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SEPARATION OF NAPHTHOQUINONES AND LIPOPHILIC VITAMINS BY GEL AND THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A separation of naphthoquinones on silica gel and on silica gel impregnated with polyethylene glycol 200 by thin-layer chromatography was compared with gel permeation chromatography (GPC) on styrene–divinylbenzene copolymer S-832-gel using tetrahydrofuran as mobile phase. Factors affecting the separations attainable are discussed, and it is concluded that GPC is a suitable method for the determination of K vitamins in natural materials.

INTRODUCTION

Naphthoquinones occur only rarely in nature, mainly in higher plants and bacteria[1]; an important group comprises the K vitamins, which are important to warm-blooded animals because they maintain normal blood-clotting properties. The K vitamins can be determined spectrophotometrically[2,3], polarographically[4] and by titrating with ceric sulphate after reduction to hydroquinone[5].

Preliminary separation of the K vitamins from accompanying compounds is carried out by column chromatography on silica gel[6] or aluminium oxide[7], and separation proper of these vitamins is performed by thin-layer chromatography (TLC) on cellulose impregnated with liquid paraffin[8], on silica gel[9], on silica gel impregnated with silver nitrate[10], and on polyamide[11]. Gel chromatography has been used in the separation of vitamin K_2 homologues with various numbers of isoprene units in the molecule, and also of mixtures of vitamins A, E and K_2 (ref. 12).

This work had as its objective the chromatographic separation of a mixture of quinones by TLC or gel chromatography. Since, in many materials, lipophilic vitamins appear together, we studied the separation of these compounds and also of some others co-extracted with them.

EXPERIMENTAL

Chemicals

Vitamin K_1 (phylloquinone), standard grade, was obtained from Merck (Darmstadt, G.F.R.); vitamin K_3 (menadione), pure grade, from Fluka (Buchs, Switzerland) and from Spofa (Prague, Czechoslovakia); vitamin K_4 (menadiol diacetate), pure grade, from Fluka; 1,2-naphthoquinone, and 1,4-naphthoquinone, pure grade, from Lachema (Brno, Czechoslovakia); vitamin D_2 (calciferol) from Calbiochem (Los Angeles, Calif., U.S.A.); vitamin A (retinyl acetate), pure grade, from Koch-Light (Colnbrook, Great Britain); cholesterol, standard grade, from Lachema; polyethylene glycol (PEG) 200 and 400 from Fluka; silica gel L 5/40 from Lachema; and Silufol silica gel sheets for TLC from Kavalier (Votice, Czechoslovakia). The light petroleum (boiling point 60–80°) used was from a Hungarian supplier, and other chemicals and solvents were of reagent grade.

Methods and equipment

The applicator for TLC was obtained from Dioptra Turnov, the development tanks and constriction pipettes from Labora, and the gel chromatograph from the Institute of Macromolecular Chemistry of the Czechoslovak Academy of Sciences (Prague).

Experimental conditions

For TLC, the sample was dissolved in benzene–ethanol (1:1) to give a concentration of *ca.* 5 mg/ml, and this solution was applied with a 5-μl micropipette. The chromatographic tank was saturated with solvent vapour at 22 $\pm$ 1° for 1 h, development was by the ascending technique, and the zones were detected by spraying the chromatogram with a 70% solution of perchloric acid and heating it at 105° for 5–10 min (see ref. 13); the colours and detection limits are shown in Table I.

To prepare a silica gel layer impregnated with polyethylene glycol (4 plates, each 17 × 23 cm), 20 g of silica gel were mixed with 100 ml of acetone, and the impregnating agent was added to the suspension by pipette; the mixture was set aside overnight, then evaporated in a rotating vacuum evaporator at 40°. Starch (2 g) and light petroleum (60 ml) were added to the impregnated gel, the mixture was stirred thoroughly for 1 min and spread on glass plates (width of slot adjustment, 0.2 mm), and the coated plates were dried at room temperature for 24 h.

For gel chromatography, the compounds being studied were separated on five columns (each 1.2 m × 8 mm) connected in series and packed with S-832 gel (a

TABLE I

DETECTION OF NAPHTHOQUINONES WITH PERCHLORIC ACID

Compound	*Colour*	*Detection limit, µg*
1,2-Naphthoquinone	Olive green	—
1,4-Naphthoquinone	Violet	3
Phylloquinone	Violet	2*
Menadione	Violet	1
Menadiol diacetate	Violet	0.4

* See ref. 13.

styrene–divinylbenzene copolymer obtained from the Czechoslovak Academy of Sciences); redistilled tetrahydrofuran was used as the mobile phase, and the samples (0.2 ml) were injected as a *ca.* 1% solution in tetrahydrofuran.

