromatograms obtained for all degradation conditions are shown in Figure 2.

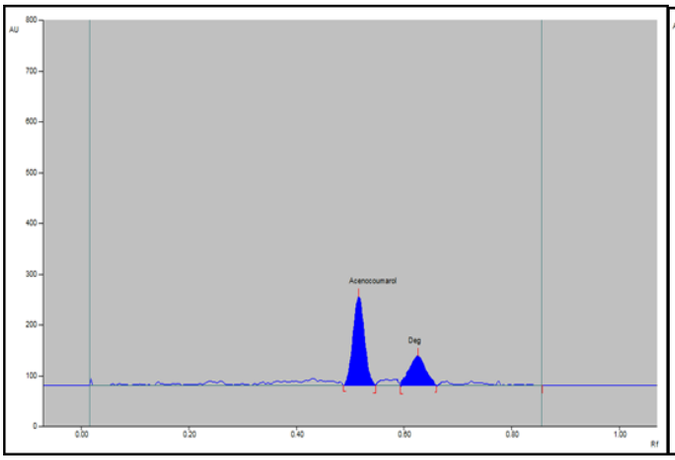


Figure 2(a)

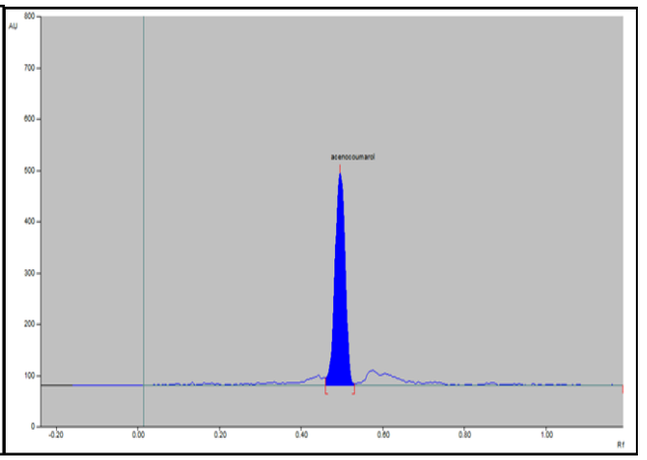
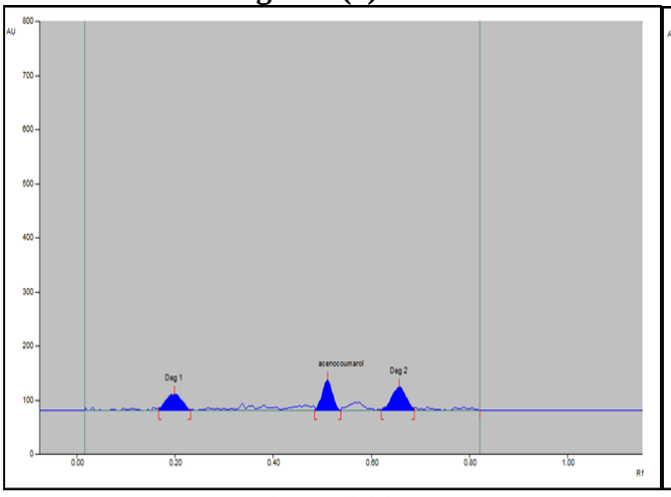


Figure 2(b)



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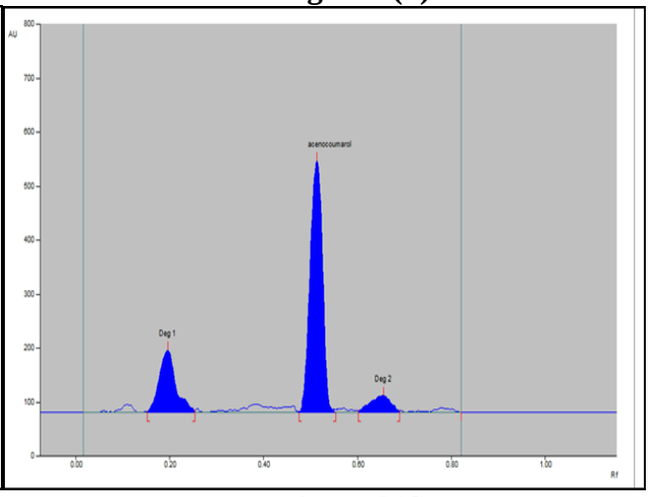

