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Lighter than Water Dispersive Liquid-liquid Microextraction Coupled with High Performance Liquid Chromatography for Determination of Cholecalciferol and Calcifediol from Plasma

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In this study, a dispersive liquid-liquid microextraction method using an extraction solvent lighter than water has been developed for the extraction and preconcentration of cholecalciferol and calcifediol from plasma samples followed by high performance liquid chromatography determination. Initially, acetonitrile and sodium chloride (NaCl) are added into the plasma as an extraction solvent and a salting-out agent, respectively. After manual shaking, the mixture is centrifuged. In the presence of sodium chloride, a two-phase system is formed. Then, a portion of the upper phase is removed and mixed with *n*-hexane at µl-level and rapidly injected into distilled water by a syringe. In this process, the analytes are extracted into the fine droplets of *n*-hexane (as an extraction solvent). Under optimal conditions, enrichment factor was obtained 92 and 94 for calcifediol and cholecalciferol, respectively. The intra- (n = 6) and inter-day (n = 4) precisions were less than or equal to 8.1% at a concentration of 10 ng ml^{-1} of each analyte. Finally, this method was applied to the analysis of the analytes in human plasma samples.

Abbreviations: DLLME, Dispersive liquid-liquid microextraction; DAD, Diode array detector; PF, Preconcentration factor; ER, Extraction Recovery; HPLC, High performance liquid chromatography; LOD, Limit of detection; LOQ, Limit of quantification; LLOQ, Lower limit of quantification; LPME, Liquid phase microextraction; QC, Quality control; RSD, Relative standard deviation

Keywords: Cholecalciferol, Calcifediol, High performance liquid chromatography, Human plasma

INTRODUCTION

Vitamins are essential to the health of humans and animals and they cannot be synthesized by these vertebrates and must be obtained from the diet. Generally, vitamins are classified to categories named fat-soluble or water-soluble according to their solubilities in solvents [1]. Fat-soluble vitamins are composed of four vitamins namely A, E, D and K [2]. Among these compounds, vitamin D has a key role in human health. Vitamin D deficiency is known as a major hidden health problem in the worldwide. There is increasing evidence that vitamin D may contribute to the pathogenesis of multiple sclerosis and influences the disease course and activity [3]. Vitamin D deficiency can lead to bone

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deformities such as rickets in children, and bone pain and tenderness as a result of a condition called osteomalacia in adults. On the other hand, excessive vitamin D intake is associated with the risk of hypercalcemia or hypercalciuria and kidney problems [4,5]. Human obtains its vitamin D needs in two forms including vitamins D_3 (cholecalciferol) and D_2 (ergocalciferol) from two sources. Cholecalciferol is biosynthesized in skin during exposure to UV light, whereas ergocalciferol is absorbed from the diet. In liver, cholecalciferol is converted to calcifediol and then it is partially converted to calcitriol, the biologically active form of vitamin D by kidneys [6,7]. The importance of vitamins determination in human fluids is due to the relationship between vitamin deficiency states and certain pathologies. Generally, fat-soluble vitamins are extracted from different matrices with various sample preparation methods including liquid-liquid extraction [8] and solid phase extraction [9, 10]. These methods suffer from time-consuming and low efficiency and preconcentration factor (PF). On the other hand, the need for high volume of toxic organic solvents limits their use as a sample preparation procedure in the determination of vitamin D_3. To overcome these disadvantages, miniaturized extraction methods were considered in determination of vitamin D_3. Liquid phase microextraction (LPME) and solid phase microextraction (SPME) are the most famous miniaturized extraction procedures introduced in the last decades. In SMPE, the analytes are extracted on a sorbent coated onto a fused silica fiber. The method was used in the extraction of vitamin D_3 from human serum [11]. However, fragility of the fibers and their price are the main disadvantages of the method. After introduction of LPME method in 1996, this method has attracted many attentions in the determination of different analytes [12]. Using LPME, high PFs can be achieved for the analytes with high partition coefficients, because the sample volume is much larger than the extraction solvent volume [13]. LPME methods are usually classified into three major groups including single drop microextraction (SDME) [14,15], hollow fiber liquid-phase microextraction (HF-LPME) [16-18] and dispersive liquid–liquid microextraction (DLLME) [19]. In DLLME, a few microliters of a water immiscible organic solvent is mixed with a water-soluble organic solvent (at ml-level) and the mixture is rapidly injected into an aqueous solution containing the analytes resulting in the formation of a cloudy solution. Then, the analytes are rapidly extracted into the fine droplets of the extraction solvent. After extraction, phase separation is performed by centrifugation and the enriched analytes in the organic phase are determined by chromatographic or spectrophotometric methods. The advantages of DLLME are the simplicity of operation, rapidity, low cost, and high extraction recoveries (ERs) and PFs [20,21]. The DLLME procedure was used in determination of vitamin D3 in human serum [22], milk [23] and foods [24]. The major disadvantage of the traditional DLLME methods is the use of halogenated solvents as the extraction solvent which are highly toxic. To resolve this disadvantage, lower density than water extraction solvents have been used. However, in this case, to collect the extraction solvent after the extraction, using special home-made extraction vessels is necessary [25,26]. Many applications of DLLME in the extraction of analytes from aqueous samples have been reported. However, its application in biological samples such as plasma is limited due to the complexity of the mentioned samples.

In the present work, a DLLME procedure using an organic solvent lighter than water has been developed for the extraction and preconcentration of cholecalciferol and calcifediol from human plasma samples. The extracted analytes were determined using high performance liquid chromatography-ultraviolet (HPLC-UV) determination. For this purpose, initially, the target analytes are extracted into a water-soluble organic solvent which it will be used as a disperser in the following DLLME method. To the best of our knowledge, this is the first study in which a DLLME method using a low density and safe solvent *e.g.* hexane is used for the selected analytes. The influence of the different experimental parameters on the yield of sample preparation step is studied and optimized. Finally, the recommended method will be applied to extract and preconcentrate the selected analytes from plasma samples prior to their determination by HPLC-DAD.

