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UV-SPECTOPHOTOMETRIC METHOD DEVELOPMENT AND VALIDATION OF SYNTHESIZED 7-HYDROXY-4-METHYL COUMARIN.

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ABSTRACT

Objectives: The objectives of present workwastoo develop and standardize UV-Spectrophotometric method for estimation of 7-Hydroxy-4-Methyl Coumarin in marketed formulation. Materials and method: UV- Spectrophotometric method was developed using 70ml water: 30ml methanol as solvent. The developed method was standardized in terms of validation parameters such as simple, sensitive, precise, linear, accurate, robust, reproducible as per ICH Q2(R1) guidelines. For estimation of 7-Hydroxy-4-Methyl Coumarin in marketed formulation this newly developed method was successfully applied. Results: 7-Hydroxy-4-Methyl Coumarin exhibits λ_{max} at 321nm in the Beer's range 2 to 10µg/ml Beer's law was obeyed. The limit of detection was found to be 0.84µg/ml and limit of quantification was found to be 2.54µg/ml. Recovery of 7-Hydroxy-4-Methyl Coumarin in marketed formulation was obtained in the range of 75-100%. All the precision range less than 2%. Conclusion:The method was found to be simple, accurate, environment friendly, reproducible and marketed API of 7-Hydroxy-4-Methyl Coumarin can be estimated.

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INTRODUCTION

Coumarin are an important class of organic compound occurring widely in nature. Synthesis of these compounds by simple methods is beingattempted because of wide variety of uses such as fixatives in food and cosmetics, liquid crystal displays, information systems, pharmaceuticals, insecticides, rodenticides and to mask disagreeable odors in industrial products such as printing inks, paints and synthetic rubber[1-8] and possessing a wide spectrum of biological activities[9-10] like antibacterial[11], antitumor, anti-HIV and anti-inflammatory properties[12]. Coumarins are usually synthesized in laboratory scale having substitution at C4 ana C7 by Pechmann condensation using an acid catalyst with several methods.

7-Hydroxy-4-Methyl coumarin is also known as 4Methylumbelliferone, Hydroxy Methyl coumarin and Dantong. It used in the biliary sphincter relaxation, strong spasmolytic, analgesic effect, continuously promote bile secretion, strengthen the gall bladder contraction and antibacterial effect. 7-Hydroxy-4-Methyl coumarin derivatives have been of great interest in medicinal chemistry for their role as potent antibacterial and antifungal agents[13].

Manyformulation of 7-Hydroxy-4-Methyl coumarin are not available on the market. The compound might be chemically modified or conjugated with other molecules for specific applications.

Many researchers has been used the solvent as Methanol for the analysis of 7-Hydroxy-4-Methyl coumarin which is more economic. For this purpose, in present study we are tried to develop a less toxic, cheap, ecofriendly spectroscopic method. The aim of this work was to develop and standardize UV-spectroscopic method development and validation of 7-Hydroxy-4-Methyl coumarin.

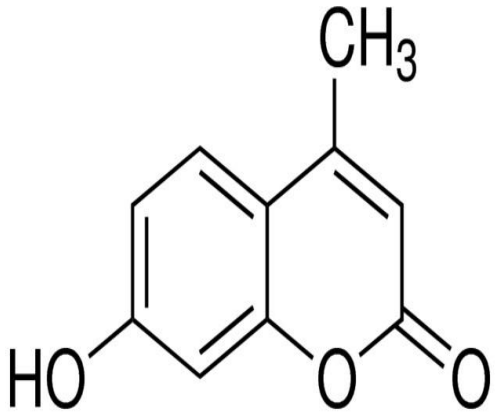

Fig.1: Structure of 7-Hydroxy-4-Methyl Coumarin

MATERIALS AND METHODS:

Reagents and chemicals:

Resorcinol, Ethyl-acetoacetate, Conc.H_2SO_4, Distilled water, Methanol, n-hexane, Ethyl acetate, Iodine.

Procedure for synthesis of 7-Hydroxy-4-Methyl coumarin:

Take 9.3ml conc. H_2SO_4 in a beaker and allow it to cool for 5-10 mins in ice bath. 2.2gm of resorcinol and 2.7ml of ethyl-acetoacetate was added to the beaker. The mixture was stirred continuously until the solution is obtained. H_2SO_4 was added slowly to above beaker so that the temperature of beaker should not rise above 10^0C. After addition of conc. H_2SO_4 it is stirred continuously for 30 mins. The mixture was poured into the crushed ice and the product separated. Yellow precipitates (M.P.186-191^{0}C) formed, filtered and washed with water, recrystallized with ethanol.

