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Original Article

Rapid, Cost Effective and Simple Analytical Method Development and Validation for Simultaneous Estimation of Thymol and Cholecalciferol by Ultraviolet Spectrophotometry

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ABSTRACT

Background: Thymol and Cholecalciferol in a combined approach can be used as antioxidant, free radical scavenging, anti-depressant, anti-inflammatory, anti-diabetic, anti-arthritis etc. So, rapid, cost effective and simple analytical method is required to estimate Thymol and Cholecalciferol simultaneously in the mixture. **Purpose:** The purpose of this work is to develop and validate Ultraviolet spectroscopic (UV) method for simultaneous estimation of Thymol and Cholecalciferol. **Materials and Methods:** Two methods were developed i.e., simultaneous equation method and Q-Absorbance ratio method. In simultaneous equation method, absorbance of Thymol and Cholecalciferol were measured at λ_{max} of 276.3 nm and 263.2 nm. While in Q-Absorbance ratio method, absorbance of Thymol and Cholecalciferol were measured at 270.1 nm (isosbestic point) and at 263.2 nm (λ_{max} of Cholecalciferol). Methanol was used as a solvent in both the methods. **Results:** The concentration of the individual drugs in the combined mixture was found in the range of 98% to 100% in both methods. Developed methods were validated as per ICH quality guidelines. The validation parameters checked were linearity, range, LOD, LOQ, precision and accuracy. **Conclusion:** Both the methods were found accurate, simple, precise and reproducible for simultaneous estimation of Thymol and Cholecalciferol. These developed analytical methods can be used for quantification of Thymol and Cholecalciferol in pharmaceutical dosage forms.

Keywords: Thymol, Cholecalciferol, UV-visible spectroscopy, Simultaneous equation method development, Q-absorbance ratio method development, Analytical method validation.

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INTRODUCTION

Thymol is colourless crystalline powder which has very less water solubility.[1] It is freely soluble in alcohols.[2] Thymol is monoterpene phenol (Figure 1 (a)) (chemical name is 2-isopropyl-5-methylphenol) has many pharmacological activities like anti-inflammatory, antioxidant, antiseptic, antibacterial, analgesic, antifungal, free radical scavenging, antitumor.

Cholecalciferol also called as Vitamin D_3 is a fat-soluble vitamin has free solubility in alcohols. Mainly it helps to regulate calcium and phosphate in body. Chemically it is (3β,5Z,7E)-9,10-secocholesta- 5,7,10(19)-trien-3-ol (Figure 1 (b)). Vitamin D deficiency is common problem because of many factors like decreased sun exposure. When circulatory Vitamin D_3 i.e., 25(OH) Vitamin D_3 level is <10 ng/mL, it is called as deficiency of Vitamin D_3. As per literature survey, Cholecalciferol can be given in many diseases as a supplementary role.[3]

Rheumatoid Arthritis (RA) is a disabling autoimmune condition distinguished by inflammation of the synovium, the production of autoantibodies, damage to cartilage and bone, and the presence of systemic symptoms. It affects approximately 1% of the global population, with a higher incidence in females. Most treatment approaches for RA primarily target the alleviation of pain, reduction of joint inflammation, and prevention of joint damage. Thymol is recognized for its anti-inflammatory properties, while Cholecalciferol plays a role in regulating the immune system. Both Thymol and Cholecalciferol have each been individually investigated for their potential therapeutic effects in managing arthritis. Our research was designed to evaluate the combined effects of Thymol and Cholecalciferol in a pharmaceutical

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formulation to explore the potential of using them together for treating RA. The objective behind this combination is to offer an alternative or supplementary treatment option that has the potential to enhance the well-being of individuals suffering from RA. As of now, there are no commercially available products that integrate Thymol and Cholecalciferol for the management of RA.

Ultraviolet (UV) Visible spectrophotometry is one of the most frequently employed technique in pharmaceutical analysis.[4] Simultaneous multi-component analysis by UV-visible molecular absorption spectrophotometry is mainly developed for the purpose of minimizing the cumbersome task of separating interferents and to allow determination of an increasing number of analytes, consequently reducing analysis time and cost.[5] Two UV methods were used for development - 1) Simultaneous equation method, 2) Q-Absorbance ratio method.

Simultaneous equation method is also termed as Vierordt's method.[6] It is possible to quantify both drugs using the simultaneous equation method if a sample contains two absorbing drugs (x and y), each of which absorbs at the wavelength maxima of the other.[6] Thymol and Cholecalciferol both can absorbs at the wavelength maxima of each other.

