Enhanced Sensitivity for the Determination of Endogenous Phylloquinone (Vitamin K_1) in Plasma Using High-performance Liquid Chromatography with Dual-electrode Electrochemical Detection

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High-performance liquid chromatography (HPLC) with dual-electrode electrochemical detection has been used, in the redox mode, to determine normal and sub-normal circulating plasma levels of phylloquinone (vitamin K_1) and concentrations down to 20 pg ml^{-1} have been measured in plasma. The coefficient of variation, for endogenous levels, was 10% (mean 330 pg ml^{-1}; $n = 6$). When compared with a single glassy carbon electrode, operated in the reductive mode, the sensitivity of the dual-electrode cell was greater by at least an order of magnitude. Thus the volume of plasma required for the assay could be reduced.

Keywords: *Phylloquinone (vitamin K_1) determination; dual-electrode electrochemical detection; high-performance liquid chromatography*

Recently, we have described an assay for the determination of normal circulating phylloquinone (vitamin K_1) levels in plasma using high-performance liquid chromatography (HPLC) with electrochemical detection (LCEC).[1] The detector consisted of a thin-layer cell containing a glassy carbon working electrode and was operated in the reductive mode. This method was about three times more sensitive than that developed by Shearer *et al.*[2] who employed HPLC with ultraviolet detection.

Our current studies required a method with even greater sensitivity, as we needed both to reduce the volume of plasma required for the assay and to determine sub-normal levels of vitamin K_1.

A relatively recent approach to improving the sensitivity, and selectivity, of LCEC is to use dual-electrode cells,[3] where the electrodes are arranged either in series,[4,5] or in parallel.[6,7] In the series configuration it has been shown that a product, formed by reduction at the first electrode, can be detected downstream at a second electrode by re-oxidation at a much lower working potential than by using reduction alone. In addition, there is no interference from oxygen at the detector electrode because the reduction reaction for this molecule is not reversible at the potentials applied.

Haroon and Hauschka[8] have shown that series dual-electrode LCEC may be used for the determination of phylloquinone and menaquinones (vitamins K_2) in biological materials. However, their studies were only preliminary and they did not extend their investigations to the determination of sub-normal plasma levels.

We have also investigated this technique, but we used our original apparatus and mobile phase, simply substituting the dual-electrode cell for the thin-layer cell. The purpose of this paper is to describe the optimisation of the conditions for the determination of vitamin K_1, by redox-mode LCEC, using a cell containing two porous graphite electrodes in series. The application of this technique to the determination of both normal and depressed circulating plasma levels of this vitamin is also described. The sensitivity achieved with the dual-electrode cell is compared with that of the thin-layer cell of our earlier method.[1]

Experimental

Chemicals and Reagents

All chemicals were of analytical-reagent grade, unless otherwise stated. Phylloquinone (an isomer mixture containing 70% *trans* and 30% *cis*) was obtained from Sigma Chemical Company. Solvents used for HPLC were of HPLC grade and obtained from Rathburn Chemicals.

The procedures used for the preparation of 95% methanol - 0.05 M acetate buffer (pH 3.0), which was used as the mobile phase and for preparing standard phylloquinone solutions, together with the deaeration procedure, have been described previously.[1]

Apparatus

LCEC was performed with a constant-flow reciprocating pump (Model 300 from Applied Chromatography Systems). In the reductive mode LCEC was performed with a Bioanalytical Systems electrochemical detection system; in the redox mode a Model 5100A Coulochem detector equipped with a dual-electrode cell (Model 5011) containing two porous graphite electrodes in series (Environmental Science Associates) was used.

Optimisation of the Conditions for LCEC

To optimise the applied potentials for LCEC, hydrodynamic voltammetry was performed by injecting fixed volumes of a standard vitamin K_1 solution and varying the applied potential in 50-mV steps. For detection in the redox mode two voltammograms were constructed: the first was obtained by keeping the potential of the downstream electrode constant at +0.2 V and varying the potential of the upstream electrode between −0.5 and −1.5 V; the second was obtained by keeping the upstream electrode constant at −1.3 V and varying the potential of the downstream electrode between −0.3 and +0.5 V.

Method for the determination of vitamin K_1 in plasma by redox-mode LCEC

Generally, about 3 ml of plasma were extracted for the assay of vitamin K_1 in the normal subjects and about 5 ml for the osteoporotic patients with a fractured neck of femur. These plasma samples were extracted with hexane and further purified by semi-preparative HPLC as described previously.[1,2] The residues from the second purification step, involving normal-phase HPLC, were dissolved in 70 µl of mobile phase with the aid of a vortex mixer; usually, 30 µl of these solutions were injected into a Spherisorb (5 µM) octyl column (25 cm × 5 mm i.d.). The flow-rate was 1 ml min^{-1} and the applied

