Simultaneous Estimation of Menaquinone-7 and Cholecalciferol in Combined Pharmaceutical Dosage Forms by Ultraviolet Spectrophotometry

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Abstract

Context: Simple, precise, easy, and accurate spectrophotometric methods are ideal for regular quality control purpose. Menaquinone – 7 (MK-7) and cholecalciferol (CHOL) are two vitamins which work in synergy to elicit pharmacological responses in body. Inspite of multiple available brands of these two vitamins in market, to the best of our knowledge, there is no spectrophotometric method available for simultaneous quantification of these two vitamins. Therefore, an attempt was made in present research to develop two simple, precise, rapid, accurate, and economical spectrophotometric methods for simultaneous estimation of MK-7 and CHOL in combined pharmaceutical dosage forms. **Materials and Methods:** Method I was based on dual-wavelength method while Method II was based on Q-absorbance ratio method. Validation of developed methods was done as per the International Conference on Harmonization guidelines, and developed methods were applied for the determination of MK-7 and CHOL in laboratory prepared admixtures as well as in-house formulated dosage form. **Results:** Beer's law was obeyed over concentration range of 5–35 μg/mL for both MK-7 and CHOL (values of correlation coefficient were close to 1). Developed methods are accurate, reproducible, precise, and robust for determination of these two vitamins (percent recoveries lies within range of 98–102% while associated relative standard deviation was <2%). **Conclusion:** Developed methods can be used for quantification of MK-7 and CHOL in pharmaceutical dosage forms during regular quality control analysis.

Key words: Cholecalciferol, Menaquinone-7, Dual-wavelength method, Q-absorption ratio method, Ultraviolet spectroscopy

Key message: Two simple, precise, accurate, robust, and economical spectrophotometric methods were developed for simultaneous quantification of CHOL and MK-7 in combined pharmaceutical dosage forms using dual-wavelength method and Q-absorption ratio method. Validation of developed methods was done as per the International Conference on Harmonization guidelines. Developed methods were found to be appropriate for regular quality control analysis.

INTRODUCTION

Cholecalciferol (CHOL), (Vitamin D$_3$ or cholecalciferol or calcidiol), is a fat-soluble vitamin chemically known as (3β,5Z,7E)-9,10-secocholesta- 5,7,10(19)-trien-3-ol [Figure 1]. It is converted into 25α-hydroxy Vitamin D$_3$ (25(OH) D$_3$), circulatory Vitamin D (VD), by 25α-hydroxylase enzyme present in liver. It is finally converted into calcitriol (1, 25α-dihydroxy Vitamin D$_3$), biologically active form of VD, in kidney tubules by the action of 1α-hydroxylase enzyme. Initially, it was thought that VD only plays a role in modulating calcium absorption and reabsorption within the body, but after the

discovery of VD receptors on almost every organ of the body, it is now proven to have many other roles to play.[1] Therefore, maintenance of adequate levels of VD in body is important. Circulatory level of 25(OH) D_3 is considered as a marker for VD sufficiency (20–30 ng/mL), insufficiency (10–19 ng/mL) or deficiency (< 10 ng/mL). Nowadays, VD deficiency is quite common due to lifestyle changes (which results in decreased sun exposure) and is reported to be associated with many diseases (particularly, diabetes, asthma, cancer, and infertility); therefore, its supplementation is usually recommended.[2,3]

