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Menadione-γ-Cyclodextrin Inclusion Complex

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Abstract. A stable, nonsublimable γ-cyclodextrin inclusion complex of menadione (Vitamin K_3) of 11.7% menadione content (molar ratio 1 : 1) was prepared in solution, in suspension, and by 'kneading'. The physical-chemical properties of the complex formed were studied by X-ray diffraction, by thermal analysis (DTG, TG, DSC, TEA), and by chiroptical studies. The biological activity of the complex was tested on baby chickens by prothrombin time determination. Reduced prothrombin times showed an enhanced bioavailability of menadione in complexed form. The stability of vitamin K_3 is greatly improved in pharmaceuticals or in veterinary products prepared with a menadione-γ-CD inclusion complex.

Key words: Menadione, γ-cyclodextrin, inclusion compounds, complex formation, vitamin, stabilization.

1. Introduction

Menadione (vitamin K_3, 2-methyl-1,4-naphthoquinone) is a synthetic analogue of the natural vitamin K_1 [1]. Its high biological activity and rather simple industrial manufacturing make it an accepted vitamin K source.

Vitamin K deficiency is followed by intensive prolongation of blood coagulation causing serious bleeding, thus menadione is of practical importance in the treatment of both humans and animals. According to present marketing data, 95% of menadione is consumed by the livestock breeding industry. Poultry raising requires large quantities of vitamin K_3 as a feed additive in premixes.

Crystalline menadione sublimes rapidly and is decomposed by light. In premixes, chemical reactions (reduction of quinone groups, reactions with amino acids) further reduce the effective menadione content. Thus, a stable biologically-active menadione preparation has long been desired.

In a previous paper [2], the preparation, complex stability, decomposition rate, and polarographic and spectral properties of the menadione-β-cyclodextrin complex were reported and a second paper [3] was dedicated to the study of its stability, physical-chemical properties, and biological effects.

In aqueous solutions, the existence of a 1 : 1 complex was proved although the crystalline menadione-β-cyclodextrin complex contained only 4.2% menadione, corresponding to a 1 : 3 molar ratio, which could not be increased in favour of menadione. The application of such a complex, while highly stable and biologically active, would not have been economically viable in large-scale production.

In the present work, the preparation and properties of the menadione-γ-cyclodextrin inclusion complex are presented.

2. Results and Discussion

2.1. PREPARATION OF THE COMPLEX

A menadione-γ-cyclodextrin inclusion complex was prepared in solution by dissolving 6.5 g (5 mM) of γ-cyclodextrin in 25 ml of distilled water at 60°C and adding dropwise 0.86 g (5 mM) of menadione dissolved in 10 ml of 96% ethanol, while vigorously stirring. The pale yellow complex started to precipitate immediately. The reaction mixture was kept at 60°C for 2 h, after which heating was stopped but stirring was continued for another 4 h. After slow cooling, the reaction mixture was kept at 5°C for 12 h. The crystals which precipitated were filtered off and dried over P_2O_3. Yield: 6.77 g. Menadione content 11.3%, determined spectrophotometrically at 338 nm.

The complex was also prepared in suspension by stirring 3.5 g (2.7 mM) of crystalline γ-cyclodextrin and 0.42 g (2.4 mM) of menadione in 5 ml of distilled water for 6 h at room temperature. The pale yellow product which formed was filtered off and dried over P_2O_5. Yield: 3.65 g, menadione content: 11.5%.

Kneading was also a suitable technique for complex preparation. 250 mg (0.192 mM) crystalline γ-cyclodextrin and 33 mg (0.192 mM) menadione were homogenized and intensively kneaded for 45 min with the dropwise addition of 1.2 ml of 96% ethanol. The product formed was dried over P_2O_5. Yield: 270 mg, menadione content: 11.5%.

The complexes prepared were analyzed by sublimation. Free menadione is sublimable and can easily be separated from the complexed vitamin K_3 which does not undergo sublimation. This phenomenon is direct evidence for complex formation by all sublimable guest compounds.

