Selective chemiluminescence method for monitoring of vitamin K homologues in rheumatoid arthritis patients

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ABSTRACT

Vitamin K is a fat-soluble vitamin involved in blood coagulation and bone metabolism. The detection and monitoring of vitamin K homologues in rheumatoid arthritis (RA) patients is a challenging problem due to the smaller concentrations of vitamin K and the presence of several interfering medications. Therefore, this study aimed to develop a new highly sensitive and selective chemiluminescence (CL) method designated to quantify vitamin K homologues in plasma of RA patients including phylloquinone (PK, vitamin K_1), menaquinone-4 (MK-4, vitamin K_2) and menaquinone-7 (MK-7, vitamin K_2). The method was based on the unique photochemical properties of vitamin K homologues that were exploited for selective luminol CL reaction. The correlation coefficients of 0.998 or more were obtained in the concentration ranges of 0.1–100 ng mL^{-1} vitamin K homologues. The detection limits were 0.03–0.1 ng mL^{-1} in human plasma for vitamin K homologues. The developed HPLC-CL system was successfully applied for selective determination of vitamin K homologues in plasma of RA patients. The developed method may provide a useful tool for monitoring vitamin K homologues in different clinical studies such as RA, osteoporosis and hepatocellular carcinoma in which vitamin K is intervented.

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1. Introduction

Vitamin K is an essential fat-soluble micronutrient which plays important roles in bone health as well as blood coagulation [1]. In nature, there are two major forms of vitamin K: vitamin K_1 (phylloquinone, PK), which is produced by plants and algae and has a phytyl side chain and vitamin K_2 (menaquinones, MKs) which is of microbial origin and comprises a family of molecules distinguished by unsaturated side chains of isoprenoid units varying in length from 1 to 14 repeats [2]. The major dietary form of vitamin K has been considered to be PK whose major source is green and leafy vegetables. In contrast, MKs are found in fermented food and in the colon where they are synthesized by the intestinal microflora [3,4].

The biological role of vitamin K is to act as a cofactor for γ-glutamyl carboxylase that catalyzes an unique posttranslational conversion of specific glutamyl residues to γ-carboxylglutamyl residues in several proteins such as plasma clotting factors II (prothrombin), VII, IX, X and protein C. These vitamin K-dependent proteins play crucial roles in blood coagulation [2]. In addition, several reports indicated the important role of vitamin K in bone metabolism [5–13]. Three vitamin K-dependent proteins, osteocalcin (bone Gla protein), matrix Gla protein and protein S, have been identified as bone matrix components produced by osteoblasts [5–7]. Moreover, there is an evidence for the association between the low circulating levels of vitamin K and the high incidence of femoral neck and vertebral fractures [8] and the low bone-mineral density [9,10]. Several studies have demonstrated that vitamin K_2 is proposed to be an effective treatment for osteoporosis and the prevention of fractures [11,12]. The importance of vitamin K in bone metabolism is further supported by studies showing that vitamin K inhibits osteoclastic bone resorption by induction of osteoclast apoptosis [13,14]. In addition to these biological activities, vitamin K_2 has been reported to induce apoptosis in hepatocellular carcinoma, leukaemia [15] and myelodysplastic syndrome cell lines [16]. Recently, it was found that MK-4 could induce apoptosis of synovial cells and thus be a novel treatment for RA [17,18]. Therefore, it is necessary to study the correlation between circulating vitamin K homologues levels in RA patients and RA biomarkers. Selective detection and monitoring of vitamin K homologues in human plasma is a challenging problem especially in patients with low levels of vitamin K homologues due to the small concentrations

of vitamin K and the presence of several interferences from plasma components and also from administered medicaments.

To date, several publications were reported for the separation and determination of vitamin K in biological samples including high performance liquid chromatography (HPLC) with ultraviolet (UV) [19,20], fluorescence [21–25], electrochemical [26,27], chemiluminescence (CL) [28] and mass spectrometric detection [29]. In initial study to detect vitamin K homologues in plasma of RA patients, it was found that vitamin K concentrations were significantly lower in RA patients that could not be detected by many of the reported methods [19–28] while others remain expensive techniques for routine assay of vitamin K concentrations [29]. Therefore, it is necessary to develop a highly sensitive and selective determination method designated for measurements of lower concentrations vitamin K homologues in the presence of any existed interferences from medications or plasma components. Luminol CL is one of the most efficient and versatile CL reactions that provide a useful tool in monitoring trace quantities of samples in complex matrices [30].

