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## Note

# Thin-layer chromatography of coumarins of medicinal and phytochemical interest on buffered layers

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A number of papers have described the separation and identification of coumarin anticoagulants by thin-layer chromatography (TLC), and silica gel G[1–5], silica gel $HF_{254}$[4], silica gel G–cellulose (1:1)[6], silica gel $HF_{254}$–cellulose (1:1)[6], MN silica gel $N/UV_{254}$[7] and Kieselgel G[8] have been suggested as adsorbents. The solvent systems utilized have ranged from binary to quaternary mixtures of various organic solvents.

Owing to their weak acidic nature, coumarin anticoagulants have also been separated with solvent systems containing small amounts of either ammonia[8], formic acid[9] or acetic acid[3,4,6,7]. A convenient variation of this approach is to replace the alkali or the acid with a buffered layer of the corresponding pH value. For instance, Daenens and Van Boven[2] proposed the system silica gel G–phosphate buffer (pH 11), whereas Gàvat *et al.*[10] separated 3-(*R*)-substituted-$^{14}$C-ring labeled-4-hydroxycoumarins on silica gel G–0.1 *N* boric acid layers.

In this study, we investigated the possibility of conducting the TLC of the five coumarin anticoagulants listed in Table I, utilizing silica gel $HF_{254}$ layers buffered to pH 2.0, 3.6 or 5.0 and developed with binary solvent systems. In addition, the applicability of these procedures to the TLC of various plant coumarins has also been evaluated.

TABLE I

COUMARIN ANTICOAGULANTS

| *Chemical name* | *Drug name* | *Molecular formula* | *Molecular weight* |
|---|---|---|---|
| 3-(α-Acetonyl-*p*-nitrobenzyl)-4-hydroxycoumarin | Acenocoumarin | $C_{19}H_{15}NO_6$ | 353.32 |
| 3,3′-Methylenebis-(4-hydroxycoumarin) | Dicumarol | $C_{19}H_{12}O_6$ | 336.29 |
| Bis-(4-hydroxy-2-oxo-2*H*-1-benzopyran-3-yl)-acetic acid ethyl ester | Ethyl biscoumacetate | $C_{22}H_{16}O_8$ | 408.35 |
| 3-(α-Ethylbenzyl)-4-hydroxycoumarin | Phenprocoumon | $C_{18}H_{16}O_3$ | 280.31 |
| 3-(α-Acetonylbenzyl)-4-hydroxycoumarin | Warfarin | $C_{19}H_{16}O_4$ | 308.32 |

## EXPERIMENTAL

### *Samples*

The natural coumarins listed in Table II were purchased from Pfaltz & Bauer

TABLE II

COUMARINS OF PHYTOCHEMICAL INTEREST

| *Chemical Name* | *Common name* | *Chemical name* | *Common name* |
|---|---|---|---|
| Coumarin | — | 6,7-Dihydroxycoumarin | Esculetin |
| 4-Hydroxycoumarin | — | 6,7-Dihydroxycoumarin-6-glucoside | Esculin |
| 7-Hydroxycoumarin | Umbelliferone | 6-Methoxy-7-hydroxycoumarin | Scopoletin |
| 7,8-Dihydroxycoumarin | Daphnetin | 6-Methoxy-7-hydroxycoumarin-7-glucoside | Scopolin |

(Stamford, Conn., U.S.A.). Acenocoumarin and ethyl biscoumacetate were donated by Ciba-Geigy Pharmaceuticals (Ardsley, N.Y., U.S.A.), dicumarol and warfarin by Abbott Labs. (Chicago, Ill., U.S.A.) and phenprocoumon by Organon (West Orange, N.J., U.S.A.). Samples for TLC were prepared by dissolving the corresponding compound in either methanol or methylene dichloride to give a final concentration of 1 mg/ml. All samples remained stable for several weeks when stored in a refrigerator and protected from light.

*Thin-layer plates*

Glass plates (10 × 20 cm) were coated with a slurry prepared by vigorous mixing of 30 g of silica gel $HF_{254}$ (E. Merck, Darmstadt, G.F.R.; according to Stahl) with 70 ml (anticoagulants) or 80 ml (plant coumarins) of the buffer solution. A Brinkmann–Desaga spreading device adjusted to provide a 0.25-mm layer was used to apply the slurry. The plates were allowed to air-dry for 20 min at room temperature followed by oven drying at 110° for 2 h. The prepared plates were cooled and stored in a desiccator until needed.

*Buffers*

Three buffer solutions were used: (a) Clark and Lubs's KCl–HCl buffer, pH 2.0 (25 ml of 0.2 *N* KCl + 5.3 ml of 0.2 *N* HCl made up to 100 ml with water)[11]; (b) Walpole's acetate buffer, pH 3.6 (46.3 ml of 0.2 *M* acetic acid + 3.7 ml of 0.2 *M* sodium acetate made up to 100 ml with water)[11]; and (c) Sørensen's phosphate buffer, pH 5.0 (98.8 ml of 1/15 *M* $KH_2PO_4$ + 1.2 ml of 1/15 *M* $Na_2HPO_4 \cdot 2H_2O$)[11].

