SIMULTANEOUS DETERMINATION OF CHOLECALCIFEROL AND 25-HYDROXYCHOLECALCEFEROL IN LIPID-BASED SELF-NANOEMULSIFYING FORMULATIONS AND MARKETED PRODUCT VI-DE 3® BY UHPLC-UV”

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Abstract:

Purpose: The purpose of the current study was to develop a selective, precise, fast and economical reverse phase ultra-high-performance liquid chromatography (UHPLC UV) advanced method and validate for the simultaneous estimation of cholecalciferol and its analogue 25-hydroxycholecalciferol in lipid-based self-nanoemulsifying formulation (SNEDDS). **Method:** The chromatographic separation was simply performed on a Dionex® UHPLC systems (Ultimate 3000, Thermo scientific) by using HSS C_{18} (2.1x50 mm, 1.8µm) analytical column. The UV detection was carried out isocratically with the mobile phase consisting of acetonitrile and methanol in the ratio of (50:50 %v/v) with a flow rate of 0.4 ml/min at 265nm. The injection volume was 1µl and the column temperature was maintained at 45°C. FDA regulatory guidelines were used to develop and validate the method. **Results:** The current developed UHPLC-UV method was found to be rapid (run time 2 min), and selective with high resolution of cholecalciferol and 25-hydroxycholecalciferol (RT=0.530 min & 1.360 min) from different lipid matrices. The method was highly sensitive (Limit of Detection and Lower Limit of Quantification were 0.13ppm & 0.51ppm, and 0.15ppm & 0.54ppm, respectively). The linearity, accuracy and precision were determined as suitable over the concentration range of 0.5-50.0 ppm for both the analytes. **Conclusion:** The proposed UHPLC-UV method can be used for the determination of cholecalciferol and 25-hydroxycholecalciferol in SNEDDS and marketed Vi-De 3® as pure forms (intact) with no interference of excipients or drug related substances.

Key words: Cholecalciferol, 25-hydroxycholecalciferol, UHPLC, Lipid-based formulation, Method validation

Introduction:

Vitamin D is a fat soluble vitamin naturally occurs in two different major molecular forms; vitamin D_2 (Ergocalciferol) and vitamin D_3 (cholecalciferol) [1]. Vitamin D_2 is naturally available in low amounts in certain foods, whereas vitamin D_3 (Fig 1) is synthesized in the skin on exposure to sunlight and has more effectiveness in humans. Calcitriol (25-hydroxycholecalciferol) is the biologically active form of vitamin D_3 which is essential for maintaining body normal calcification of the skeleton and bone mineralization. [2, 3]. Cholecalciferol is a poorly water soluble compound belongs to the class II in biopharmaceutics classification system [4].

Vitamin D_3 chemically described as $C_{27}H_{44}O$ or (3S,5Z,7E)-9,10-Secocholesta-5,7,10-trien-3-ol with a melting point between 83°C and 86°C [5]. Vitamin D3 is taken by consumers regularly as a supplement to support enhance calcium absorption and function within the body. As with many steroid compounds, when vitamin D_3 breaks down in the body, the subsequent molecules are passed into the nucleus of certain cells and determine/change which genes are going to be turned on or off. Many reports advise that the steroid hormone similarity of vitamin D may deliver some anticancer activity. Vitamin D_3 is found structurally similar to that of steroidal hormones like testosterone, cortisol and cholesterol, despite being a secosteroid. Physician and researchers may prescribe or use Vitamin D for osteomalacia, which is caused by underminalized bones [6].

Vitamin D_3 is only available as tablets and alcoholic liquids in the market (oral drops Vi-De 3® and Vidrop® which contain 4500 IU/ml and 2800 IU/ml). More importantly, about 65% of ethanol is used as a solvent in the marketed formulation Vi-De 3® which produce unpalatable taste and lead to harmful impact on the health due to high risk of alcohol side effect [7]. The

recommended daily doses of vitamin D3 ranges from 400 to 600 IU in healthy volunteers. According to the pharmacokinetics, it is a poorly water soluble drug with solubility of (1.03×10^{-6}) Mole (log p 9.1) [8] and it has low, variable and incomplete absorption. Therefore, it is a suitable candidate for lipid-based formulation, which could improve the aqueous solubility and oral absorption rate [9, 10].

Many methods have been described in the literature for the determination of cholecalciferol (vitamin D3) and its analogue 25-htdroxycholecalciferol (25-OH-vitamin D3) individually and in combination with other drugs [11-14]. However, there is no HPLC method developed for the simultaneous determination of these compounds, which is required for both *in vitro* and *in vivo* studies. Therefore, the present work describes a fast, precise, linear and accurate reversed phase UHPLC-UV method for the simultaneous determination of cholecalciferol along with its analogue 25-hydroxycholecalciferol in lipid-based dosage form (SNEDDS).

An intend to develop a UHPLC method for simultaneous determination with greater sensitivity and faster elution, the present analytical method was validated following the ICH guidelines [15].

Within the scope of the current method, cholecalciferol and its analog were assayed and validated using an advanced UHPLC system, which reduced the time of analysis and the use of excess solvent. Apart from reducing time and solvent, the current instrument method allows the system to reduce high back pressure without any harmful effect to the analytical column which can make the column last longer. The method described here is successfully applied to the analysis of lipid-based formulations containing cholecalciferol and marketed product Vi-De 3® with no interference from dosage form excipients.

