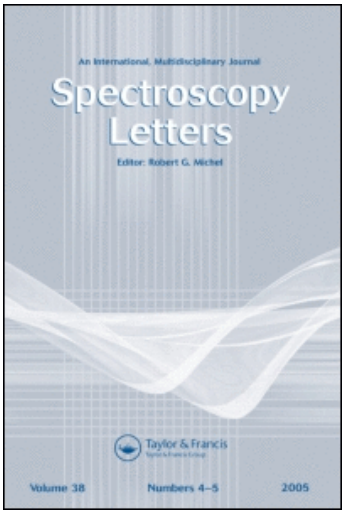

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ESR Determination of Vitamin K_1 Following Photoexitation of a Frozen Solution

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ESR DETERMINATION OF VITAMIN K_1 FOLLOWING PHOTOEXITATION OF A FROZEN SOLUTION

KEY WORDS: Phylloquinone, Photoexitation, Biradicals, ESR Spectrum

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ABSTRACT

It was found that the photoexitation of a frozen solution of vitamin K_1 (phyllonaphtoquinone) gave rise to the formation of biradicals with a very bright spectra ($\Delta H \sim 200$ G), typical for randomly oriented radical pairs with a summary spin of $S = 1$. This spectra was not overlaped by those of the other radical products generated following the photolysis of vitamin K_1 in different matrices. This method is proposed for the determination of vitamin K_1 in biological samples and was successful in following the accumulation of vitamin K_1 in mouse liver following subcutaneous injection.

INTRODUCTION

Vitamin K_1 (2-methyl-3-phytyl-1,4-naphoquinone plays a vital role in the biosynthesis of active prothrombin and other calcium-binding proteins involved in blood clotting (1-3). It also serve as a component in respiratory electron transfer chains (4) and in bacterial photosynthetic reaction centres(5). Therefore the in-

vestigation of the vitamin K_1 concentration in tissues and its metabolism are very important tasks for biochemistry and medicine.

Most current methods for the determination of vitamin K_1 in foods, blood,plasma and tissue homogenates use HPLC (6,7). This method is very sensitive, permiting the determination of vitamin K_1, menadion and vitamin K_1 epoxide in nanogram quantities (6).However, these methods necessitate the use of special equipment and very pure solvents and often require prior purification of the sample to be analyzed.The determinations may be impeded by the presence of other quinones with isoprenoid chains (e.g. ubiquinones,plastoquinones and alpha-tocopherolquinones). As a result, despite their sensitivity, the applicability of current HPLC-based determinations are limited by these restrictions and can not be easily and universally employed.

MATERIALS AND METHODS

Vitamin K_1 was of 99% purity. Ethylbenzene and ethylether, used as solvents, were of spectroscopic grade purity. Photolysis was carried out in quartz tubes using a high pressure mercury lamp DRSh-1000, glass filtered to allow the passage of light only between the region 425-455 nm.

ESR spectra were recorded using an ESR-20 spectrometer (Inst. Chem. Phys., Acad.Nauk USSR) and a BRUKER ER-220. Vitamin K_1 standards were prepared in ethylbenzene. The method was capable of detection down to concentrations of around 10^{-7}g/ml with an error in determination of around 10 % at 5 x 10^{-5} g/ml.

RESULTS AND DISCUSSION

Previously, we demonstrated that photolysis of vitamin K_1 in solution results in the formation of biradicals which are produced after the intramolecular transition of a hydrogen atom from the CH-group to the proximal carbonyl of the naphtoquinone ring. The

biradicals are formed with high quantum yield and have ESR spectra which are typical for randomly oriented paramagnetic species with a summary spin $S = 1$ (Figure 1). When biradicals are generated after irradiation with light of a wavelength near the carbonyl $n \rightarrow \pi^*$ transition band ($\lambda \sim 420$ nm), the whole spectrum is due to absorbtion by the biradicals and is composed of intensive bands corresponding to their perpendicular and parallel orientations in a magnetic field ("perpendicular"-AA' and "parallel"-BB' components of the spectrum). In the centre of the spectrum there is a weak singlet, R, belonging to the naphosemiquinone radical. The dipole-dipole interaction constant is about 20 mT.and hence, the biradical ESR spectrum does not interfere with those of other radicals generated following photolysis of vitamin K_1 in different matrices. In the half-field a forbidden signal, with $\Delta M_s = 2$, is observed.

At 77 K biradicals were seen to be thermally unstable. By kinetically analyzing the annealing process of the biradicals, it was found that there were two species desintegrating with characteristic speeds. It was assumed that the biradicals were distinguishable by the existance of two discrete isomers as in the first isoprene unit there were cis- and trans-chain conformations at the γ-carbon atoms. The average distance between unpaired electrons in the two types of biradicals, as determined by ESR analysis of dipole-dipole interaction constants D, demonstrated that they differ insignificantly, being equal to 0,531 and 0,540 nm. respectively. However, the rates of annealing by these two species is significantly different. The destruction of the biradicals occured as the sum of two first order reactions and the ratio between the rate constant of the fast reaction to the slow was 10:1. New paramagnetic species did not arise on annealing of the biradicals. Differences between the cis- and trans-forms could be observed in experiments involving static microwave saturation of irradiated samples in the cavity of the ESR spectrometer. When the microwave power was changed from 0,01 to 150 mWt, the constant, D, fell from 18,7 to 17,7 mT.