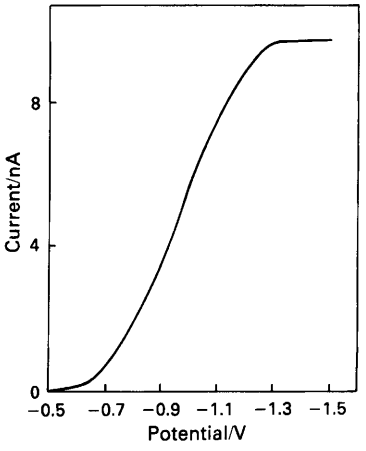

Fig. 1. Cathodic hydrodynamic voltammogram for 1-ng injections of phylloquinone. Downstream electrode held constant at +0.2 V

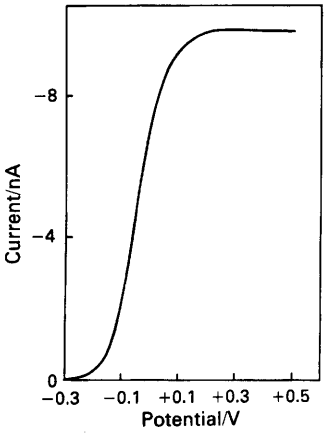

Fig. 2. Anodic hydrodynamic voltammogram for 1-ng injections of phylloquinone. Upstream electrode held constant at −1.3 V

potentials were −1.3 V at the upstream electrode and 0 V at the downstream electrode.

Calibration, effect of dissolved oxygen and precision in redox-mode LCEC

Calibration graphs were constructed by injecting between 50 pg and 1 ng of vitamin K_1 dissolved in the mobile phase. Peak heights were measured from the chromatograms and graphs of peak height *versus* amount injected were constructed. This procedure was performed both without deaeration and with deaeration by the method previously described.[1]

To determine the precision of the assay six 3-ml aliquots of plasma were taken from the same normal subject (female, aged 53 years); these were extracted and measured by the method described above.

Results and Discussion

Optimisation of Redox-mode LCEC Conditions

The principle of operation of the series dual-electrode cell, for redox-mode detection of vitamin K_1, is as follows: the first electrode (upstream electrode) is held at a potential where the quinone form of the vitamin is reduced to the hydroquinone form; this is then detected at the second electrode (downstream electrode) where the hydroquinone is reoxidised to the quinone.[8]

In order to determine the optimum potentials to apply to the two electrodes, two hydrodynamic voltammograms were constructed (Figs. 1 and 2).

It is apparent from these voltammograms that the maximum current occurred at a potential of −1.3 V at the upstream electrode and +0.2 V at the downstream electrode. However, when these conditions were used for the analysis of some plasma samples an interfering pre-peak overlapped with the vitamin K_1 peak. This interference could be reduced significantly by simply decreasing the applied potential of the downstream electrode below +0.2 V. The results shown in Table 1 were with an applied potential of 0 V, although in more recent studies potentials of 0 to +0.1 V have been found suitable (results not given).

When the redox-mode LCEC system was being used on a routine basis it was found beneficial to leave the mobile phase recycling overnight with the detector set at the operating potentials. This procedure eliminated the time required to stabilise when first activating the electrochemical detector and also reduced the risk of losing sensitivity.

Occasionally, the sensitivity decreased to an unacceptable level; this may have been due to adsorption of the hydroquinone form of vitamin K_1 and/or co-extracted plasma compounds, on to the surface of the electrodes. However, the sensitivity could often be restored by recycling the mobile phase overnight with the downstream and upstream electrodes set at −0.5 and +1.3 V, respectively. If this procedure was unsuccessful, the cell was detached from the HPLC system and treated by one, or both, of the following procedures: in the first the cell was flushed sequentially by means of a syringe, with water, 6 M nitric acid, water and finally methanol; in the second, the cell was flushed sequentially with water, tetrahydrofuran, 2 M sodium hydroxide solution, water and finally methanol.

Calibration, Effect of Dissolved Oxygen and Precision in Redox-mode LCEC

The calibration graph of peak height *versus* mass of vitamin K_1 injected was linear in the range 50 pg–1 ng; there was no change in the slope of the graph when the mobile phase was deaerated. Therefore, all subsequent investigations were carried out without deaerating the mobile phase.

The coefficient of variation for six plasma samples, taken from the same normal subject, was 10% and the mean value was 330 pg ml^{-1}; this was not included in the results given in Table 1.

Comparison of the Sensitivities of a Single Glassy Carbon Electrode Cell (TL5) and a Dual Porous Graphite Electrode Cell

When compared with a single glassy carbon electrode cell, operated in the reductive mode, the peak height obtained with a dual-electrode cell was greater by at least an order of magnitude (Fig. 3). This is perhaps not surprising as the surface areas of the porous graphite electrodes are both greater than that of the glassy carbon electrode; therefore, higher concentrations of vitamin K_1 would be expected to be electrolysed.

It should be mentioned that the detection system, incorporating the TL5 cell, provides current ranges below that used in our investigations, *i.e.*, 5 nA; however, these ranges could not be used for the assay, to amplify further the vitamin K_1 signal, because the base line was found to drift too rapidly; there was also interference from oxygen that was present in the injected sample.

