Table III—Ascorbic Acid Analysis

Product	Amount Declared per Dose, mg.	Mean Amount (mg.) $\pm$ SD^a	
		TCE[b] Method	Independent Method
A	100	115.13 $\pm$ 2.78	111.81 $\pm$ 11.37[c]
B	100	97.77 $\pm$ 5.36	102.98 $\pm$ 6.38[c]
C	75	77.98 $\pm$ 2.78	79.32 $\pm$ 2.99[c]
D	75	76.32 $\pm$ 2.09	80.74 $\pm$ 2.65[c]
E	50/5 ml.	54.10 $\pm$ 0.44	53.81 $\pm$ 0.20[d]
F	60/0.6 ml.	61.41 $\pm$ 0.52	62.00 $\pm$ 0.21[d]

[a] Calculated on basis of five doses; each assayed by TCE and independent method. [b] Tubular carbon electrode method. [c] *Reference 8.* [d] *References 9* and *10.*

Several compounds commonly used as antioxidants undergo oxidation at carbon electrodes (7), but no interference by these compounds with the ascorbic acid assay is expected for two reasons:

1. The concentrations of antioxidants is usually low and will not be detectable in the samples diluted (usually 1 : 10,000) for analysis.

2. The half-wave potentials for most (7) antioxidants differ sufficiently from that of ascorbic acid to permit separation of the waves.

The absence of interferences in the multivitamin products used in this study is indicated by the fact that the shapes of the current–voltage curves for the products were identical to those for standard ascorbic acid solutions.

The vitamin content of the products studied is given in Table I. No alterations of the ascorbic acid current–voltage curves resulted from the other vitamins contained in these products. Table III shows the mean ascorbic acid content of these products determined by assaying five doses of each. Aliquots of each dose were assayed using both the tubular carbon electrode and the methods indicated in the table. The data for the solid dosage forms show variations in the content of the tablets as well as the relative precision of the methods used, while the data for the liquid dosage forms indicate only the relative precision of the methods. From this study it may be concluded that the tubular carbon electrode method gives comparable or superior precision and accuracy when compared to other methods commonly used in the analysis of ascorbic acid. The reproducibility of the response of the tubular carbon electrode was determined by assaying a single sample dilution five times. A standard deviation of the mean of 0.91% was found.

A recorder trace of limiting current *versus* time for a series of standard ascorbic acid solutions is shown in Fig. 3. This recorder trace indicated the time required to assay a single sample. Once the sample solutions had been prepared, between 25 and 30 ascorbic acid determinations could be performed each hour. Although automation was not employed in the present study, the flowing stream methodology could readily be incorporated in automated or semi-automated systems.

In comparison to the tubular carbon electrode method, the iodine titration was less specific while the colorimetric methods involved several manipulative steps and thus required a much longer time. In summary, it is concluded that the method introduced in this paper has comparable precision, accuracy, and freedom from interferences when compared with methods presently available for the analysis of ascorbic acid in multivitamin products. In addition, the method is faster and less complicated to perform.

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TLC of Coumarin Anticoagulants

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Abstract ☐ A simple, rapid, and reproducible TLC procedure for the separation and identification of five 4-hydroxycoumarin derivatives used as anticoagulants and rodenticides is presented.

Keyphrases ☐ Coumarin anticoagulants—TLC separation and identification ☐ Anticoagulants, coumarin—TLC separation and identification ☐ TLC—separation, identification of coumarin anticoagulants