Q-Absorbance ratio method is a modification of simultaneous equation method. This method also called as Absorption ratio method.[6] According to this method, the ratio of absorbance at any two wavelengths for a substance, which obeys Beer's law, is a constant value independent of the concentration and path length. This constant is termed as Q-value or "Hufner's Quotient". This method uses the ratio of absorbance at two selected wavelengths, one at isosbestic point and other being the λ_{max} of one of the two drugs.

Structure of Thymol and Cholecalciferol contains conjugated double bond system showing UV activity. There is no Ultraviolet (UV) spectrophotometric analytical method was developed previously to estimate Thymol and Cholecalciferol simultaneously in the mixture. Therefore, simple, rapid, cost effective, accurate, precise, sensitive ultraviolet spectrophotometric analytical method for simultaneous estimation of these two drugs were developed and validated as per ICH quality guidelines.

MATERIALS AND METHODS

Chemicals and Reagents

Thymol was purchased from Raw pharma biz Pvt. Ltd.,; Cholecalciferol was purchased from Research Lab Fine Chem Industries and methanol (99.8%) AR grade was purchased from Loba Chemie Pvt. Ltd.

Instruments and apparatus

Ultraviolet-visible spectrophotometer of model Shimadzu UV-1900i was used. Absorbances were recorded with a pair of matched quartz cells of 1 cm. Mettler Toledo ME204/A04 weighing balance was used. All glassware were treated with sulphuric acid and chromic acid mixture and rinsed with double distilled water and then dried in hot air oven.[7]

Preparation of stock solution and working solution for wavelength selection

Thymol and Cholecalciferol stock solutions were prepared separately by adding 10 mg of drug in 10 mL volumetric flask and made up the volume with methanol up to denoted mark to get 1000 µg/mL of concentration. The volumetric flask was sonicated for 5 min. 1 mL solution was withdrawn from above stock solution by calibrated glass pipette and added to 10 mL volumetric flask and volume was made up with methanol up to 10 mL to get 100 µg/mL solutions of Thymol and Cholecalciferol separately. Further dilutions of these solutions were done with a methanol to get the concentration of 24 µg/mL of Thymol and 6 µg/mL of Cholecalciferol separately. These solutions were independently scanned in Ultraviolet spectrophotometer from 400 nm to 200 nm to obtain spectra for identifying maximum

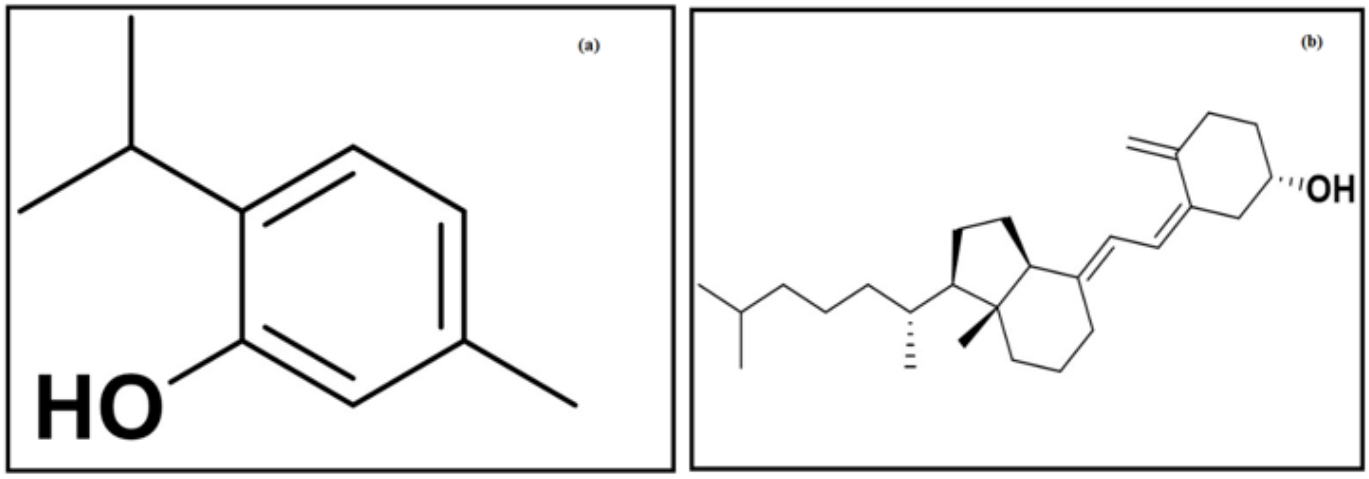

Figure 1: Chemical structure of (a) Thymol (b) Cholecalciferol.

wavelength (λ_{max}) of both drugs. Overlay spectra of Thymol and Cholecalciferol were formed and isosbestic point (iso-absorptive point) of these drugs was recorded.