Vitamin K (VK) is a fat-soluble vitamin. Since Henrik Dam discovered and named VK, its research has undergone tremendous transformation. Initially, it was thought to play a critical role in the synthesis of coagulation factors only, but now it is proven to have many other extrahepatic roles to play, particularly in regulation of bone metabolism, prevention of vascular calcification, and prevention of cardiometabolic disorders.[4,5] Among various forms of VK, menaquinone-7 (MK-7) attracts special attention due to is higher plasma half-life within the body which enables it to circulate within the body for longer duration of time so as to perform various extrahepatic roles.[6] Chemically, MK-7 is 2-[(2E,6E,10E,14E,18E,22E)-3,7,11,15,19,23,27-heptamethyloctacosa-2,6,10,14,18,22,26-heptaenyl]-3-methylnaphthalene-1,4-dione [Figure 2].
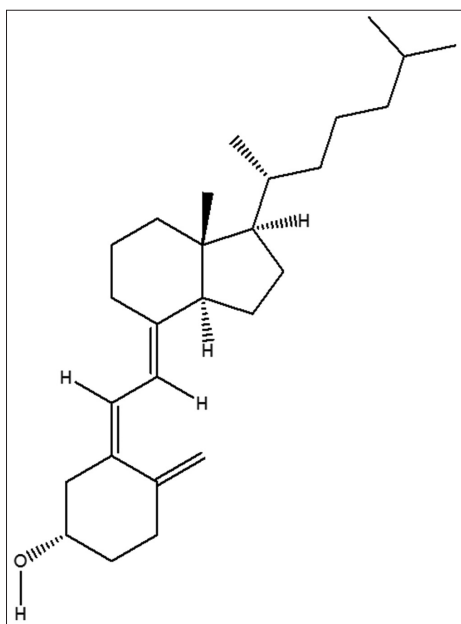

Figure 1: Chemical structure of cholecalciferol

Both CHOL and MK-7 are reported to work in synergism within the body because MK-7 activate various proteins expressed as the result of VD receptor activation by VD, particularly, osteocalcin (plays crucial role in bone mineralization and glucose metabolism), and matrix Gla protein (responsible for inhibition of vascular calcification).[7] This is the reason why multiple products containing combination of these two vitamins are existing in market. Multiple analytical methods have been developed for analysis and quantification of CHOL and MK-7 in biological matrices, but all these methods are very time consuming and lack application at industrial level for routine analysis of batch to batch variations.[8-10] Till now, only one research describes the simultaneous determination of these two vitamins in various pharmaceutical dosage forms through ultra-high-performance liquid chromatography (UHPLC) and high-performance liquid chromatography (HPLC).[11] In this research, author compared the developed methods on UHPLC with HPLC and concluded that HPLC takes a lot of time to analyze and hence UHPLC usage is better over HPLC for routine analysis, but UHPLC is a costly method of analysis and requires especially skilled staff, therefore, there is always a need of economical, robust, and easy method for simultaneous estimation of these vitamins for regular quantity control purposes which can be affordable even by small and medium scale industries. To the best of our knowledge, there is no ultraviolet (UV) spectrophotometric method reported for simultaneous quantification of these two vitamins in pharmaceutical dosage form. Therefore, aim of present research is to develop and validate rapid, easy, sensitive, and precise UV spectrophotometric methods for simultaneous determination of CHOL and MK-7 in various pharmaceutical dosage forms. UV spectrophotometry was used as a technique of choice for analysis because both VD3 and MK-7 have conjugated double bond system in their structure which makes them UV active. Validation of developed methods was also done as per the International Conference on Harmonization (ICH) quality guidelines (ICH Q2 R1, 2005)[12] and was found to comply with specified acceptance criteria.

MATERIALS AND METHODS

Instruments

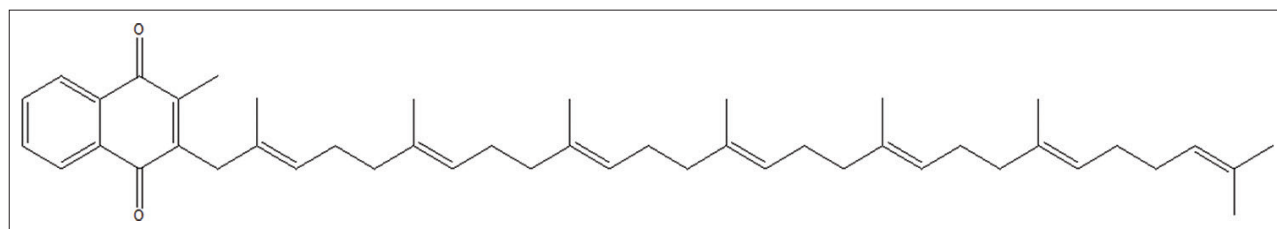Double beam UV-visible spectrophotometer (Shimadzu, Kyoto, Japan), model Pharma spec UV-1700 with 1 cm quartz

Figure 2: Chemical structure of manaquinone-7

cells was used. The instrument had automatically checked the wavelength accuracy of 0.1 nm.

