Flow injection determination of vitamin K_3 by a photoinduced chemiluminescent reaction

The Analyst

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The determination of vitamin K_3 using the coupling between a photochemical reaction and a chemiluminescent reaction in a flow system is described. The method is based on the photooxidation of ethanol sensitized by vitamin K_3 to yield hydrogen peroxide, which is monitored through the chemiluminescent reaction with luminol catalysed by hematin. The new approach allows the determination of vitamin K_3 in a wide concentration range (1×10^{-7}–5×10^{-4} mol l^{-1}) with a throughput of 30 samples h^{-1}. The applicability of the method was demonstrated by the determination of vitamin K_3 in pharmaceutical preparations.

Introduction

Compounds in the vitamin K series are 2-methyl-1,4-naphthoquinones, which are substituted with side-chains at carbon 3. The two principal natural classes are the phylloquinones (K_1 type) synthesized in plants and the menaquinones (K_2 type) of bacterial origin. Several synthetic analogues and derivatives have been used, most related to or derived from menadione (K_3), which lacks a side-chain substituent at position 3. The K vitamins are insoluble in water but soluble in solvents used to dissolve fat-soluble substances. In a sense, the menadione-type compound is a synthetic provitamin, since K_3 is converted into menaquinone by addition of the side-chain in the liver. The interest in vitamin K_3 has also grown because of its anticancer activity.[1,2]

A variety of manual methods have been developed for the determination of menadione using different techniques, such as spectrophotometry,[3,4] polarography,[5] fluorimetry[6,7] and stripping voltammetry.[8,9] Liquid chromatography with different detection systems has also been used. Early methods used ultraviolet spectrophotometric detectors.[10] Electrochemical[11] and fluorimetric detection offer increased sensitivity and selectivity, but the quinone form of vitamin K_3 lacks native fluorescence and has to be reduced to its hydronaphthoquinone form prior to fluorescence detection. Post-column chemical ($NaBH_4$ or Zn metal) reduction,[12,13] photochemical reduction[14] and electrochemical reduction[15] have been described. Gas–liquid chromatographic methods with flame ionization detection have been reported, but are not sufficiently sensitive.[16]

Recently, two fluorimetric methods have been reported for the determination of a water-soluble derivative of vitamin K_3, menadione hydrogensulfite (MSB). In the first procedure, the photochemical reduction of MSB sensitized by acetone is carried out in an 18 μl cell with UV radiation from the light source of the spectrofluorimeter.[17] The second method uses a flow injection assembly provided with a solid-phase reactor with immobilized zinc, where MSB is hydrolysed in alkaline medium and reduced by zinc in an acidic medium.[18] The reported detection limits were 4.4×10^{-9} and 2.9×10^{-8} mol l^{-1}, respectively. Both procedures were applied to the determination of menadione in pharmaceutical formulations.

In the last few years there has been increased interest in using chemiluminescence (CL) to determine trace and ultratrace concentrations of inorganic and organic species.[19,20] The most notable characteristics of CL methods are the higher sensitivity and greater dynamic concentration range that can be measured than with other analytical methods. CL analysis is also useful because the instrumentation necessary is very simple. Furthermore, the introduction of flow injection analysis (FI) has made CL methods even more attractive because it is possible to mix sample and reagents rapidly with high reproducibility.[21,22] Recently, it has been established that the irradiation of photoreactive analytes leads to the formation of species that can be detected by CL. However, until now, such photochemical–CL detectors have rarely been used in simple hydrodynamic systems such as FI.[23,24]

This paper presents a sensitive FI method for the determination of vitamin K_3 through a photoinduced chemiluminescent reaction. It is based on the photooxidation of ethanol sensitized by vitamin K_3. The hydrogen peroxide formed in the photochemical reaction is monitored by means of the CL reaction with luminol and hematin. The proposed method has been applied satisfactorily to the determination of vitamin K_3 in commercial pharmaceutical preparations.

Experimental

Reagents

All chemicals were of analytical-reagent grade and ultrapure water obtained from a Milli-Q system (Millipore Ibérica, Madrid, Spain) was used throughout.

A stock standard solution of menadione (172.2 mg l^{-1}) was prepared by dissolving the product from Sigma (St. Louis, MO, USA) in ethanol (Merck, Darmstadt, Germany).

A hematin stock standard solution (5×10^{-5} mol l^{-1}) was prepared by dissolving 3 mg of hematin (Sigma) in 1 ml of 0.1 mol l^{-1} sodium hydroxide and diluting to 100 ml with ultrapure water. This solution was freshly prepared each day.