In this research, we developed a selective luminol CL method specially designated for trace determination of vitamin K homologues in plasma samples of RA patients. The method was based on the unique photochemical characteristics of vitamin K that was exploited for selective determination by mixing with luminol without addition of oxidant and/or catalyst. The coexistence of oxidant and enhancer could significantly increase the light output and duration kinetics of luminol CL. Accordingly, this will allow the development of more sensitive and selective analytical methodology for the determination of vitamin K homologues. Furthermore, high selectivity and sensitivity is a necessity for monitoring of plasma vitamin K concentrations in RA patients specially in the presence of trace concentrations and many interfering medications.

2. Experimental

2.1. Materials and reagents

MK-4 and MK-7 were kindly provided by Eisai Pharmaceuticals (Tokyo, Japan). Luminol were obtained from Nacalai Tesque (Kyoto, Japan). Isoluminol and *N*-(4-aminobutyl)-*N*-ethylisoluminol (ABEI) were from Tokyo Chemical Industry (Tokyo, Japan). 8-Amino-5-chloro-7-phenylpyrido[3,4-*d*]pyridazine-1,4-(2*H*,3*H*)dione (L-012) and DHPA was from Wako Pure Chemical Industries (Osaka, Japan). Vitamins K_1 and K_3 (2-methyl-1,4-naphthoquinone), silver nitrate, ammonium persulfate and acetonitrile were from Kanto Chemical (Tokyo). Superoxide dismutase (SOD) from bovine erythrocytes was purchased from Sigma (St. Louis, MO, USA). Sodium hydroxide (NaOH) was from Merck (Darmstadt, Germany). 2-Methyl-3-pentadecyl-1,4-naphthoquinone as an internal standard was synthesized in our laboratory according to the previous reported method [31]. Stock solutions of vitamin K and internal standard ($100 \mu mol L^{-1}$) were prepared in ethanol and stored in the dark at $-30 °C$. These solutions were diluted appropriately with ethanol to prepare the working solutions. Stock solutions of luminol analogues ($0.3 mmol L^{-1}$) were prepared in $100 mmol L^{-1}$ NaOH*aq* solution and diluted before analysis. Imidazole was from Tokyo Chemical Industry; imidazole was recrystallized from acetonitrile before use. Distilled water was obtained using Simpli Lab-UV (Millipore, Bedford, MA, USA) water device. Other chemicals were of extra pure grade.

2.2. CL time profile for vitamin K photoproducts with L-012

The CL profiles of vitamin K homologues photoproducts with L-012 were assessed by a batch method with Sirius luminometer (Berthold, Germany). The procedures was as follows; a $10 \mu mol L^{-1}$ of vitamin K homologues, dissolved in ethanol, was mixed with a mixture of imidazole-HNO$_3$ ($500 mmol L^{-1}$, pH 9.0) and acetonitrile (10:90) and then, it was UV irradiated in a photoreactor composed of a PTFE tubing ($5.0 m \times 0.25 mm$ i.d.) coiled around the quartz well of Philips TUV-SE low-pressure mercury lamp (16 W, 254 nm, Philips, Poland). In a small test tube, $100 \mu L$ of photoproducts was added and placed in the luminometer then $100 \mu L$ of $3 \mu mol L^{-1}$ L-012 in $100 mmol L^{-1}$ NaOH*aq* was injected automatically, and the produced CL was measured for 600 s. The total CL intensity was defined as the area under the CL delay curve. Data was expressed as the mean of triplicate measurements.

2.3. HPLC-CL system and chromatographic conditions

The HPLC system consisted of two LC 10AS liquid chromatographic pumps (Shimadzu, Kyoto), a Rheodyne 7125 injector (Cotati, CA, USA) with a $20-\mu L$ sample loop, Capcellpack ODS UG 120 ($35 mm \times 1.5 mm$ i.d., $5 \mu m$, Shiseido, Tokyo) column that was maintained at ambient temperature ($25 °C$) followed by an online photoreactor composed of a PTFE tubing ($5.0 m \times 0.25 mm$ i.d., GL Sciences, Tokyo) coiled around the quartz well of Philips TUV-SE low-pressure mercury lamp (16 W, 254 nm), a CLD-10A CL detector (Shimadzu), a UNI noise cleaner (Union Corp., Gunma, Japan) and SIC chromatorecorder (System Instruments, Tokyo, Japan). Temperature of the photoreactor was maintained at $25 °C$ by an oven (Jasco, Tokyo). The chromatographic separation was carried out by isocratic elution with a mixture of imidazole-HNO$_3$ ($500 mmol L^{-1}$, pH 9.0) and acetonitrile (10:90). $3-\mu mol L^{-1}$ L-012 in $0.1 mol L^{-1}$ NaOH*aq* was used as a single post column CL reagent. The flowrates of the mobile phase and the CL reagent were set at 0.3 and $0.7 mL min^{-1}$, respectively.