Experimental

Materials

All chemicals used in the present study were obtained from commercial suppliers. Cholecalciferol and 25-hydroxycholecalciferol (purity > 98.5%) were obtained as a gift sample from Riyadh Pharma Co. Ltd. Riyadh, KSA. Moringa oleiferra oil (MO, cold pressed), Imwitor 988 (I988, medium chain mono- & di-glycerides) and HCO-30 (non-ionic surfactant of hydrogenated castor oil) were used to prepare self-nanoemulsifying lipid formulations (SNEDDS), supplied by Sasol Germany GmbH, Werk Witten, Germany and Nikkol Chemical Co, Japan. The marketed product Vi-De 3® was purchased from the local pharmacy in Riyadh, KSA. The high purity Milli-Q water was obtained through a Milli-Q Integral Water Purification System (Millipore, Bedford, MA). All other reagents used in the study were of analytical grade and used as such without any further purification.

Methods

UHPLC instrumentation

After a successful UHPLC chromatographic separation it is optimized with respect to the stationary/ mobile phase compositions, flow-rate, sample volume, temperature of column and detection wavelength in UV. The study was performed on highly sensitive UHPLC system that consisted of a Dionex ® UHPLC binary solvent manager equipped with a Dionex® automatic sample manager and a Photodiode Array (PDA) eλ detector obtained from Thermo scientific, Bedford, MA, USA. The isocratic mixer of mobile phase consisting of acetonitrile and methanol

in the ratio of (50:50%v/v) with a flow rate of 0.4 mL/min, delivered through an Acquity® UHPLC HSS C_{18} column (2.1x50 mm, 1.8 µm) kept at 45 ºC. The total run time was 2 min. The mobile phase was regularly prepared as fresh and degassed continuously by an online degasser within the UHPLC system. The detector wavelength was set at 265 nm and the injection volume was 1.0 µl.

Preparation of stock solution, calibration standards and QC samples

Standard stock solution was prepared by dissolving accurately weighted 50 mg cholecalciferol and its analogue 25-hydroxycholecalciferol each powder in 50 ml methanol, resulting in a solution containing 1000 ppm (1000µl/ml). For the purpose of calibration, the standard solutions of cholecalciferol & 25-hydroxycholecalciferol at six points were prepared by appropriate serial dilutions in methanol to cover the concentration range of 0.5-50.0 ppm (µg/ml). These standards solutions were freshly prepared and used immediately. Calibration curves were achieved for both cholecalciferol & 25-hydroxycholecalciferol by plotting peak area in comparison to standard drug concentration and regression equations were computed thereby. Three quality control (recovery studies) samples with the selected concentration levels (0.688, 5.55, 22.00 ppm) were prepared from stock solution to obtain the desired range. For recovery studies, samples were prepared by spiking the self-nanoemulsifying lipid-based formulation (SNEDDS) with known amount of cholecalciferol & 25-hydroxycholecalciferol, and then diluting the mixture with appropriate volume of methanol.

Vi-De3® sample preparation (commercial product)

Three 10 mL ample bottles (each bottle having cholecalciferol 45000 I.U., 1.125mg by Novartis, Switzerland) were accurately weighed and mixed using vortex mixture for 10 minutes. A standard unit dose (400-600 I.U) amount was weighed and located to a 50 mL volumetric flask and dissolved in methanol and then stirred and sonicated until its dissolved completely. The final solution was diluted to the working range (within calibration concentrations) for application of the developed method. The samples were filtered after dilution, through a 0.22 µm disposable nylon filter (Bellefonte, PA, USA) into amber colored glass auto sampler vials for UHPLC analysis.

Formulation Matrix effect

Analyte and excipients under goes spectral overlapping which is very common in dosage and it can be a major drawback if the drug compound is analysed directly, which affects the sensitivity, accuracy and precision of the method [16]. Thus, it was necessary to execute UHPLC spectral scanning for both drug-free and drug-containing SNEDDS formulations to examine any possible excipients-drug interference. Within the method development of the current study, cholecalciferol was loaded in lipid-based dosage form at many low-high concentrations in order to check the extent of the matrix effect.

Method Validation

The current established UHPLC protocol has been validated in terms of systems suitability, linearity, accuracy, precision, selectivity, and recovery according to the standard method validation guidelines by ICH [17]. The precision and accuracy acceptance criteria were evaluated by consecutively injecting the standard and sample solutions and the amount of drugs

(µg/ml), percentage content, standard deviations, percentage coefficient of variation (%CV determined precision of the method) were then calculated.

Linearity and range

Appropriate volume of cholecalciferol & 25-hydroxycholecalciferol stock solution (1000ppm) was utilized in the preparation of six non-zero standard drug concentrations covering the calibration range of 0.5-50.0 ppm. Three different quality control samples were prepared by spiking known concentrations of cholecalciferol & 25-hydroxycholecalciferol within the same detection range (0.5-50.0 ppm).

Each standard solution from 0.5ppm to 50.0ppm has been inserted as six replicates everyday on three successive days for validation. Calibration solutions were loaded in order to lower to higher concentrations in each validation run and the other samples were dispersed casually through the run.

The linearity of the results were statistically calculated by employing linear regression equation and correlation coefficient (R^2) [18].

Specificity

Specificity of the method was required to assess the matrix effect by comparing peak areas between the drug and different SNEDDS formulations.

The specificity of the method was evaluated through the whole assay period using drug free lipid formulation (representative SNEDDS) samples and cholecalciferol & 25-

hydroxycholecalciferol analyte. The retention times of the drug free lipid constituents were matched with that of cholecalciferol & 25-hydroxycholecalciferol analyte. In addition, towards the establishment of the method, specificity was also studied by determination of the intact drug in terms of resolution (*R*) among the drug peak and the nearby interference peak if present.

Accuracy and precision

The Intra-day accuracy and precision of the proposed method were evaluated by analyzing six replicates of each six cholecalciferol & 25-hydroxycholecalciferol standards within the same day. Similarly, the Inter-day accuracy and precision were also obtained during the three consecutive days using six replicates analysis of the low, medium and high quality control samples. The complete precision and accuracy of the method was specified as relative standard deviation (RSD) and as % drug recovered, respectively.