Figure 2(d)

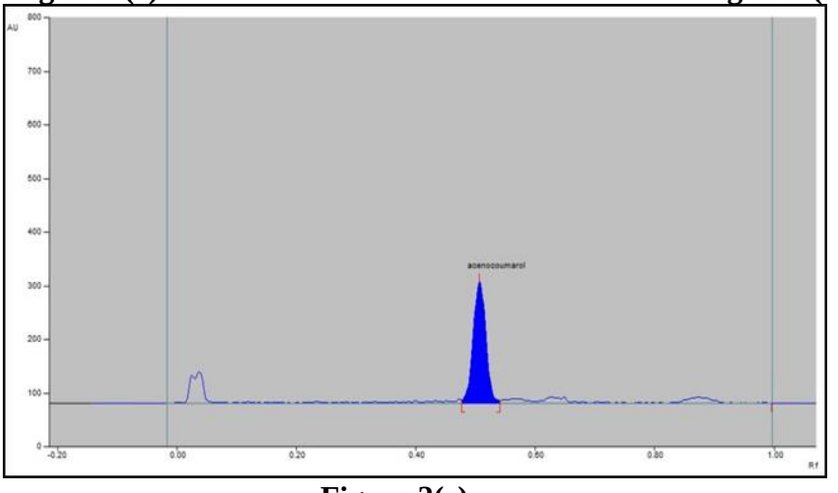

Figure 2(e)

Figure 2: Chromatograms obtained from forced degradation studies. (a) Acidic hydrolysis (1N HCl at 80°C for 5 hr); Deg (Rf: 0.61), ACC (Rf: 0.50 ± 0.03). **(b)** Alkaline hydrolysis (1N NaOH at 80°C for 5 hr); ACC (Rf: 0.50 ± 0.03). **(c)** Photolytic degradation (exposed to sunlight for 10 hrs); Deg 1 (Rf : 0.20), ACC (Rf : 0.50 ± 0.03), Deg 2 (Rf : 0.67). **(d)** Oxidative degradation (6% H_2O_2 at 80°C for 4 hrs); ACC (Rf: 0.50 ± 0.03), Deg1 (Rf : 0.20), Deg 2 (Rf : 0.67). **(e)** Thermal degradation (110°C for 5 hrs), ACC (Rf : 0.50 ± 0.03).

Table 3: Summary of validation parameters

Parameters	Results
Linearity Range	200-1000 ng/spot
Correlation Co-efficient (R^2)	0.9954
Precision (%RSD)	
Repeatability (n=7)	
Repeatability of measurement	0.30
Repeatability of sample application	0.45
Intermediate precision	
Intraday precision (n=3)	0.50 – 0.85
Inter day precision (n=3)	0.80 – 1.32
% Recovery	98.36 – 101.28 %
Limit of Detection (LOD)	26 ng/spot
Limit of Quantification (LOQ)	78.80 ng/spot

Table 4: Summary of forced degradation study of Acenocoumarol

Stress type	Stress conditions	% Drug degradation
Acid hydrolysis	1N HCl at 80^0C for 5 hr	33.26%
Alkali hydrolysis	1N NaOH at 80^0C for 5 hr	1.61%
Oxidative degradation	6% H_2O_2 at 80^0C for 4 hr	30.28%
Photolytic degradation	Direct sunlight for 10 hr	64.22%
Thermal degradation	Dry heat at 110^0C for 5 hr	3.20%

3.2. Selection of Wavelength

For the determination of wavelength, 15 µg/ml solution of ACC was scanned between 200 – 700 nm and spectrum was recorded against methanol as blank in UV-visible spectrophotometer. The standard solution of ACC was scanned and absorbance maximum (λ_{max}) was found at 290 nm which was selected as detection wavelength for ACC.