2.4. RA Patients and sample collections

Samples were collected from RA patients at Sasebo Chuo hospital (36–69 years) representing different disease stages from (1–4) with morbidity period (4–22 years). Patients received tocilizumab as anti-rheumatic agent besides some non-steroidal anti-inflammatory drugs such as etodolac and loxoprofen. Also, a group of healthy subjects (35–60 years) was assessed for their circulating level of vitamin K homologues. Blood samples were collected from a convenient forearm vein into tubes containing ethylenediamine tetraacetic acid. The samples were centrifuged at $3000 \times g$ for 10 min at room temperature then the plasma was separated and stored at $-30 °C$ in dark until analysis. The present experiments were approved by the Ethics Committee of the School of Pharmaceutical Sciences, Nagasaki University, and performed in accordance with established guidelines.

2.5. Sample preparation

Exactly $500 \mu L$ of donated human plasma sample was placed in a brown screw-capped tube. An aliquot of $500 \mu L$ of $3.8 ng mL^{-1}$ solution of internal standard (2-methyl-3-pentadecyl-1,4-naphthoquinone) in ethanol was added. Extra ethanol (1 mL) was then added to denature protein. The sample was extracted using 5 mL of hexane with shaking for 5 min before centrifuging at $3000 \times g$ for 5 min. The supernatant was separated and applied to a Sep-Pak silica cartridge (Waters, Milford, MA, USA), which was washed with 10 mL of hexane. The analytes were eluted with 5 mL of hexane/diethyl ether (97:3, v/v). The eluate was evaporated under reduced pressure, and the residue was dissolved with $50 \mu L$ of ethanol then injected to the HPLC system.
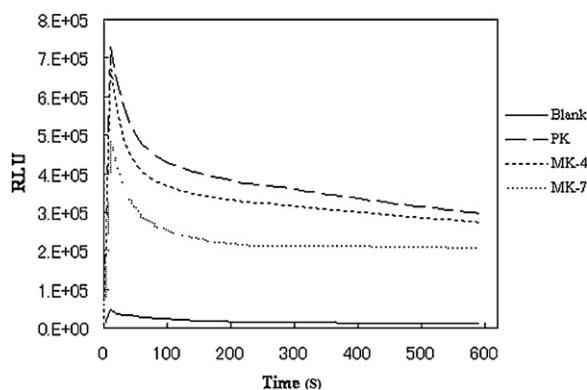
Fig. 1. Time profiles of CL emission obtained by UV irradiation products from vitamin K homologues with 3 $\mu mol\,L^{-1}$ of L-012.

2.6. Method validation

The validation was performed according to US-FDA guidance for industry on bioanalytical method validation [32]. The validation studies were carried out using pooled plasma sample pre-analyzed for vitamin K content before spiking of standard vitamin K homologues. Three days of calibration curves with six replicates of plasma samples spiked with a mixture of standard vitamin K homologues (MK-4, PK and MK-7) over concentration range of $0.1-100\,ng\,mL^{-1}$ and internal standard ($3.8\,ng\,mL^{-1}$) were performed to assess linearity of the proposed method.

To evaluate intra-day and inter-day precision and accuracy, three sets of plasma samples spiked with three different concentration levels of vitamin K homologues (MK-4, PK and MK-7); low ($0.2\,ng\,mL^{-1}$), middle ($10\,ng\,mL^{-1}$) and high ($100\,ng\,mL^{-1}$) with $3.8\,ng\,mL^{-1}$ internal standard. The assay was performed with five replicates at three successive days.

The recovery was determined by using pooled plasma samples spiked with standard mixture of vitamin K homologues (MK-4, PK and MK-7) in triplicate at the three concentration levels 0.2, 10 and $100\,ng\,mL^{-1}$. The obtained results were compared with true concentration of the standard.