Limit of Detection (LOD) and Lower Limit of Quantification (LLOQ)

The method for determination of LOD and LLOQ is very common in analytical methods that exhibit base line noise. The detection and quantification levels were resolute by sequential dilutions of cholecalciferol & 25-hydroxycholecalciferol stock solutions in order to get signal to noise (S/N) ratio of at least ≈ 3:1 for LOD and ≈ 10:1 for LLOQ. The LOD and LLOQ are the two concert characteristics in analytical method development and worked for peak height measurements.

Statistical analysis

The standard error of mean (SEM ±) were used to express the data. The significance of data was calculated by applying one-way ANOVA. P values *<0.05* were considered significant.

Results and discussion

UHPLC peak separation

Separation and detection of cholecalciferol & 25-hydroxycholecalciferol peaks without any minimum interference was ideal by the developed UHPLC assay. The chromatographic results of UHPLC technique in the current analysis show that both cholecalciferol & 25-hydroxycholecalciferol can be determined well enough in the self-emulsifying lipid formulations (SNEDDS) within the highest sensitivity and selectivity of this analytical procedure. Fig. 3 shows the representative chromatograms of blank sample (3A), standard solution of cholecalciferol & 25-hydroxycholecalciferol (3B, 10ppm), drug-free lipid SNEDDS sample (3C), drug-containing lipid SNEDDS sample (3D). The cholecalciferol & 25-hydroxycholecalciferol analytes were well separated from the solvent peak (used as mobile phase as well as sample dilutions) at retention time of 0.530 min and 1.357 min, while there were no interference peaks detected in the sample formulation (Fig. 3 A-D). The total chromatographic run time was ≈2 min, where the cholecalciferol & 25-hydroxycholecalciferol peaks were of good shape and completely yielded.

ICH method validation

The precision and accuracy of the current method showed an excellent workability of the method. The precision was 20 % or better for LLOQ (%RSD) and 15 % or better for the

remaining concentrations and the acceptable accuracies were 100 ± 20 % or better for LLOQ and 100 ± 15 % or better for the remaining concentrations.

Linearity and range

A six-point standard calibration curve was constructed to see the linearity within the concentration range. The peak area response of cholecalciferol & 25-hydroxycholecalciferol was linear over the concentration range between 0.5 and 50.0 ppm (Fig.2). The result of linear regression gives the following mean equation:

y = 0.0833x -0.0052 for cholecalciferol and y = 0.0866x -0.0034 for 25-hydroxycholecalciferol

Where *y* and *x* denote: the peak area and the concentration of the analyte respectively. This result shows an excellent linearity (approaching a straight line function) over the interval studied for both cholecalciferol and 25-hydroxycholecalciferol. The correlation coefficient (r) was recorded as 0.9993 for cholecalciferol and 0.9991 for 25-hydroxycholecalciferol (Fig 2) [18].

Accuracy and precision

The Intra-day and Inter-day accuracies were calculated as the % of drug recovered after analyzing six replicates of the quality control samples at five nominal concentration levels. The Intra-day and inter-day accuracies were found in between 100.20% and 104.87% respectively.

The results from the drug recovery studies confirm that the accuracy of the assay method was within the acceptable limits according to the ICH guidelines.

Precision

Repeatability: Five concentrations of cholecalciferol and 25-hydroxycholecalciferol (0.6875, 1.375, 5.50, 11.00 and 44.00ppm) were analysed three times Intra-day. The developed method was found to be precise as the Intra-day standard deviation (SD) values (Table 1) of six replicate analyses were within the range of 0.012-0.14ppm. Within the analytical concentration range of 0.5-50.0 ppm, %CV values were less than 4.87%. The good percentage recoveries were obtained confirming the repeatability of the methods.

Intermediate precision: Five concentrations of cholecalciferol and 25-hydroxycholecalciferol mentioned previously were repeated Inter-daily on three different days for the analysis. The Inter-day (Table 1) accuracies of six replicates during the three consecutive days were between 0.042 and 0.31 ppm, whereas the %CV values were less than 3.85%. These low values of both SD and %CV during the Intra-day and Inter-day analysis thus met the acceptance criteria of precision for the proposed method.

Specificity

The specificity of the developed UHPLC method was investigated in order to measure the cholecalciferol & its analogue 25-hydroxycholecalciferol response in the available dosage forms. Specificity was established by determining the purity of the compound's peak using a PDA detector. In addition, the nearest resolving peak was taken as determinant for the resolution

factor of the drug peak. The developed method was found to be specific for cholecalciferol & 25-hydroxycholecalciferol without having any possible interference from the excipients. The result from a sample of SNEDDS solubility in dispersion studies (cholecalciferol & 25-hydroxycholecalciferol loaded SNEDDS formulation) in Fig. 3D shows that there were no degradation products present in the sample containing cholecalciferol & 25-hydroxycholecalciferol. It seems that cholecalciferol & 25-hydroxycholecalciferol compounds can be recovered completely from the lipid SNEDDS formulation. Therefore, the *R* value in this assay can be calculated based on the availability of the cholecalciferol & 25-hydroxycholecalciferol peaks only. Additionally, no significant interfering peaks were observed in randomly selected drug free lipid formulation samples at cholecalciferol & 25-hydroxycholecalciferol retention times (Fig. 3C), which recommends that the compounds can be analyzed predominantly from SNEDDS formulations.