*Solvent systems*

All solvents were of analytical-reagent grade and were utilized without further purification. The ratios of the components in each system are expressed by volume.

*Chromatography*

A 3-$\mu$l sample of each reference compound was applied by means of a Lang–Levy micropipet, 2 cm from the lower edge of the plate and at intervals of about 1 cm. The plates were developed by the ascending technique to a distance of 14 cm from the point of application. The chromatographic chamber was fully lined with a piece of Whatman No. 1 filter-paper and pre-saturated with solvent vapor for at least 5 min prior to the introduction of the plate. All operations were conducted at room temperature (25–27°).

*Detection*

The developed chromatograms were thoroughly dried in a current of moving air and then examined under long- (365 nm) and short- (254 nm) wavelength UV light. Any visible spot was outlined by dotting the surface of the adsorbent with a dissecting needle. More permanent detection was achieved by exposing the chromatoplates to iodine vapor in a closed chamber or by spraying them with freshly prepared diazotized sulfanilic acid reagent[7].

## RESULTS AND DISCUSSION

Table III gives the $R_F \times 100$ values of the coumarin anticoagulants, and Table IV gives the $R_F \times 100$ values of the coumarins of phytochemical interest. These values are averages of several individual determinations.

TABLE III

$R_F \times 100$ VALUES OF COUMARIN ANTICOAGULANTS ON BUFFERED LAYERS

| *Compound* | *Buffer pH 2.0* | | | | | *Buffer pH 5.0* | | | | *Buffer pH 3.6* | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *1** | *2* | *3* | *4* | *5* | *3* | *6* | *7* | *8* | *8* | *9* | *10* | *11* | *12* | *13* | *14* |
| Dicumarol | 15 | 18 | 4 | 8 | 13 | 16 | 37 | 23 | 40 | 4 | 17 | 29 | 4 | 1 | 16 | 6 |
| Phenprocoumon | 56 | 83 | 45 | 53 | 39 | 44 | 63 | 60 | 34 | 61 | 68 | 62 | 54 | 29 | 61 | 52 |
| Acenocoumarin | 23 | 55 | 19 | 32 | 20 | 25 | 49 | 35 | 10 | 42 | 46 | 46 | 35 | 9 | 43 | 36 |
| Ethyl biscoumacetate | 0 | 2 | 0 | 3 | 0 | 0 | 1 | 1 | 0 | 1 | 4 | 18 | 0 | 0 | 4 | 0 |
| Warfarin | 34 | 70 | 35 | 47 | 26 | 36 | 57 | 49 | 14 | 54 | 58 | 58 | 48 | 18 | 51 | 41 |

* Solvent systems: 1, benzene–diethyl ether (2:1); 2, chloroform–diethyl ether (2:1); 3, *n*-hexane–diethyl ether (1:3); 4, *n*-hexane–dioxane (4:5); 5, toluene–acetone (5:1); 6, chloroform–diethyl ether (2:3); 7, *n*-hexane–ethyl acetate (1:1); 8, toluene–diethyl ether (1:4); 9, benzene–ethyl acetate (1:3); 10, benzene–isopropanol (6:1); 11, *n*-hexane–diethyl ether (1:10); 12, *n*-hexane–dioxane (3:1); 13, toluene–acetone (3:1); 14, toluene–dioxane (3:1).

TABLE IV

$R_F \times 100$ VALUES OF COUMARINS OF PHYTOCHEMICAL INTEREST

| *Compound* | *Buffer pH 2.0* | | | | | | *Buffer pH 5.0* | | | *Buffer pH 3.6* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *1** | *2* | *3* | *4* | *5* | *6* | *4* | *5* | *7* | *1* | *5* | *8* | *9* | *10* | *11* |
| Coumarin | 58 | 86 | 80 | 46 | 56 | 59 | 49 | 54 | 45 | 67 | 58 | 63 | 61 | 60 | 32 |
| 4-Hydroxy-coumarin | 37 | 52 | 49 | 5** | 23 | 32 | 11 | 18 | 26 | 31 | 19 | 29 | 7 | 15 | 18 |
| Umbelliferone | 46 | 71 | 71 | 30 | 37 | 40 | 32 | 31 | 37 | 54 | 34 | 49 | 52 | 48 | 15 |
| Daphnetin | 17*** | 30*** | 46*** | 8*** | 27*** | 23*** | 20*** | 23 | 18 | 17*** | 25 | 18*** | 12*** | 21*** | 9*** |
| Esculetin | 16 | 26 | 45 | 6 | 17 | 21 | 13 | 14 | 13 | 13*** | 14 | 15*** | 11*** | 17*** | 3*** |
| Esculin | 0 | 7 | 14 | 0 | 0 | 11** | 0 | 0 | 0 | 2 | 1 | 0 | 0 | 1 | 0 |
| Scopoletin | 31 | 70 | 67 | 15 | 27 | 34 | 19 | 23 | 3 | 33 | 22 | 33 | 28 | 39 | 5 |
| Scopolin | 1 | 9 | 5 | 0 | 0 | 6 | 0 | 0 | 0 | 4 | 0 | 2** | 2 | 6** | 1 |