Determination of Normal and Sub-normal Circulating Vitamin K_1 Levels by Redox-mode LCEC

Fig. 4 shows the chromatograms obtained with the dual-electrode cell for a normal subject and an osteoporotic patient with a fractured neck of femur. In both cases well defined

Table 1. Endogenous plasma levels of phylloquinone in normal subjects and osteoporotic patients with a fractured neck of femur

Normal subjects				Osteoporotic subjects			
Subject's initials	Age	Sex	Phylloquinone plasma concentration/ pg ml^{-1}	Subject's initials	Age	Sex	Phylloquinone plasma concentration/ pg ml^{-1}
P.F.	51	F	950	L.W.	69	F	70
A.S.	57	F	460	M.M.	81	F	30
J.C.	59	M	430	D.M.	64	F	70
M.C.	64	F	130	I.B.	75	F	140
B.C.	64	F	80	B.J.	83	F	110
A.G.	53	F	90	W.B.	75	F	110
F.M.	69	M	330	L.T.	88	F	70
B.S.	54	F	120	B.C.	63	F	20
H.K.	69	M	240	S.W.	80	M	80
F.R.	69	M	580	J.F.	81	F	60
L.M.	65	F	430	E.C.	81	F	80
B.P.	62	F	530	E.L.	72	F	250
B.D.	62	F	210	E.H.	80	F	40
J.O.	72	M	520	M.T.	74	F	40
J.M.	81	F	540	E.H.	79	F	80
				E.W.	86	F	310
Mean			376	Mean			98

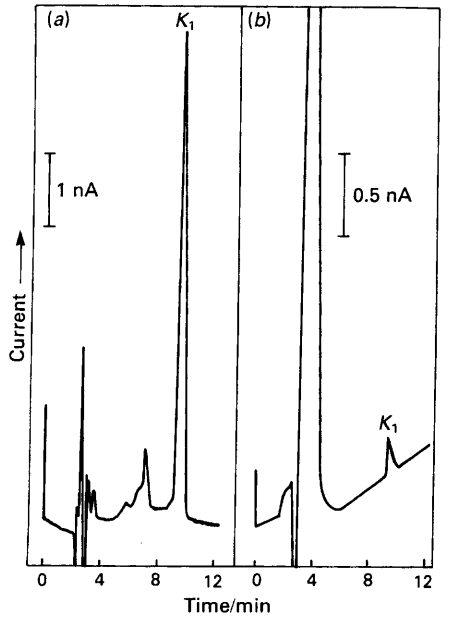

Fig. 3. Chromatograms obtained for 1-ng injections of phylloquinone using LCEC in: (*a*) redox mode; and (*b*) reductive mode

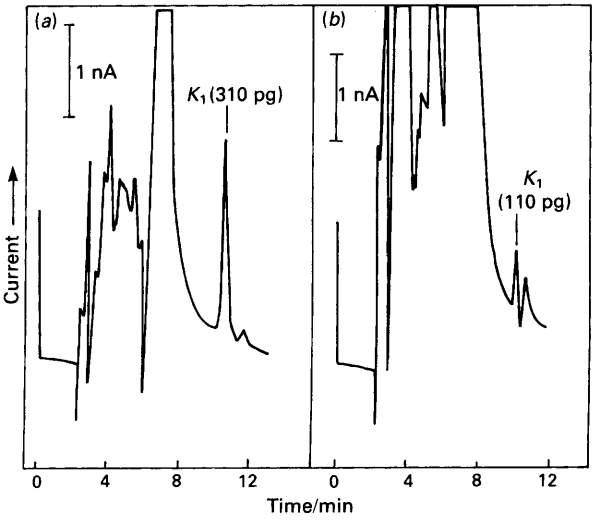

Fig. 4. Chromatograms of plasma samples obtained by LCEC in the redox mode for: (*a*) a normal subject (endogenous concentration of 240 ng ml^{-1} in plasma); and (*b*) a patient with osteoporosis and fractured neck of femur (endogenous concentration of 60 pg ml^{-1} in plasma)

peaks were obtained; the injected volumes were equivalent to only 1.3 and 1.8 ml of plasma, respectively.

The limit of detection of this method is obviously a function of both plasma volume and vitamin K_1 concentration. Under the conditions described earlier, for this particular study, down to 50 pg of vitamin K_1 could be measured; therefore, an injected sample containing the vitamin K_1 from 2.5 ml of plasma was required to measure 20 pg ml^{-1}.

The results obtained for circulating vitamin K_1 levels in the osteoporotic patients, and the age matched normals, are summarised in Table 1; these results and their possible clinical significance have been discussed elsewhere.[9,10] We are also investigating vitamin K_1 levels in other disorders of bone and envisage that this work will be reported at a later date. However, it is clear that the technique described in this paper can be applied to other nutritional aspects of vitamin K_1, particularly where low plasma levels may occur.

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