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Fluorometric Determination of Menadione with 3-Amino-2(1*H*)-quinolinethione. III[1,2]

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A fluorometric method for the determination of menadione (K_3) with 3-amino-2(1*H*)-quinolinethione (AQT) was developed. The method is based on the reaction of K_3 with AQT, followed by extraction of a red fluorescent product (excitation maxima, 533 and 573 nm; emission maximum, 600 nm) with carbon tetrachloride after making the reaction mixture strongly basic. The calibration curve was linear in the range from 0.01 to 1.0 μg/ml of K_3. This method was applicable to the determination and detection of K_3 contaminating phytonadione.

Keywords——organic reagent; fluorometry; menadione; 3-amino-2(1*H*)-quinolinethione

Many colorimetric methods are available for the determination of menadione (K_3); these methods involve reactions with cysteins,[3] 2,4-dinitrophenylhydrazine,[4] ethyl cyanoacetate,[5] acetylacetone,[6] titanium (III) sulfate,[7] piperidine or malononitrile,[8] and thiosemicarbazide.[9]

On the other hand, only a few fluorometric methods, which are generally more sensitive than colorimetry, have been reported as far as we are aware. Kofler[10] reported a fluorometric method based on the condensation with *o*-phenylenediamine. Although this method was highly specific, the procedure was somewhat cumbersome. Veronese *et al.*[11] described a method for the determination of sodium salts of the diphosphoric and disulfuric esters of 2-methyl-1,4-naphthohydroquinone (MNHQ). Fluorometric methods based on the formation of MNHQ derivatives with sodium dithionite and with cysteine hydrochloride were reported by Damme and Nève[12]; these derivatives gave a determination limit of 0.04 or 0.065 μg/ml of K_3, respectively.

We have already reported the selective fluorometric determination of 1,4-naphthoquinone (NQ) with 3-amino-2(1*H*)-quinolinethione (AQT).[13] In this paper, we describe a sensitive and selective fluorometric method for determination of K_3, based on the reaction of K_3 with AQT, followed by extraction of the fluorescent product with carbon tetrachloride (CCl_4) after making the reaction mixture strongly basic. This procedure, which has a determination limit of 0.01 μg/ml of K_3, is more sensitive than the fluorometric method reported by Damme and Nève.[12]

Experimental

Reagents and Materials——K_3 Solution: K_3 (recrystallized from petroleum ether, mp 106—107 °C) was dissolved in EtOH at a concentration of 100 μg/ml. The solution was diluted with EtOH to an appropriate concentration before use.

AQT Solution: AQT (synthesized by the method described previously[13]) (0.2 g) was dissolved in 100 ml of EtOH.

Uranine solution was used as a reference to assess the sensitivity of the fluorophotometer. Phytonadione (K_1), K_3, and 4-amino-3-methyl-1-naphthol (K_5) were purchased from Wako Pure Chemicals, Ltd. 2,3-Dimethyl-1,4-naphthoquinone (DMNQ) was synthesized according to the previous method.[14] CCl_4 was purified by refluxing with ethanolic KOH solution, followed by distillation. All other chemicals and solvents were of reagent grade.

Apparatus——Fluorescence spectra were taken on a Hitachi 650-10S spectrofluorophotometer equipped with a Hitachi 050 recorder, and routine fluorescence readings were made on a Hitachi FPL-2 fluorophotometer. Infrared (IR) spectra were obtained in the indicated state with a Hitachi 270-30 spectrometer. Mass spectra (MS) were taken on a JEOL JMS-D300 spectrometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were determined in the indicated solvent with a JEOL PS-100 spectrometer.