EXPERIMENTAL

Chemicals and Solutions

Analytes including cholecalciferol and calcifediol with purity higher than 99% were obtained from Sigma-Aldrich

(St. Louis, Missouri, USA). The analytical-grade solvents used as extraction solvents in DLLME including *n*-hexane, toluene, *n*-hexanol, and *n*-octanol were from Merck (Darmstadt, Germany). The analytical-reagent grade acetonitrile (ACN), methanol, tetrahydrofuran (THF), and acetone tested as extraction/disperser solvents were obtained from Merck. Analytical-reagent grade hydrochloric acid, sodium hydroxide, and sodium chloride (NaCl) were obtained from Merck. ACN and HPLC-grade water were purchased from Chemlab (Zedelgem, Belgium). Individual stock solutions of the analytes at a concentration of 50 mg l^{-1} were prepared in acetone and stored in a refrigerator at 4 °C. Fresh working standard solutions with lower concentrations were daily prepared by appropriate dilutions of the stock solution. Structures and physical properties of the analytes are summarized in Table 1.

Instrumentation

A Hewlett-Packard 1090-II HPLC (Palo Alto, CA, USA) equipped with a DAD, and a Rheodyne 7725 injector equipped with a 10-µl sample loop were used for separation and determination of the analytes. An Alltech Alltima analytical C18 column (150 × 4.6 mm) (Fisher Scientific, Massachusetts, USA) packed with 5 µm particles was used for the separation. The mobile phase was ACN delivered at a flow rate of 0.7 ml min^{-1}. Monitoring of the analytes was done at 265 nm for cholecalciferol and 260 nm for calcifediol. Data acquisition and processing were done using ChemStation software. A D-7200 centrifuge from Hettich (Kirchlengern, Germany) was used in the sample preparation.

Real Samples

One plasma sample was obtained from the Iranian Blood Transfusion Organization (Tabriz, Iran) and kept frozen at -20 °C until analysis. The preliminary tests indicated that it was free of the analytes. Therefore, it was used in the optimization step as blank plasma (matrix). Also, ten plasma samples were provided from ten volunteers from Plasma Medical Laboratory (Tabriz, Iran).

Microextraction Procedure

A 1 ml analytes-free plasma (blank plasma) spiked with 20 ng ml^{-1} of each analyte or real plasma sample was transferred into a 10-ml glass test tube. Then, 0.15 g sodium chloride (15%, w/v) and 1.5 ml ACN were added to the tube and the mixture was shaken manually for 1 min. The mixture was centrifuged at 4000 rpm for 5 min and 0.9 ml of the supernatant phase was removed and mixed with 0.1 ml pure ACN and 33 µl *n*-hexane. The mixture was rapidly injected into 5 ml distilled water placed in a home-made extraction vessel. In this step, a cloudy solution consisting of very fine droplets of *n*-hexane dispersed into the aqueous phase was formed and the analytes were extracted into the droplets. The mixture was then centrifuged for 5 min at 4000 rpm, which led to the dispersed droplets of the extraction solvent (*n*-hexane) to collect on the top of the aqueous phase. To transfer the collected organic phase into the narrow section of the extraction vessel, about 2 ml distilled water was slowly injected into the vessel by a 5-ml glass syringe through the septum. Then, whole of the collected phase (10 ± 0.5 µl) was transferred into a home-made microtube with the conical bottom (42 × 7 mm) and evaporated to dryness under a stream of nitrogen at room temperature. The residue was re-dissolved in 10 µl ACN and whole of the solution was injected into the separation system for the analysis.

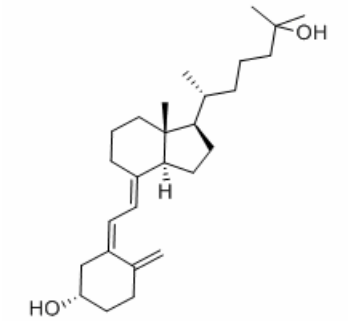
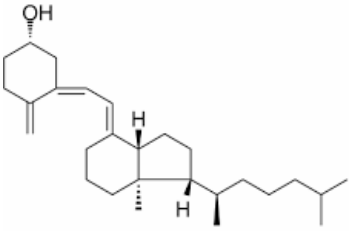
RESULTS AND DISCUSSION

There are different factors affecting the efficiency of the developed method. Some of them are selection of a suitable extraction solvent and its volume, dispersive solvent and its volume, ionic strength, pH, and centrifuging rate and time. It is very important to optimize them in order to obtain high ERs and EFs, and low limits of detection (LODs). In this study "one-variable-at-a-time" strategy was used for optimizing the mentioned parameters.

Optimization of Parameters in Extraction of the Analytes from Plasma

Selection of extraction solvent type and volume. In the present work, the extraction solvent used for extraction of the target analytes from plasma samples is used as a disperser solvent in the following DLLME procedure. Therefore, this solvent should be miscible in both of the aqueous phase and DLLME extractant, and suitable for extraction of the analytes from plasma samples along with

Table 1. Structures and Physical Properties of the Analytes [27,28]

Analyte	Structure	MW[a]	$logK_{o/w}$[b]	Bp[c]	Mp[d]	LD_{50}[e]
Calcifediol		400	4.3	496	83	5-50 for rat
Cholecalciferol		384	7.5	496	84	325 for rat 42 for male 619 for female

[a]Molecular weight (g mol^{-1}). [b]log *n*-octanol/water partition coefficient. [c]Boiling point. [d]Melting point. [e]Lethal dose 50% (mg kg^{-1}).

the capability of proteins precipitation. Considering these requirements, methanol, ACN, acetone, and THF were evaluated as the extraction solvents in this step. For this purpose, 1.5 ml of each above-mentioned solvent was tested separately. The obtained results indicated that among the tested solvents only methanol and ACN participated proteins efficiently and formed a two–phase system in the presence of sodium chloride. Base on the results in Fig. 1, ACN has the high extraction efficiency towards the analytes compared to methanol. It can be attributed to better dispersion of the extraction solvent used in DLLME in the presence of ACN compared to methanol. Therefore, it was selected as the extraction solvent for the further experiments.