Preparation of sample mixtures

Marketed preparation of Thymol and Cholecalciferol as a combination is not available in the market. Therefore, a mixture of these two drugs as a sample solution was prepared. Thymol (10 mg) and Cholecalciferol (2.5 mg) were added in 10 mL volumetric flask and volume was made up with methanol up to 10 mL. This solution was sonicated for 5 min. Samples were withdrawn from above solution and diluted to obtain different concentrations like 8 µg/mL, 16 µg/mL, 24 µg/mL of thymol and 2 µg/mL, 4 µg/mL, 6 µg/mL of Cholecalciferol respectively.

Analysis of prepared mixtures by Simultaneous equation method

In this method, absorbance was observed at λ_{max} of same drug and λ_{max} of another drug of the combination. So, Thymol and Cholecalciferol was analysed at two different wavelengths in increasing drug concentrations. Absorptivity was calculated by following equation:

$$\text{Absorptivity} = \frac{\text{Absorbance}}{\text{Concentration}} \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Absorbance of sample mixture solution was recorded at selected wavelengths of Thymol and Cholecalciferol. The concentration of the two drugs in mixture was calculated by using equations given below:[8]

$$Cx=(A2\,ay1 - A1\,ay2)/(ax2\,ay1 - ax1\,ay2) \ldots\ldots (2)$$

$$Cy=(A1\,ax2 - A2\,ax1)/(ax2\,ay1 - ax1\,ay2) \ldots\ldots (3)$$

Where,

ax1: Absorptivity of Thymol at λ1,

ax2: Absorptivity of Thymol at λ2,

ay1: Absorptivity of Cholecalciferol at λ1,

ay2: Absorptivity of Cholecalciferol at λ2,

A1: Absorbance of mixture at λ1,

A2: Absorbance of mixture at λ2,

Cx: Concentration of Thymol (µg/mL),

Cy: Concentration of Cholecalciferol (µg/mL).

Analysis of prepared mixtures by Q-Absorbance ratio method

In this method, absorbance was observed at isosbestic point and λ_{max} of one of the drugs of combination. Wavelength of Cholecalciferol was selected according to accuracy of results. So, Thymol and Cholecalciferol was analysed at two different wavelengths in increasing drug concentrations. Absorptivity was calculated by following equation 1. On the basis of absorptivity results, Q value of Thymol (Qx), Cholecalciferol (Qy) and sample mixture (Qm) was calculated.

Absorbance of sample mixture solution was recorded at isosbestic point and at wavelength of Cholecalciferol. The concentration of the two drugs in the mixture was calculated by using equations given below:

$$Cx=[(Qm - Qy)/(Qx - Qy)]\times(A/ax1) \ldots\ldots\ldots\ldots\ldots (4)$$

$$Cy=[(Qm - Qx)/(Qy - Qx)]\times(A/ay1) \ldots\ldots\ldots\ldots\ldots (5)$$

Where,

Qm: A2/A1 (Q value of sample mixture),

Qx: ax2/ax1 (Q value of Thymol),

Qy: ay2/ay1 (Q value of Cholecalciferol),

ax1: Absorptivity of Thymol at λ1,

ax2: Absorptivity of Thymol at λ2,

ay1: Absorptivity of Cholecalciferol at λ1,

ay2: Absorptivity of Cholecalciferol at λ2,

A1: Absorbance of mixture at λ1,

A2: Absorbance of mixture at λ2,

Cx: Conc. of Thymol (µg/mL),

Cy: Conc. of Cholecalciferol (µg/mL).

Validation of Methods

The developed methods were validated in accordance with the ICH Q2 (R1) guideline.[9] The parameters checked were linearity, range, accuracy, precision, Limit of Detection (LOD) and Limit of Quantification (LOQ).

Linearity and range

Thymol concentrations of 2 µg/mL, 4 µg/mL, 8 µg/mL, 12 µg/mL, 16 µg/mL, 20 µg/mL, 24 µg/mL, 28 µg/mL, 32 µg/mL, 36 µg/mL, 40 µg/mL and Cholecalciferol concentrations of 0.5 µg/mL, 1 µg/mL, 2 µg/mL, 4 µg/mL, 6 µg/mL, 8 µg/mL, 10 µg/mL, 12 µg/mL. 14 µg/mL were prepared in methanol separately. Calibration curve at λ_{max} 276.3 nm, 264.2 nm and isosbestic point 270.1 nm were plotted for both the drugs to measure linearity and range.