3.3. Calibration curve of ACC

Calibration curve of peak area versus concentration is shown in Figure 3. Linearity was determined from regression line equation.

3.4. Validation of Developed HPTLC Method

3.4.1. Linearity and Range

The linearity and range for ACC was found in the range of 200–1000 ng/spot. Correlation coefficient for calibration curve of ACC was found to be 0.9954. The regression line equation for ACC is as following: y = 6.3383 x + 856.84.

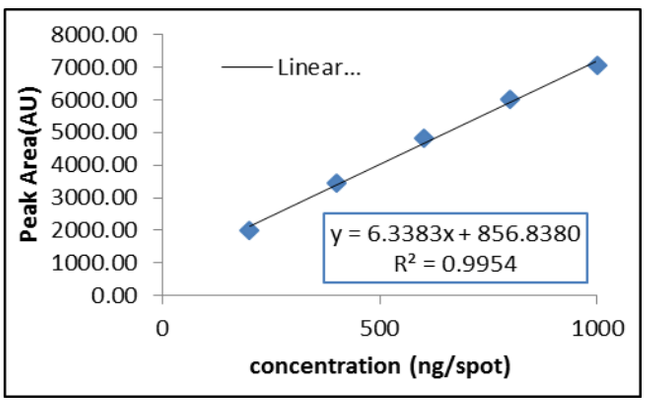

Figure 3: Calibration curve of ACC (200 – 1000 ng/spot)

3.4.2. Precision

The repeatability of sample application and for measurement of peak area of ACC was found to be 0.45 and 0.30 respectively. The % RSD for intra-day and inter-day precision of ACC was found to be in range of 0.50 – 0.85 and 0.80 – 1.32 respectively.

3.4.3. Specificity

Comparison of chromatograms of standard ACC and ACC from tablet formulation showed identical R_f values of 0.50 ± 0.03 (n=3). Comparison of UV absorbance spectra scanned at peak start (s), peak apex (m) and peak end (e) positions of individual spots of ACC showed a high degree of correlation confirmed the purity of the corresponding spots. Overlain spectra of ACC and sample ACC (Tablet) is given in figure 4.

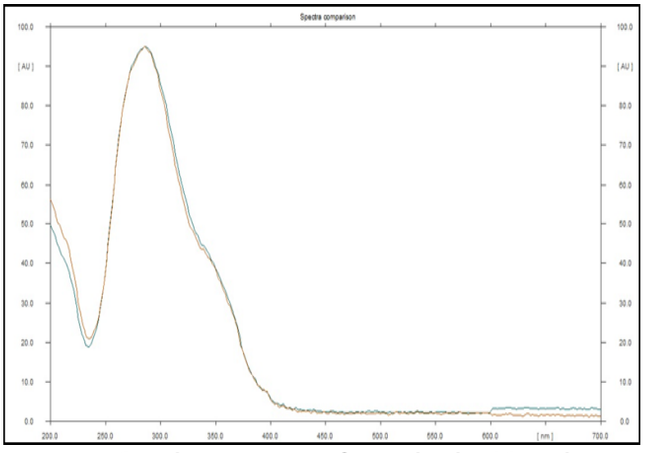

Figure 4: Overlain spectra of standard ACC and sample ACC (Tablet)

3.4.4. LOD and LOQ

The LOD and LOQ for Acenocoumarol were found to be 26 ng/spot and 78.80 ng/spot respectively.

3.4.5. Accuracy

Accuracy of the method was confirmed by recovery study from marketed formulation at three level of standard addition. The % recovery of ACC was found to be in range of 98.36 - 101.28%. Results of recovery study are given in Table 2.

3.5. Analysis of marketed Formulation

The drug content was found to be 102.0 ± 0.59 %. There was no interference from the excipients commonly present in the tablet.

The summary of results of validation parameters is given in Table 3.