The separation and identification of coumarin derivatives used as anticoagulants and rodenticides are of interest to the forensic toxicologist and the analytical chemist. Internal hemorrhages found in corpses upon autopsy is often considered presumptive evidence of death arising from intoxication by coumarin anticoagu-
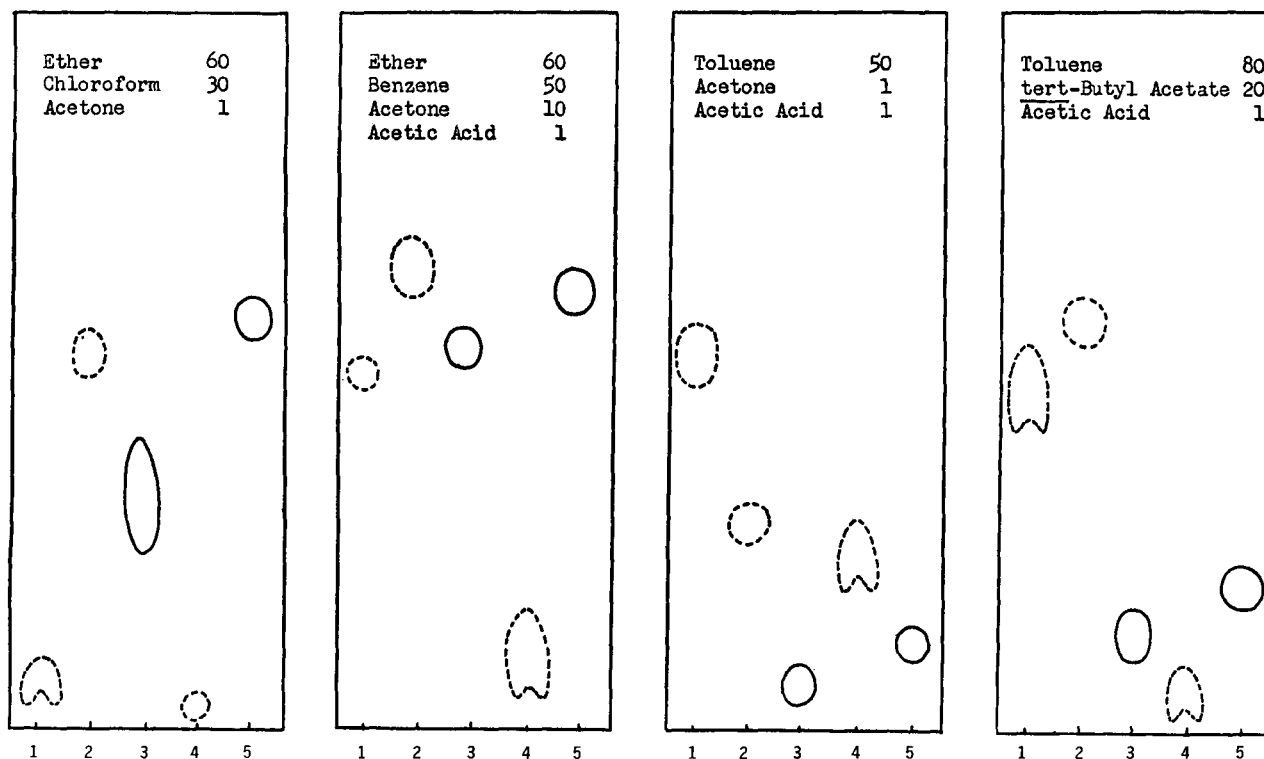
Figure 1—*TLC of 4-hydroxycoumarin anticoagulants on silica gel HF_{254}. Key: 1, dicumarol; 2, phenprocoumon; 3, acenocoumarol; 4, ethyl biscoumacetate; and 5, warfarin. Dotted outlines indicate that the spot is fluorescent under 365-nm. UV light.*

lants. Several commercial rodenticides contain between 0.5 and 1% of a coumarin derivative as the active ingredient (1). In these instances, the problem resides in the proper characterization and identification of these substances by sensitive and reliable procedures (2).

TLC (2–7) and paper chromatography (1, 7–9) have been applied to the analysis of coumarin anticoagulants following their isolation from biological materials and commercial products. TLC has proven particularly advantageous for routine work owing to its simplicity, speed, and high resolving power, coupled with a great versatility and small sample requirement (2–7).

The only work dealing with the TLC analysis of the five coumarin derivatives listed in Table I appears to be that of Kamm (3). In this study, separations were accomplished on silica gel G plates with either of two solvent mixtures: (*a*) *n*-butanol–amyl alcohol (1:1) saturated with 25% ammonia, or (*b*) incorrectly reported as dioxane–toluene–isopropanol–25% ammonia (1:2:1:4: 2) [the correct composition is dioxane–xylene–toluene–isopropanol–25% ammonia (1:2:1:4:2)[1]]. Chromatography with these solvents requires several hours, and spots are generally elongated. Furthermore, the two-dimensional chromatographic procedure proposed by Kamm for the resolution of a mixture of various coumarin anticoagulants is also considerably lengthy (approximately 7 hr.).

The present article reports on the TLC of five coumarin anticoagulants (Table I) on layers of silica gel GF_{254} and silica gel HF_{254} by the ascending technique in conjunction with several solvent systems and detection procedures.

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[1] Personal communication from Dr. G. Kamm, University of Marburg, Germany.