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters. For the determination of a method's robustness, a number of method parameters, for example, photoreactor coil length and temperature, imidazole buffer pH and concentration, CL reagent concentration and flow rate, are varied within a realistic range, and the quantitative influence of the variables is determined.

3. Results and discussion

3.1. The unique photochemical properties of vitamin K

To prove the method selectivity, determination of vitamin K homologues was pursued directly using batch method. The CL time profile of vitamin K homologues; PK, MK-4 and MK-7 were examined by batch method using Sirius luminometer. At first, the vitamin K homologue was UV irradiated in the presence of imidazole buffer and the photoproducts were collected then mixed with luminol derivative, L-012. The CL time profile of vitamin K homologues after UV irradiation is shown in Fig. 1. A strong CL was emitted after addition of L-012 and continued for more than 10 min indicating a long-lived CL. The photoproducts had strong enhancement effect on luminol CL. Additionally, the CL profile was similar to that obtained by standard DHPA referring to the formation of stabilized semiquinone radical of DHPA [33]. Moreover, the CL was

extremely quenched in the presence of SOD that was used as a selective scavenger for $O_2^{\bullet -}$. Accordingly, stabilized semiquinone radical and $O_2^{\bullet -}$ were the main species involved in the CL reaction. The generated photoproducts could initiate selectively luminol CL reaction. Although the photochemically initiated luminol chemiluminescence can react with various compounds, this reaction is unique for vitamin K compounds due to the simultaneous generation of oxidant (ROS) and catalyst/enhancer (DHPA) from the on-line photochemical reaction that could significantly increase the light output and duration kinetics of luminol CL. However, other organic compounds need addition of oxidant and/or catalyst to produce CL in this reaction hence the method considered selective for vitamin K homologues determinations. The principle was adopted for selective monitoring of vitamin K homologues in RA patients.

3.2. Optimization of the selective CL method

In order to obtain the best sensitivity and selectivity, the conditions of photochemical and CL reactions were optimized. The main goal of photochemical reaction detection schemes is to provide a useful way of transforming analyte to compounds with enhanced detectability. The simultaneous generation of different photoproducts including ROS and phenolic products such as DHPA offers an interesting strategy that could be coupled with luminol CL reaction. The reaction yields of photoproducts are highly dependent on residence time in the photoreactor, temperature of photochemical reactor, pH and composition of the mobile phase [34]. Therefore, the photochemical reaction parameters were optimized in order to get the maximum sensitivity and selectivity for the proposed CL method. At first, the effect of PTFE coil length ranging from 2.0 to 8.0 m that corresponds to 20–80 s of residence time in the photoreactor on CL intensity and detection sensitivity (S/N ratio) was studied. It was found that CL and S/N ratio increased as the length of the reaction coil increased till 5 m coil length due to the increase in UV irradiation time then it decreased. The reason for that is the decomposition of the photoreactive products (ROS and DHPA) by increasing the residence time. Since the maximum CL intensity and detection sensitivity was obtained by 50 s of UV irradiation, 5 m coil length was selected for subsequent experiments. Moreover, the effect of photoreactor temperature from 20 to 50 °C on CL intensity and S/N ratio was investigated. Both of CL intensity and S/N ratio was increased by increasing photoreactor temperature till 25 °C then decreased. This may be attributed to the decomposition of photoreactive products by increasing the temperature. Therefore, 25 °C was selected as the optimal.