Material and reagents

CHOL was purchased from Stabicoat vitamins, India, and had a claim to contain 500,000 IU of CHOL/g. MK-7 was purchased from Shanghai Cee Bio Co Ltd, China, and had a claim to contain 1% MK-7 w/w. Ethanol was purchased from Changshu Hongsheng Fine Chemical Co. Ltd., China, and had a purity claim of 99.99%. Aerosil was purchased from Wacker Chemie AG, Germany. Magnesium stearate was purchased from Nitika Pharmaceuticals Specialities Pvt., Ltd., India. Talcum was purchased from Golcha Associated, India. Sodium lauryl sulfate was purchased from Aarti Industries Ltd., India. Polyvinylpyrrolidone K-30 was purchased from Boai Pharmaceuticals Ltd., China. Mannitol was purchased from Shijiazhuang Huaxu Pharmaceutical Co., Ltd., China. Lactose monohydrate was purchased from Saputo Ingredients, Canada.

Spectra characterization and selection of wavelength

Eight hundred milligrams of CHOL and 1 g MK-7 were individually weighed in previously calibrated volumetric flask (100 mL) and were extracted with ethanol using ultrasonication for 15 min in ultrasonic bath. After extraction volume was made up to 100 mL with analytical grade ethanol. Stock solutions were subjected to filtration so as to remove the stabilizers (filter suitability, for probable adsorption, was previous checked using control solutions of both CHOL and MK-7. It was found to be suitable for usage because percent recovery of both CHOL and MK-7 following filtration was lie in between 98% and 102%). Resultant solutions had concentration of 100 µg/mL for both CHOL and MK-7. Suitable dilutions of both stock solutions were made with ethanol so as to made solutions of 10 µg/mL for both CHOL and MK-7. Absorption spectra of both the solutions were recorded from 200 to 400 nm using ethanol as blank. The overlay spectrum was observed for selection of the suitable wavelengths for each method [Figure 3].

Linearity

Method I dual-wavelength method

Standard solutions of both CHOL and MK-7 were individually made in ethanol over a concentration range of 5 µg/mL–35 µg/mL after appropriate dilution of 100 µg/mL stock solution. Absorbance values for CHOL were recorded at 222 nm and 241.5 nm (had zero absorbance difference for MK-7), while absorbance values of MK-7 were recorded at 249 and 282 nm (had zero absorbance difference for CHOL).[13] The concentration of CHOL was determined from regression equation obtained by plotting

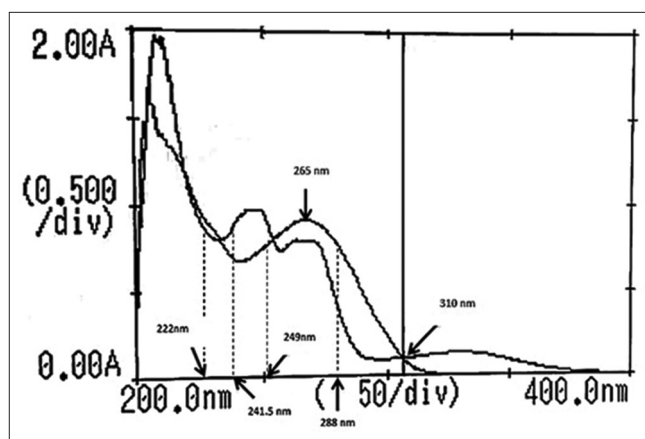**Figure 3:** Zero-order absorption spectra of 10 µg/ml each of cholecalciferol (CHOL) and manaquinone-7 (MK-7) in ethanol. Absorption maxima of CHOL and MK-7 waa observed at 265 nm and 248 nm, respectively

the difference in absorbance at 222 nm and 241.5 nm against corresponding concentrations and concentration of MK-7 was determined from regression equation obtained by plotting difference in absorbance at 249 nm and 282 nm, respectively.