Determination of K_3——An aliquot of sample solution (5 ml), 30% (w/v) H_2SO_4 (2 ml) and 0.2% (w/v) AQT solution (2 ml) were placed in a 50 ml-centrifuge tube without a stopper. The mixture was heated on a boiling water bath for 90 min; the tube was not stoppered during the reaction. The reaction mixture was transferred into a separatory funnel by rinsing with distilled water. A 30% NaOH solution (4 ml) was added to the funnel to make the mixture strongly basic. After cooling to room temperature, the basic solution was shaken vigorously with 10 and 5 ml portions of CCl_4 for 5 min each. Then each extract was filtered through a small cotton plug and washed with CCl_4. The organic layers were combined and diluted with CCl_4 to 20 ml. The relative fluorescence intensity of the CCl_4 solution was measured with a Hitachi FPL-2 fluorophotometer (primary filter, No. 546; secondary filter, No. 61).

The sensitivity of the fluorophotometer was adjusted to give a reasonable fluorescence intensity with a uranine solution (primary filter, No. 436; secondary filter, No. 53).

Defection of K_3 in K_1——First, K_1 (2 mg) was dissolved in EtOH (5 ml) in a 50-ml centrifuge tube without a stopper, then 30% (w/v) H_2SO_4 (2 ml) and 0.2% AQT solution (2 ml) were added. The mixture was heated by the same method as described above. The reaction solution was alkalized with 30% NaOH (4 ml) and extracted with CCl_4 (10 ml). Then the fluorescence of the CCl_4 layer was observed with the naked eye under irradiation with a Toshiba FI-3S UV lamp.

Condensation Product of AQT and K_3——A solution of AQT (44 mg, 2.5×10^{-4} mol) in EtOH (30 ml) was mixed with K_3 (43 mg, 2.5×10^{-4} mol) and 60% (w/v) H_2SO_4 (15 ml), and the whole was allowed to stand for 2 weeks at room temperature. After dilution with H_2O, the red crystals formed were collected by filtration, washed with H_2O and recrystallized from EtOH to give 6-methyl-7-thia-8,14-diazabenzo[*a*]naphthacen-5-one (**1**) (34.3 mg, 42%) as red needles, mp 256—257 °C. *Anal.* Calcd for $C_{20}H_{12}N_2OS$: C, 73.15; H, 3.68; N, 8.53. Found: C, 72.73; H, 3.45; N, 8.55. IR $\nu_{\max}^{\mathrm{KBr}}$ cm^{-1}: 1626 (C=O), 1304 (C=N). MS *m/e*: 328 (M^+), 300 ($M^+ - CO$). ^{1}H-NMR (C_5D_5N): 2.22 (3H, s, CH_3), 7.55—8.95 (9H, m, Ar-H).

Results and Discussion

Fluorescence Spectra

Figure 1 shows the uncorrected fluorescence excitation and emission spectra of the product formed from the reaction of K_3 with AQT in CCl_4 solution under the standard conditions of the procedure described in the experimental section. Fluorescence excitation maxima were observed at 533 and 573 nm (emission, at 600 nm); the emission miximum was the same at each excitation maximum. The shapes of these spectra were the same as described previously.[13]

Effect of Reagent Concentrations

The fluorescence intensity was significantly dependent on the concentration of sulfuric acid, but concentrations ranging from 25% (w/v) to 40% (w/v) gave the maximum intensity (Fig. 2); a 30% (w/v) sulfuric acid solution was used in the standard procedure.

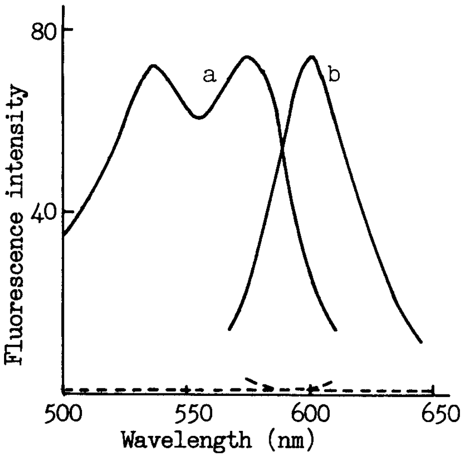

Fig. 1. Excitation and Emission Spectra of the Reaction Product in CCl_4

Menadione taken: 0.5 μg/ml. ——, reaction product; ----, reagent blank. a, excitation spectrum; b, emission spectrum.

