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Paper Chromatography of Vitamin K_1 and Related Compounds with some Observations on Products of Ultra-violet Irradiation

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Although a number of methods for the determination of vitamin K_1 and other naphthoquinones have been described [1] each has certain limitations. In searching for a simple, sensitive, and specific method, we have directed our attention to filter-paper chromatography, since the naphthoquinones have a characteristic fluorescence on filter-paper [2]. In this report the R_F values of a series of compounds related to vitamin K_1 are given in three different solvent systems along with the fluorescent colors observed under ultra-violet light. At the same time some observations of the effect of ultra-violet irradiation on these compounds are presented.

EXPERIMENTAL

Chromatography. Ascending technique and Whatman No. 1 filter-paper were used throughout. The filter-paper was soaked in 5 % (v/v) solution of Dow Corning Silicone No. 1107 in *cyclo*hexane [3] or chloroform and allowed to dry in air. Unless otherwise noted, 10 μg of the naphthoquinones in ethanol solution were applied to the paper with a 1 μl Carlsberg pipette; the dimer of menadione was in acetone solution. Vitamin K_1 was used as a control substance and run on all chromatograms. These were developed overnight (16—20 hrs) at room temperature under black cloaks to prevent photolysis.

Materials. Vitamin K_1 and vitamin K_1-oxide were products of Merck & Co., Inc. Menadione and phthiocol were products of Hoffmann-La Roche & Co., Inc. The dimer of menadione was prepared by irradiation of menadione [4]. The other naphthoquinones, recently synthesized and examined biologically [5] were kindly donated by Hoffmann-La Roche & Co.

Solvents. Preliminary observations suggested that the substances were more stable in acidified solvents, as chromatography of a single compound in a non-acidified alcohol-water system often resulted in the development of two spots. Of thirty solvent systems examined, only three proved useful, and these are listed in Tables 1 and 2.

Detection of the substances. The developed chromatograms were dried at room temperature, held before a Philips No. 57202 E 170 ultra-violet lamp (3 665 Å) and the fluorescent colors noted. After an exposure of 45—60 sec. to the ultra-violet light, the color

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of the fluorescence was again noted and the spot outlined. The fluorescent color brought out by spraying with a solution of 2 *N* KOH in ethanol was also recorded. In quantitative measurements the outlined spot was traced on millimeter paper and the area obtained by direct counting.

Irradiation. The compounds and the vitamin K_1 control were applied to the coated filter-paper, allowed to dry, and then held before the ultra-violet lamp (3 665 Å) for 2—3 minutes. Identical quantities of the same substances which were not irradiated, were placed on the same paper. It was chromatographed and dried in the manner described and then examined under the lamp for fluorescence.

RESULTS

The R_F values presented in Tables 1 and 2 were obtained from chromatograms run on the same batch of silicone-coated paper and in fresh solvents. The latter was especially important, for as the solvents aged, the R_F values diminished, although their relative values remained the same.

The R_F values of the various naphthoquinones in those solvent systems that seemed most useful are listed in Table 1 together with a notation of their fluorescent colors. It may be seen that as the length of the side-chain increased, the R_F values decreased. However, compounds containing 25 and 30 carbons (X and XI respectively) were not significantly different. Vitamin K_1 and its oxide had the same R_F values.

"Before activation" denotes the fluorescent colors emitted by the chromatographed substance when initially held before the ultra-violet lamp. This fluorescence was clearly evident after the substance was chromatographed and less obvious when the sample was first applied to the paper. Exposure to the ultra-violet lamp for 45—60 seconds induced a permanent change in the fluorescent color of most compounds and an increase in the area of fluorescence. The fluorescent colors are tabulated under "After activation". Spraying with alcoholic KOH after activation produced yet other colors, and these are listed in the last column. The fluorescence after activation was stable for at least four months.

Quantitative chromatographic measurements of vitamin K_1 were carried out in the ethyl alcohol and *iso*propyl alcohol systems. The fluorescence after activation (*i.e.*, the green fluorescence) was clearly visible in concentrations as low as 0.5 μg, while the fluorescence before activation (*i.e.*, the red fluorescence) was not apparent to the unaided eye until 5 μg were present. The color change produced by activating quantities greater than 10 μg was restricted to the periphery of the spot, the central portion retaining its red fluorescence. Spraying KOH on quantities less than about 2.5 μg obliterated the fluorescence.

In ethyl alcohol quantities greater than 5 μg developed as elliptical spots; in *iso*propyl alcohol spots were elliptical at concentrations above 40 μg. In both solvents 80 μg or more of the vitamin developed as long ellipses which began at the point of application and swept forward.

Between 1.5 and 50 μg, the log of the concentration was linearly related to the area of the spot. In concentrations of 0.5 μg, the area was not related to the concentration of the vitamin, but to the unaided eye the intensity of fluorescence appeared to be related to concentration.

Table 1. R_F values and fluorescence of 2-methyl-1,4-naphthoquinone and its derivatives.