Limit of Detection (LOD) and Lower Limit of Quantification (LLOQ)

The LLOQ for cholecalciferol in the present assay was 0.51 µg/ml (ppm) which was assessed to be the lowermost concentration in the standard curve that can be measured with acceptable accuracy and precision for the cholecalciferol & 25-hydroxycholecalciferol analytes with S/N ratio of (84.9). To the best of our knowledge, this concentration was relatively low as compared to other available analytical methods that developed using the similar instruments. Conversely, LOD was 0.13 ppm with S/N ratio of (31.9). Both LLOQ and LOD were experimentally verified by six replicate injections of cholecalciferol & 25-hydroxycholecalciferol standard concentrations (Table 3).

Matrix effect

The valuations of matrix effect denoted an integral part of authentication for quantitative analysis of drug in SNEDDS within the present analytical technique development. The effects of matrix were assessed at three concentration levels (0.688, 5.55, & 22.00ppm) for cholecalciferol & 25-hydroxycholecalciferol. In the present analysis, efficiency of process suggested that the proposed method was practically free from relative matrix effects for the determination of cholecalciferol & 25-hydroxycholecalciferol in lipid-based SNEDDS formulation.

Suitability of the systems

Systems suitability parameters were taken into consideration to confirm the highest precision of the systems. The variation (%RSD) in the peak surface area from six replicates injections of cholecalciferol and 25-hydroxycholecalciferol were 0.023% and 0.036%, which proved that the system is precise. The results of other chromatographic parameters such as peak tailing and theoretical plate numbers (shows column efficiency) are shown in Table 4. The overall analysis results show the acceptable performance of the system as the %RSD and the tailing peak are not more than 2.0 % and 1.53 respectively along with the theoretical plates, which are not less than 2000.

Application

Within the scope of the current research, the developed UHPLC method has been successfully used for the quantification of cholecalciferol & 25-hydroxycholecalciferol compounds in the studies of equilibrium solubility, and dynamic dispersion of the representative self-nanoemulsifying lipid-based formulations (SNEDDS) [19]. A dispersion profile is shown in Figure 4 as an example of the method application. The data represented an immediate release of

cholecalciferol & 25-hydroxycholecalciferol from a SNEDDS formulation BSO: I988(7:3)/HCO30(50/50%w/w) in simulated intestinal media (FeSSIF). It can be indicated from the results that the current method quantified more than 97.5% cholecalciferol & 25-hydroxycholecalciferol, which has absolute release immediately after dispersion and stayed in solution during 24 hrs time period. Comparatively, the present assay method has evaluated and reported extensive validation parameters as per ICH guidelines. The method has been proved to be acceptable, precise, accurate, and adequately sensitive and thus demands to be in use for further analytical studies.

Determination of cholecalciferol in commercial product

In order to assess the applicability of the UHPLC-UV method, the determination of cholecalciferol was performed in its Vi-De 3 commercial product. The procedure of sample preparation was carried out as described under Vi-De 3 sample preparation in the experimental section. Six replicate determinations for cholecalciferol were performed. Satisfactory results were obtained in a good agreement with the label claimed. The obtained results were validated by spiking cholecalciferol standards of three concentration levels to Vi-De3 samples. The recoveries were obtained in the range of 100.20–101.80% Table 2. Figure 3E shows the UHPLC chromatograms of cholecalciferol in Vi-De 3® marketed solution. The chromatograms did not show any interference, as no detectable matrix peak was eluted in the retention time of cholecalciferol. Nevertheless, there are slight changes in the retention time (≈0.150min variation) of the analyzed compounds; this could be due to the matrix effect of the column.

Conclusion

The developed UHPLC analytical method provides a reliable, reproducible and specific assay for cholecalciferol & 25-hydroxycholecalciferol in pure and available pharmaceutical formulations. The described method is sensitive enough to detect as low as 0.13 ppm and exclusively offer a rapid determination of cholecalciferol & 25-hydroxycholecalciferol (peak at 0.530 min & 1.360 min within 2 min run time). No significant interferences were recorded by the formulation excipients, diluents and or degradation products. The validation method allows quantification of cholecalciferol & 25-hydroxycholecalciferol in pure and pharmaceutical formulations in the range between 0.5 to 50.0 ppm. The established method satisfactorily fulfills all the criteria of the system suitability, peak integrity, and resolution of the drug peak. The overall results clearly stated that the current method is attractive due to the good selectivity for quantitative determination of cholecalciferol & 25-hydroxycholecalciferol in self-nanoemulsifying lipid-based formulation and commercial product.

Conflict of interests

The authors have declared that there is no conflict of interest.

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Tables

Table 1: Evaluation of accuracy and precision of the proposed method for the simultaneous determination of cholecalciferol & 25-hydroxycholecalciferol, by Intra- and Inter-day assay

Drug name	Assay Type	Nominal Conc. (µg/mL)	Measured Conc. ±SD (µg/mL)	%Precision	%Accuracy	%CV
Cholecalciferol	Intra-day	0.6875	0.6931±0.0552	0.82	100.80	7.96
		1.375	1.408±0.012	2.40	102.40	0.85
		5.50	5.55±0.05	0.91	100.90	0.90
		11.00	11.06±0.07	0.55	100.50	0.63
		44.00	44.12±0.14	0.27	100.30	0.32
	Inter-day	0.6875	0.7101±0.0731	3.29	103.29	10.29
		1.375	1.399±0.042	1.75	101.75	3.00
		5.50	5.67±0.06	3.09	103.09	1.06
		11.00	11.13±0.07	1.18	101.18	0.63
		44.00	44.09±0.11	0.20	100.20	0.25
25-hydroxy-cholecalciferol	Intra-day	0.6875	0.721±0.033	4.87	104.87	4.58
		1.375	1.386±0.045	0.80	100.80	3.25
		5.50	5.66±0.05	2.91	102.91	0.88
		11.00	11.21±0.08	1.91	101.91	0.71
		44.00	44.19±0.09	0.43	100.43	0.20
	Inter-day	0.6875	0.714±0.081	3.85	103.85	11.34
		1.375	1.402±0.060	1.96	101.96	4.28
		5.50	5.62±0.12	2.18	102.18	2.14
		11.00	11.04±0.31	0.36	100.36	2.81
		44.00	44.33±0.24	0.75	100.75	0.54

Table 2. Recovery study of cholecalciferol & 25-hydroxycholecalciferol in spiked lipid-based formulation samples and marketed product Vi De3®.