Thin layer chromatography of synthesized 7-Hydroxy-4-Methyl coumarin:

In thin layer chromatography, stationary phase is thin adsorbent material as silica gel which was coated on to a glass slide. The sample was spotted onto one end of the TLC plate and placed vertically into a closed chamber containing mobile phase. In 1st trial, ethyl acetate: n- hexane (8:2 ratio), in 2nd trial, ethyl acetate: n-hexane (2:8 ratio) and in 3rd trail, ethyl acetate: methanol (8:2 ratio) was taken as mobile phase. The mobile phase travels up the plate by capillary forces and sample components migrate varying distance based on their affinities for the stationary and mobile phase. When the solvents riches the top of the plate then the plate was removed from the iodine chamber and dried. The separated components appear as spots on the plate.

Melting point:

By placing a sample of synthetic 7-Hydroxy-4-Methyl coumarin into a capillary and sealing the other end with a flame, the melting point of the capillary was determined. After attaching this capillary to the thermometer and placing it in theThiel's tube filled with liquid paraffin, it was uniformly heated. Tube the sample begins to melt, and this was identified as the sample's melting point.

Selection of wavelength:

In water: methanol (70:30), several 7-Hydroxy-4-Methy coumarin concentrations were created. In a UV-spectrophotometer, 7-Hydroxy-4-Methyl coumarin 10µg/ml of working standard solution was scanned between 200 and 400nm, with maximum absorption occurring at 321nm.

Preparation of stock solution:

0.1g of synthesized 7-Hydroxy-4-Methyl coumarin was precisely weighed and dissolved in 70 ml of water and 30 ml of methanol using sonication transfer 1ml of above solution to a 100 ml volumetric flask containing water: methanol (70:30). The standard stock solution had a concentration of 10µg/ml. The standard stock solution was used to make additional a dilutions.

Preparation of calibration curve:

From the stock solution, serial dilutions were made concentration ranges of 2-10 From µg/ml. After analyzing the three sets of solutions, the absorbance at 321nm was determined. Plotting the linearity curve with concentration on the x-axis and absorbance on the y-axis allowed for the calculation of linearity regression.

Metod development and validation:

Water: methanol (70:30ml) was used as solvent because 7-Hydroxy-4-Methnol coumarin was soluble in it. The suggested approach was verified in accordance with the rules provided by the ICH Q2 (R1).Thecreated method was validated in accordance with the ICH recommendations to demonstrate that it was appropriate to use method parameters for the validation of analytical techniques.

Specificity and selectivity:

The method was determined to be selective since 7-Hydroxy-4-Methyl coumarin had the highest absorbance at 321nm and the solvent, water: methanol (70:30ml), did not have an absorbance at that wavelength indicating that this approach was specific.

Linearity:

Linearity was examined in a range of 2-10µg/ml. 100mg of 7-Hydroxy-4-Methyl coumarin was accurately weighed and transport to a clean and dried volumetric flask, with the remaining volume being adjusted with water: methanol (70:30ml). Using solvent in methanol: water, a volume of 100ml was created by pipetting out 1ml of the stock solution mentioned above and transferring it into a volumetric flask. To test the linearity, several dilutions of this solution are made.

LOD and LOQ:

Limit of detection is the concentration at which analyte in the test sample was detected. Limit of quantification is the concentration at which analyte in the test sample is quantified. Following formulae are used to calculate LOD and LOQ

LOD = 3.3×standered deviation of regression/slope

LOQ = 10×standered deviation of regression/slope

Precision:

Precision was determined by making by preparing three duplicates of a solution of 2µg/ml, 6µg/ml and 10µg/ml of 7-Hydroxy-4-Methyl coumarin, and its absorbance was measured at 321nm, and the percentage RSD computed.

To experiments were performed to determined the method's precision:

1) Intraday precision.
2) Interday precision.

For intraday precision, three duplicates of the solution with concentrations 2µg/ml, 6µg/ml and 10µg/ml of drugwasexamined, and at different time intervals, % RSD was calculated. Three replicates of a solution having concentrations of 2µg/ml, 6µg/ml and 10µg/ml of drug evaluated for interday precision, and % RSD was competed at different time intervals.

Ruggedness:

In order to verify reproducibility, ruggedness was tested using the same suggested procedure with a different analyzer.

Robustness:

7-Hydroxy-4-Methyl coumarin was determined by performing recovery experiment. % mean recovery of sample was determined by preparing different levels of the sample solutions as 50%, 100%, 150%.