The mobile phase compositions play an important role in the photochemical reaction kinetics and reaction pathways [34]. Because imidazole plays an important catalytic role in this photochemical reaction for the generation of DHPA, therefore imidazole was selected as a buffer for the HPLC mobile phase. A mixture of imidazole–HNO$_3$ buffer/acetonitrile (1:9) was sufficient for complete separation of all the studied vitamin K homologues and the selected internal standard. The effect of imidazole concentration on the CL intensity and S/N ratio was examined in the range $50-800\,mmol\,L^{-1}$. It was found that both CL intensity and S/N ratio increased by increasing concentration till $500\,mmol\,L^{-1}$ then decreased. Therefore, $500\,mmol\,L^{-1}$ of imidazole–HNO$_3$ solution was chosen. Since the photochemical reaction proceeds in alkaline medium and it was significantly affected by pH of the medium, the imidazole buffer pH was optimized. Although, the useful pH range of imidazole buffer is 6.2–7.8, higher pH ranges were necessary for generation of high yields of the photoproducts (DHPA and ROS). In addition, a Capcellpack ODS-UG-120 column that characterized by the stability at high pH ranges was used. A buffer solution with pH 6.0–9.5 was investigated for its effect on CL intensity and S/N ratio (Fig. 2). It was found that both CL intensity and S/N ratio increased
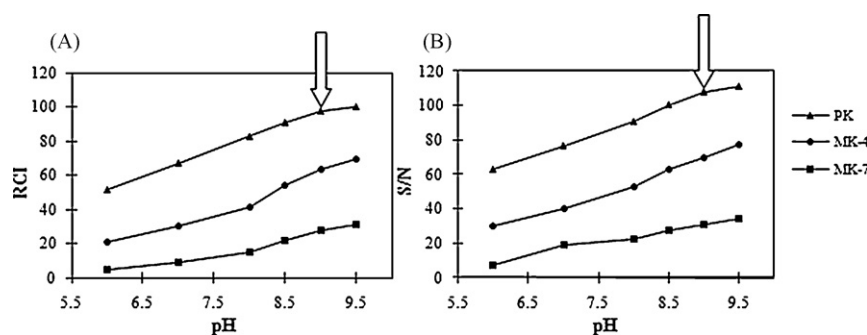
Fig. 2. Effects of pH of imidazole–HNO$_3$ buffer on (A) CL intensity and (B) S/N ratio. Sample concentration was 0.5 µg mL^{-1} for each vitamin K.

as a function of pH. However, pH of 9.0 was selected for subsequent work for column durability reasons.

In order to increase quantum yield and light intensity produced by the proposed CL method, several luminol derivatives were investigated. Several luminol derivatives were known for their efficient and strong CL properties including; luminol, isoluminol, ABEI and L-012 [35]. Therefore, these luminol derivatives were selected for studying their effect on CL intensity and S/N ratio in the proposed photo-induced luminol CL method (Fig. 3). Among them, L-012 gave the best CL intensity and S/N ratio. Additionally, L-012 was reported to give high sensitivity and selectivity for the detection of O$_2^{\bullet-}$ [36]. Therefore, L-012 was chosen for the determination of vitamin K homologues. The method was further optimized for the effect L-012 concentration on CL intensity and S/N ratio (Fig. 4). It was found that CL intensity increased linearly as a function of L-012 concentration. However, increasing L-012 concentration increased the background noise. The best S/N ratio was found at 3 µmol L^{-1} of L-012. This may be attributed to the presence of interferences that have poor catalytic effect at low concentration of L-012. Therefore, the use of low concentration of L-012 reagent enabled high selectivity of the proposed method. On the other hand, it was reported that luminol CL reaction took place in alkaline medium and the degree of alkalinity has great influence on CL intensity [35] and the activity of a chemical enhancer is strongly dependent upon pH of the CL reaction [37]. Therefore, the effect of pH of solvent for L-012 on CL reaction was examined. L-012 was dissolved in NaOH solution of different concentrations ranging from 1 to 250 mmol L^{-1} (pH 11–13.4). CL intensity and S/N ratio reach maximum value when the concentration of NaOH was 100 mmol L^{-1} then it decreased. Thus, the concentration of 100 mmol L^{-1} NaOH (pH 13) was the optimal. Finally, the effect of flow rate of L-012 solution was investigated. It was found that CL intensity increased by the increase in flow rate and reached maximum at 0.7 mL min^{-1} whereas the noise remain constant then CL intensity decreased. This was probably due to the decrease in the reaction time between L-012 and the generated reactive CL species, as the light output of luminol CL reaction is time dependent. Therefore, flow rate of 0.7 mL min^{-1} was selected.

3.3. Validation of the proposed method

To assess linearity of the proposed method, calibration curves were prepared by plotting peak area ratio of vitamin K and internal standard against the vitamin K homologue concentration in plasma. Calibration curve, retention time, calibration range, correlation coefficient and detection limit were recorded for each analyte (Table 1). The calibration curves were linear in the range 0.1–100 ng mL^{-1} plasma with good correlation coefficients (>0.998). The detection limits (S/N = 3) obtained with the proposed method were 0.03, 0.04 and 0.1 ng mL^{-1} plasma for PK, MK-4 and MK-7, respectively. The proposed method was found to be 15–1000 times more sensitive than HPLC-UV methods [19,20], 2–9 times

compared with HPLC-FL [21–25], 2–5 times compared with HPLC-EC [26,27], 3–4 times with HPLC-PO-CL method [28] and with sensitivity similar to LC–MS method [29]. However, the high cost and the need for technical experience make LC–MS less convenient for routine assay of vitamin K in plasma of RA patients. Fig. 5A shows a typical chromatogram of a standard mixture of vitamin K homologues and internal standard analyzed by the proposed HPLC-CL method and under the optimum conditions. Vitamin K homologues and the internal standard were separated efficiently and all peaks were eluted within 45 min.