Drug name	**Amount added (µg/mL)**	**Amount found ±SD[a] (µg/mL)**	**%Error**	**%Recovery**	**%RSD**
(SNEDDS) Cholecalciferol	0.688	0.708±0.034	2.91	102.91	4.80
	5.55	5.62±0.05	1.26	101.26	0.89
	22.00	22.14±0.22	0.64	100.64	0.99
(SNEDDS) 25-hydroxy-Cholecalciferol	0.688	0.698±0.024	1.45	101.45	3.44
	5.55	5.60±0.06	0.90	100.90	1.07
	22.00	22.09±0.17	0.41	100.41	0.77
(Vi De®) Cholecalciferol	1.00	1.01±0.03	1.00	101.00	2.97
	5.00	5.09±0.16	1.80	101.80	3.14
	25.00	25.05±0.38	0.20	100.20	1.52

SD, standard deviation: [a]Mean of three measurements.

Table 3. Statistical data of the regression equation for the determination of cholecalciferol & 25-hydroxycholecalciferol obtained from the proposed method.

Parameters	**Cholecalciferol**	**25-hydroxy--Cholecalciferol**
Linearity range	0.5–50 ppm	0.5–50 ppm
Intercept[a]	0.0052	0.0034
Slope[a]	0.0833	0.0866
Correlation coefficient (r^2)	0.9993	0.9991
Limit of detection (LOD)	0.13 ppm	0.15
Limit of quantification (LOQ)	0.51ppm	0.54

[a]mean of three measurements

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415**Table 4**: Systems suitability parameters of cholecalciferol & 25-hydroxycholecalciferol

Parameters	Cholecalciferol	25-hydroxy Cholecalciferol
Retention Time (minutes)	1.36	0.53
Theoretical Plates	3135	2010
Tailing Factor	1.25	1.53
Peak Area %RSD	0.023	0.036

416

417**Table 5:** Determination and % recovery of cholecalciferol in commercial products Vi-De3®

Real sample	**Manufacturer**	**Amount claimed/fortified**	**Found (µg/ml) ±SD**	**% of labelled claim**
Cholecalciferol 45000 I.U/10ml	Novartis, Switzerland	1125 µg	1110.32±34.75	98.70

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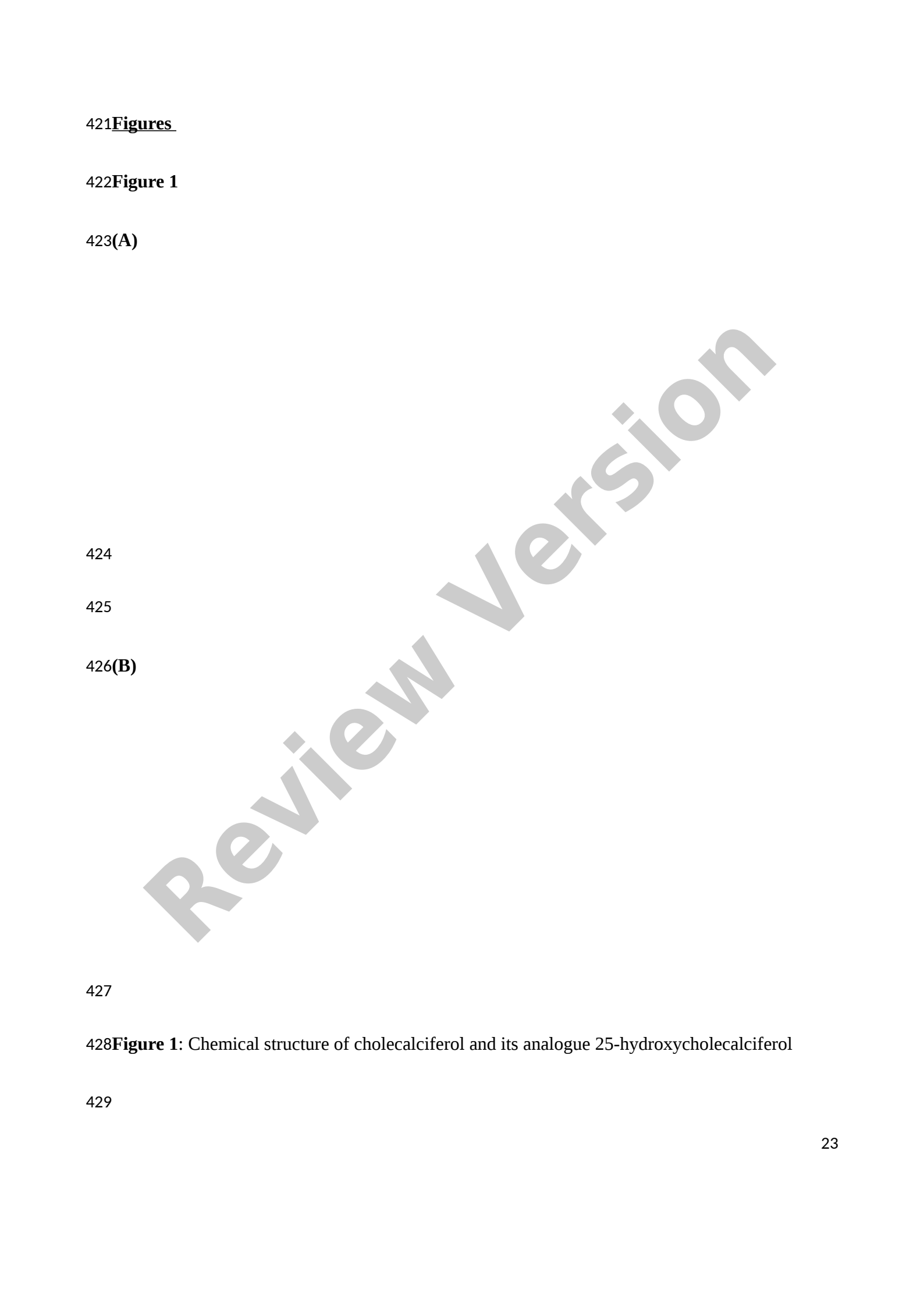
Figures