* Solvent systems: 1, benzene–isopropanol (8:1); 2, methylene dichloride–methanol (8:1); 3, toluene–acetone (1:2); 4, toluene–ethyl acetate (3:2); 5, toluene–ethyl formate (1:1); 6, toluene–methanol (3:1); 7, toluene–1-butanol (5:1); 8, benzene–isopropanol (6:1); 9, benzene–ethyl acetate (1:2); 10, toluene–acetone (1:1); 11, *n*-hexane–isopropanol (10:1).

** Slight tailing.

*** Tailing.

A comparison of the present procedure with an earlier one carried out on non-buffered layers[3] indicated that it can serve as an advantageous alternative. All five coumarin anticoagulants examined were readily and effectively separated by the buffer–solvent mixtures examined. Spots appeared rounded, well defined and easily detectable under short-wavelength UV light. Dicumarol was the only compound to exhibit a slight tendency to elongate, but this behavior was observed only on layers buffered with Walpole's pH 3.6 solution.

From published reports[3–7], it appears that satisfactory separations of coumarin anticoagulants can be obtained with solvent systems that contain small concentrations of an organic acid. The use of acidic layers not only obviates the necessity for the acid but also permits TLC with binary mixtures of common organic solvents. A simpler and satisfactory procedure is that of French and Wehrli[4], who suggested the combination of silica gel G with benzene–glacial acetic acid (20:3). In our experience, this method differed from others in that the coumarins were separated as very narrow, horizontally elongated spots, and a very uniform layer is required for optimal separation. Slight imperfections in the layers caused both distortion in the shape of the spots and some overlapping.

The $R_F$ values of the coumarin anticoagulants varied in a characteristic and predictable manner in the three buffer systems. The mobility of each compound increased with decreasing molecular weight and decreasing number of oxygenated functional groups (see Table I). Their positions in order of increasing $R_F$ values were ethyl biscoumacetate > dicumarol > acenocoumarin > warfarin > phenprocoumon. The only exception was found for the combination of Sørensen's pH 5.0 phosphate buffer with toluene–diethyl ether (1:4), where dicumarol migrated further than phenprocoumon.

Of the eight simple coumarins examined, seven have been reported to occur in higher plants[12]. The remaining one (4-hydroxycoumarin) was included in order to evaluate the behavior of a 4-hydroxyl substituent on the migration of the coumarin ring.

None of the buffer–solvent mixtures tested was able to separate all eight simple coumarins using one-dimensional TLC. The dihydroxycoumarins daphnetin and esculetin, which differ only in the pattern of substitution, migrated almost identically in most of the TLC systems and, furthermore, showed a tendency to tail considerably, especially in those solvents which contained a lower alcohol. The coumarin monoglucosides esculin and scopolin consistently remained at or near the point of sample application in all systems used.

The most satisfactory solvent for the TLC of simple coumarins was toluene–ethyl formate (1:1). In this system, the reference compounds migrated as round, well defined spots, but again the glucosides did not show any appreciable movement. Stahl and Schorn[13] have separated esculetin, esculin, scopoletin and 4-methylumbelliferone on silica gel G layers buffered with 0.3 *M* sodium acetate. The order of separation of these compounds according to decreasing $R_F$ values was 4-methylumbelliferone > scopoletin > esculin > esculetin. In contrast, we observed that acidic layers will separate esculetin ahead of esculin and that the former will tail considerably in most of the solvents tested.

The following conclusions could be drawn for the coumarin derivatives: (a) if the coumarin ring bears no substituent or a 7-hydroxyl group, the compound will

have the highest $R_F$ value; (b) in Walpole's buffer of pH 3.6 with *n*-hexane–isopropanol (10:1), 4-hydroxycoumarin moves further than the 7-hydroxy isomer; in the remaining systems, 7-hydroxycoumarin appears in front of its 4-hydroxy counterpart; (c) in those solvents which contain a lower alcohol, the order of migration of the coumarins with the highest $R_F$ values was, in decreasing order, coumarin > 7-hydroxycoumarin > scopoletin > 4-hydroxycoumarin; (d) the presence of a methoxyl group in the coumarin ring lowered the $R_F$ value to a greater extent than the presence of a hydroxyl group; (e) 4-hydroxycoumarin trailed behind 7,8-dihydroxycoumarin and 6-methoxy-7-hydroxycoumarin in solvents that contain acetone or an ester; and (f) all simple coumarins, except coumarin, 4-hydroxycoumarin and scopolin, were detectable under long-wavelength UV light; coumarin and 4-hydroxycoumarin became visible under short-wavelength UV light; scopolin remained invisible under these conditions and its detection required exposure of the chromatoplates to iodine vapor for a few minutes.

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