Table I—4-Hydroxycoumarin Anticoagulants

Drug Name	Chemical Name
Acenocoumarol	3-(α-Acetonyl-*p*-nitrobenzyl)-4-hydroxycoumarin
Dicumarol[a]	3,3′-Methylenebis[4-hydroxycoumarin]
Ethyl biscoumacetate	(4-Hydroxy-2-oxo-2*H*-1-benzopyran-3-yl)acetate
Phenprocoumon	3-(α-Ethylbenzyl)-4-hydroxycoumarin
Warfarin	3-(α-Acetonylbenzyl)-4-hydroxycoumarin

[a] Formerly named bishydroxycoumarin.

EXPERIMENTAL

Materials—Acenocoumarol[2], ethyl biscoumacetate[3], dicumarol[4] (bishydroxycoumarin), warfarin[4], phenprocoumon[5,6], and coumarin[7] were used. Phenprocoumon was also obtained from the commercial tablets as follows. Tablets sufficient to yield 20 mg. of coumarin derivative were reduced to a fine powder in a mortar, the powder being thoroughly extracted by trituration and shaking with methylene dichloride–methanol (9:1). The solution was filtered and evaporated to dryness *in vacuo*. The residue was dried over anhydrous silica gel for 24 hr. All solvents and chemicals were of analytical reagent grade and were used without further purification.

Preparation of Plates—The plates (20 × 20 cm.) were coated with silica gel GF_{254}[8] or silica gel HF_{254}[8] according to the procedure of Randerath (11). The layers (250 μ thick) were activated at 110° for 3 hr. and stored in a desiccator over anhydrous silica gel for 1 hr. prior to use.

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[2] Sintrom, Geigy Pharmaceuticals, Ardsley, N. Y.
[3] Tromexan, Geigy Pharmaceuticals, Ardsley, N. Y.
[4] Abbott Laboratories, Chicago, Ill.
[5] Liquamar, Organon Inc., West Orange, N. J.
[6] Marcoumar, obtained from Dr. G. Kamm, University of Marburg, Germany.
[7] Pfaltz & Bauer, Inc., Flushing, N. Y.
[8] E. Merck, Brinkmann Instruments Inc., Westbury, N. Y.

Table II—Solvent Systems for TLC of Coumarin Anticoagulants

Solvent	Composition[a]
1	Ether–acetic acid (99:1)
2	Ether–chloroform–acetone (60:30:1)
3	Ether–ethyl acetate–acetic acid (80:30:1)
4	Ether–benzene–acetone–acetic acid (60:50:10:1)
5	Ether–benzene–acetone (60:30:10)
6	Ether–benzene–acetic acid (60:40:1)
7	Toluene–acetone–acetic acid (50:1:1)
8	Toluene–ethyl acetate–acetic acid (20:5:1)
9	Toluene–p-dioxane–acetic acid (50:10:1.5)
10	Toluene–ether–acetic acid (50:7:1)
11	Toluene–tert-butyl acetate (80:20)
12	Toluene–tert-butyl acetate–acetic acid (80:20:1)
13	Benzene–ethyl acetate–acetic acid (70:30:0.5)

[a] All ratios are expressed in volume.

Table III—$R_f \times 100$ Values of Coumarin Anticoagulants on Silica Gel HF$_{254}$

Compound	Solvent System												
	1	2	2[a]	3	4	5	6	7	8	9	10	11	11[a]	12	13
Coumarin[b]	87	58	61	62	62	58	50	24	46	47	34	40	35	40	47
Dicumarol	60	9	6	41	52	12	54	44	68	56	53	17	13	41	41
Phen-procoumon	95	60	53	68	64	66	58	24	55	40	29	40	35	49	46
Aceno-coumarol	83	42	31	56	51	50	44	7	30	26	9	7	7	12	31
Ethyl biscoum-acetate	10	3	2	11	11	4	52	24	25	24	15	0	0	5	14
Warfarin	91	84	54	65	57	58	22	11	35	32	10	14	11	16	37

[a] Silica gel GF$_{254}$ plates. [b] Internal standard.

Chromatographic Procedure—Compounds were applied (3 µl. of a 0.2% w/v solution in chloroform) by means of a micropipet, at 2 cm. from the lower edge of the plate and at intervals of about 1.5 cm. Coumarin was used as the internal standard. The plates were developed to a distance of 12 cm. from the point of application in a rectangular chromatographic chamber fully lined with Whatman No. 1 filter paper and saturated with solvent vapors for 15 min. prior to use. All operations were conducted at ambient temperature (25–26°).