Figure 1

(A)



(B)

Figure 1: Chemical structure of cholecalciferol and its analogue 25-hydroxycholecalciferol

Figure 2

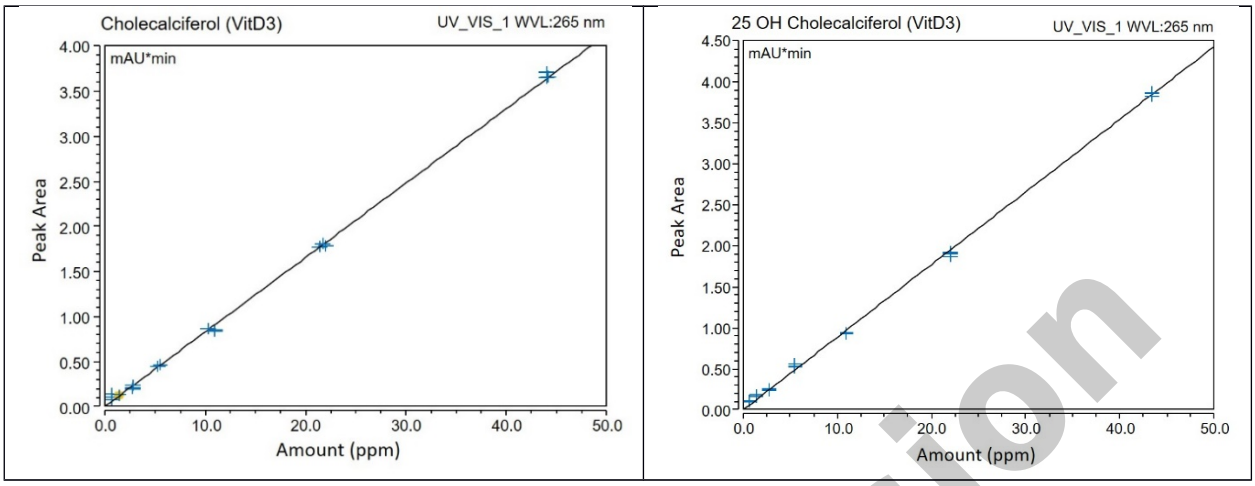
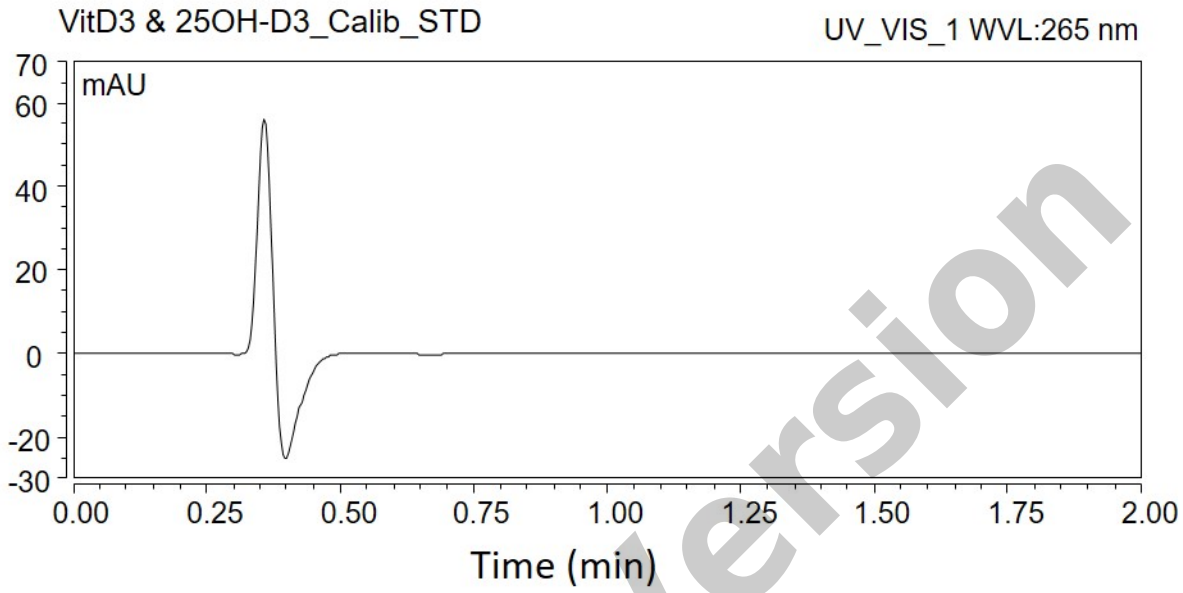


Figure 2: UHPLC **c**alibration curve of cholecalciferol and 25-hydroxycholecalciferol in Methanol/ACN (50/50%v/v)