The intra and inter-day accuracy and precision of the proposed method was evaluated at three different concentration levels of vitamin K homologues (MK-4, PK and MK-7); low (0.2 ng mL^{-1}), middle (10 ng mL^{-1}) and high (100 ng mL^{-1}) (Table 2). It was found that the accuracy of the determination were 91.2–105.9%, 89.5–104.1% and 90.7–107.3 for PK, MK-4 and MK-7, respectively. The relative standard deviations intra-day (n = 5) and inter-day (n = 3) were 2.7–7.2% and 2.6–6.1%, respectively so good reproducibility was obtained. Moreover, the extraction recovery experiments were carried out using pooled plasma samples spiked with three concentration levels of standard vitamin K homologues, 0.2, 10 and 100 ng mL^{-1}. It was found that a percentage recovery of 81.3–90.5% was obtained for vitamin K homologues. Additionally, the usage of vitamin K analog, 2-methyl-3-pentadecyl-1,4-naphthoquinone, as an internal standard improved largely the accuracy of vitamin K homologues determination.

The robustness of an analytical procedure refers to its capability to remain unaffected by small and deliberate variations in method parameters without changes in quantitation. Seven parameters were selected from the analytical procedure to be examined in the robustness testing: photoreactor coil length and temperature, imidazole buffer pH and concentration, NaOH concentration, L-012 concentration and flow rate (Table 3). It was found that none of these variables had a significant effect on the determination of vitamin K homologues. This provides an indication of the reliability of the proposed method during normal usage so the developed HPLC-CL method considered robust.

3.4. Selective measurements of vitamin K homologues in plasma of RA patients

In order to assess the applicability of the developed HPLC-CL method, it was applied for the selective measurements of vitamin K homologues in RA patients as well as healthy human subjects. A typical chromatogram of plasma extract from RA patient determined by the proposed HPLC-CL method is shown in Fig. 5B. It is clear from the chromatogram that PK, MK-4 and MK-7 can be determined selectively without interference from interfering medications and plasma components. The chromatogram indicates the high sensitivity and selectivity of the proposed method. Plasma cir-
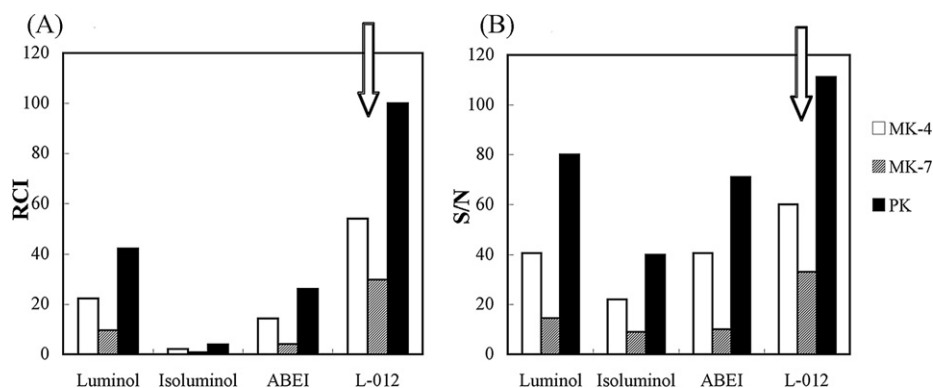
Fig. 3. Effects of different luminol derivatives on (A) CL intensity and (B) S/N ratio. Samples concentrations were 0.5 µg mL^{-1} from each vitamin K.