No. of compound	Formula of group in 3-position or designation of compound	Solvent system			Color of fluorescence		
		Ethyl-alcohol-acetic acidwater (75 : 25 : 225)	*Iso*propyl alcohol-acetic acidwater (600 : 25 : 375)	*n*-Propyl alcohol-acetic acidwater (600 : 25 : 375)	Before activation	After activation	After activation and spraying with KOH
I	—H (menadione)	0.71	0.82	0.89	red	blue	green
II	(dimer of menadione)	0.73	0.88	0.97	red	blue	green
III	—OH (phthiocol)	0.63	0.72	0.80	red	red	(cherry red in visible light)
IV	$-CH_2-CH=C(CH_3)-CH_2-CH_2-CH=C(CH_3)-CH_3$	0.55	0.66	0.73	red	green	orange
V	$-CH_2-CH=C(CH_3)-[CH_2-CH_2-CH=C(CH_3)]_2-CH_3$	0.49	0.58	0.68	red	green	orange
VI	$-CH_2-CH=C(CH_3)-[CH_2-CH_2-CH_2-CH(CH_3)]_2-CH_3$	0.42	0.46	0.54	red	green	orange
VII	$-CH_2-CH=C(CH_3)-[CH_2-CH_2-CH=C(CH_3)]_3-CH_3$	0.31	0.40	0.47	red	green	orange
VIII	$-CH_2-CH=C(CH_3)-(CH_2)_3-CH(CH_3)-(CH_2)_3-CH(CH_3)-(CH_2)_3-CH(CH_3)-CH_3$ (vitamin K_1)	0.19	0.28	0.36	red	green	orange
IX	(the 2,3-oxide of vitamin K_1)	0.19	0.27	0.38	red	green	orange
X	$-CH_2-CH=C(CH_3)-(CH_2-CH_2-CH_2-CH(CH_3))_4-CH_3$	0.12	0.16	0.26	red	green	orange
XI	$-CH_2-CH=C(CH_3)-CH_2-(CH_2-CH=C(CH_3)-CH_2)_4-CH_2-CH=C(CH_3)-CH_3$ (vitamin K_2)	0.14	0.18	0.30	red	green	orange

Table 2. R_F values and fluorescence of irradiated 2-methyl-1,4-naphthoquinone and its derivatives.

No. of compound	Formula of group in 3-position or designation	Solvent system: Ethyl alcohol-acetic acidwater (750 : 25 : 225)	Solvent system: *Iso*propyl alcohol-acetic acidwater (600 : 25 : 375)	Solvent system: *n*-Propyl alcohol-acetic acidwater (600 : 25 : 375)	Color of fluorescence: Before activation	Color of fluorescence: After activation	Color of fluorescence: After activation and spraying with KOH
I	—H (menadione)	0.00 0.73	0.00 0.80	0.00 0.89	blue red	blue blue	blue green
II	(dimer of menadione)	0.00 0.74	0.00 0.89	0.00 0.93	blue red	blue blue	blue green
III	—OH (phthiocol)	0.63	0.74	0.81	red	red	(cherry red in visible light)
IV	$-CH_2-CH=C(CH_3)-CH_2-CH_2-CH=C(CH_3)-CH_3$	0.00 0.51 0.72 0.81	0.00 0.63 0.81 0.85	0.00 0.73 0.82 0.89	blue none none green	blue green green green	blue orange none orange
V	$-CH_2-CH=C(CH_3)-[CH_2-CH_2-CH=C(CH_3)]_2-CH_3$	0.00 0.49 0.68 0.77	0.00 0.57 0.78 0.85	0.00 0.66 0.80 0.91	blue none none green	blue green green green	blue orange none orange
VI	$-CH_2-CH=C(CH_3)-[CH_2-CH_2-CH_2-CH(CH_3)]_2-CH_3$	0.00 0.43 0.62 0.72	0.00 0.49 0.73 0.87	0.00 0.51 0.78 0.89	blue none none green	blue green green green	blue orange none orange
VII	$-CH_2-CH=C(CH_3)-(CH_2-CH_2-CH=C)_3-CH_3$	0.00 0.34 0.62 0.77	0.00 0.41 0.71 0.86	0.00 0.44 0.79 0.89	blue none none green	blue green green green	blue orange none orange
VIII	$-CH_2-CH=C(CH_3)-(CH_2)_3-CH(CH_3)-(CH_2)_3-CH(CH_3)-(CH_2)_3-CH(CH_3)-CH_3$ (vitamin K_1)	0.00 0.18 0.49 0.74	0.00 0.28 0.54 0.85	0.00 0.33 0.62 0.87	blue none none green	blue green green green	blue orange none orange
IX	(the 2,3-oxide of vitamin K_1)	0.00 0.19 0.49 0.75	0.00 0.17 0.47 0.83	0.00 0.28 0.53 0.87	blue none none green	blue green green green	blue orange none orange
X	$-CH_2-CH=C(CH_3)-(CH_2-CH_2-CH_2-CH)_4-CH_3$	0.00 0.13 0.39 0.76	0.00 0.17 0.47 0.83	0.00 0.28 0.53 0.87	blue none none green	blue green green green	blue orange none orange
IX	$-CH_2-CH=C(CH_3)-CH_2-(CH_2-CH=C(CH_3)-CH_2)_4-CH_2-CH=C(CH_3)-CH_3$ (vitamin K_2)	0.00 0.12 0.28 0.72	0.00 0.22 0.38 0.84	0.00 0.31 0.49 0.88	blue none none green	blue green green green	blue orange none orange