Investigating the ACN volume was performed using various volumes of ACN in the range of 1.0-2.5 ml (at 0.5-ml intervals). It is obvious that volume of the separated ACN phase is related to the initial volume of the ACN used. In volumes of 1.0, 1.5, 2.0 and 2.5 ml of ACN, the collected phase volume was 0.7, 0.9, 1.3 and 1.9 ml, respectively. In all cases, 1 ml of the collected phase was removed and used in the following DLLME procedure. In the cases of 1.0 and 1.5 ml, the collected phase was entirely removed and diluted with pure ACN to 1 ml before performing the DLLME procedure. According to the obtained results, higher analytical signals were obtained at 1.5 ml of ACN and it was selected as a suitable volume for the next steps.

Salt addition. Salting-out effect has been commonly employed for enhancing extraction efficiency. In general, salt addition can decrease the solubility of analytes in the aqueous phase (and can also reduce the solubility of the organic solvents in water) and at the same time can reinforce partitioning the analytes into the organic phase. To evaluate the effect of this parameter, different weights of sodium chloride including 0.00, 0.05, 0.10, 0.15, 0.20 and 0.25 g were tested. After performing the proposed method,

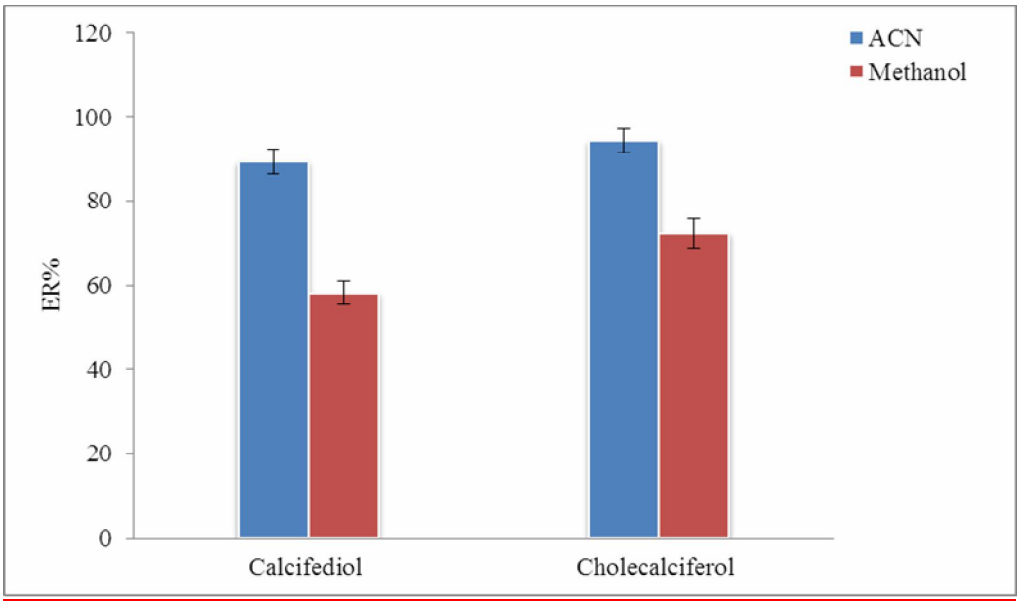


Fig. 1. Effect of chemical identity of extraction solvent in extraction of the analytes from plasma sample. Extraction conditions: blank plasma sample, 1 ml spiked with the analytes at 20 ng ml^{-1} (each analyte); extraction solvent volume, 1.5 ml; NaCl weight, 0.15 g; and extraction solvent in DLLME procedure (volume), *n*-hexane (33 µl). In both steps, centrifugation rate of 4000 rpm and centrifugation time of 5 min were used. The error bars indicate the standard deviations of three repeated determinations.