Weighed accurately 100 mg 7-Hydroxy-4-Methyl coumarin was transferred into clean 100 ml volumetric flask and volume is made upto the mark using water: methanol (70:30) as 7-Hydroxy-4-Methyl coumarin is soluble in it. Three replicates of concentration wasmade at each level and recovery study was performed.

RESULT AND DISCUSSION

Thin Layer Chromatography:

To check the purity of compound TLC was performed and its result are shown in the fig.2(ab

Rf value of 7-Hydroxy-4-Methyl coumarin = $\frac{\textit{Distance travelled by solute}}{\textit{Distance travelled by solvent}}$

$=\frac{3.5}{5.5}= 0.63$

Rf value of resorcinol = $\frac{4.2}{5.5} = 0.80$

Trial No.1Fig.2(a).

separation of compound by using ethyl-acetate:n-hexane(2:8) is not clear as shown in fig.2(a)

Rf value of 7-Hydroxy-4-Methyl coumarin = $\frac{\textit{Distance travelled by solute}}{\textit{Distance travelled by solvent}}$

$= \frac{4.7}{5.5} = 0.85$

Trial.no.2fig.2(b)

Separation of compound by using ethyl-acetate: Methanol (8:2) is proper as shown in fig.2(b)

Melting point:

The melting point of 7-Hydroxy-4-Methyl coumarin was done to check purity of compound and was found to be 189^0C.Sothe synthesized compound was supposed to be a pure.

Method development:

UV-1800 model was used for development of UV-Spectrophotometric method using water:methanol(70: 30) as a solvent. Maximum absorbance of 7-Hydroxy-4-Methyl coumarin was formed at 321nm. Details of method developed was presented in Table: 1

Table1: Developed method parameters.

SI. No.	Parameters	Specifications
1.	Method	Spectrometric
2.	Instrument	UV
3.	Model	1800
4.	Make	Shimadzu
5.	Software	UVProbe
6.	Synthesized drug	7-Hydroxy-4-Methyl coumarin
7.	Solvent	Water: Methanol
8.	Scanning wavelength range	400-200nm
9.	λ_{max}	321nm

Method validation:

Developed method was validated interms of validation parameters such as specificity, selectivity, linier range, precision, robustness, ruggedness and reproducibility as per ICH guidelines.

Specificity and Selectivity:

7-Hydroxy-4-Methyl coumarin was showed maximum absorbance at 321nm and water:methanol solvent didn't show absorbance at 321nm. Hence the method was found to be specific and selective.

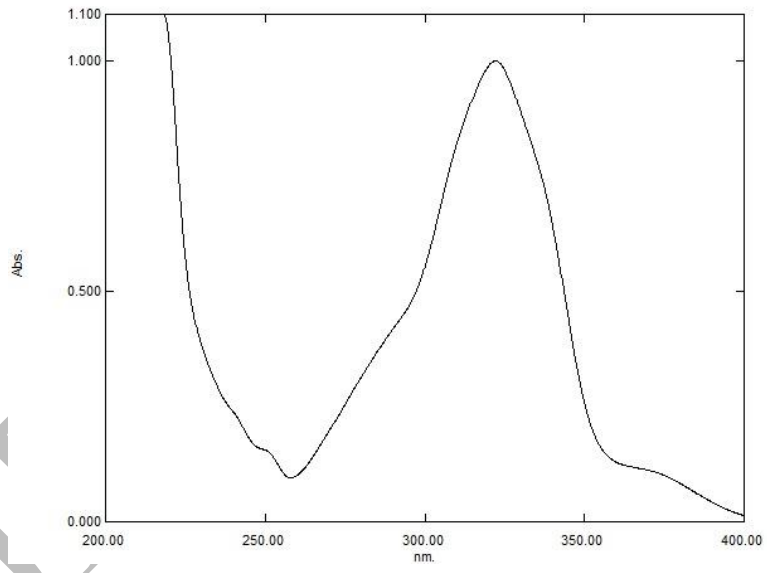

Fig 4: UV-Spectrum of 7-Hydroxy-4-Methyl coumarin.

Linearity:

Linearity was found in range of 2-10 µg/ml. The linearity graph is showed in fig 5. The linearity and range is showed in Table 2 and the calibration curve is given in fig.6.