Some 40 mg of the product was weighed into a subliming pot and heated at 120°C in vacuo for 10 min. Sublimed free menadione from the cold finger was dissolved in 96% ethanol, the

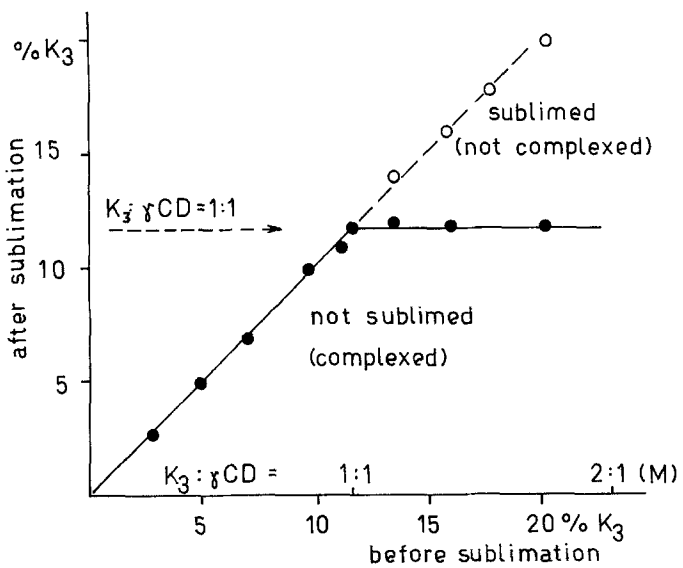

Fig. 1. Complexed (not subliming) and free (subliming) menadione content of menadione-γ-cyclodextrin complexes prepared by kneading at different molar ratios.

rest (menadione-γ-cyclodextrin complex) was dissolved in 50% ethanol and analyzed spectrophotometrically at 333 mm.

Complex-forming reactions in both solution and suspension led to the precipitation of the 1 : 1 molar complex (containing 11.7% menadione) with traces of free vitamin K_3. The complex prepared by kneading contained 0.3% free menadione, and no ethanol could be detected by GLC.

Many attempts have been made to prepare a complex with a higher menadione ratio. That was the reason why three different techniques were tested for complex formation. When γ-cyclodextrin was kneaded with less than 11.7% menadione, all of the guest compound underwent complexation and only traces of free vitamin K_3 could be detected. Applying higher than 1 : 1 molar ratios in the kneading process, the amount of complexed menadione never exceeded 11.7% (the theoretical 1 : 1 molar ratio). The remaining menadione above 11.7% stayed in a free, sublimable form and was adsorbed on the surface of complex crystals (Figure 1).

2.2. SOLUBILITY OF THE COMPLEX

Table I illustrates the results of solubility tests.

Table I. 5 mg menadione or equivalent amounts of its 3.5% menadione containing β-cyclodextrin complex (143 mg) or its 11.5% menadione containing γ-cyclodextrin complex (43.5 mg) were stirred in 5 ml of Sörensen buffer solution (pH = 7.5) for 1 h at 37°C.

	Amount dissolved	
	Menadione mg/100 ml	Complex mg/100 ml
menadione	21.3	–
menadione-β CD	82.2	2342.1
menadione-γ CD	31.0	269.6

2.3. CHIROPTICAL PROPERTIES

Detailed studies of the circular dichroism of the menadione-β-cyclodextrin complex have recently been published [5]. The results demonstrate that the phenyl moiety of the naphthoquinone molecule is embedded in the cyclodextrin cavity and that the long axis of the guest molecule lies parallel to the cyclodextrin symmetry axis.

The wider cavity diameter of the γ-cyclodextrin ring was expected to host the whole menadione molecule, thus providing further protection against chemical reactions. The analogous shape of the circular dichroism spectra of menadione-β-cyclodextrin, menadione heptakis-(2,6-di-O-methyl-)β-cyclodextrin and menadione-γ-cyclodextrin complexes (Figure 2) indicates that the location of the menadione molecule in the different cyclodextrin cavities must be similar. In the case of the γ-cyclodextrin complex, it is again the aromatic

ring only which is inside the cyclodextrin cavity. Lower absolute intensity values of the circular dichroic maxima show a weaker interaction between the electronic transitions of the host and the induced dipoles of the guest molecules, as compared to the β-complex.