LOD and LOQ

LOD and LOQ were calculated by assessing signal to noise ratio according to equations stated in ICH Q2 (R1).[9] Following equations were used:

$$LOD = 3.3 \times \sigma/S \ldots\ldots\ldots\ldots\ldots (6)$$

$$LOQ = 10 \times \sigma/S \ldots\ldots\ldots\ldots (7)$$

Where, σ=Standard deviation of response, S = Slope of calibration curve.

Precision

The precision of developed methods was performed in terms of repeatability, intra-day, and inter-day precision.[9] Repeatability measurement was carried separately for 6 times by analyzing solution containing 16 µg/mL of Thymol and 4 µg/mL of Cholecalciferol. Intraday and interday precision was determined with standard solution of Thymol (8, 16, 24 µg/mL) and Cholecalciferol (2, 4, 6 µg/mL) for 3 times. Percentage Relative Standard Deviation (RSD) was calculated. Percentage Relative Standard Deviation (RSD) should be less than 2%.[10]

Accuracy

"Standard addition method" was used to determine the accuracy of both the developed methods.[11] Standard addition at three addition levels i.e. 50% Low Concentrations (LC), 100% Intermediate Concentrations (IC) and 150% High Concentrations (HC) were carried out as per ICH quality guidelines. (2) Percent recoveries were calculated as per following formula:

$$\%\,\text{Recovery} = [\text{Conc.(spiked)} - \text{Conc.(unspiked)} \times 100] / \text{Conc.(added)} \ldots\ldots\ldots (8)$$

RESULTS

Selection of wavelengths

The λ_{max} of Thymol and Cholecalciferol were obtained at 276.3 nm (Figure 2 (a)) and at 263.2 nm (Figure 2 (b)) respectively. After overlay plot of these drugs, isosbestic point was determined to be at 270.1 nm (Figure 2 (c)).

Analysis by Simultaneous equation method

According to equation 1, Thymol showed absorptivity of 0.0177 (ax1) and 0.0085 (ax2), while Cholecalciferol showed absorptivity of 0.0470 (ayl) and 0.0579 (ay2) at 276.3 nm and 263.2 nm respectively (Table 1).

After calculation by equation 2 and 3, concentrations of Thymol and Cholecalciferol were observed in a range of 98 to 100% respectively in all three different sample mixtures (Table 2).

Analysis by Q-Absorbance ratio method

In this method, the values of absorptivity measurement for Thymol were 0.0148 (ax1) and 0.0085 (ax2), and for Cholecalciferol were 0.0537 (ayl) and 0.0579 (ay2) at 270.1 nm and 263.2 nm respectively (Table 3). The Qx and Qy were observed to be 0.5768, and 1.0771 respectively. The Qm of samples 1, 2 and 3 was observed to be 0.8143, 0.8138 and 0.8142 respectively. After calculation by equations 4 and 5, similar to the results of the simultaneous equation method, concentrations of Thymol and Cholecalciferol were observed in a range of 98 to 100% respectively in all three different sample mixtures (Table 4).

Validation parameters

Linearity and Range

Correlation coefficients values were observed close to value 1. R^2 value of Thymol reported at 276.3 nm, at 263.2 nm and at 270.1 nm were 0.9998, 0.9993 and 0.9997 respectively. For Cholecalciferol, R^2 reported at 276.3 nm, at 263.2 nm and at 270.1 nm were 0.9992, 0.999 and 0.9992 respectively (Figure 3 (a)-(f)). The regression equation is reported in Table 5.

LOD and LOQ

LOD of Thymol observed at 276.3 nm, 263.2 nm, 270.1 nm were 0.64 µg/mL, 1.19 µg/mL and 0.72 µg/mL respectively, while LOQ of Thymol observed at 276.3 nm, 263.2 nm, 270.1 nm were 1.96 µg/mL, 3.62 µg/mL and 2.19 µg/mL respectively.

LOD of Cholecalciferol at 276.3 nm, 263.2 nm, 270.1 were observed as 0.48 µg/mL, 0.55 µg/mL and 0.48 µg/mL respectively and LOQ of cholecalciferol at 276.3 nm, 263.2 nm and 270.1 nm were observed as 1.46 µg/mL, 1.67 µg/mL and 1.47 µg/mL respectively (Table 5).

Precision

The results of repeatability, intra-day and inter-day precision are depicted in Table 6. Repeatability (in %RSD) of Thymol and Cholecalciferol were observed in a range of 0.4320 to 0.9936% at 276.3 nm, 263.2 nm, 270.1 nm. For Intraday Precision, Inter-day Precision, % RSD was found to be <2% which conforms to ICH Q2 (R1).