Method II Q-absorbance ratio method

Standard solutions containing 5–35 µg/mL of CHOL and MK-7 were prepared separately in ethanol. The absorption spectrum of prepared solutions was recorded over 200–400 nm using ethanol as blank. Absorbance values of both the drugs were recorded at 310 nm (λ_{iso}, isoabsorptive point) and 265 nm (λ_{max} of CHOL). Concentrations of both the drugs were obtained by Q-ratio method using following equations:[13]

$$C_{MK-7} = [(Q_m - Q_y)/(Q_x - Q_y)] \times (A/A_{MK-7}) \qquad Eq.~(1)$$

$$C_{CHOL} = [(Q_m - Q_x)/(Q_y - Q_x)] \times (A/A_{CHOL}) \qquad Eq.~(2)$$

Where, C_{CHOL} and C_{MK-7} are the concentrations of CHOL and MK-7 in µg/mL, respectively. Q_m is a ratio of absorbance of sample at λ_{310nm} to absorbance of sample at λ_{265nm}; Q_x is ratio of absorptivity of MK-7 at λ_{265nm} to absorptivity of MK-7 at λ_{310nm}; Q_y is ratio of absorptivity of CHOL at $\lambda_{265\,nm}$ to absorptivity of CHOL at λ_{310nm}; A_{CHOL} is absorptivity of CHOL at λ_{310nm}; A_{MK-7} is absorptivity of MK-7 at λ_{310nm}; and A is absorptivity of sample at λ_{310nm}.

Analysis of prepared mixtures and tablets

Analysis of prepared mixtures

Different mixtures of CHOL and MK-7 were prepared in a laboratory containing different concentrations of both the drugs. Zero-order absorption spectrum of all mixtures was taken, and absorbance at 222 nm, 241.5 nm, 249 nm, and 282 nm was recorded for Method I and for Method

II, absorbance at 265 nm and 310 nm was recorded. The concentration of both CHOL and MK-7 was calculated from respective regression equations. All measurements were done in a replicate of six ($n = 6$).

Analysis of prepared tablets

In house, tablets were prepared with common tablet excipients using direct compression technology and were used for analysis. Twenty tablets were taken and were crushed in mortar pestle to obtain fine powder. Accurately weighed powder mixture corresponding to 80 mg of CHOL (1000 µg) and 120 mg of MK-7 (1200 µg) was separately transferred to 100 mL previously calibrated volumetric flask. 80 mL of ethanol was added to individual volumetric flask and was sonicated in ultra-sonication bath for 30 min. Volume was made up to 100 mL with ethanol and solutions were filtered. Filtered solutions were suitably diluted with ethanol to get 10µg/mL of CHOL and 12µg/mL of MK-7, respectively. Prepared mixtures were analyzed at previously selected wavelengths, and corresponding concentrations were calculated. All measurements were done in a replicate of six ($n = 6$).

Method validation

Validation of proposed methods was done as per the ICH Q2 (R1) 2005[12] guidelines for prescribed ICH parameters such as accuracy, precision, linearity, limit of detection (LOD), limit of quantification (LOQ), and specificity of developed methods for determination of CHOL and MK-7 in the presence of various excipients.

Linearity

From 100 µg/mL stock solution of CHOL and MK-7, separately aliquot portions of 0.5–3.5 mL were withdrawn and transferred to previously calibrated 10 mL volumetric flask. Volume was adjusted up to the mark with ethanol to obtain concentration range of 5 µg/mL to 35 µg/mL for both CHOL and MK-7, respectively. The absorbance of prepared solutions was recorded at 222 nm, 241.5nm, 249 nm, and 282 nm for Method I and for Method II, absorbance was recorded at 265 nm and 310 nm, and value of correlation coefficients was determined.

Accuracy

"Standard addition method" was applied to determine the accuracy of proposed methods and percent recovery at each addition level was calculated. Standard addition was done at three levels: 80% low concentration (LC), 100% intermediate concentration (IC), and 120% high concentration (HC) as per the ICH guidelines.[12] All measurements were done in a replicate of six ($n = 6$). Percent recoveries were calculated as per following formula:

$$\text{Recovery (\%)} = [(A–B)/C] \times 100 \qquad \text{Eq. (3)}$$

Where,
A = Total estimated amount of drug
B = Amount of drug present on pre-analysis basis
C = Amount of drug added.

