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Qualitative and Quantitative Aspects of Using Eshtblausalz B to Visualise 4-Hydroxycoumarin and Some of its Biologically Active Derivatives on Thin-Layer Chromatographs

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Qualitative and quantitative aspects of the analysis of 4-hydroxycoumarin and some of its biologically active derivatives (dicumarol, Tromexan^R, warfatin, 3-(ω-bromoacetyl)-4-hydroxycoumarin and 3-(ω-bromoacetyl)-4-hydroxy-7-bromocoumarin) were studied. The coumarin derivatives can be sharply separated by TLC on silica gel HF_{254} using Eshtblausalz B for detection. Azo compounds of coumarine derivatives show that a linear relationship exists between spot-eluate absorbances and the amonts applied from 10 to 30 μg. The new rapid and sensitive method was obtained for qualitative and quantitative analysis of 4-hydroxycoumarin derivatives.

We believe that any new contribution which improves the existing analytical methods for coumarins, furocoumarins, and other derivatives of α-pyrone should be of considerable practical value. To justify this view let us remind the reader that the α-pyrone structure occurs not only in numerous physiologically important plant constituents[1], but also in many synthetic agents which produce useful effects in animals and man[2]. Our own interest in such compounds centers around the hydroxylated coumarins, because several of these compounds have been introduced into human therapy[3].

The methods currently used to detect and quantitate compounds containing the coumarin ring system are based on composite procedures including chromatography — especially GLC[4,5] — and other methods of separation, color-forming reactions[6] and colorimetry, ultraviolet and visible absorption and emission (fluorescence) spectrophotometry[7], and electrochemical recording by potentiometry[8] and polarography[9–11]. We have reported previously[12] that several coumarin derivatives can be sharply separated by TLC on silica gel, and detected on chromatograms by exciting their fluorescence. This procedure has been thereafter successfully used with the parent 4-hydroxycoumarin and its 7-hydroxylated derivative, with anticoagulant drugs belonging to the 4-hydroxycoumarin series (dicumarol. Tromexan^R, warfarin), and with two agents producing multiple biological effects — fungicidal, insecticidal, rodenticidal —, 3-(ω-bromoacetyl)-4-hydroxycoumarin and its 7-brominated derivative[13].

Meanwhile we have modified our original procedure by introducing a different mode of visualization. Instead of exciting fluorescence we now produce visible colorations by reacting the chromatographically separated coumarin derivatives, in situ, with Echtblausalz B (EBB), a double salt containing 4,4'-bis(diazonium)-3,3'-dimethoxybiphenyl dichloride and $ZnCl_2$. The modified procedure seems to offer some advantages which will be described in the next Section.

RESULTS

We have summarized in Table I some of the data showing the merits of EBB detection. In order to obtain the data presented, fluorescence excitation and the development of color were carried out, in succession, on the same chromatograms so that the results are directly comparable. Two series of chromatograms were prepared using the same stationary and mobile phases, one series with a layer of 0.25 mm, the other with 0,50 mm thickness.

The layer thickness had practically no influence on the migration speed of any compound tested, as shown by the almost equal R_f values*, but it did affect the sensitivity of detection in both modes of visualization. On the other hand, the mode of visualization also influenced the detection sensitivities, except in one instance, namely with 3-(ω-bromoacetyl)-7-bromo-4-hydroxycoumarin. With all other compounds the use of EBB distinctly lowered the minimum detectable mass (m. d. m.), this increasing the detection sensitivities. Finally, the compounds tested produced different nuances of blue and could

TABLE I

Comparison of Fluorescence Excitation and Color Development as Modes of Visualization for 4-Hydroxycoumarin Derivatives on TL Chromatograms

Chromatography. — Stationary phase: silica gel; **I**, commercial[a], 0.25-mm layer on aluminum (DC Alufolien Kiesel-gel 60 HF_{254}) — **II**, laboratory-made, 0.5-mm layer on glass [crystalline powder HF_{254} (Stahl's type 60)[a] was used]. — Mobile phase: benzene-acetic acid-ethylmethylketone, 8 : 1 : 1 all solvents of highest purity grade[a].