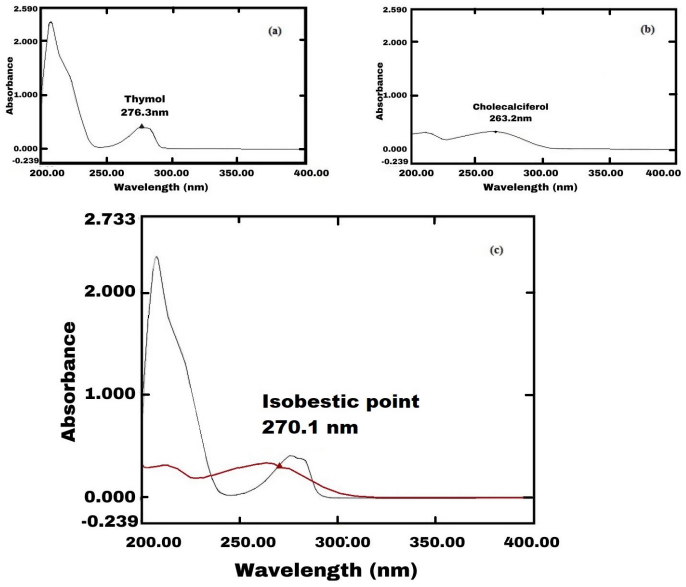

Figure 2: (a) Spectra of Thymol (b) Spectra of Cholecalciferol and (c) Overlay spectra of Thymol and Cholecalciferol.

Table 1: Absorptivity measurement values for Simultaneous equation method.

Drug Name	Conc. (µg/mL)	Absorbance		Absorptivity (absorbance/conc.)	
		At λ1=276.3 nm	At λ2=263.2 nm	At λ1=276.3 nm	At λ2=263.2 nm
Thymol	4	0.0742	0.0358	0.0185	0.0089
	8	0.1368	0.0676	0.0171	0.0084
	12	0.2159	0.1053	0.0178	0.0088
	16	0.2829	0.1359	0.0177	0.0085
	20	0.3550	0.1748	0.0177	0.0087
	24	0.4229	0.2042	0.0176	0.0085
	28	0.4917	0.2355	0.0176	0.0084
	32	0.5579	0.2663	0.0174	0.0083
	36	0.6261	0.2972	0.0174	0.0082
	Mean			ax1=0.0177	ax2=0.0085
CHL	2	0.1007	0.1243	0.0503	0.0621
	4	0.1967	0.2403	0.0491	0.0600
	6	0.2764	0.3386	0.0460	0.0564
	8	0.3707	0.4546	0.0463	0.0568
	10	0.4520	0.5599	0.0452	0.0559
	12	0.5514	0.6795	0.0459	0.0566
	14	0.6547	0.8154	0.0468	0.0582
	16	0.7429	0.9091	0.0464	0.0568
	18	0.8490	1.0402	0.0471	0.0577
	Mean			ay1=0.04704	ay2=0.0579

CHL: Cholecalciferol.

Table 2: Analysis of prepared mixtures by Simultaneous equation method.

Sample Mixture No.	Mixture conc. (µg/mL)		Absorbance at 276.3 nm	Absorbance at 263.2 nm	Found conc. (µg/mL)		Found conc. (%)	
	Thymol	CHL	A1	A2	Thymol (Cx)	CHL (Cy)	Thymol (%)	CHL (%)
1	8	2	0.2326	0.1815	7.9275	1.9647	99.09 ±0.03	98.24 ±0.04
2	16	4	0.469	0.3662	15.9668	3.9683	99.79 ±0.05	99.20 ±0.03
3	24	6	0.7054	0.551	23.9984	5.9747	99.99 ±0.01	99.58 ±0.06

Data represented as mean±SD. n=3; n is number of replicates.CHL: Cholecalciferol.

Accuracy

The values of accuracy at LC, IC and HC in methanol by simultaneous equation method and Q-absorbance ratio method are mentioned in (Table 7 (a) and Table 7 (b)) respectively. The values of per cent recovery of both the methods were found to be in the limit of 98-101%.[7,9] which conforms to ICH Q2 (R1) guidelines.

DISCUSSION

The λ_{max} of Thymol and Cholecalciferol was obtained near to reported range. So, drug analysis was carried out at observed λ_{max} and isosbestic point of Thymol and Cholecalciferol. Simultaneous equation method and Q-Absorbance ratio method has extremely few technical difficulties, so selected for development.

Table 3: Absorptivity measurement values for Q-Absorbance ratio method.