Specificity

The specificity of developed methods was checked by determining the concentration of CHOL and MK-7 in the presence of various excipients used in preparation of tablets, namely, mannitol, lactose, magnesium stearate, sodium lauryl sulfate, talcum, Aerosil, and polyvinylpyrrolidone K30. The solution of CHOL and MK-7 (each at 10 µg/mL) was prepared, with or without excipients in ethanol. All solutions were scanned from 200 nm to 400 nm using ethanol as blank and were analyzed for any change or shift in absorbance values at respective wavelength. In another study, from independent stock solutions of CHOL and MK-7 (each having 100 µg mL concentration), a sample was prepared to contain 15 µg/mL concentration of each of the drug with or without excipients and was analyzed for its content. All measurements were done in a replicate of six ($n = 6$).

Precision

Repeatability of developed methods was determined by preparing different concentrations of CHOL and MK-7 from independent stock solutions (same as was selected for accuracy studies). The absorbance of prepared solutions was determined at wavelengths selected for both the methods and percent recoveries were calculated. All measurements were done in a replicate of three ($n=3$). Intermediate precision was determined by studying the intraday and interday variations. Different drug concentration levels in triplicates ($n = 3$) were prepared at 3 different times of the day and were observed for intraday variations. Same procedure was repeated for 3 consecutive days so as to study interday variation. Percent relative standard deviation (%RSD) was determined to evaluate intraday and interday precision of developed methods.

LOD and LOQ

LOD and LOQ were calculated by evaluating signal to noise ratio, i.e., 3.3 for LOD and 10 for LOQ, using equations mentioned in ICH Q2 (R1), 2005[12]

$$\text{LOD} = 3.3 \times \sigma/S \qquad \text{Eq. (4)}$$

$$\text{LOQ} = 10 \times \sigma/S \qquad \text{Eq. (5)}$$

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

Robustness

Robustness of the methods was determined by assessing the stability of CHOL and MK-7 in ethanol after storing their solutions in amber colored flasks for 12 h. Three different concentration levels were prepared (same as were used in accuracy studies) and were observed for their respective absorbance at interval of 6 h and 12 h. All measurements were done in a triplicate ($n = 3$) and percent recovery was calculated.

RESULTS AND DISCUSSION

Development of simple, accurate, rapid, and sensitive method is the requirement for routine quantitative analysis of pharmaceutical samples which otherwise requires tedious procedure for sample preparation, costly analytical technique, and especially skilled workforce. UV spectroscopic analysis is a cost effective and less time-consuming alternative to UHPLC and HPLC.[12] It is evident from Figure 3 that CHOL and MK-7 show strong spectral overlap which interferes in direct spectrophotometric measurements of these two vitamins. We selected ethanol for method development and validation due to the good solubility of both CHOL and MK-7 in it, which aid in appropriate extraction of both vitamins from their stabilized forms after ultrasonication for appropriate time period. Simultaneous equation method was not found to be suitable method for simultaneous determination of these two vitamins due to less difference in their λ_{max} which results in significant interference of both components at each other's λ_{max} [(A2/A1)/(ax2/ax1) or (ay2/ay1)/(A2/A1) lies within 0.1–2.0].[13] Therefore, in present research, we suggest that dual-wavelength method and Q-absorbance ratio method can be simple, sensitive, rapid, and accurate methods for simultaneous determination of CHOL and MK-7 in pharmaceutical sample. These methods are cost effective, simple, reliable, and precise for regular quality control purposes.

Method I dual-wavelength method

As per absorption spectra are shown in Figure 3, the absorbance values of CHOL were similar at 249 and 282 nm; therefore, these two wavelengths were selected for determination of MK-7. Similarly, MK-7 had similar absorbance values at 222 nm and 241.5 nm. Therefore, these two wavelengths were selected for determination of CHOL. Calibration plots for both CHOL and MK-7 were constructed by plotted their respective concentrations (on X-axis) versus absorbance difference at selected wavelengths (on Y-axis). The two vitamins obey Lambert Beer law over 5–35 µg/mL with good linear correlation coefficients [Table 1]. The linear regression equations obtained for determination of CHOL and MK-7 were as follows:

$$A_{MK-7} = 0.0231C_{MK-7} + 0.0054 \qquad \text{Eq. (6)}$$

$$A_{CHOL} = 0.0162C_{CHOL} + 0.0123 \qquad \text{Eq. (7)}$$

Where, A_{MK-7} and A_{CHOL} are absorbance of MK-7 and CHOL, respectively, and C_{CHOL} and C_{MK-7} are concentration of CHOL and MK-7, respectively.