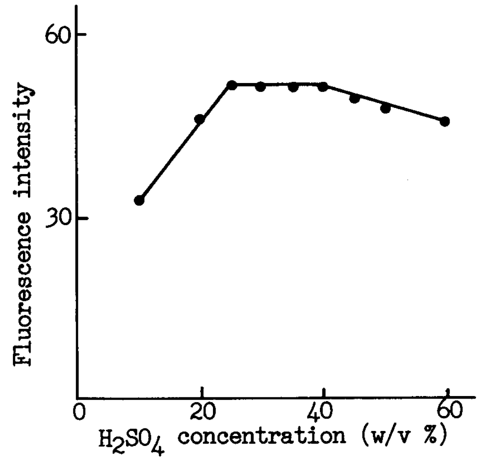

Fig. 2. Effect of H_2SO_4 Concentration on the Fluorescence Intensity

Menadione taken: 0.3 μg/ml.

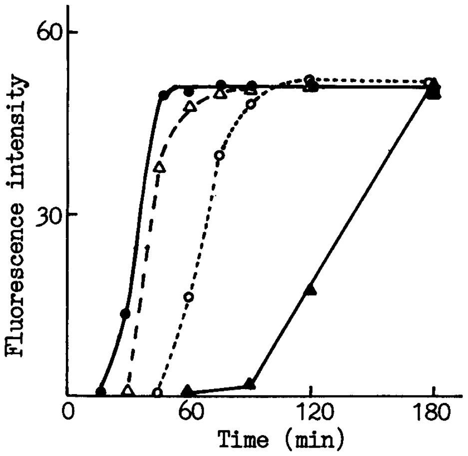

Fig. 3. Effect of Reaction Temperature on the Development of Fluorescence

Menadione taken: 0.3 μg/ml. —●—, on a boiling water bath; --△--, at 95 °C, ---○---, at 90 °C; —▲—, at 82 °C.

TABLE I. Effect of Extracting Solvent on the Determination of Menadione[a)]

Solvent	λ_{ex}[b)] (nm)		λ_{em}[c)] (nm)	R.F.I.[d)]
Carbon tetrachloride	533	573	600	100
Methyl isobutyl ketone	527	558	599	82
Chloroform	530	567	607	70
m-Xylene	537	572	614	68
Benzene	538	573	614	65
1,2-Dichloroethane	531	564	607	62

a) Menadione taken: 0.3 μg/ml. *b*) Excitation maxima, uncorrected. *c*) Emission maximum, uncorrected. *d*) Relative fluorescence intensity.

TABLE II. Fluorescence Characteristics of Vitamin K and a Related Compound

Compound[a)]	λ_{ex}[b)] (nm)		λ_{em}[c)] (nm)	R.F.I.[d)]
Phytonadione (K_1)	—	—	—	0
Menadione (K_3)	533	573	600	100
4-Amino-3-methyl-1-naphthol (K_5)	533	574	600	76
2,3-Dimethyl-1,4-naphthoquinone	—	—	—	0

a) Compound taken: 0.3 μg/ml. *b*) Excitation maximum, uncorrected. *c*) Emission maximum, uncorrected. *d*) Relative fluorescence intensity.

As the fluorescence intensity was found to be constant in the range of 1.5—3 ml of 0.2% AQT solution, 2 ml of the AQT solution was added. The AQT solution could be stored without change for two weeks in the dark.

Effects of Reaction Temperature and Time

The fluorescence intensity was constant on a boiling water bath for 50—180 min (Fig. 3). The rate of the fluorescence development increased markedly with increase in temperature, as shown in Fig. 3. The conditions described in the experimental section were chosen from the viewpoints of ease of temperature control and speed of operation.

Effects of Solvents and Other Conditions

The red fluorescent product produced in this method was extracted with solvent from the strongly alkalized reaction mixture. This method has the advantage of keeping in the aqueous layer the large excess of AQT, which tended to decrease the fluorescence intensity of the organic layer. Therefore, 4 ml of 30% sodium hydroxide solution was added to the reaction mixture.