3.6. Forced Degradation Study

The degradation study indicated that ACC was susceptible to acidic, oxidative and photolytic degradation while it was stable to alkaline, neutral and thermal (dry heat) conditions. ACC gets degraded into one degradation product in acidic and two degradation products under oxidative and photolytic conditions. The chromatogram of acid degraded sample of ACC showed one degradation product at Rf 0.61.

The chromatograms of the photo and oxidative degraded sample of ACC showed two additional peaks at Rf 0.20 and 0.67 respectively.

The results for stress degradation study are summarized in Table 4.

4. CONCLUSION

The proposed stability indicating HPTLC method for the estimation of Acenocoumarol was precise, accurate, and selective. The method was found to be linear in wide range of concentration. The developed method was free from interference due to the excipients present in tablet dosage form. From the results obtained, it is concluded that suggested methods showed high sensitivity, reproducibility, accuracy and specificity. As the method could effectively separate the drug from their degradation products; therefore, it can be employed as a stability indicating one**.**

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6. REFERENCES

1. Moffat AC, Osselton MD, Widdop B. Clarke' Analysis of Drugs and Poisons. Vol II. 3rd ed. UK: Pharmaceutical Press; 2005. p. 572,573.
2. Indian Pharmacopoeia. Vol III. 6th ed. Ministry of Health and Family Welfare Government of India. Ghaziabad: Indian Pharmacopoeia Commission; 2010. p. 1777-1779.
3. British Pharmacopoeia. Vol I. Medicines & Healthcare Products Regulatory Agency (MHRA). United Kingdom: The Stationery Office; 2007. p. 43-44.
4. ICH Harmonised Tripartite Guideline. Stability Testing of New Drug Substances and Products Q1A (R2). International conference on Harmonisation. Geneva: Switzerland; 2003. p. 1-24.
5. Mehta AS, Gurupadayya BM, Gopinath B, Manohara YN, Chandra A. Spectrophotometric estimation of acenocoumarol in tablets. Indian Journal of Pharmaceutical and Educational Research. 2008;42(4):310-13.
6. Mandrupkar SN, Nagras MA, Mulgund SV. Development and validation of spectrophotometric method of acenocoumarol in bulk and tablet dosage form. International Journal of Pharmacy and Pharmaceutical Science. 2012;4(4):288-289.
7. Shah KD, Sahoo U, Sen DB, Zanwar A, Seth AK. Development and validation of spectrophotometric method of acenocoumarol in bulk and tablet dosage form. International Journal of Pharmacy and Pharmaceutical Science. 2013;4(3):369-376.
8. Mandrupkar SN, Mulgund SV, Nagras MA. Development of validated stability indicating rp-hplc method for estimation of acenocoumarol in bulk and tablet dosage form. International Journal of Pharmaceutical Science Review and Research. 2012;16(1):101-106.
9. Ceresole R, Rosasco MA, Forastieri CC, Segall AI. HPLC determination of acenocoumarol and its major thermal degradation product. Journal of Liquid Chromatography & Related Technologies. 2008;31:179–187.
10. Mulgund SV, Gawale BD. Stability indicating LC method for determination of acenocoumarol and characterisation of its acid catalysed degradation product by LC-MS. International Journal of Pharmaceutical Research and Applied Science. 2013;2(1):1-8.
11. Mogale AS, Ganure AL, Khatale PN, Tuljapure D. Development and validation of hptlc method for estimation of acenocoumerol in bulk drug and dosage form. American Journal of PharmTech Research. 2013;3(2):566-573.
12. Pommier F, Sioufi A, Godbillon J. Determination of acenocoumarol in human plasma by capillary gas chromatography with mass-selective detection. Journal Chromatography B: Biomedical Sciences and Applications. 1994;654(1):35-41.
13. Kollroser M, Schober C. Determination of coumarin-type anticoagulants in human plasma by HPLC electrospray ionization tandem mass spectrometry with an ion trap detector. Clinical Chemistry. 2002;48:84-91.
14. ICH Harmonised Tripartite Guideline. Validation of Analytical procedures: Text and Methodology Q2 (R1). International conference on Harmonisation. Geneva: Switzerland; 2005. p. 1-13.