Method II Q-absorbance ratio method

From overlay spectra [Figure 3], λ_{iso} for both CHOL and MK-7 was observed at 310 nm while the λ_{max} of CHOL and MK-7 was observed at 265 nm and 248 nm, respectively. Using λ_{iso} at 310 nm and λ_{max} of CHOL at 265 nm gave the best results in regard to selectivity. Absorbance values of both CHOL and MK-7 were obtained over 5–35 µg/mL at 265 nm and 310 nm, individually. Absorptivity values of both drugs were determined, and average values were calculated. Obtained absorption ratios were used to develop following equations to calculate concentration of both CHOL and MK-7 in given sample:

$$C_{MK-7} = [(Q_m - 17.452)/(6.614 - 17.452)] \times (A/0.00545) \quad \text{Eq. (8)}$$

$$C_{CHOL} = [(Q_m - 6.614)/(17.452 - 6.614)] \times (A/0.00266) \quad \text{Eq. (9)}$$

Where, C_{CHOL} is concentration of CHOL in µg/mL, C_{MK-7} is concentration of MK-7 in µg/mL, Q_m is ratio of absorbance of sample at 310 nm to absorbance of sample at 265 nm, and A is absorbance of sample at 310 nm.

To check the selectivity of developed methods, we prepared laboratory mixtures of both CHOL and MK-7 in known ratios. These mixtures were analyzed by developed dual-wavelength and Q-absorbance ratio methods, and percent recovery and %RSD were calculated. Percent recoveries were found to be within 98–120% and %RSD was found to be <2%, shows that both methods are selective for CHOL and MK-7 [Table 2]. Developed methods were also applied for determination of CHOL and MK-7 in prepared tablet dosage form and were found to have in close agreement with the label claim [Table 2]. There was no difference among both developed methods for determination of MK-7 and CHOL (*t*-test, $P = 0.848$ at 95% Confidence interval [CI]). Hence, both of the methods can be used for routine quality control analysis.

Method validation

Linearity

Both methods were found to have good linearity over a concentration range of 5–35 µg/mL. Values of correlation coefficients were close to 1, indicating good linearity of developed methods[13] (Q-absorbance method, R^2 for MK-7 = 0.9963 and R^2 for CHOL = 0.998; Dual-wavelength method, R^2 for MK-7 = 0.9995 and R^2 for CHOL = 0.9997) [Table 1].

Table 1: Linear regression and analytical parameters of proposed methods for simultaneous estimation of MK-7 and CHOL