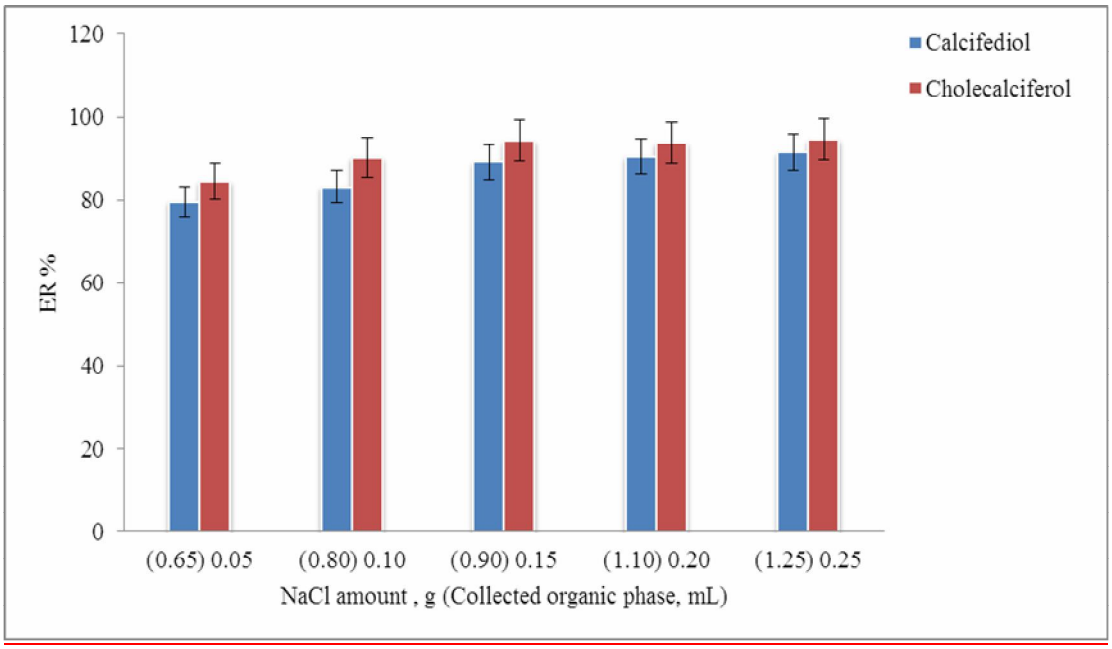


Fig. 2. Optimization of NaCl weight.
Extraction conditions: the same as those used in Fig. 1, except extraction solvent was 1.5 ml ACN.

analytical signals for each analyte are shown in Fig. 2. The results illustrate that addition of salt increases analytical signals up to 0.15 g partially and then they remain nearly constant. It is noted that the two-phase system was not formed when sodium chloride was not added and the method became useless. Also, the collected phase volume was 0.65, 0.80, 0.90, 1.1, and 1.25 mLl when 0.05, 0.10, 0.15, 0.20 and 0.25 g sodium chloride were added, respectively. Considering the results in Fig. 2, 0.15 g sodium chloride was used in the further studies.

Optimization of Parameters in DLLME Step

Selection of extraction solvent type and volume. In each DLLME method, selecting a suitable organic solvent as an extractant is a critical parameter in order to reach favorable EFs and good sensitivity for the target analytes. In this work, the extraction solvent should satisfy some characteristics such as: (a) higher or lower density than water; (b) extraction capability of the analytes; (c) immiscible with water; and (d) good chromatographic behavior. Moreover, most of the high-density extraction solvents are chlorinated solvents which are toxic. On the other hand, low-density solvents are often safer than the chlorinated solvents, therefore, the use of low-density organic solvents are preferred in most extractive methods. In this study, toluene (d = 0.86 g ml^{-1}), *n*-hexane (d = 0.65 g ml^{-1}), *n*-octanol (d = 0.82 g ml^{-1}), and *n*-hexanol (d = 0.81 g ml^{-1}) were tested as extraction solvents. To achieve a similar volume of the collected phase, different volumes of the above-mentioned extraction solvents were selected. It was found that to obtain 10 µl of the collected phase 64, 56, 42 and 33 µl of toluene, *n*-hexanol, *n*-octanol, and *n*-hexane, respectively, should be used. According to the obtained results in Fig. 3, among the tested solvents *n*-hexane gives the highest analytical signals. It can be attributed to more solubility of the analytes in *n*-hexane compared to other tested extraction solvents. On the other hand, low viscosity of *n*-hexane can lead to better dispersion into the aqueous solution, and hence high contact area can be obtained. So, it was used for the further experiments.

Optimization of extraction solvent volume. Optimization of extraction solvent volume is another parameter in a microextraction process which can affect performance of the method. Low volumes of extraction solvent lead to obtain high EFs. This enhances analytical signals and improves LODs of the method. On the other hand, keeping the sample size at a constant volume and changing the extraction solvent volume will change the volume ratio of sample to the extraction phase and hence ERs are improved. Evaluation of extraction solvent volume effect was done by performing the developed method using different volumes of *n*-hexane including 30, 33, 40, 45 and 50 µl. It is remarkable of the collected phase volume increased from 7 to 28 µl with increasing volume of the extraction solvent in the mentioned range. The extraction solvent volume of 33 µl was the minimum volume required to reach 10 µl of the collected organic phase volume. It is noted that when 30 µl of *n*-hexane was used, the volume of the collected phase was about 7 µl, which its removal was difficult and a little variation in the volume of the collected phase led to significant differences in analytical signals and hence repeatability of the method was reduced.

Study of ionic strength. Salting-out effect is an effective parameter on the basis of electrolyte/non-electrolyte interaction, in which the solubility of non-electrolyte can be decreased less soluble in the presence of high concentrations of salt. This phenomenon has been used in most extraction methods. Generally, addition of a salt decreases solubility of the analytes in an aqueous sample and enhances their partitioning into an organic phase. In this method, ionic strength of the aqueous sample was studied by adding different concentrations of sodium chloride (0-10%, w/v), whereas the other experimental conditions (except volume of the extraction solvent) were kept constant. It is noted that addition of salt increases the volume of collected phase because of decrease in the extraction solvent solubility into the aqueous phase. The experiments were performed using different volumes of the extraction solvent to achieve 10 µl of the collected organic phase volume (33, 28, 22 and 15 µl for 0.0, 2.5, 5.0, and 10%, w/v, sodium chloride, respectively). Plot of ER% *vs.* ionic strength (Fig. 4) shows that addition of salt has no significant effect on the analytical signals. Therefore, in the following, all experiments were performed without salt addition.