Developing Systems—The chromatographic behavior of the various coumarin anticoagulants was studied utilizing five types of solvent systems: (a) single solvents: acetone, p-dioxane, ether, ethyl acetate, isopropanol, methanol, tert-butanol, cyclohexanone, chloroform, tert-butyl acetate, and cyclohexane; (b) binary solvent systems: acetone–chloroform, acetone–methanol, acetone–benzene, acetone–ether, acetone–tert-butyl acetate, acetone–toluene, ether–ethyl acetate, ether–chloroform, ether–methylene dichloride, ether–toluene, ether–p-dioxane, ether–ethyl benzoate, ether–isopropyl palmitate, ether–tert-butyl acetate, ethyl acetate–methylene dichloride, ethyl acetate–cyclohexane, ethyl acetate–benzene, methanol–tert-butyl acetate, toluene–ethyl benzoate, toluene–tert-butyl acetate, toluene–isopropanol, toluene–p-dioxane, benzene–tert-butyl acetate, and benzene–methanol; (c) ternary solvent systems: ether–acetone–chloroform, ether–methanol–benzene, ether–ethyl acetate–methanol, ether–ethyl acetate–benzene, ether–acetone–benzene, toluene–ace-tone–ether, toluene–p-dioxane–ether, toluene–p-dioxane–acetone, and toluene–ether–methanol; (d) basic solvent systems: ethyl acetate–pyridine (99:1), acetone–pyridine (99:1), and ether–pyridine (99:1); and (e) acidic solvent systems: cyclohexane–ethyl acetate–acetic acid, toluene–ethyl acetate–acetic acid, toluene–ether–acetic acid, toluene–acetone–acetic acid, toluene–p-dioxane–acetic acid, toluene–isopropanol–acetic acid, ether–acetic acid, ether–methanol–acetic acid, ether–ethyl acetate–acetic acid, ether–acetone–acetic acid, ether–acetone–benzene–acetic acid, ether–benzene–acetic acid, acetone–acetic acid, acetone–formic acid, ethyl acetate–acetic acid, p-dioxane–formic acid, ethyl acetate–methylene dichloride–acetic acid, benzene–isopropanol–acetic acid, benzene–ethyl acetate–acetic acid, p-dioxane–ethyl acetate–acetic acid, p-dioxane–ether–acetic acid, and toluene–tert-butyl acetate–acetic acid.

In solvent types (b), (c), and (e), the ratios of each component were varied to achieve a satisfactory separation and well-defined spots.

Detection—Following development, the chromatoplates were dried in an oven at 70° for 5 min. and then viewed under 254- and 365-nm. UV light. Compounds were also detected by exposing the chromatograms to iodine vapors for 5 min. in a closed chamber. A more permanent record was obtained by spraying the plates with 2,6-dichloroquinonchloroimide reagent (I) (2). Alternatively, anisaldehyde-sulfuric acid reagent (II) (12) and diazotized sulfanilic acid reagent (III) (2, 8) were also employed.

RESULTS AND DISCUSSION

Table II presents those solvents found most suitable for the separation and identification of the five coumarin anticoagulants examined. Table III gives the average $R_f \times 100$ values for the different compounds. Table IV summarizes the colors obtained for each coumarin examined with the various chromogenic reagents as well as their fluorescences under long wavelength UV light.

Of the two adsorbents used, silica gel GF$_{254}$ and silica gel HF$_{254}$, better separations and slightly higher R_f values were obtained with silica gel HF$_{254}$. Moreover, the time of development with this adsorbent (approximately 20 min. with most of the solvents) was shorter.

Excessive streaking of materials on the plates was observed with the majority of the binary systems and with all the basic solvents tested. In the first case, the addition of 0.1–1% of either formic acid or glacial acetic acid improved the chromatography and resulted in almost circular spots. Sharpness of separation was decreased with higher concentrations of acid.

Occasionally, ethyl biscoumacetate and warfarin were accompanied by a blue fluorescent spot with a higher R_f than either of the two coumarins. This finding confirms a previous report indicating that degradation products of warfarin, arising from the action of light on this compound during its TLC analysis, may be detected on thin-layer plates (7). Since, in our experience, these additional spots were only observed with sample solutions in p-dioxane and not in chloroform, it is assumed that the solvent used in the preparation of the sample for chromatography can also contribute to the degradation of the solute. Welling et al. (7) previously showed that solutions of warfarin in either 1,2-ethylene dichloride or acetone are light sensitive. On the other hand, we observed that if warfarin or any of the other coumarin anticoagulants are spotted on a chromatoplate, allowed to remain on the starting line for a few hours, and then developed, several additional spots with higher and lower R_f values can be detected under 365-nm. UV light or after visualization with