Parameters	Dual-Wavelength method		Q- absorbance ratio method	
	MK-7	**CHOL**	**MK-7**	**CHOL**
λ_{max} (nm)	Difference in absorbance at 249 nm and 282 nm	Difference in absorbance at 222 nm and 241.5 nm	310 nm (λ_{iso}) and 265 nm (λ_{max})	
Beer's law range	5–35 µg/mL	5–35 µg/mL	5 – 35 µg/mL	5–35 µg/mL
Regression equation y=mx+c)	$A_{MK-7}=0.0231C_{MK-7}+0.0054$	$A_{CHOL}=0.0162C_{CHOL}+0.0123$	$C_{MK-7} = [(Q_m - 17.452) /(6.614 - 17.452)]$ x (A/0.00545)	$C_{CHOL} = [(Q_m - 6.614) /(17.452 - 6.614)]$ x (A/0.00266)
Correlation coefficient (R^2)	0.9995	0.9997	0.9963 (at λ_{iso})	0.998 (at λ_{iso})
Precision [%Recovery (%RSD)]				
Repeatability*				
LC	99.23 (0.835)	100.55 (1.156)	100.06 (1.383)	99.72 (1.105)
IC	100.49 (0.725)	99.86 (1.374)	99.97 (1.803)	99.94 (1.006)
HC	99.30 (0.467)	100.32 (1.068)	99.34 (1.104)	99.56 (1.180)
Intraday†				
LC (morning)	99.33 (0.925)	100.38 (1.565)	99.59 (1.936)	99.86 (0.688)
IC (morning)	100.88 (0.900)	99.50 (1.399)	100.18 (1.589)	100.36 (0.532)
HC (morning)	99.63 (0.329)	99.90 (0.986)	99.64 (1.632)	100.66 (1.028)
LC (afternoon)	98.93 (1.053)	100.15 (0.713)	99.67 (1.049)	99.73 (0.736)
IC (afternoon)	101.72 (0.623)	99.50 (1.870)	99.60 (1.642)	99.04 (0.933)
HC (afternoon)	100.40 (0.189)	99.80 (0.974)	99.33 (1.497)	99.26 (0.476)
LC (evening)	99.59 (0.506)	100.15 (1.296)	101.04 (1.487)	100.35 (1.119)
IC (evening)	101.78 (0.541)	99.60 (1.725)	100.01 (1.954)	98.87 (0.263)
HC (evening)	99.80 (0.716)	99.62 (0.650)	100.47 (1.821)	100.26 (0.806)
Interday†				
LC (day 1)	98.93 (0.883)	99.23 (0.870)	100.20 (1.262)	100.75 (1.019)
IC (day 1)	100.64 (0.724)	100.22 (0.533)	100.79 (0.958)	100.99 (0.458)
HC (day 1)	100.45 (1.232)	99.99 (1.134)	99.22 (1.353)	99.34 (1.041)
LC (day 2)	99.86 (1.707)	100.83 (0.520)	10.20 (1.207)	101.26 (0.727)
IC (day 2)	100.72 (0.623)	99.29 (1.891)	99.02 (0.811)	99.13 (0.982)
HC (day 2)	100.34 (0.094)	99.80 (1.488)	100.86 (1.481)	99.18 (0.490)
LC (day 3)	99.79 (1.160)	100.72 (0.021)	99.63 (1.202)	99.89 (1.163)
IC (day 3)	100.60 (0.672)	99.60 (1.420)	101.01 (0.107)	99.08 (1.247)
HC (day 3)	99.30 (0.874)	100.46 (0.161)	100.11 (1.440)	100.01 (1.772)
LOD (µg/mL)	0.542	0.422	1.294	0.810
LOQ (µg/mL)	1.644	1.279	3.923	2.456

*n=6, †n=3, LC=21.6 µg/mL, IC=24 µg/mL, HC=26.4 µg/mL, %RSD: Percent relative standard deviation, LOD: Limit of detection, LOQ: Limit of quantification. MK-7: Menaquinone – 7, CHOL: Cholecalciferol, LC: Low concentration, IC: Intermediate concentration, HC: High concentration

Accuracy

The mean percent recovery values of both the methods were found to be in range of 98–102% and %RSD associated with all the measurements was < 2%, which complies with ICH Q2 (R1) 2005[12] recommendations for accurate analytical method. The validity and reliability of both the methods were accessed by "standard addition method," values of the mean % recovery (%RSD) for LC, IC, and HC with dual-wavelength method were found to be 99.56 (0.789), 100.37 (0.416), and 99.44 (0.243) for MK-7 and 100.78 (0.727), 99.96 (1.226), and 100.22 (1.073) for CHOL. Values of mean

Table 2: Determination of MK-7 and CHOL in laboratory prepared mixtures and tablets by proposed methods and their statistical comparison

Parameters	Dual-Wavelength method [%Recovery (%RSD)]		Q-absorbance ratio method [%Recovery (%RSD)]	
	MK-7	CHOL	MK-7	CHOL
Specificity (n=6)	99.61 (0.456)*	100.95 (0.980)*	99.86 (1.050)*	99.86 (0.711)*
Laboratory mixtures (n=6)	99.56 (0.214)*	100.06 (0.901)*	100.35 (1.220)*	100.20 (0.771)*
Tablets (n=6)	99.48 (0.775)*	100.02 (1.214)*	99.92 (1.426)*	98.89 (0.840)*

*t-test, P=0.848 at 95% CI, non–significant. CI: Confidence interval, MK-7: Menaquinone–7, CHOL: Cholecalciferol, %RSD: Percent relative standard deviation

% recovery (%RSD) for LC, IC, and HC with Q-absorbance method were found to be 100.02 (1.500), 99.74 (1.304), and 100.08 (1.028) for MK-7 and 98.67 (1.320), 99.44 (1.570), and 99.63 (0.986) for CHOL.