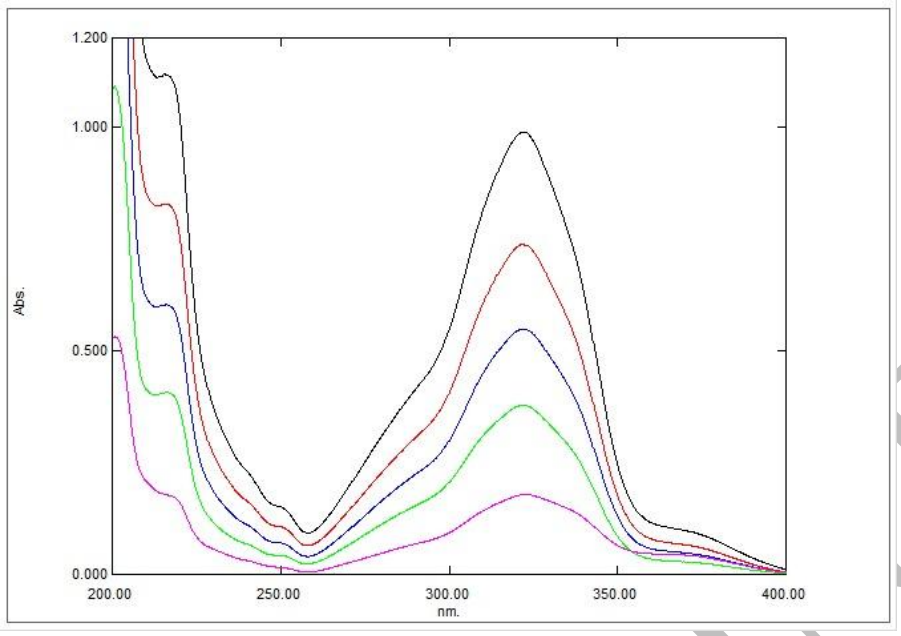

Fig 5: Linearity graph of 7-Hydroxy-4-methyl coumarin.

Table no 2: Linearity and range data of 7-Hydroxy-4-Methyl coumarin.

SI. No.	Concentration(µg/ml)	Absorbance
1	2	0.176
2	4	0.372
3	6	0.548
4	8	0.734
5	10	0.984
	r^2	0.996
	Slope	0.996
	LOD	0.8425
	LOQ	2.5461

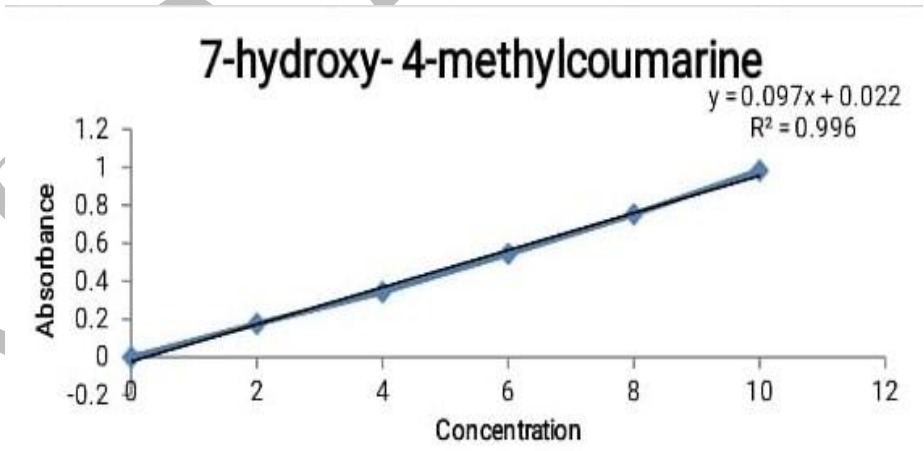

Fig 6: Linearity curve of 7-Hydroxy-4-Methyl coumarin.

Precision:

System precision: As stated in the procedure for figuring out system precision 7-Hydroxy-4-Methyl coumarin solution was replicated three times, or 2µg/ml. As per the procedure, 10µg/ml, was created to measure the system precision, and the absorbance of each solution was measured 321nm. After computation the percentage RSD was discovered to be less than 2% (Table 3)

Table 3: System precision data of 7-Hydroxy-4-Methyl coumarin.

Concentration (µg/ml)	Absorbance	Standard deviation	% RSD
2	0.334	0.0015	0.3
6	0.569	0.0015	0.27
10	0.713	0.001	0.14

*=average absorbance of 6 replicates.

Intraday precision:

Three duplicates of the 7-Hydroxy-4-Methyl coumarin solution with concentration of 2µg/ml, 6µg/ml, 10µg/ml were made and examined for intraday precision. The percentage RSD was computed on the same day at various time intervals, and it was shown to be less than 2%(Table 4)

Table 4: Intraday precision data of 7-Hydroxy-4-Methyl coumarin.