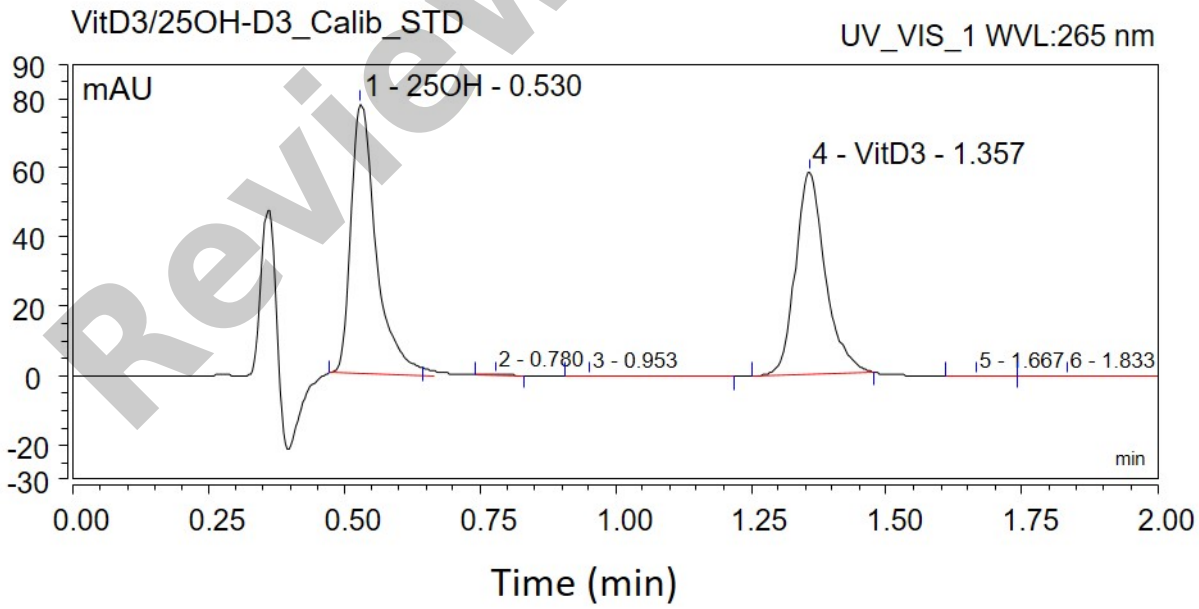
435**Figure 3**

436**(3A)**



437

438**(3B)**



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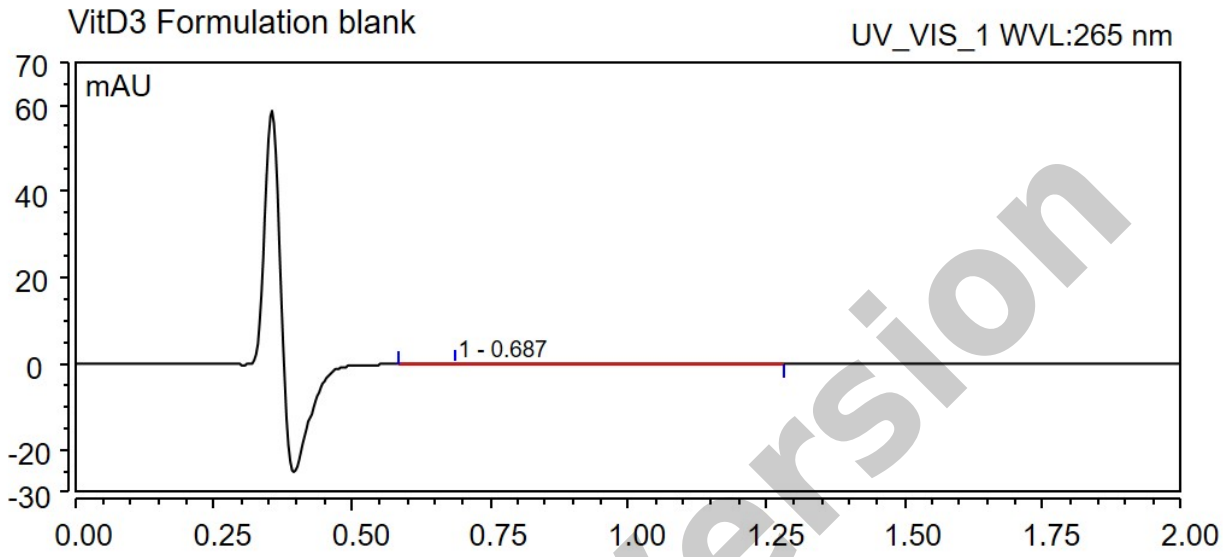
440