Study of pH. In order to evaluate the effect of aqueous phase pH on the extraction efficiency of the developed method different experiments were performed in the pH range of 4-8 by adding appropriate volumes of 0.1 M

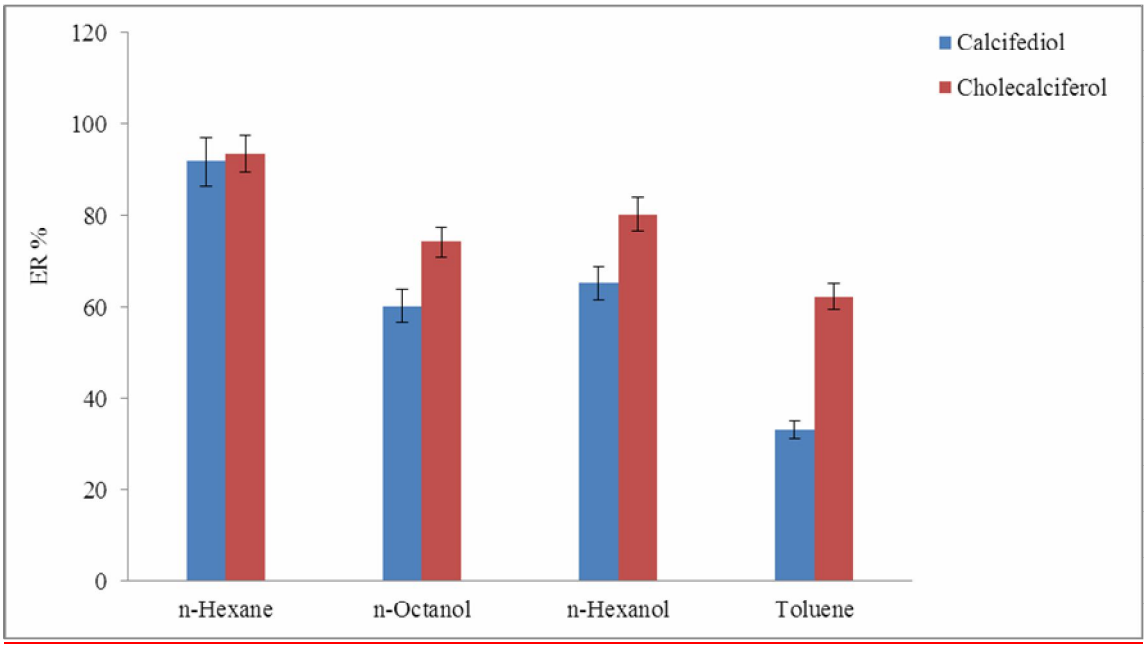


Fig. 3. Selection of extraction solvent type in DLLME step.
Extraction conditions: are the same as those used in Fig. 2, except 0.15 g NaCl was used.

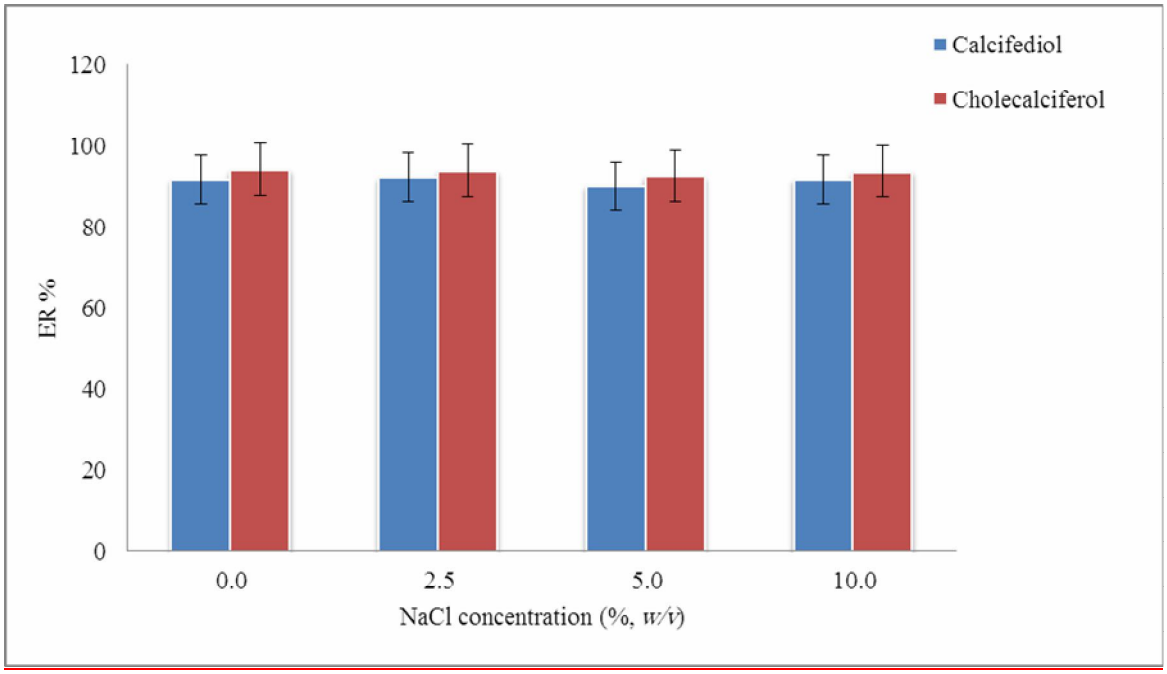


Fig. 4. Salt addition study in DLLME.
Extraction conditions: the same as those used in Fig. 5, except extraction solvent which was *n*-hexane.

hydrochloric acid or sodium hydroxide solution to the aqueous phase. According to the obtained results, best extraction efficiency was obtained for the analytes in the pH range of 6-8, which covers the pH value of de-ionized water. So, the new experiments were carried out without pH adjustment.

Optimization of centrifugation rate and time. During centrifugation, the dispersed fine droplets of the extractive phase are collected on top of the aqueous phase in the extraction vessel. The effects of centrifugation time and rate were examined at the ranges of 3-10 min and 2000-5000 rpm, respectively. It is noted that the separated phase volume (ACN or *n*-hexane) was the same in different centrifugation times and rates. According to the obtained results, centrifugation time and rate had no significant effect on the performance of the method in the tested ranges. It is obvious that at low centrifuging times and speeds (< 2000 rpm) the collection of organic phase can not be complete and low analytical signals can be obtained. However, 5 min and 4000 rpm were selected as centrifuging time and rate, respectively, in this study.