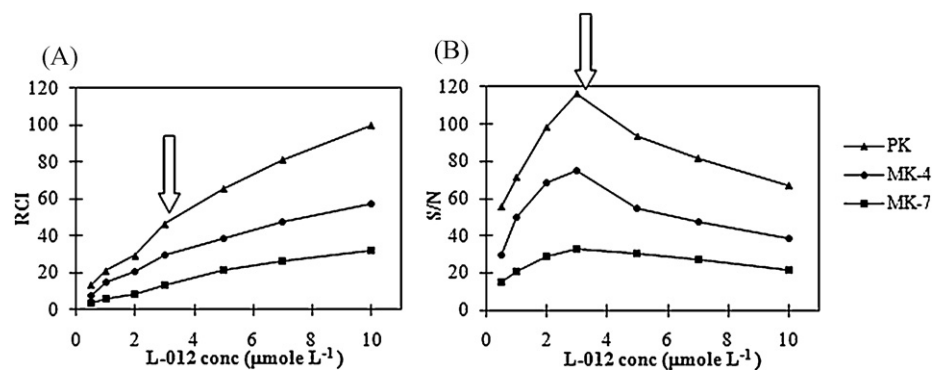**Fig. 4.** Effects of concentration of L-012 reagent on (A) CL intensity and (B) S/N ratio. Samples concentrations were 0.5 µg mL^{-1} from each vitamin K.

Table 1
Retention times, calibration curves and detection limits for vitamin K homologues in human plasma.

Compound	t_R (min)	Calibration curve[a] ($n = 3$)				Detection limit[b] (ng mL^{-1})
		Range (ng mL^{-1})	Slope[c] ($\pm$SE)	Intercept[c] ($\pm$SD)	r	
PK	11.2	0.1–100	0.05 ($\pm$0.01)	1.1 ($\pm$0.25)	0.998	0.03
MK-4	4.7	0.1–100	0.04 ($\pm$0.01)	0.93 ($\pm$0.20)	0.999	0.04
MK-7	39.4	0.3–100	0.02 ($\pm$0.005)	0.67 ($\pm$0.17)	0.998	0.10

[a] Peak area ratio of vitamin K homologues and internal standard versus concentration (ng mL^{-1}).
[b] Detection limit at a S/N ratio of 3.
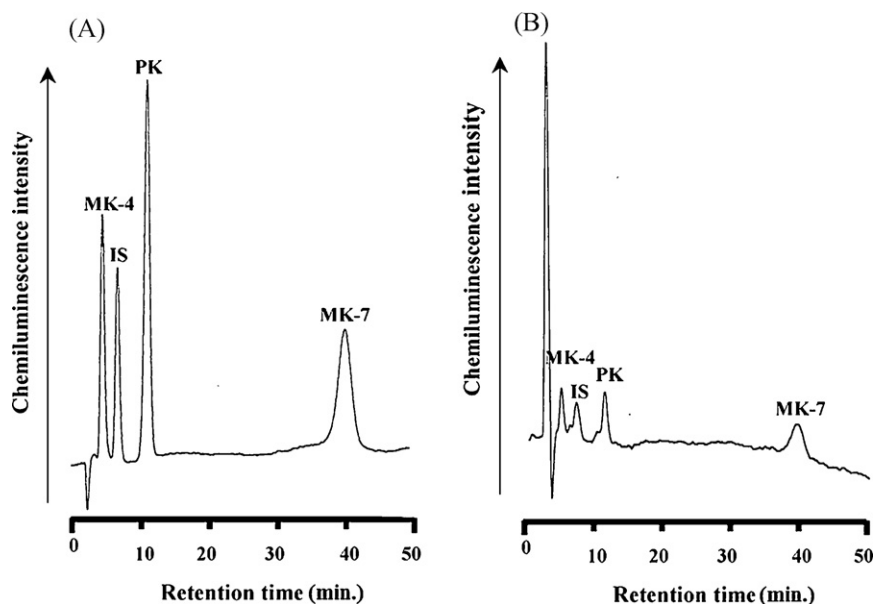[c] Data presented as mean $\pm$ SD of three experiments.

Fig. 5. A typical chromatogram of (A) a mixture of 0.5 µg mL^{-1} standard vitamin K homologues and internal standard (B) extract of plasma sample of RA patient spiked with internal standard and analyzed by the proposed HPLC-CL method.

Table 2
Accuracy and precision of the proposed method for the determination of vitamin K homologues in human plasma.