441**(3C)**



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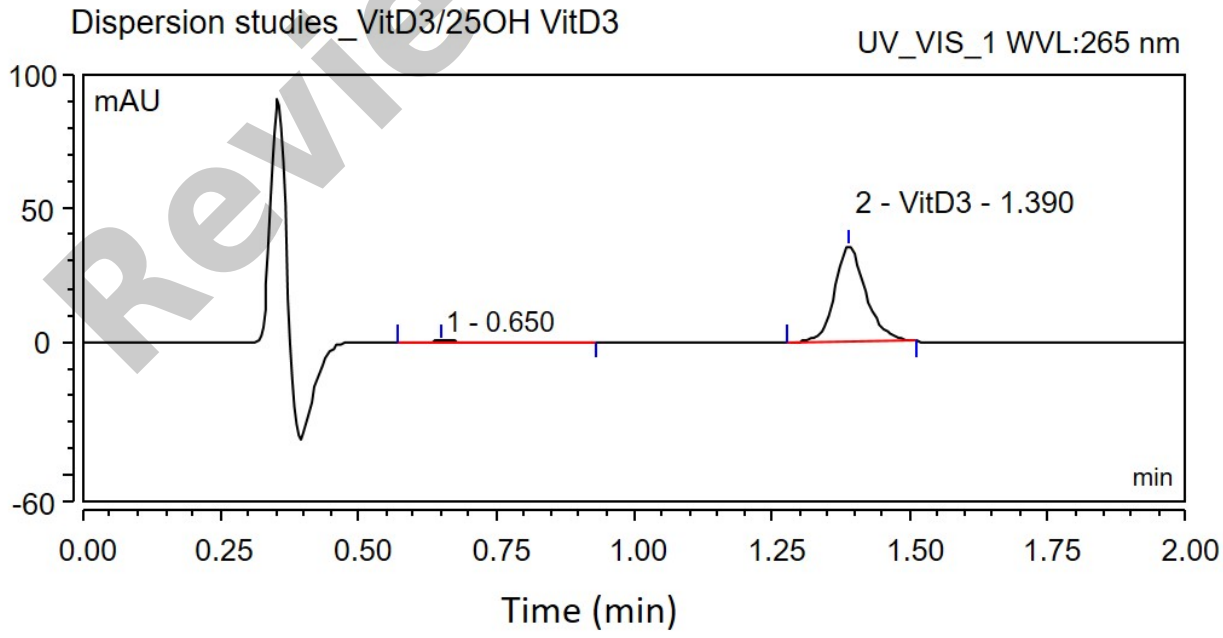
443

444**(3D)**



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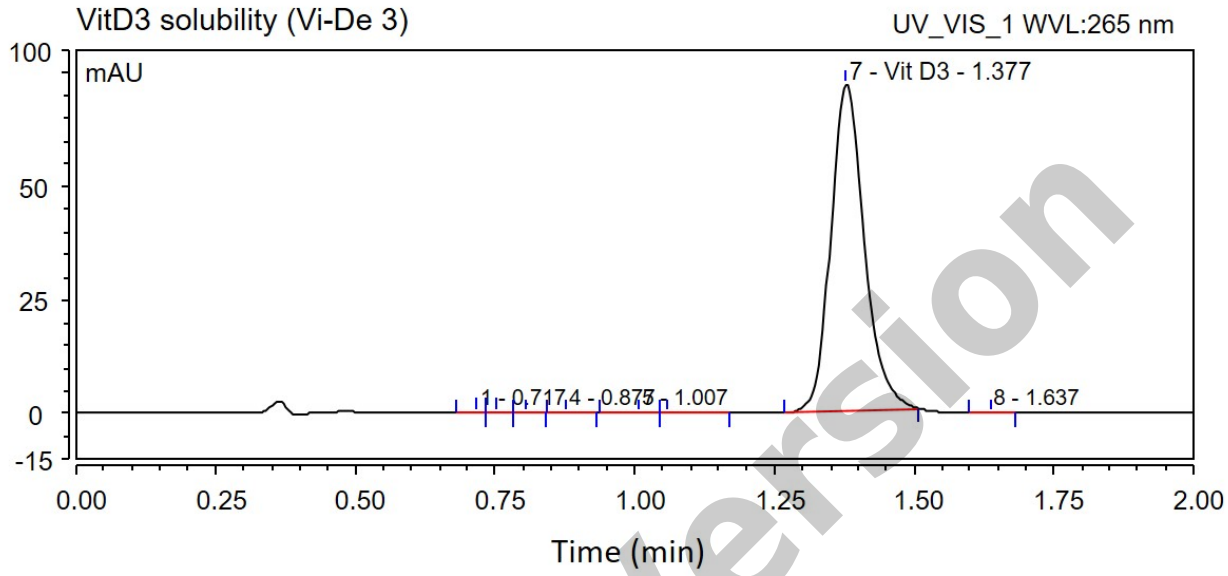
446

447**(3E)**



448

449**Figure 3**: UHPLC chromatograms of blank sample (A), standard solution of cholecalciferol and

45025-hydroxycholecalciferol at concentration 10 ppm (B), drug-free lipid formulation sample

451(SNEDDS, BSO:I988(7:3)/HCO30 (50/50%w/w) (C), and drug-containing lipid formulation

452(SNEDDS in dispersion studies) sample (D) and marketed product Vi-De3® (E)

453

Figure 4

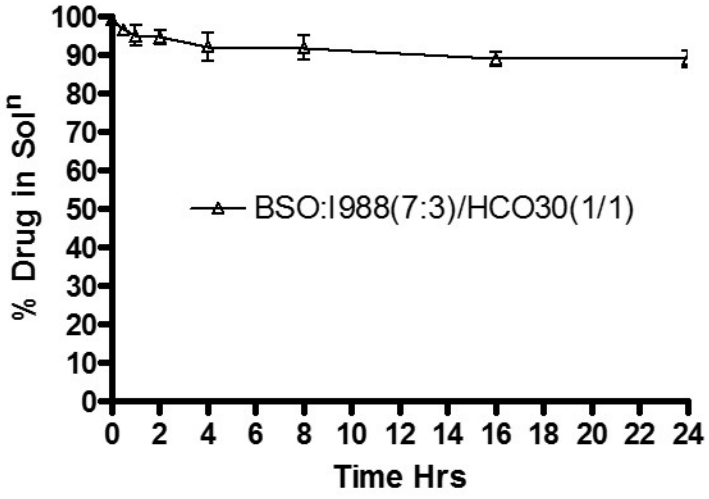


Figure 4: % Drug release of cholecalciferol from a SNEDDS dosage form [standard dose of vitamin D3 in BSO:I988 (7:3)/HCO30(1/1)] in simulated intestinal media. Arithmetic means (n=3) are shown, standard deviations are within the symbols