A luminol stock standard solution (1×10^{-3} mol l^{-1}) was prepared by dissolving 17.72 mg of luminol (Fluka, Buchs, Switzerland) in 100 ml of 0.1 mol l^{-1} phosphate buffer (pH 12). Working standard solutions were prepared daily from the stock standard solution by appropriate dilution with the buffer.

Apparatus

Measurements of the emitted light were performed on a Bio Orbit (Turku, Finland) luminometer connected to a personal computer fitted with Bio Orbit software. A Gilson (Villiers-le-Bel, France) Minipuls peristaltic pump was used to pump all reagent solutions. Two rotary valves (Omnifit, Cambridge, UK) were also used. Except for the pump tubing (Tygon), PTFE tubing (0.5 mm id) was used throughout the manifold. The cell (Hellma, Müllheim, Germany) had an inner volume of 25 µl and exposed an aperture of 1.5 × 8.5 mm to the adjacent photomultiplier tube.

The photoreactor was a 60 cm length of PTFE tubing (0.5 mm id) coiled around a rod-shaped low pressure mercury discharge lamp (Spectronic, Westbury, NY, USA). This assembly was housed in a metal box covered with aluminium foil to increase the photon flux by reflection.

A timer synchronized to the injection system allowed the flow to be stopped at any delay time and for any length of time.

Results and discussion

Photochemical reaction between menadione and ethanol

Photochemical reactions between 9,10-anthraquinones and hydrogen atom-donating substrates have been widely studied.[25–29] The primary step is the absorption of a photon by menadione. After excitation, menadione rapidly decays to its excited singlet state (S_1) by internal conversion. Most of the S_1 menadione molecules undergo intersystem crossing to the triplet state and subsequent internal conversion to the lowest excited triplet state (T_1). Once formed, the T_1 state of menadione rapidly extracts a hydrogen atom from ethanol to produce two radical products, the semiquinone form of menadione and α-hydroxyethyl radical. These two radical intermediates have two possible reaction paths depending on the experimental conditions. Under aerobic conditions, both intermediates will react with molecular oxygen to produce hydrogen peroxide, menadione and the oxidized substrate; that is, ethanol is oxidized, oxygen is reduced and menadione is regenerated. This photochemical scheme is called photooxidation, because ethanol is oxidized by oxygen in the presence of the sensitizer (menadione):

$$Q\ (S_0) \xrightarrow{h\nu} Q\ (S_1) \xrightarrow{\text{ISC}} Q\ (T_1)$$
$$Q\ (T_1) + CH_3CH_2OH \longrightarrow QH^{\bullet} + CH_3\dot{C}HOH$$
$$2\ QH^{\bullet} + O_2 \longrightarrow 2Q + H_2O_2$$
$$2\ CH_3\dot{C}HOH + O_2 \longrightarrow 2\ CH_3CHO + H_2O_2$$

In contrast, in an anaerobic environment, the two radical intermediates rapidly disproportionate and produce the oxidized substrate and the reduced quinone (dihydromenadione). This photochemical reaction pathway can be referred to as photoreduction, because menadione is reduced:

$$CH_3\dot{C}HOH + Q \longrightarrow QH^{\bullet} + CH_3CHO$$
$$2\ QH^{\bullet} \longrightarrow QH_2 + Q$$

Two approaches can be used for determining vitamin K_3 through the photochemical reaction with ethanol: either the hydrogen peroxide produced in the photooxidation pathway or the dihydromenadione formed in the photoreductive pathway can be measured.

To evaluate the rate at which oxygen destroys the reduced vitamin K_3, oxygen was introduced after the photoreduction step. Dihydromenadione was very rapidly oxidized back to menadione, demonstrating that efficient deoxygenation is a requirement for the photoreduction pathway.

Because of the difficulty involved in deoxygenating sample and reagents in FI systems, the photooxidation pathway was selected for the FI determination of vitamin K_3. The hydrogen peroxide generated in the photochemical reactor was monitored through the CL reaction with luminol catalysed by hematin.

CL reaction

In order to measure the maximum CL intensity, the mixing order of the reagents and the time profile of the CL were examined with the stopped-flow system. It was found that the emission intensity was dependent on the way in which the reagent solutions were mixed. The most intense CL signal was obtained when the solution containing menadione and ethanol once photolysed was mixed with the alkaline buffer before the addition of hematin and luminol. On the other hand, the light emission occurred at high speed and reached the maximum intensity within a few seconds. This clearly suggests that the sample and the CL reagents should be mixed as close as possible to the detection flow cell.