Drug Name	Conc. (µg/mL)	Absorbance		Absorptivity (absorbance/conc.)		
		Isosbestic point λ1=270.1 nm	At λ2=263.2 nm	At λ1=270.1 nm	At λ2=263.2 nm	
Thymol	4	0.062	0.0358	0.0155	0.0089	
	8	0.1152	0.0676	0.0144	0.0085	
	12	0.1813	0.1053	0.0151	0.0088	
	16	0.2368	0.1359	0.0148	0.0085	
	20	0.2984	0.1748	0.0149	0.0087	
	24	0.3544	0.2042	0.0148	0.0085	
	28	0.4119	0.2355	0.0147	0.0084	
	32	0.4674	0.2663	0.0146	0.0083	
	36	0.5227	0.2972	0.0145	0.0083	
	Mean			ax1= 0.0148	ax2= 0.0085	Qx=ax2/ax1 =0.5768
CHL	2	0.1154	0.1243	0.0577	0.0621	
	4	0.2237	0.2403	0.0559	0.0600	
	6	0.3152	0.3386	0.0525	0.0564	
	8	0.423	0.4546	0.0528	0.0568	
	10	0.5173	0.5599	0.0517	0.0559	
	12	0.6311	0.6795	0.0526	0.0566	
	14	0.75	0.8154	0.0536	0.0582	
	16	0.847	0.9091	0.0529	0.0568	
	18	0.968	1.0402	0.0538	0.0578	
	Mean			ay1= ay1=	ay1= 0.0579	Qy=ay2/ ay1=1.0771

CHL: Cholecalciferol.

Table 4: Analysis of prepared mixtures by Q-Absorbance ratio method.

Sample Mixture No.	Mixture conc. (µg/mL)		Absorbance at 270.1 nm (Isosbestic point)	Absorbance at 263.2 nm	Qm	Found conc. (µg/mL)		Found conc. (%)	
	Thymol	CHL	A1	A2		Thymol (Cx)	CHL (Cy)	Thymol (%)	CHL (%)
1	8	2	0.2229	0.1815	0.8143	7.9044	1.9687	98.80 ±0.03	98.44 ±0.04
2	16	4	0.45	0.3662	0.8138	15.9874	3.9664	99.92 ±0.03	99.16 ±0.01
3	24	6	0.6767	0.551	0.8142	23.9987	5.9764	99.99 ±0.015	99.61 ±0.04

Data represented as mean±SD. n=3; n is number of replicates.CHL: Cholecalciferol.

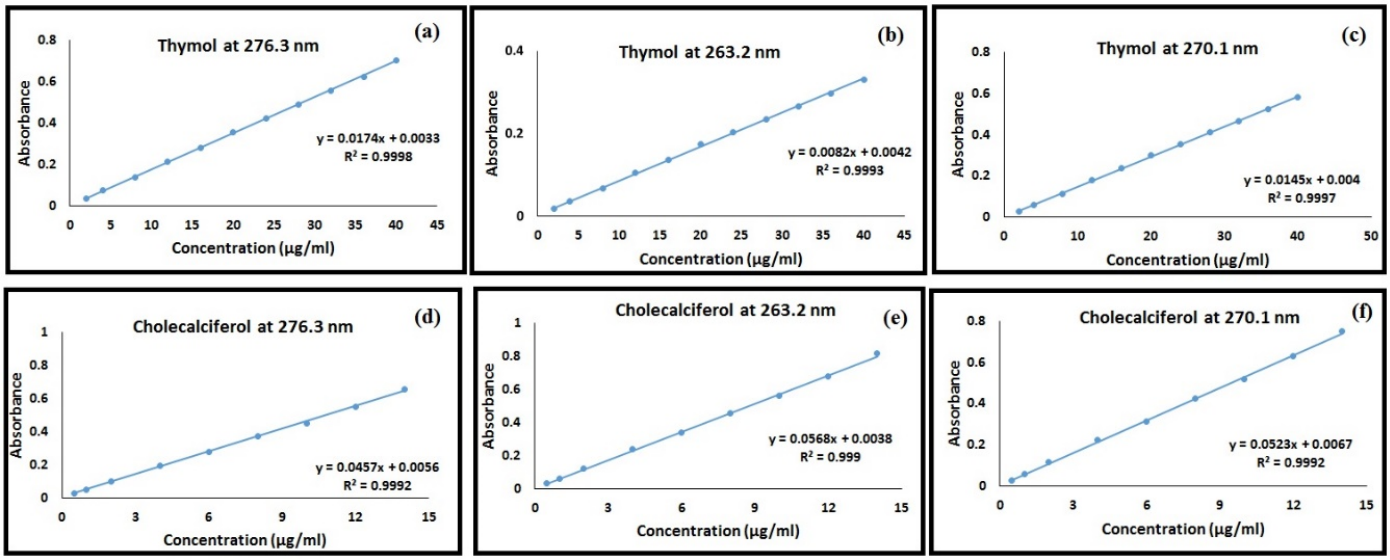

Figure 3: Calibration curve of (a) Thymol at 276.3 nm, (b) Thymol at 263.2 nm, (c) Thymol at 270.1 nm, (d) Cholecalciferol at 276.3 nm, (e) Cholecalciferol at 263.2 nm and (f) Cholecalciferol at 270.1 nm.