Concentration (µg/ml)	Absorbance		Standard deviation	% RSD
	Abs 1hr	0.334	0.0015	0.3
2	Abs 4hr	0.333	0.001	0.3
	Abs 8hr	0.335	0.0015	0.46
	Abs 1hr	0.569	0.0015	0.27
6	Abs 4hr	0.564	0.001	0.18
	Abs 8hr	0.569	0.0015	0.27
	Abs 1hr	0.713	0.001	0. 14
10	Abs 4hr	0.712	0.0015	0.21
	Abs 8hr	0.714	0.001	0.14

Precision for interday three replicates of 7-Hydroxy-4-Methyl coumarin solution with concentration of 2µg/ml, 6µg/ml, 10µg/ml were generated and examined. Three consecutivedaysworthof % RSD calculation made, and the results showed that the % RSD was less than 2%(Table5)

Table 5: Interday precision data of 7-Hydroxy-4-Methyl coumarin.

Concentration (µg/ml)	Absorbance		Standard deviation	% RSD
	Day 1	0.334	0.002	0.6
2	Day 2	0.337	0.002	0.59
	Day 3	0.335	0.002	0.59
	Day 1	0.569	0.001	0.18
6	Day 2	0.565	0.002	0.36
	Day 3	0.568	0.002	0.35
	Day 1	0.715	0.002	0.28
10	Day 2	0.713	0.001	0.14
	Day 3	0.712	0.002	0.28

*= average absorbance of three replicates.

Ruggedness:

Ruggedness was examined by performing the same proposed method by different analyst to check reproducibility. % RSD was obtained less than 2% which indicates that the method developed was rugged. (Table 6)

Table 6: Ruggedness data of 7-Hydroxy-4-Methyl coumarin.

Concentration (µg/ml)	Absorbance		Standard deviation	% RSD
	Analyst 1	0.334	0.0014	0.42
2	Analyst 2	0.333	0.0007	0.21
	Analyst 1	0.569	0.0014	0.25
6	Analyst 2	0.567	0.0007	0.12
	Analyst 1	0.715	0.0007	0.1
10	Analyst 2	0.714	0.0014	0.2

*= average absorbance of three replicates.

Robustness:

Water: Methanol used as a solvent because 7-Hydroxy-4-Methyl coumarin was soluble in it. Maximum absorbance of a drug was found at 321nm. Robustness is performed with sonication for 5 min and by changing the wavelength 320nm, 321nm, 322nm. The % RSD was obtained to be less than 2% (Table 7).

Table no 7: Robustness data of 7-Hydroxy-4-Methyl coumarin.

Concentration (µg/ml)	Absorbance			Standard deviation	% RSD
	Change in Wavelength	320	0.335	0.001	0.3
2	Sonication	321	0.335	0.001	0.3
	5 min	322	0.335	0.001	0.3
	Change wavelength	320	0.569	0.001	0.18
	Sonication	321	0.569	0.001	0.18
6	5 min	322	0.569	0.001	0.18
	Change in wavelength	320	0.713	0.0006	0.08
10	Sonication	321	0.713	0.0006	0.08
	5 min	322	0.714	0.0006	0.08

Accuracy:

Accuracy was determined by performing recovery experiments in which determination of percent mean recovery of sample by standardization method at three different levels 50%, 100%, 150% of the sample solutions were prepared. The precent recovery was found in the range of 100-106%(Table 8)

Table 8: Recovery data of 7-Hydroxy-4-Methyl coumarin.

Total Concentration (µg/ml)	Standard Cocentration (µg/ml)	Sample Cocentration (µg/ml)	Absorbance (321nm)		Concentration Y=mx+c (µg/ml)	Sample Concentration Difference(µg/ml)	% Recovery
			Standard	Sample			
	1	1	0.335	0.334	1.99	0.99	99%
2(50%)	1	1	0.336	0.332	1.97	0.97	97%
	1	1	0.334	0.333	1.99	0.99	99%
	1	3	0.567	0.578	4.07	3.07	76.75%
4(100%)	1	3	0.569	0.577	4.05	3.05	76.25%
	1	3	0.566	0.576	4.06	3.06	76.5%
	1	5	0.712	0.718	6.05	5.05	84.16%
6(150%)	1	5	0.714	0.719	6.04	5.04	84%
	1	5	0.716	0.717	6.00	5.00	83.33%

CONCLUSION

As per ICH Q2(R1) guidelines the present analytical method was validated and it specific acceptance criteria. It is concluded that the analytical method was simple, sensitive, precise, linear, accurate, robust, reproducible

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ABBREVIATIONS

µ : microgram
LOD : Limit of detection
LOQ : Limit of quantification
UV : Ultra violet spectrophotometer.

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