Table IV—Color Reactions and Fluorescences of 4-Hydroxycoumarin Anticoagulants

Compound	I[b] Immediately	I[b] After 10 min.	I[b] After 1 hr.	II[c]	III[d]	Fluorescence[e]
Coumarin[a]	Bluish-green	Pale-blue	Blue	—	—	Pale-violet-blue
Dicumarol	Pale-violet	Violet-brown	Blackish-brown	—	Orange-yellow	Whitish-pink
Phenprocoumon	Blue	Purple	Purplish-brown	—	Pale-yellow	Violet-blue
Acenocoumarol	Yellow	Reddish-brown	Blackish-brown	—	Pale-yellow	—
Ethyl biscoumacetate	Pale-orange	Emerald-green	Greenish-blue	Pink	Orange-yellow	Whitish-blue
Warfarin	Pale-orange	Purple, then reddish-brown	Reddish-brown	Pink-violet	Pale-yellow	

[a] Internal standard. [b] 2,6-Dichloroquinonchloroimide reagent. [c] Anisaldehyde–sulfuric acid reagent. [d] Diazotized sulfanilic acid reagent. [e] Under 365-nm. UV light.

detecting reagents. Degradation was perceived by the gradual appearance of a yellow color at the point of sample application.

In conjunction with the R_f values, further verification of the identity of the anticoagulants considered in this study was ascertained by spraying the chromatograms with either I or II. The first reagent gave characteristic colors with each of the coumarins, whereas the latter only reacted with ethyl biscoumacetate (pink color) and warfarin (pink-violet color). In addition, certain spots were easily identified by their characteristic appearance under 365- and 254-nm. UV light after development with Solvent 2, 4, 7, or 12 (Table II and Fig. 1). As little as 1 mcg. of sample could be detected with iodine vapors, I, or II. The detection limit for phenprocoumon was about 2-3 mcg. III was the least sensitive of all the reagents tested.

The excellent one-dimensional chromatographic separation obtained with the majority of solvents obviates the use of the two-dimensional technique for the resolution of a mixture of coumarin anticoagulants. In general, separation was better than has been reported in other laboratories and the time of development with the solvents employed was much shorter.

This work may be useful as an adjunct tool for the rapid identification and confirmation of coumarin anticoagulants in experimental pharmacology, biochemistry, and quality control as well as in forensic and clinical medicine.

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ACKNOWLEDGMENTS AND ADDRESSES

Fluorometric Determination of Ethinyl Estradiol in Tablets

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Abstract ☐ A fluorometric procedure, based on the Liebermann–Burchard reaction, was developed for assaying ethinyl estradiol in tablets. A chloroform extract of ethinyl estradiol is reacted with acetic anhydride and sulfuric acid, and the resulting fluorophor is measured at 400 nm. while exciting at 324 nm. Fluorescence *versus* concentration is linear up to 10 mcg./ml.; the lower limit of detection is 0.5 mcg./ml. under the conditions studied. Replicate analyses showed good agreement, and an average recovery of $100.0 \pm 0.94\%$ was obtained for 10 analyses of a synthetic mixture. Assay results on eight different commercial samples (0.01–0.50 mg./tablet) are reported.

Keyphrases ☐ Ethinyl estradiol tablets—fluorometric analysis, Liebermann–Burchard reaction ☐ Spectrophotofluorometry—analysis, ethinyl estradiol tablets, Liebermann–Burchard reaction

Commercially available tablets of ethinyl estradiol are usually of very low dosages (0.01–0.05 mg./tablet). Consequently, quantitative analysis of these products has been difficult. The USP (1) colorimetric procedure, a modification of the Kober reaction, has several disadvantages:

1. Ethinyl estradiol is only slightly soluble in isooctane, so it may not be completely extracted in the USP procedure. Indeed, the first supplement to the USP XVIII, official in November 1971, modified the original isooctane extraction by using an isooctane–chloroform mixture instead.

2. The method involves numerous transfers and extractions, making it both time consuming and cumbersome.

3. There are several very critical variables in the color formation (moisture, purity of reagents, preparation of the sulfuric acid–methanol reagent, *etc.*).

Other colorimetric procedures (2–6), which are also variations of the Kober reaction, suffer similar handicaps.

UV (7) and GLC (8, 9) methods for determining ethinyl estradiol also have been reported. These methods require extensive cleanup procedures. Furthermore, the GLC methods usually involve derivatization.

The sensitivity of fluorescence prompts an investigation of its application to the analysis of ethinyl estradiol. Fluorescence methods reported to date (10–14), however, are again extensions of the Kober reaction. Little work has been done on the natural fluorescence of ethinyl estradiol or other forms of induced fluorescence.