Table 5: Regression characteristics, LOD and LOQ.

Parameters	Thymol			CHL		
	276.3 nm	263.2 nm	270.1 nm	276.3 nm	263.2 nm	270.1 nm
Range (µg/mL)	2-40	2-40	2-40	0.5-14	0.5-14	0.5-14
Regression equation	y=0.0174x +0.0033	y=0.0082x +0.0042	y=0.0145x +0.004	y=0.0457x +0.0056	y=0.0568x +0.0038	y=0.0523x +0.0067
Correlation coefficient (R^2)	0.9998	0.9993	0.9997	0.9992	0.999	0.9992
LOD (µg/mL)	0.64	1.19	0.72	0.48	0.55	0.48
LOQ (µg/mL)	1.96	3.62	2.19	1.46	1.67	1.47

For Simultaneous equation method, λ1 is 276.3 nm and λ2 is 263.2 nm. For Q-Absorbance ratio method, λ1 is 270.1 nm and λ2 is 263.2 nm.CHL: Cholecalciferol.

Table 6: Precision parameters.

Parameters	Thymol				CHL			
	Conc. (µg/mL)	276.3 nm	263.2 nm	270.1 nm	Conc. (µg/mL)	276.3 nm	263.2 nm	270.1 nm
Repeatability	16	0.2832 ±0.0020	0.1372 ±0.0006	0.2352 ±0.0023	4	0.1966 ±0.0016	0.2413 ±0.0019	0.2238 ±0.0019
		%RSD 0.7053	%RSD 0.4320	%RSD 0.9936		%RSD 0.8360	%RSD 0.7704	%RSD 0.8364
Intraday Precision	8	0.1361 ±0.0013	0.0663 ±0.0008	0.1155 ±0.0010	2	0.1003 ±0.0009	0.1231 ±0.0009	0.1158 ±0.0014
		%RSD 0.9608	%RSD 1.1722	%RSD 0.9039		%RSD 0.8995	%RSD 0.7373	%RSD 1.2127
	16	0.2854 ±0.0024	0.1367 ±0.0011	0.2366 ±0.0011	4	0.1959 ±0.0022	0.2378 ±0.0011	0.2278 ±0.0013
		%RSD 0.8265	%RSD 0.8047	%RSD 0.4674		%RSD 1.1370	%RSD 0.4418	%RSD 0.5490

Parameters	Thymol				CHL			
	Conc. (µg/mL)	276.3 nm	263.2 nm	270.1 nm	Conc. (µg/mL)	276.3 nm	263.2 nm	270.1 nm
	24	0.4251 ±0.0028	0.2051 ±0.0007	0.3555 ±0.0023	6	0.2754 ±0.0011	0.3355 ±0.0028	0.3148 ±0.0023
		%RSD 0.6523	%RSD 0.3516	%RSD 0.6471		%RSD 0.3994	%RSD 0.8452	%RSD 0.7261
Interday Precision	8	0.1352 ±0.0018	0.0667 ±0.0010	0.1352 ±0.0014	2	0.1011 ±0.0011	0.1223 ±0.0014	0.1028 ±0.0006
		%RSD 1.3241	%RSD 1.5025	%RSD 1.0072		%RSD 1.0944	%RSD 1.1454	%RSD 0.6176
	16	0.2853 ±0.0018	0.1355 ±0.0011	0.2834 ±0.0019	4	0.1937 ±0.0027	0.2368 ±0.0016	0.1948 ±0.0018
		%RSD 0.6309	%RSD 0.8293	%RSD 0.6623		%RSD 1.3863	%RSD 0.6841	%RSD 0.9204
	24	0.4246 ±0.0031	0.2068 ±0.0011	0.4235 ±0.0024	6	0.2763 ±0.0014	0.3365 ±0.0025	0.2805 ±0.0048
		%RSD 0.7217	%RSD 0.5304	%RSD 0.5707		%RSD 0.4976	%RSD 0.7425	%RSD 1.6936

For Simultaneous equation method, λ1 is 276.3 nm and λ2 is 263.2 nm. For Q-Absorbance ratio method, λ1 is 270.1 nm and λ2 is 263.2 nm.Data represented as mean absorbance±SD. n=6 for Repeatability; n=3 for Intraday and Interday precision; n is number of replicates.CHL: Cholecalciferol.