Method Validation

Several criteria for method validation have been considered according to the international guidelines in validation of bioanalytical method. These parameters are included LOD, limit of quantification (LOQ), linearity, selectivity, accuracy, precision, stability, EF and ER [29, 30].

Linearity and calibration curves. To obtain linearity of the developed method, matrix-matched calibration curves were constructed by plotting peak areas *versus* the analytes concentrations. Coefficient of determination (r^2) was computed from the mean of three calibration curves plotted in 3 different days. The LOD and LOQ values were evaluated on the basis of signal-to-noise ratios (S/N) of 3 and 10, respectively. The S/N was estimated using measurement of the peak height relative to baseline noise, and peak height values were consequently converted into concentrations through height of the analyte peaks at lower limit of quantification (LLOQ). The LLOQ was reported as the lowest concentration on the calibration curve that could be determined with a precision of ≤ 20% and accuracy between 80 and 120%. The obtained results are summarized in Table 2. Good linearities are obtained with r^2 higher than or equal to 0.9941. The LODs, LOQs, and LLOQs values are low, allowing that the proposed method can be used in determination of the target analytes in plasma samples.

Selectivity. The interference of drugs that can potentially be available in plasma was studied to evaluate matrix exogenous substances by spiking blank plasma with 10 mg l^{-1} of each drug. The drugs tested in selectivity assay were antiarrhythmic drugs (metoprolol, propranolol, carvedilol, and verapamil), anti-inflammatory drugs (ibuprofen, acetaminophen, sodium diclofenac and naproxen), antiepileptic drugs (carbamazepine and valproic acid), and antidepressants (nortriptyline, clomipramine, and fluoxetine). Responses of the analytes at LLOQ concentrations of the analytes were compared with the responses of the samples spiked with the mentioned drugs. No interference from the drugs was observed at the retention times of the studied analytes. These results indicate that the proposed method is selective for the analysis of the desired analytes in plasma samples. This good selectivity can be resulted from two extraction methods used in the proposed procedure as well as selectivity of the separation technique, *e.g.*, HPLC-DAD.

Precision and accuracy. Precision and accuracy are defined as the nearness of the individual measurements of an analyte and deviation of mean test results from supposed concentration, respectively. In this section, under the optimized conditions, precision of the method was assessed for both intra- and inter-day precisions. To assess precision, quality control samples were prepared at three concentrations including 15, 50 and 250 ng ml^{-1} of each analyte and analyzed on the same day (intra-day assay, n = 6). Inter-day precision was studied at a concentration of 15 ng ml^{-1} of each analyte in different days (n = 4). The results in Table 2, expressed as relative standard deviation (RSD), show that RSD% are in the ranges of 3.9-6.3 and, 7.3-8.1% for intra- and inter-day precisions, respectively. Accuracy of the method was determined by added-found method using five replicate determinations at 15 ng ml^{-1} level (each analyte), and the obtained deviations were less than 8% for both analytes.

Calculation of PF and ER. PF is defined as the ratio of the analyte concentration in the collected organic phase (C_{col}) and the initial concentration of the analyte (C_0) in the

Table 2. Quantitative Features of the Developed Method for the Selected Analytes

Analytes	LOD[a]	LOQ[b]	LLOQ[c]	LDR[d]	R^2 [e]	RSD (%) [f]				EF ± SD[g]	ER ± SD[h]
						Intra-day			Inter-day		
						(1)	(2)	(3)			
Calcifediol	3.2	11	5.6	11-2000	0.9941	5.2	4.8	3.9	7.3	92 ± 5	92 ± 5
Cholecalciferol	1.9	6.4	3.9	6.4-2000	0.9976	6.3	5.9	6.0	8.1	94 ± 4	94 ± 4

[a]Limit of detection (S/N = 3**)** (ng ml^{-1}). [b]Limit of quantification (S/N = 10) (ng ml^{-1}). [c]Lower limit of quantification (S/N = 5) (ng ml^{-1}). [d]Linear dynamic range (ng ml^{-1}) (n = 7). [e]Coefficient of determination. [f]Relative standard deviation (n = 6, C = 15 (1), 50 (2) and 250 (3) ng ml^{-1}) for intra- and (n = 4, C = 15 ng ml^{-1}) for inter-day precisions. [g]Enrichment factor ± standard deviation (n = 3). [h]Extraction recovery ± standard deviation (n = 3).

Table 3. The Obtained Concentrations (ng ml^{-1}) of the Selected Analytes in the Plasma Samples Obtained from Different Persons with the Developed and Enhanced Chemiluminescence Methods. Triplicate Determinations were Used in Each Case and the Results are Given as Mean Concentration ± Standard Deviation

Sample	Method	Calcifediol	Cholecalciferol
Plasma 1	ECL[a]	-	20 ± 0.76
	Developed method	ND[b]	19 ± 0.29
Plasma 2	ECL	-	23 ± 0.65
	Developed method	ND	21 ± 0.79
Plasma 3	ECL	-	20 ± 0.38
	Developed method	ND	21 ± 0.42
Plasma 4	ECL	-	19 ± 0.36
	Developed method	ND	19 ± 0.22
Plasma 5	ECL	-	24 ± 0.74
	Developed method	ND	24 ± 0.46
Plasma 6	ECL	-	57 ± 1.2
	Developed method	14 ± 0.2	60 ± 1.5
Plasma 7	ECL	-	42 ± 0.38
	Developed method	ND	41 ± 0.61
Plasma 8	ECL	-	37 ± 0.63
	Developed method	6 ± 0.1	39 ± 0.65
Plasma 9	ECL	-	9.2 ± 0.17
	Developed method	ND	10 ± 0.49
Plasma 10	ECL	-	24 ± 0.34
	Developed method	9 ± 0.4	25 ± 0.54

[a]Enhanced chemiluminescence. [b]Not detected.