The buffer constituents (carbonate, borate and phosphate) had little influence on the emission time response. Phosphate buffer provided the highest peak and this was used in all subsequent experiments.

Flow injection manifold

Because the photochemical and CL reactions must be conducted at different pH values, the FI configuration used for the determination of vitamin K_3 was designed to provide the best conditions for each reaction in order to measure the stronger signal.

The FI manifold finally adopted is shown in Fig. 1. The sample and ethanol were injected simultaneously into the carrier streams with the aid of two rotary valves and then synchronously merged before they reached the irradiated reactor. When the sample zone was located in the photochemical reactor, the flow was stopped and the irradiation was carried out with the UV lamp. After the irradiation, the pump was triggered by the timer and the solution emerging from the photoreactor (containing the H_2O_2 generated in the photochemical reaction) was merged 10 cm downstream with a stream of phosphate buffer (pH 12) and merged another 10 cm downstream with the reagent stream (luminol plus hematin) at a PTFE Y-confluence in front of the flow cell. The distance from the Y-confluence to the inlet point of the detector was set at about 5 cm, which is as short as it can possibly be. The tubing of the reagent and buffer streams and that from the photoreactor to the flow cell were covered with black insulating tape to prevent a fibre optic effect introducing stray light into the detector.

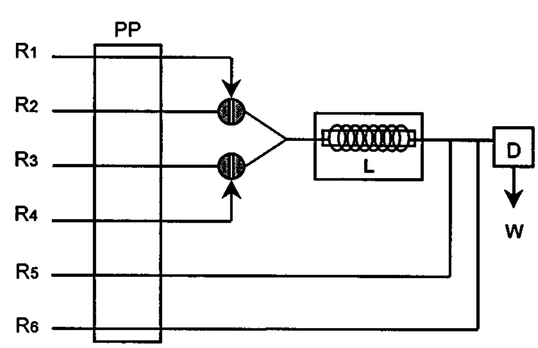

Fig. 1 Flow injection manifold for the determination of vitamin K_3. PP = peristaltic pump; R_1 = sample; R_2 and R_3 = 0.05 mol l^{-1} phosphate buffer of pH 7.5; R_4 = ethanol; R_5 = 1 mol l^{-1} phosphate buffer of pH 12; R_6 = 2×10^{-4} mol l^{-1} luminol and 6×10^{-6} mol l^{-1} hematin; L = photoreactor; D = detector; W = waste.

Influence of photochemical reaction variables

The effect of pH on the photooxidation of ethanol sensitized by vitamin K_3 is shown in Fig. 2. The rate of the photochemical reaction increased with increasing pH, although alkaline media must be avoided since vitamin K_3 undergoes a photodegradation reaction.[30] A pH of 7.5 was chosen for further experiments. The concentration of the phosphate buffer used as carrier (R_2 and R_3 in the manifold) must be such that the solution in the photoreactor is buffered at pH 7.5; however, its concentration should be as low as possible so that the buffering capacity of the added buffer stream can ensure the optimum alkaline conditions of the CL reaction. A 0.05 mol l^{-1} phosphate buffer of pH 7.5 was used as the carrier.

The development of the photochemical reaction with the length of the irradiation time is shown in Fig. 3. From the kinetic curves, it can be seen that the maximum CL was obtained with a 1 min irradiation time, and this value was selected in subsequent work.

Influence of CL reaction variables

The reaction between hydrogen peroxide and luminol catalysed by hematin only gives CL in a basic solution.[31] The emission intensity increased when the pH in the flow cell increased from 8 to 12, above which it became constant. A high-capacity phosphate buffer (1 mol l^{-1}) of pH 12 was used as an alkalinizing stream so that the pH, after mixing with the other streams, was as close as possible to the optimum value.

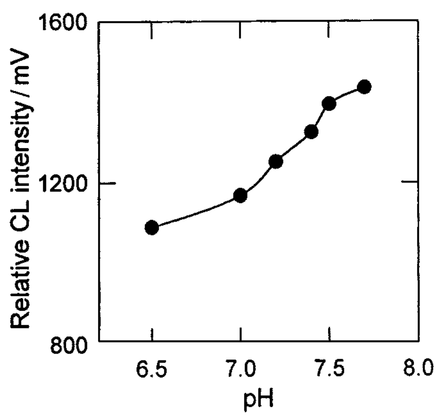

Fig. 2 Effect of pH on the photochemical reaction. [Menadione] = 5×10^{-5} mol l^{-1}. Concentrations of luminol and hematin as in Fig. 1.