The identities of the naphthoquinones and K vitamins were confirmed by IR and UV spectrophotometry.

RESULTS AND DISCUSSION

A benzene–ethyl acetate (97:3) development system[14] has been used successfully in the TLC of naphthoquinones on silica gel, and a similarly good separation of these compounds can also be achieved by development with benzene alone. The addition of a polar component (ethyl methyl ketone) to the benzene mobile phase impairs the separation of naphthoquinones from each other, but improves their separation from products of decomposition. The separation values thus obtained are shown in Table II. The lower R_F of 1,2-naphthoquinone compared with 1,4-naphthoquinone on silica gel is due to interaction (chelation) between 1,2-naphthoquinone and the adsorbent. Good separation of the compounds studied was also obtained on silica gel impregnated with polyethylene glycol 200, with benzene–light petroleum (1:1) as mobile phase; the experimental arrangement was that used for the TLC of lipophilic K vitamins[15] (see Table II).

A similar separation of the compounds was achieved by TLC on silica gel impregnated with polyethylene glycol 400.

Naphthoquinones, cholesterol and some lipophilic vitamins were also separated by gel chromatography (see Table II). Molecular weights were calculated from the elution volumes by using the equation[16]

$$\log M = a + b(V_0/V_e), \tag{1}$$

where M is the molecular weight, a and b are constants, V_0 is the limiting elution volume and V_e is the elution volume. Calibration by means of compounds of known molecular weight yielded a regression equation in the form

$$\log M = 1.084 + 2.162(V_0/V_e), \tag{2}$$

TABLE II

CHROMATOGRAPHIC SEPARATION OF NAPHTHOQUINONES AND VITAMINS

Compound	$R_F \times 100$ *for TLC**			*Relative zone velocity*** *in gel chromatography*	*Mol. wt.*	
	S_1	S_2	S_3		*True*	*Calc.*
1,2-Naphthoquinone	10	48	25	0.556	158.2	193.4
1,4-Naphthoquinone	28	61	54	0.525	158.2	165.7
Phylloquinone	61	80	75	0.723	450.7	443.9
Menadione	30	65	59	0.528	172.2	172.1
Menadiol diacetate	13	59	41	0.608	255.7	248.4
Calciferol	8	53	30	0.712	396.7	420.3
Retinyl acetate	20	52	47	0.657	328.5	320.0
Cholesterol	9	46	28	0.691	386.7	378.7

* The TLC systems used were: S_1, Silufol/benzene; S_2, Silufol/benzene–ethyl methyl ketone (3:1); S_3, silica gel with 0.25 ml of PEG 200 per 200 g/benzene–light petroleum (1:1).

** Ratio of V_0/V_e, where V_0 is the limiting elution volume (47 counts; 1 count = 2.7 ml) and V_e is the elution volume.

($t = 12.73$; $t_{0.01;5} = 4.03$ at $n = 7$, where t is Student's characteristic and n is the number of compounds in the set tested). The calculated and true molecular weights are also shown in Table II.

Under the given experimental conditions, 1,2-naphthoquinone behaves as though it were a compound of higher molecular weight than 1,4-naphthoquinone. Differences in the elution volume found for positional isomers can be explained by the formation of solvation complexes with the solvent (see Fig. 1). Čoupek and co-workers[17,18] infer from the behaviour of phenols, bisphenols and dicarbo-*closo*-dodecaboranes that this phenomenon depends on the polarity of the molecule and on the proton-acceptor behaviour of the solvent. Accordingly, the more "polar" 1,2-naphthoquinone has a smaller elution volume than 1,4-naphthoquinone. It may be assumed that interaction takes place at the site of electron depletion in 1,2-naphthoquinone.

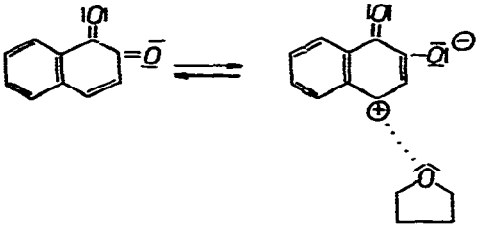

Fig. 1. Mechanism of formation of solvation complexes.

The different positions of hydroxyl groups also affect the difference between the calculated and true molecular weights of calciferol (*trans*-hydroxyl group); in this instance, the difference is larger than for cholesterol (*cis*-hydroxyl group).

Gel chromatography is particularly suitable for the determination of K vitamins in natural materials. The fact that these vitamines are oxidized readily in alkaline medium vitiates saponification of the sample and thus removal of such accompanying materials as triglycerides and diglycerides of higher molecular weight.

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