Table 4. Results of Assays to Check the Samples Matrices Effect for the Selected Analytes. Analytes' Contents of the Samples were Subtracted

Analyte		Found (ng ml^{-1}) ± SD (Mean relative recoveries ± SD)			
	Added (ng ml^{-1})	Plasma 1	Plasma 2	Plasma 3	Plasma 4
Calcifediol	10	9.2 ± 0.4	9.3 ± 0.2	9.1 ± 0.3	9.1 ± 0.4
		(92 ± 4)	(93 ± 2)	(91 ± 3)	(91 ± 4)
	30	28 ± 2	29 ± 0.3	28 ± 0.5	29 ± 2
		(93 ± 7)	(96 ± 1)	(93 ± 2)	(96 ± 7)
Cholecalciferol	10	9.2 ± 0.8	9.1 ± 0.7	9.2 ± 0.2	9.2 ± 0. 4
		(92 ± 8)	(91 ± 7)	(92 ± 2)	(92 ± 4)
	30	28 ± 1	28 ± 1	29 ± 0.4	29 ± 0.4
		(93 ± 4)	(93 ± 4)	(96 ± 2)	(96 ± 1)

sample solution.

$$PF = \frac{C_{col}}{C_0} \quad (1)$$

C_{col} is obtained from the calibration graph plotted as peak areas versus concentrations of the analytes in standard solution prepared in ACN at different concentrations. These standards were injected directly into the analysis system. ER is defined as the percentage of the total analyte amount (n_0) which is extracted into the collected organic phase (n_{col}),

$$ER = \frac{n_{col}}{n_0} \times 100 = \frac{C_{col} \times V_{col}}{C_0 \times V_{aq}} \times 100 = EF \times \frac{V_{col}}{V_{aq}} \times 100 \quad (2)$$

where V_{col} and V_{aq} are volumes of the collected phase and sample solution, respectively. Under the optimal experimental conditions, PFs and ERs were 92 and 94, and 92 and 94% for calcifediol and cholecalciferol, respectively (Table 1).

Robustness. The effect of small variations in the method parameters on the results should be studied during method validation. The trustiness of an analysis with respect to the deliberate variations in method parameters can be evaluated by robustness testing. The robustness of an analytical method is a measure of its capacity to remain unaffected by small and planned changes in the method conditions. To evaluate the robustness, small deliberate variations in method parameters were performed and quantitative influence of the variables was determined. In this study, the effects of the following parameters including ACN volume (1.4, 1.5 and 1.6 ml), NaCl weight (0.14, 0.15 and 0.16 g), and *n*-hexane volume (32, 33 and 34 µl) were studied. The obtained results showed that small changes applied in test conditions had no significant effect on the analysis results.

Stability. Stability of the analytes in plasma samples was investigated with the spiked samples at two concentrations including 10 and 20 ng ml^{-1} (each analyte, n = 3) in different experimental conditions. In this work, stability assessment was conducted under short–term temperature and freeze-thaw conditions. Aliquots of each concentration were kept at room temperature (24 °C) for 6 h and analyzed for room temperature stability study. The freeze-thaw stability of the analytes was also determined after three freeze and thaw (-20 to 24 °C) cycles according to the following conditions. No peaks were observed in the

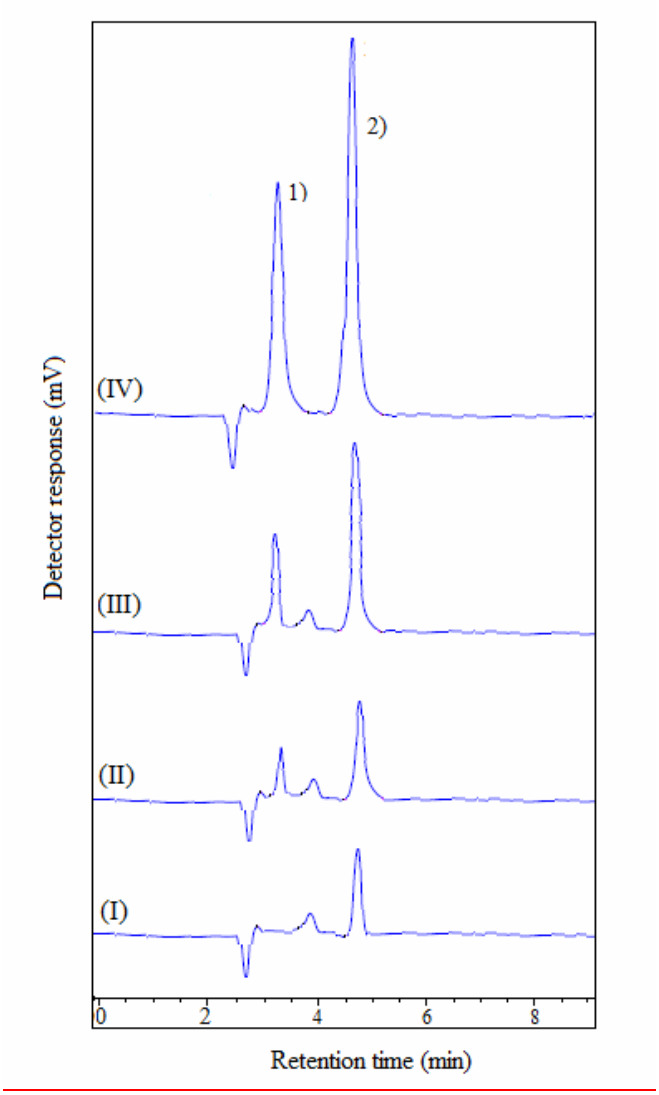


Fig. 5. Typical HPLC-DAD chromatograms of (I) un-spiked plasma sample (plasma 1 in Table 3) after performing the method, (II) plasma sample 1 spiked at a concentration of 10 ng ml^{-1} (each analyte) after performing the developed method, (III) plasma sample 1 spiked at a concentration of 30 ng ml^{-1} (each analyte) after performing the developed method, and (IV) direct injection of a standard solution of the analytes at a concentration of 10 mg l^{-1} of each analyte prepared in ACN. In the case of plasma sample, the proposed method was performed on it and the final solution was injected into the separation system. Detection wavelength was 265 nm. Peaks identification: 1 is calcifediol and 2 is cholecalciferol. For chromatographic conditions, see experimental section.