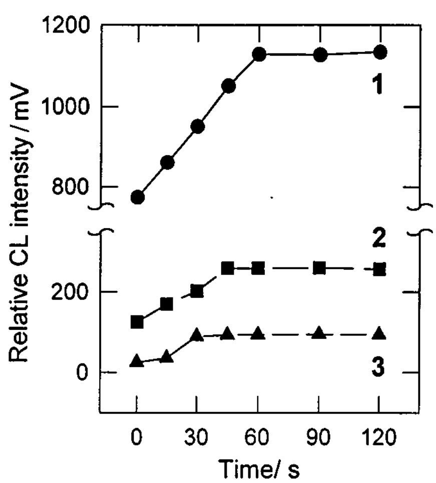

Fig. 3 Influence of the irradiation time on the peak height. [Menadione] = (1) 5×10^{-5}, (2) 1×10^{-5} and (3) 1×10^{-6} mol l^{-1}. Concentrations of the reagent streams as in Fig. 1.

The concentrations of luminol and hematin were studied using separate channels for each reagent. The luminol and hematin concentrations which give the maximum signal can be deduced from Fig. 4 and 5, respectively. A solution containing 2×10^{-4} mol l^{-1} luminol and 6×10^{-6} mol l^{-1} hematin was selected as the reagent stream for subsequent experiments.

Influence of FI parameters

After selecting the optimum conditions for the photochemical and CL reactions, we also found that the CL intensity was related to the parameters of the FI system, such as the volumes of vitamin K_3 and ethanol injected and the total flow rate.

The volumes of vitamin K_3 solution and ethanol injected varied between 35 and 300 μl. The peak height increased with increasing volume up to 125 μl for vitamin K_3 and 270 μl for ethanol, above which it remained virtually constant. A sample volume of 125 μl and a volume of 270 μl for ethanol were chosen.

Determination of the total flow rate is important because the speed of the CL reaction and the time lapsed from the confluence of the irradiated sample and luminol–hematin streams until the solution leaves the flow cell must be matched as closely as possible. A total flow rate of 8.5 ml min^{-1} provided a constant and maximum signal when the flow rates of each solution were the same and the distance between the confluence point and the inlet of the flow cell was 5 cm.

Analytical features

Under optimum conditions, the effect of the concentration of vitamin K_3 on the CL intensity was studied by measuring the

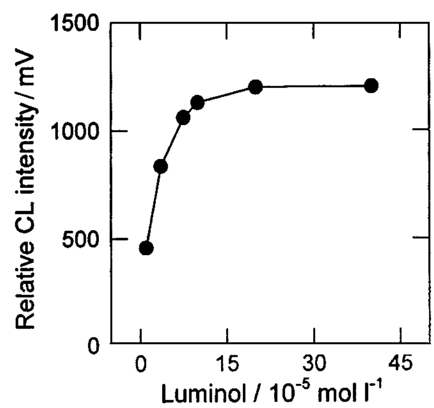

Fig. 4 Influence of luminol concentration on the peak height. Sample injected, 125 μl of 5×10^{-5} mol l^{-1} menadione. Concentration of the other reagent streams as in Fig. 1.

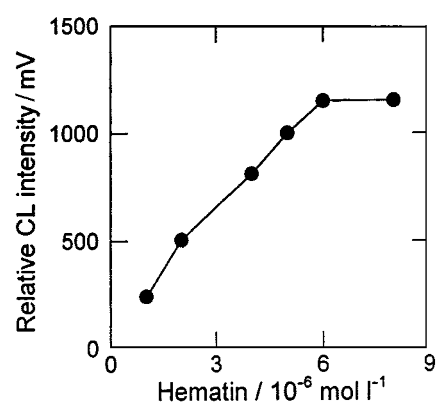

Fig. 5 Influence of hematin concentration on the peak height. Other conditions as in Fig. 4.

peak height when 125 μl of vitamin K_3 solution of different concentrations were injected. The calibration graph gave a straight line from 1×10^{-7} to 5×10^{-4} mol l^{-1} with a correlation coefficient of 0.999. The relative standard deviations (RSDs) of 10 injections of each solution containing 4×10^{-7} and 7×10^{-6} mol l^{-1} of vitamin K_3 were 1.23 and 0.46%, respectively. The detection limit calculated according to IUPAC recommendations[32] was 2.03×10^{-9} mol l^{-1}. The sample throughput was 30 h^{-1}. The reproducibility of the method was studied by analysing, on five different days, 10 identical solutions of menadione (2.0×10^{-6} mol l^{-1}). Every day three injections of each solution were made; the RSD was 1.20%.