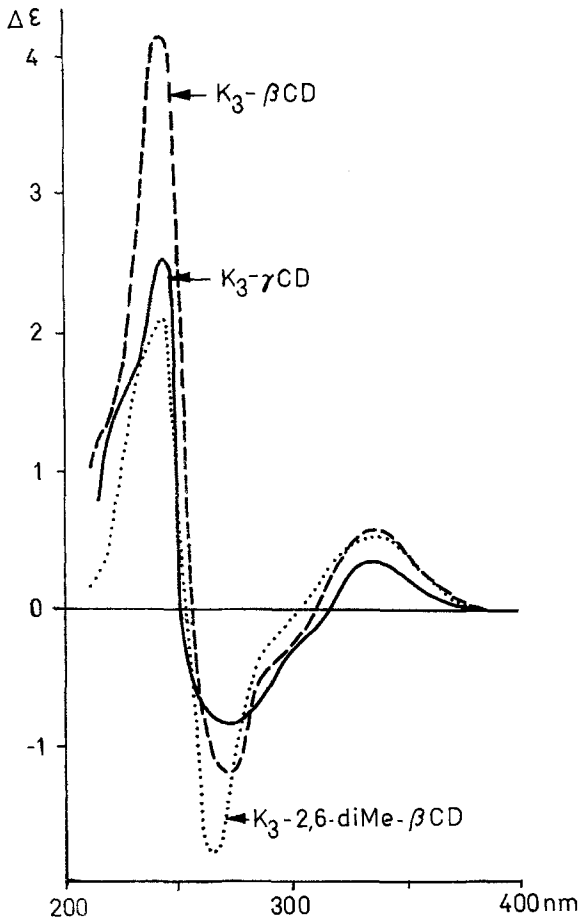

Fig. 2. Circular dichroic spectra of menadione-cyclodextrin complexes.

The stability constant of the complex in aqueous solution was calculated according to Benesi and Hildebrand [6].

$$\frac{\Delta\varepsilon}{\Delta\varepsilon_K - \Delta\varepsilon} = K \cdot c_D$$

where c_D = concentration of γ-cyclodextrin and $\Delta\varepsilon$ = circular dichroism. Here $\Delta\varepsilon_K$ corresponds to the limiting value of the circular dichroism, when the whole amount of menadione in solution is in a complexed form, and was graphically determined from a $\Delta\varepsilon^{-1}$ vs. $[\mathrm{CD}]^{-1}$ graph. The stability constant calculated was found to be $K_c = 100 \pm 20$ (25 °C). The stability constant of the γ-complex also indicates a looser association between the two molecules.

2.4. THERMOANALYTICAL STUDIES

The results of thermal analysis emphasize large stability differences in free and complexed forms of menadione. Measurements were carried out on the Du Pont 990 Thermal Analysis System. The DTG and TG curves are presented in Figure 3. Menadione rapidly sublimes over 50°C and melts between 103–107°C. The peak at 135°C on the DTG curve is attributed to the evaporation of free menadione but was not observed in the complex sample.

The TEA curve of the complex and that of the physical mixture are similar to those published in [3] on menadione and β-cyclodextrin. A small peak at 185°C indicates a