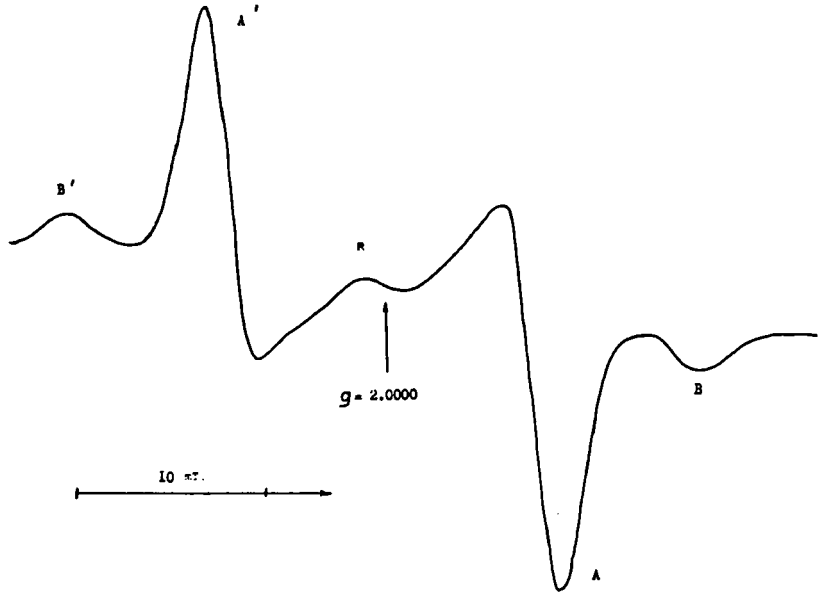

Fig.1. ESR spectrum of irradiated sample of vitamin K_1, 18 hours post-injection. Microwave power is 0.01 mWt

Accordingly, the two types of biradicals display different relaxation charac-teristics. By determination of the initial concentration of biradicals atthe extreme values of saturated microwaves it was posible toevaluate the relationship between the cis- and trans-forms in non-irradiated vitamin K_1. The concentration of the cis-form was 2-3 times higher than the trans-form in all the solvents used.

It has been previously demonstrated that the concentration of a paramagnetic species can be evaluated from the area under its ESR absorption curve (9). Such a calculation can be performed when the intensity of the perpendicular component, own line width, Lorentz and Gaussian widths between the points of maximum slope and the constant of the fine structure D, are known. However, it is more expedient to carry out a quantitative analysis of vitamin K1 following simultaneous photolysis of standards, of known

concentration, and the sample to be analysed, keteris parabus. The concentration of vitamin K_1 is then determined by comparing the relative intensities of the perpendicular components of the spectrum of the sample with those of the standards at the same time after irradiation to avoid the introduction of corrections due to decays of the spectra (8). It is of note that, due to the sensitivity of this method, vitamin K_1 can be detected at the clear surface interfaces even in opaque frozen samples.

To illustrate the feasiblity of this method for the analysis of biological samples we employed it to study the hepatic accumulation of vitamin K_1 in mice following subcutaneous injection Vitamin K1 was administered at 150mg/kg and animals sacrificed at specific time points following injection. Livers were harvested, homogenised and lipids and vitamin K_1 extracted using chloroform and ether (1:10).The resulting extract was evaporated and resuspended in ethylbenzene and 0.1 ml of this solution was used for subsequent analysis. Separate experiments demonstrated that the presence of up to 20 % lipogenous substances (comprised of a mixture of ethers of oleic, linoleic, linolenic and arachidonic acids) did not impede the determination of the vitamin K_1 concentration in the sample.

Figure 1.shows an example of the ESR spectra obtained from samples 18 hours post-injection. These results demonstrate that 6.0 mg of vitamin K1. 80 % of the subcutaneous injection. has accumulated in the liver by 18 hours.

Although this method is somewhat less sensitive than its HPLC-based counterparts it gains in expediency and can be successfully used for biochemical investigations. It should however be noted that this method permits the determination of vitamin K_1 in quinoid form but not in reduced forms (semiquinone or hydroquinone). In addition. the water soluble tetrasodium diphosphate form of vitamin K_1. often is used in medicine as a blood clotting agent. also could not be determinated using this method. The most important

characteristic of this method however is that it permits the determination of vitamin K_1 concentrations in the presence of other quinones with isoprenoid chains. Our investigations revealed that no biradicals were detected following photolysis of frozen solutions of ubiquinones Q-6, Q-9, Q-10 and a variety of α-tocopherol-quinones.

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