The proposed method shows high versatility since it is possible to work at different sensitivity ranges depending on the needs of the analysis. If a high degree of sensitivity is required, it is simply necessary to increase the irradiation time. It should be noted that it is not necessary to stop the flow, thus maintaining a high throughput (60 samples h^{-1}) although with lower sensitivity (concentration range 10^{-6}–10^{-3} mol l^{-1}).

Applications

In order to study the validity of the method, it was applied to the determination of vitamin K_3 in two commercial pharmaceutical formulations: Vitaendil CKP (Waserman, Barcelona, Spain) and Caprofides Hemostático (Fides-Rottapharm, Valencia, Spain). Previously, the influence of foreign substances that can commonly accompany menadione in pharmaceuticals was studied. Solutions of vitamin K_3 and the foreign compound were mixed to obtain samples containing 1.72 μg ml^{-1} of vitamin K_3 and various concentrations of each interferent. The interferent concentrations tested and the relative errors produced were as follows: lactose, 720 μg ml^{-1}, 1.3%; saccharin, 410 μg ml^{-1}, 1.1%, sucrose, 171 μg ml^{-1}, 2%; sodium cyclamate, 100 μg ml^{-1}, 1.7%; aminocaproic acid, 66 μg ml^{-1}, 2.2%; starch, 500 μg ml^{-1}, 1.2%; benzyl alcohol, 500 μg ml^{-1}, 1.6%; and sorbitol, 500 μg ml^{-1}, 1.1%. Ascorbic acid and sulfite were found to interfere because they react with the H_2O_2 generated in the photochemical reaction; however, this does not limit the applicability of the method because this effect can be minimized by retaining these anions on an Ionenaustauscher III (Merck) anion exchanger minicolumn when the sample is passed through the column prior to injection into the flow system.

For the determination of vitamin K_3 in Caprofides Hemostático, an amount of drops equivalent to 0.1 mg of menadione was transferred into a 25 ml calibrated flask and diluted to the mark with ethanol. If the solution becomes cloudy it should be filtered before injection into the FI system.

For analysing the Vitaendil CKP formulation, several tablets were powdered. The powder equivalent to 0.5 mg of menadione was shaken with about 10 ml of water in a water-bath at 60 °C for 5 min in order to separate the water-soluble components. After separating the residue by filtration it was washed twice with 2 ml of water and dissolved in 25 ml of ethanol. This solution was decoloured with activated charcoal and injected into the flow system.

The data in Table 1 show that the vitamin K_3 contents measured by the proposed method were in good agreement with the values supplied by the manufacturers and with those obtained by a manual fluorimetric method. The recoveries obtained by adding menadione to each pharmaceutical formulation are given in Table 2.

Table 1 Determination of vitamin K_3 in pharmaceutical preparations

Pharmaceutical preparation[a]	Content/mg per tablet or ml	Amount found/mg	
		Proposed method[b]	Reference method
Caprofides Hemostático	0.10	0.10 ± 0.02	0.16
Vitaendil CKP	5.0	4.96 ± 0.04	5.01

[a] Composition of samples: Caprofides Hemostático (amount per ml), menadione 0.1, aminocaproic acid 200, esculin 1, hesperidin 5, creatinine 0.05, saccharin 0.5, ethanol 5 and sodium cyclamate 5 mg; Vitaendil CKP (amount per tablet), menadione 5, ascorbic acid 75, rutoside 25, sucrose 174 and lactose, 58 mg. [b] Average values of four determinations ± *s*.

Table 2 Recoveries of vitamin K_3 added to real pharmaceutical formulations

Sample	Amount added/mg	Amount found/mg	Recovery (%)[a]
Caprofides Hemostático	0.2	0.21	105 ± 2.6
	0.5	0.48	96 ± 1.9
	1.0	0.98	98 ± 1.7
Vitaendil CKP	1.0	0.95	95 ± 2.7
	2.5	2.58	103 ± 1.3
	5.0	4.97	99 ± 1.6

[a] Average values of four determinations ± *s*.

Conclusions

The on-line coupling of a photochemical reaction and a CL reaction in a flow injection system allowed us to develop a sensitive method for determining vitamin K_3. The photochemical reaction selected was the photooxidation of ethanol sensitized by vitamin K_3. The hydrogen peroxide formed in this photochemical reaction was monitored through the CL reaction with luminol catalysed by hematin.

The proposed method provides a simple approach for the determination of vitamin K_3 at nanogram levels. The dynamic range of the method is very wide (1×10^{-7}–5×10^{-4} mo l^{-1} menadione) with a throughput of 30 samples h^{-1}. The method is useful for determining vitamin K_3 in pharmaceuticals.

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