Sample	Concentration (ng mL^{-1})	Intra-day assay ($n = 5$)		Inter-day assay ($n = 3$)	
		Accuracy (%)	Precision (RSD%)	Accuracy (%)	Precision (RSD%)
PK	0.2	91.2	7.2	105.9	4.7
	10	99.8	5.1	92.4	5.0
	100	100.7	4.7	101.1	3.5
MK-4	0.2	89.5	5.5	104.1	6.1
	10	93.6	6.4	96.3	4.4
	100	95.3	2.7	100.8	2.9
MK-7	0.2	100.5	7.6	107.3	6.0
	10	96.1	4.9	98.6	2.6
	100	90.7	5.3	94.7	3.8

Table 3
Robustness of the developed HPLC-CL method.

Parameter	% recovery ± S.D.		
	PK	MK-4	MK-7
No variations	95.4 ± 3.5	93.1 ± 4.4	92.4 ± 5.5
Coil length			
4.5 m	89.3 ± 4.5	92.6 ± 3.9	91.7 ± 4.0
5.5 m	95.5 ± 5.2	96.3 ± 4.8	95.0 ± 6.1
Photoreactor temp.			
20 °C	91.7 ± 0.51	90.4 ± 3.7	88.4 ± 5.1
30 °C	97.5 ± 3.5	99.2 ± 2.5	94.6 ± 5.6
Buffer conc.			
0.45 mol L^{-1}	95.7 ± 2.9	94.8 ± 3.6	90.3 ± 5.7
0.55 mol L^{-1}	97.9 ± 3.8	96.3 ± 4.7	93.6 ± 4.9
Buffer pH			
pH = 8.5	95.7 ± 2.9	94.8 ± 3.6	90.3 ± 5.7
pH = 9.5	97.9 ± 3.8	96.3 ± 4.7	93.6 ± 4.9
L-012 conc.			
2 μmol L^{-1}	89.7 ± 5.9	90.8 ± 3.3	91.9 ± 3.4
4 μmol L^{-1}	96.5 ± 3.5	96.8 ± 3.1	93.6 ± 4.5
NaOH conc.			
80 mmol L^{-1}	91.2 ± 3.2	90.1 ± 4.5	92.0 ± 2.8
120 mmol L^{-1}	92.8 ± 4.1	90.9 ± 5.2	94.6 ± 2.5
Flow rate			
0.6 mL min^{-1}	95.4 ± 2.9	95.0 ± 4.9	90.1 ± 5.0
0.8 mL min^{-1}	96.1 ± 3.7	97.7 ± 5.1	94.3 ± 4.3

culating levels of vitamin K homologues of RA patients and healthy subjects were determined (Table 4). It was found that the circulating levels of vitamin K$_2$ homologues (MK-4 and MK-7) of RA patients are significantly lower than those of healthy ones. The obtained results for the determination of vitamin K concentrations in healthy subjects were quite similar to those obtained by the reported methods [27–29]. On the other hand, some of these methods could not quantify lower concentrations of vitamin K homologues in RA patients that may be attributed to the lower sensitivity and selectivity of these methods. These results suggest that the accuracy of quantitative determination of vitamin K homologues by developed HPLC-CL method is sufficient for clinical investigations of vitamin K homologues in RA patients.

Table 4
Plasma circulating levels of vitamin K homologues of healthy subjects and RA patients.

Subjects	No. of subjects and sex	MK-4 (ng mL^{-1})	MK-7 (ng mL^{-1})	PK (ng mL^{-1})
Healthy subjects	7 F, 8 M	1.43 ± 0.27	2.93 ± 0.33	2.24 ± 0.34
RA subjects	8 F, 2 M	0.56 ± 0.15	0.9 ± 0.12	1.87 ± 0.35

F, female; M, male.

4. Conclusion

A new highly sensitive and selective HPLC method with luminol CL detection was developed and validated for selective determination of vitamin K homologues in RA patients. The method does not need the addition of oxidant or any catalyst; therefore, it allows high sensitive and selective quantitation of low concentrations of vitamin K homologues in the presence of co-administered medicaments. The proposed HPLC-CL method was 2–1000 times more sensitive compared with the reported methods [21–28] and with similar sensitivity to LC–MS method [29]. However, the simplicity and low cost make the developed method more superior to LC–MS method in routine assay of vitamin K homologues. The proposed HPLC-CL method was successfully used to measure vitamin K homologues in plasma of RA patients as well as healthy subjects. It was found that vitamin K$_2$ concentrations were significantly lower in RA patients than those in healthy subjects. Finally, the proposed method may provide a useful tool for monitoring vitamin K homologues in different clinical studies such as RA, osteoporosis and hepatocellular carcinoma in which vitamin K is supposed to be one of targets for the intervention.

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