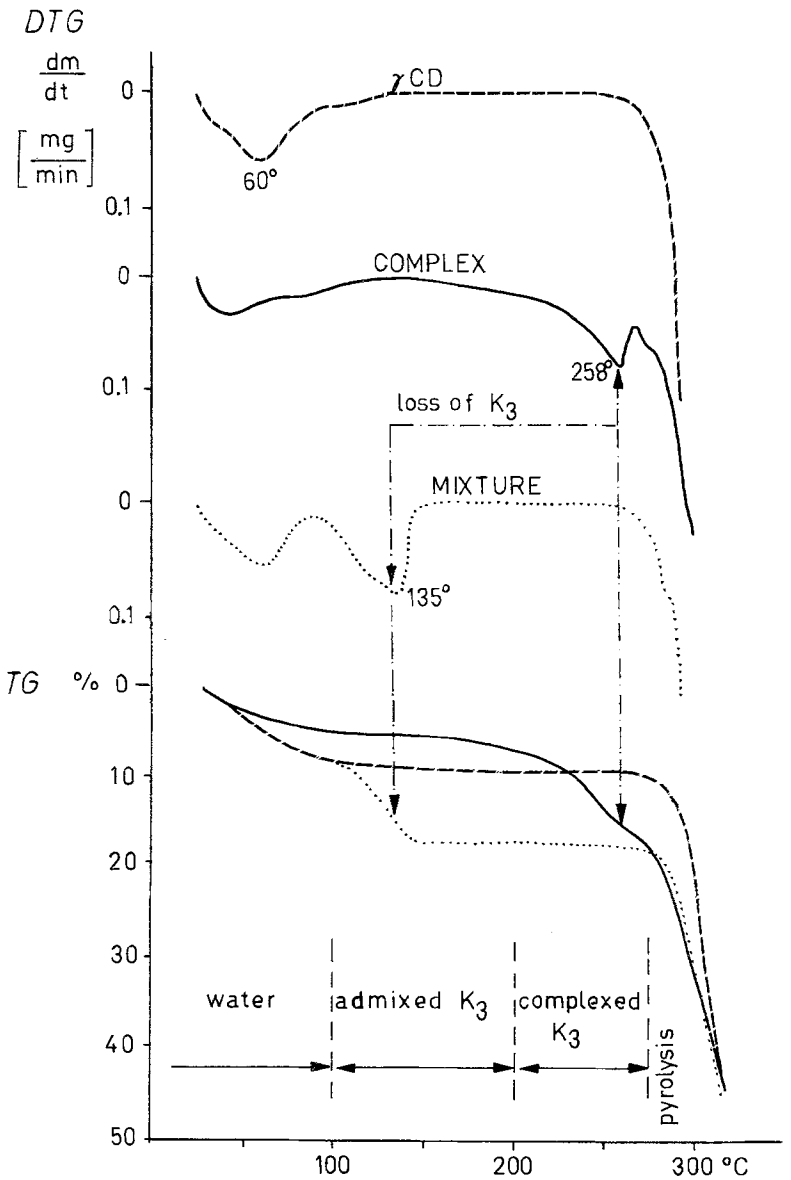

Fig. 3. Thermoanalytical (DTG and TG) curves of menadione-γ-cyclodextrin complex.

spontaneous complex formation on heating. Menadione from the complex sample starts to evaporate to a small extent at temperatures over 100°C, while larger quantities are only released over 200°C.

An attempt has been made to find a correlation between the thermoanalytical properties and the method of complex preparation. Although the samples showed some differences in binding forces and, thus, in the stability of the complexes, these differences did not correlate with the different methods.

2.5. STABILITY IN THE PRESENCE OF AMINOACIDS

The menadione-γ-cyclodextrin complex is intended for use as a feed additive in stock-breeding. The chemical stability of menadione and its γ-cyclodextrin complex was thus tested in a 'premix modelling' amino acid mixture at 60°C [3]. The menadione concentration of the mixture and menadione sublimed were determined separately. Results are given in Figure 4.

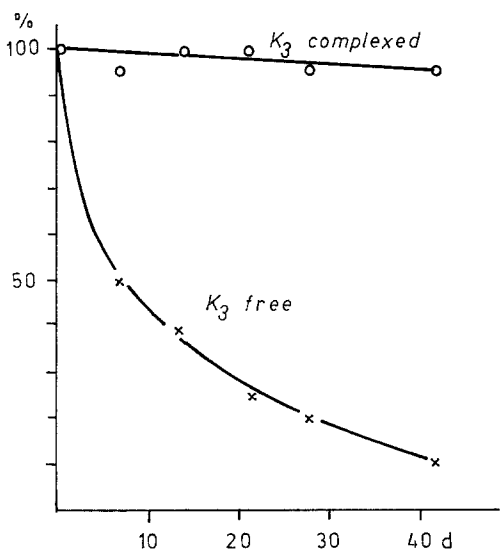

Fig. 4. Stability of menadione in 'premix modelling' amino acid mixture at 60°C, menadione content 20 mg/g. (The menadione content of the samples is expressed as a percentage of the original menadione content.)