chromatograms of both cases indicating that the analytes are degraded under exposure of light or oxidation by oxygen. Therefore, it is recommended that plasma samples are analyzed immediately after sampling.

Analysis of Real Samples

The applicability of the proposed method in analysis of the analytes was studied on ten plasma samples which cholecalciferol contents of them were simultaneously

Table 5. Comparison of the Proposed Method with other Methods in the Extraction and Determination of the Selected Analytes

Method	Sample	Analyte	LOD[a]	LDR (ng ml^{-1})[b]	R^{2} [c]	RSD (%)[d]	PF[e]	RR (%)[f]	Extraction time (min)	Sample size	Ref.
VA-LLE-HPLC-MS/MS[g]	Human Serum	Cholecalciferol	1.5	-	0.996	5.2	-	84-102	~ 2.0	250 µl	[32]
		Calcifediol	1.7	-	0.998	10.2		89-106			
	Breast milk	Cholecalciferol	1.9	-	0.998	4.9	-		27	4.0 ml	
		Calcifediol	2.1	-	0.999	7.9					
DLLME-HPLC-DAD[h]	Vegetable samples	Cholecalciferol	0.4	1-100	0.09906	-	24	89-105	15	0.2-2.0 g	[33]
SPE-HPLC-DAD[i]	Human Serum	Cholecalciferol	2.5	8-200	0.9949	3.5	-	99-100	~ 8.0	0.5 ml	[34]
		Calcifediol	2.1	7-200	0.9994	4.8		99-100			
LDS-DLLME-HPLC-UV[j]	Milk and yoghourt samples	Cholecalciferol	0.9	2-500	0.9950	7.5	274	97	12	8 ml	[35]
DLLME-HPLC-DAD[k]	Human plasma	Cholecalciferol	1.9	6.4-2000	0.9976	3.9-5.2	92-94	91-96	11	1.0 ml	Present work
		Calcifediol	3.2	11-2000	0.9941	5.9-6.3		91-96			

[a]Limit of detection (ng ml^{-1}). [b]Linear dynamic range (ng ml^{-1}). [c]Coefficient of determination. [d]Relative standard deviation. [e]Preconcentration factor. [f]Relative recovery. [g]Vortex-assisted-liquid-liquid extraction-high performance liquid chromatography-tandem mass spectrometry. [h]Dispersive liquid-liquid microextraction-high performance liquid chromatography-diode array detection. [i]Solid phase extraction-high performance liquid chromatography-diode array detection. [j]Low density solvent based-dispersive liquid-liquid microextraction- high performance liquid chromatography-ultraviolet detector. [k]Dispersive liquid-liquid microextraction-high performance liquid chromatography-diode array detection.

analyzed by the enhanced chemiluminescence method in Elecsys analyzer with Cobas test systems [31]. All samples were analyzed in triplicate. The obtained results in Table 3 show that there is a good agreement between the results of methods regarding cholecalciferol, indicating that the developed method is useful in clinical tests. It should be noted that there is no routine test for calcifediol determination in medical laboratories. Therefore, assessment of the method accuracy by a standard method was not feasible in the case of calcifediol. To evaluate

matrix effect in different samples, added-found method was used. The samples were spiked with the analytes at two concentration levels (10 and 30 ng ml^{-1} of each analyte) and the proposed method was applied to them (three times for each concentration). The obtained concentrations for the analytes in the samples are listed in Table 4. The results show that the concentrations found and mean relative recoveries obtained by the developed method are in the ranges of 9.1-9.3 and 28-29 ng ml^{-1} and 91-93 and 90-96%, for the spiked concentrations of 10 and 30 ng ml^{-1}, respectively. Figure 5 shows the typical HPLC-DAD chromatograms of a standard solution (direct injection), and one un-spiked plasma sample after performing the method.

Comparison of the Developed Method with other Approaches

Table 5 indicates the LOD, LDR, r^2, RSD, extraction time, relative recovery, PF and sample size of the proposed method and the other methods for the extraction and determination of the analytes from different samples. As seen, the RSD values of the proposed method are better than or comparable with those reported for the other methods. The LODs for the presented method are comparable with those of the mentioned methods. On the other hand, the developed method has wider LRs compared to the other methods. The method has high PFs compared to other methods except LDS-DLLME-HPLC-UV procedure. On the other hand, the method has, comparable extraction time with other approaches except for VA-LLE-HPLC-MS/MS method. These results show that the presented method is a rapid, sensitive, efficient, and reliable technique for the extraction and preconcentration of the analytes from plasma samples.

CONCLUSIONS

In the present study, for the first time, a DLLME method using an extraction solvent lighter than water was proposed for the extraction and preconcentration of cholecalciferol and calcifediol in plasma samples. In this method, the analytes were extracted into a water-miscible extraction solvent which was used as a disperser solvent in the following DLLME procedure. The experimental results demonstrated that this technique exhibits many advantages such as high ERs and PFs, low LODs, short extraction time, and good repeatability. In views of the simplicity, speed, and efficiency offered by this method, it is recommended for analyzing the analytes in plasma samples.

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