Table 7 (b): Accuracy (%Recovery) by Q-Absorbance ratio method.

Level of % Recovery	Working conc. in mixture (µg/mL)		Spiked conc. of mixture (µg/mL)		Abs at 270.1nm	Abs at 263.2nm	Amount recovered (µg/mL)		% Recovery	
	Thymol	CHL	Thymol	CHL	Thymol	CHL	Thymol	CHL	Thymol	CHL
50%	12	3	6	1.5	0.5070	0.4119	18.01	4.46	100.10 ±0.05	99.16 ±0.06
100%	12	3	12	3	0.6776	0.5514	24.07	5.97	100.58 ±0.02	99 ±0.04
150%	12	3	18	4.5	0.8456	0.6880	30.04	7.45	100.14 ±0.06	99.44 ±0.05

Data represented as mean±SD. n=3; n is number of replicates.CHL: Cholecalciferol

Table 7 (a): Accuracy (%Recovery) by Simultaneous equation method.

Level of % Recovery	Working conc. in mixture (µg/mL)		Spiked conc. of mixture (µg/mL)		Abs at 276.3 nm	Abs at 263.2 nm	Amount recovered (µg/mL)		%Recovery	
	Thymol	CHL	Thymol	CHL	Thymol	CHL	Thymol	CHL	Thymol	CHL
50%	12	3	6	1.5	0.5276	0.4100	17.99	4.44	99.94 ±0.02	98.75 ±0.03
100%	12	3	12	3	0.7068	0.5514	24.1	5.97	100.83 ±0.02	99 ±0.06
150%	12	3	18	4.5	0.8886	0.6844	30.3	7.41	101 ±0.01	98.88 ±0.04

Data represented as mean±SD. n=3; n is number of replicates.CHL: Cholecalciferol.

Absorptivity values were calculated by taking the mean of nine estimations in both the methods.[9] It is reported that sample mixture concentration calculated by simultaneous equation method should be in a range of 98 to 102%. Practically our results investigated the sample mixture concentration within this range.

In terms of linearity, accuracy, precision, LOD, and LOQ, the proposed method was validated. Linearity is the ability of a method to elicit test results that are directly proportional to the analyte concentration within a given range.[12] Both the developed methods for simultaneous estimation of Thymol and Cholecalciferol were found linear, which denotes a good correlation between absorbance and concentration of solution.

According to ICH Q2 (R1), % RSD of repeatability, Intra-day Precision and Inter-day Precision should be less than 2%. All of them for Thymol and Cholecalciferol were found within range.

Results for accuracy are within limits as specified by ICH quality guidelines. Both methods are sensitive enough to detect and quantify small amounts of Thymol and Cholecalciferol in a sample.

CONCLUSION

All factors together point to the conclusion that both the Simultaneous Equation Method and the Q-Absorbance Ratio Method are rapid, cost-effective, simple, accurate, precise for the estimation of Thymol and Cholecalciferol simultaneously in the mixture. Hence, the proposed method can be recommended for simultaneous determination of Thymol and Cholecalciferol in routine quality control analysis.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

UV: Ultraviolet; **mg:** Milligram; **µg:** Microgram; **mL:** Millilitre; **nm:** Nanometre; **SD:** Standard Deviation; **RSD:** Relative Standard Deviation; **LOD:** Limit of detection; **LOQ:** Limit of Quantitation; **ICH:** International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use; **Conc.:** Concentration; **CHL:** Cholecalciferol; **LC:** Low concentration; **IC:** Intermediate concentration; **HC:** High concentration.

SUMMARY

The present research work was for the development and validation of two UV-spectrophotometric methods for simultaneous estimation of Thymol and Cholecalciferol in the mixture. The first method was the Simultaneous equation method in which λ1 was 276.3 nm (λ_{max} of Thymol) and λ2 was 263.2 nm (λ_{max} of Cholecalciferol) and another method was Q-Absorbance ratio method in which λ1 was 270.1 nm (Isosbestic point) and λ2 was 263.2 nm (λ_{max} of Cholecalciferol). Methods were validated as per ICH guidelines. R^2 values were observed close to 1. Methods are sensitive enough to detect and quantify small amounts of Thymol and Cholecalciferol in the sample. Methods were found to be precise (% RSD was found to be <2%). The percent recovery values of both methods were found to be in the limit of 98-101%. Thus, both methods were found to be accurate, precise, rapid, cost-effective and simple and can be used for routine analysis for the estimation of Thymol and Cholecalciferol simultaneously in the mixture.

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