2.6. X-RAY DIFFRACTION ANALYSIS OF THE COMPLEX

The X-ray powder diffraction technique is one of the most reliable tools for examining crystalline cyclodextrin inclusion complexes [4]. Diffractograms of menadione, γ-cyclodextrin, their physical mixture, and that of the menadione-γ-cyclodextrin complex were recorded on a Philips powder diffractometer using Cu$K\alpha$ radiation. Characteristic reflections of the samples are listed in Table II.

Table II. Characteristic reflections of menadione, γ-cyclodextrin and their inclusion complex in the $\theta = 4$ to 20° range.

Menadione	γ – CD	Menadione + γ CD mixture	Menadione – γ CD complex
	5.1 ⇒	5.1 ⇒	
		6.0	6.0
9.2 ⇒			
	9.4	9.4	
	10.2	10.2	
			10.4
11.7	11.7	11.7	11.7
	12.4	12.4	
	13.9	13.9	
			14.0
			14.7
15.2			
	15.4	15.4	
			⇒ 15.8
16.2 ⇒			
	16.3	16.3	
	18.8 ⇒	18.8 ⇒	
		19.8	19.8

A reduction in, or even the disappearance of, the characteristic maxima in the powder diagram of the guest and host molecules together with new peaks in the diffraction pattern of the complex are interpreted as being due to the formation of an inclusion complex.

The two strong maxima at 9.2 and 16.2°, which are characteristic of menadione, are not present in the diffractogram of the complex. The intensive maximum at 11.7° is also markedly reduced. The strong peaks at 5.1 and 18.8° which are characteristic of γ-cyclodextrin, disappear completely, while a very intense new maximum appears in the powder diagram of the complex at 15.8°.

2.7. BIOLOGICAL ACTIVITY OF THE COMPLEX

Chickens on a vitamin K-free diet develop the Dam syndrome [7] characterized by an intensive change of clinical parameters such as prothrombin time and blood coagulation time.

Baby chickens were fed on a vitamin K-free diet for 15 days (Quick and Stefanini [8]). The drinking water contained 0.1% benzoic acid to prevent the formation of vitamin K-synthesizing gut flora. After 15 days, the birds were treated orally with a 1.32 μg menadione or a 12.1 μg menadione-γ-cyclodextrin complex (corresponding to 1.32 μg menadione) daily dose respectively.

Prothrombin times were measured following György and Pearson [1]. Blood coagulation was induced by thrombokinase and, in another series of experiments, by chicken-brain thromboplastine. Results of prothrombin time determinations are given in Tables III and IV. The menadione-γ-cyclodextrin was able to restore the prothrombin values and cure the vitamin K deficiency in the same way as menadione itself. The prothrombin times of

Table III. Results of prothrombin time determination in thrombokinas-etreated plasma samples (N = number of chickens)

Groups	N	Prothrombine time (s)	Prothrombine time (%)
control	6	35.3 ± 2.0	100.0
hypovitaminotic	11	41.5 ± 5.6	117.6
menadione treated	6	29.2 ± 3.0	82.9
complex treated	5	26.9 ± 7.8	76.2

Table IV. Prothrombin times determined in brain thromboplastine treated samples

Groups	N	Prothrombine time (s)	Prothrombine time (%)
control	7	31.0 ± 3.2	100.0
menadione treated	6	20.6 ± 4.1	66.4
complex treated	8	18.4 ± 2.6	59.4

menadione-treated chickens exceeded those of complex-treated ones in both experiments, showing a higher bioavailability of menadione in the complexed form.

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