Specificity

The specificity of developed methods was accessed to know their ability to estimate concentration of both MK-7 and CHOL in the presence of various excipients. The absorption spectrum of placebo solution is shown in Figure 4. Placebo solution has no absorption over selected wavelengths of both the methods. Hence, no interference of excipients was observed during estimation of MK-7 and CHOL in developed tablets, and good percent recoveries were observed with lesser %RSD associated with measurements [Table 2].

Precision

The precision of developed methods was accessed in terms of repeatability, intraday, and interday precision. The precision of both methods was evaluated in terms of percent recovery and %RSD. In each case, percent recovery was found to be in range of 98–102% and % RSD was found to be <2% which comply with ICH Q2 (R1) 2005[12] [Table 1].

LOD and LOQ

LOD and LOQ were calculated as per formulae described in ICH Q2 (R1) 2005 guidelines[12] (mentioned in the experimental section) and are mentioned in Table 1. Results show that developed methods are sensitive to detect and quantify even small amount of these two vitamins in sample. The sensitivity of both methods was not found to be statistically different from each other when evaluated by t-test (P = 0.178 at 95% CI).

Robustness

Solutions of CHOL and MK-7 were found to be stable over a period of 12 h when stored in amber colored flasks (percent recovery lies within a range of 98–120%). Percent recoveries (%RSD) of MK-7 at 6th h time interval for LC, IC, and HC were found to be 98.86 (0.468), 99.13 (0.728), and 98.76 (0.671) (for dual-wavelength method) and 100.34 (1.522), 100.16 (1.550), and 99.48 (1.798) (for Q-absorbance ratio method). Percent recoveries (%RSD) of MK-7 at 12th h time
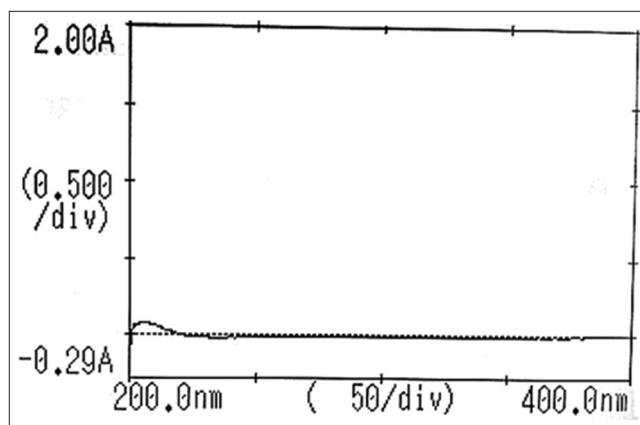

Figure 4: Zero-order absorption spectra of placebo solution

interval for LC, IC, and HC were found to be 98.59 (0.512), 98.47 (0.589), and 98.38 (0.385) (for dual-wavelength method) and 100.64 (0.809), 99.50 (1.355), and 99.55 (1.726) (for Q-absorbance ratio method). Percent recoveries (%RSD) of CHOL at 6th h time interval for LC, IC, and HC were found to be 100.03 (1.570), 98.88 (0.360), and 99.15 (1.070) (for dual-wavelength method) and 100.11 (0.991), 99.84 (0.463), and 99.77 (1.511) (for Q-absorbance ratio method). Percent recoveries (%RSD) of CHOL at 12th h time interval for LC, IC, and HC were found to be 99.01 (0.693), 98.47 (0.362), and 98.40 (0.285) (for dual-wavelength method) and 100.86 (0.025), 99.81 (0.932), and 98.81 (0.685) (for Q-absorbance ratio method). Since percent recoveries were within a range of 98–102%, it means developed methods are robust and are not susceptible to minute variations in experimental conditions.

CONCLUSION

Developed dual-wavelength and Q-absorbance ratio method can be successfully used for spectrophotometric determination of MK-7 and CHOL in combined dosage forms. These methods were found to be easy, simple, rapid, and precise which involves measurement of absorbance of samples at selected wavelengths followed by few simple calculations. Developed methods were validated as per the ICH Q2 (R1) 2005 guidelines. Sample recoveries were found to be in range of 98–102% with associated %RSD <2% in prepared dosage form which suggest non-interference of formulation

excipients. Developed methods can be successfully used for regular quality control estimation of various pharmaceutical dosage forms containing combination of these two vitamins.

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