In order to select the best extractant, various immiscible solvents listed in Table I were tested for the extraction of the red fluorescent product. The highest fluorescence intensity was obtained when CCl_4 was used as the extractant, and no change of the intensity in CCl_4 was detected for about 24 h. The other experimental conditions, extraction times and shaking time, were optimized, and the recommended procedure was proposed.

Calibration Curve and Precision

Sample solutions containing K_3 at various concentrations were assayed by the recommended procedure. The calibration curve was linear in the range from 0.01 to 1.0 μg/ml of K_3. By repeated determinations ($n = 10$) of sample solutions containing 0.3 μg/ml of K_3, the coefficient of variation was calculated to be 3.7%.

Fluorescence Characteristics of Vitamin K

The recommended procedure was tested on vitamin K and a related compound. The fluorescence characteristics and relative fluorescence intensities of fluorescent products formed by reaction of these compounds with AQT are listed in Table II. 2,3-Disubstituted NQs such as K_1 and DMNQ did not fluoresce, in contrast with K_3 and K_5. These results are in agreement with those described in the previous paper.[13)]

Detection and Recovery of Menadione in Phytonadione

Table II shows that only K_1 in the class of vitamin K compounds does not fluoresce in the recommended procedure. In this section, we aimed at minimizing the amount of sample required and getting a suitable lower limit of detection of K_3 in K_1. It was found that 0.5 μg of K_3 in K_1 (2 mg) was detectable in terms of the red fluorescence of the CCl_4 layer viewed with the naked eye under irradiation with the UV lamp.

On the other hand, 1 μg or 5 μg of K_3 added to K_1 (500 μg) was recovered with a coefficient of variation of 4.0% or 1.6% ($n = 10$), respectively, according to the standard procedure for the determination of K_3.

Fluorescent Product

Akatsuka and Yoshinaga[15)] have already described the reaction of K_3 with *o*-aminothiophenol, and obtained 6-methyl-5*H*-benzo[*a*]phenothiazin-5-one as red needles.

To elucidate the reaction pathway and the structure of the fluorescent product, we carried out the reaction of K_3 with AQT at room temperature by the method described in the experimental section and obtained the intermediate compound, 6-methyl-7-thia-8,14-diazabenzo[*a*]naphthacen-5-one (**1**), as red needles. From the elemental analysis data, the fragment at *m*/*e* 300 [loss of CO (28) from the molecular ion (*m*/*e* 328)] in the MS, the strong absorption at 1626 cm^{-1} (carbonyl group) in the IR spectrum, and the single signal at 2.22 ppm due to the methyl protons in the ^{1}H-NMR spectrum (in deuteriopyridine), the structure **1** was proposed.

The fluorescence excitation and emission maxima (λ_{ex}^{max}: 533 and 573 nm; λ_{em}^{max}: 600 nm) under the assay conditions were shifted to higher wavelengths from those (λ_{ex}^{max}: 399, 420 and 472 nm; λ_{em}^{max}: 533 nm) of **1** in CCl_4 solution. When an ethanolic solution of **1** was treated with 0.2% AQT solution according to the determination method for K_3, the CCl_4 layer showed a red fluorescence and the spectra were identical with those of the fluorescent product obtained

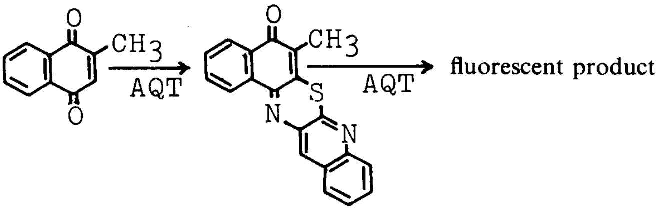

Chart 1

by the recommended procedure. From these results, it is assumed that the fluorescent product is a condensation compound of **1** with AQT, and that the fluorescence reaction proceeds as shown in Chart 1.

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References and Notes

1) This work was presented at the 99th Annual Meeting of the Pharmaceutical Society of Japan, Sapporo, August 1979.
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