Table 2 shows the R_F values of the same compounds that had been chromatographed after irradiation. Phthiocol showed one fluorescent spot, menadione and its dimer two, and the other compounds four. With the exception of phthiocol, all irradiated compounds showed one stationary spot ($R_F = 0.00$) of blue fluorescence that was not altered by the KOH spray. One of the components of each irradiated substance had an R_F value corresponding to that of its non-irradiated counterpart (*cf.* Tables 1 and 2): thus, the R_F value of non-irradiated vitamin K_1 in ethyl alcohol, *iso*propyl alcohol, and *n*-propyl alcohol were 0.19, 0.28 and 0.36, respectively; the corresponding R_F values of one component of the irradiated vitamin were 0.18, 0.28, and 0.33. In addition to the stationary spot and to the spot with the same R_F value as the non-irradiated substance, every irradiated compound with long alkyl side chains (Nos. IV—XI) showed two other spots. One fluoresced only after activation, a fluorescence that was eliminated by spraying with KOH. The fourth and most mobile component fluoresced green without prolonged activation. The R_F values of the third and fourth spots were inversely related to the length of the side-chain on the parent compound.

DISCUSSION

The results presented in Table 1 show that reversed phase paper chromatography is applicable to the separation and identification of naphthoquinones. Their R_F values were inversely related to the length of the side-chain in the 3-position up to a 30 carbon grouping. The sensitivity of the method, which was 0.5 μg for qualitative analysis and 1.5 μg for quantitative analysis, could probably be increased by instrumental measurements of fluorescence. In view of the relative roundness of the spots, the *iso*propyl alcohol system is probably the most useful, especially for quantitative studies.

Of the three different fluorescent colors exhibited by some of the compounds, the fluorescence observed "after activation" appeared to be the most intense. It was visible to the unaided eye when concentrations as low as 0.5 μg of the fluorescing compound were present. This fluorescence was in all likelihood due to a photochemical product of the original naphthoquinone. The original compound was probably represented by the fluorescence "before activation" which required the presence of about 5 μg of the compound for visibility. The fluorescence after the KOH spray was intermediate in intensity.

It is known that photo-activation of naphthoquinones can induce dimerization [4,6] and oxidation [6], but what substances are produced under the conditions described here is unknown. It is not clear that irradiation of menadione produced its dimer, for in all observations the R_F values of the migrating spot of menadione were slightly less than the dimer. It is of further interest that the dimer itself could be further photo-activated to another compound or compounds with $R_F = 0.00$. This latter spot appeared on chromatograms of all the irradiated substances with the exception of phthiocol.

All irradiated compounds revealed a component with R_F values indistinguishable from the non-irradiated compound. These probably represent the unchanged, original substance. That these spots, in some cases, did not fluoresce

"before activation" (compounds IV—XI, Table 2) in contrast with the same non-irradiated compounds (Table 1) can probably be ascribed to the presence of quantities less than 5 μg.

In addition to the stationary spot and the one presumably representing the unchanged compound, naphthoquinones with long alkyl side chains (IV —XI), when irradiated and chromatographed, produced two other fluorescing spots. Of these, the one of smaller R_F had notable fluorescent properties: it did not fluoresce until "after activation", and the fluorescence was quenched by KOH. Both characteristics are consistent with the presence of quantities less than 2.5 μg. This spot may represent an intermediate product in the change from the original to the main fluorescing product of ultra-violet irradiation, the substance with the highest R_F value. Its fluorescent color was not quenched by KOH, which suggests a concentration greater than 2.5 μg. Irradiation may well have produced other compounds the fluorescence of which the eye could not detect or which did not fluoresce at all.

SUMMARY

Vitamin K_1 and some related compounds were chromatographed on silicone-coated filter-paper. In the three solvent systems studied, R_F values were, in general, related to the length of the side-chain in the 3-position. By their fluorescence in ultra-violet light, quantities as low as 0.5 μg were visible to the unaided eye. The area of fluorescence was linearly related to the log of the concentration from 1.5 to 50 μg. The R_F values of the fluorescent spots resulting from ultra-violet irradiation of the same compounds were also determined.

REFERENCES

1. Dam, H. *Vitamins and Hormones* **6** (1948) 28.
2. Green, J. P. *Nature*, **174** (1954) 369.
3. Kritchevsky, T. H. and Tiselius, A. *Science* **114** (1951) 299.
4. Madinaveitia, J. *Rev. acad. cienc. exact. fés. quim y nat. Madrid* **31** (1934) 617; *Chem. Abstracts* **29** (1935) 5438.
5. Isler, O., Rüegg, R., Studer, A. and Jürgens, R. *Hoppe-Seylers Z. physiol. Chem.* **295** (1953) 290.
6. Hooker, S. C. *J. Am. Chem. Soc.* **58** (1936) 1212.

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