Detection. — FLU: fluorescence excited at 360 nm; EBB: spraying with a 0.25% solution of Echtblausalz B[b] in 0.05 M NaOH, followed by drying at room temperature. Positive or negative outcomes were judged by inspection. Figures represent averages from three experiments.

Serial number[c]	R_f		Substituents in the 4-hydroxycoumarin ring system			Minimum detectable mass/μg			
						FLU		EBB[d]	
	on I	on II	Position 3		Position 7	I	II	I	II
1	0.28	0.27	H	□		2.0	2.0	1.0	0.3
2	0.92	0.88	(4'-Hydroxycoumarin-3'-yl)methyl	○	H	1.0	0.5	0.2	0.2
3	0.23	0.22	(3'-Oxo-1'-phenyl)butyl warfarin	△	H	1.0	0.5	0.5	0.2
4	0.42	0.41	ω-Bromoacetyl	×	H	1.0	1.0	0.5	0.5
5	0.46	0.45	ω-Bromoacetyl		Br	2.0	2.0	2.0	2.0
6	0.15	0.15	H	▲	OH	5.0	5.0	1.0	0.5

[a] All-supported silica gel, Stahl s crystalline powder, and the solvents used in preparing the mobile phase were from E. Merck, Darmstadt, W. Ger. — [b] From FLUKA AG, Buchs, Switzerland. — [c] Compounds were applied to the plates in form of solutions in 96% ethanol, except for compd. 3 which was dissolved in acetone. — [d] The coloration of spots was in different shades of blue.

be distinguished at a glance, which may enable rapid identification by comparison with color standards and is an additional advantage of using EBB.

Quantitative relationships that can be derived directly from chromatograms, or after eluting the stains, are presented graphically in Figure 1. The relationship, spot-area vs. amount of compound applied, shows that the colored-spot areas as they result from the EBB treatment depend linearly on the amount of compound applied to the plate, and this relationship is valid up to 30 μg except for 4-hydroxy and 4,7-dihydroxycoumarins for which linearities extend only to 20 μg. Similarly, the relationship, absorbance vs. ammount of compound applied, shows that a linear relationship exists between spot-

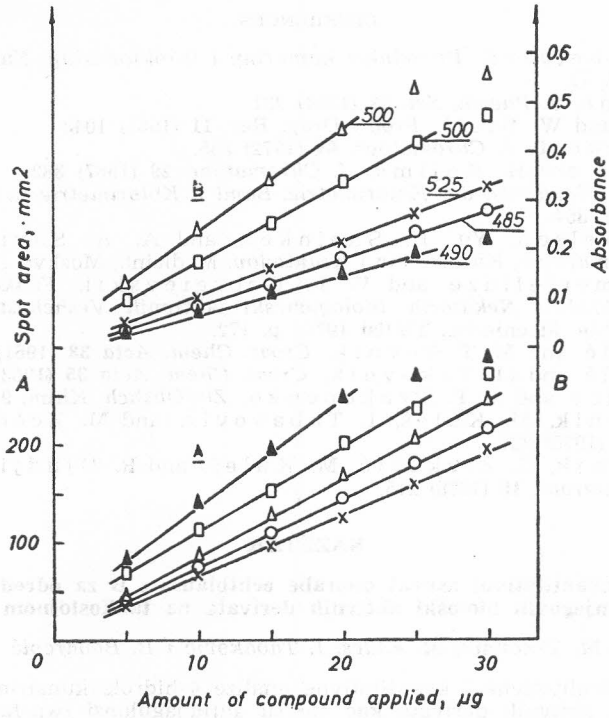

Figure 1. Quantitative evaluation of TL chromatograms prepared with some 4-hydroxycoumarin derivatives on 0.50 mm silica gel plates and visualized with Echtblausalz B For a description of materials and procedures see legend of Table I. Increasing amounts of each compound were applied onto the baseline of the same plate and the front of developing solvent was allowed to ascend 15 cm above this line in all runs. Points on the graphs represent averages from three experiments: , 4-hydroxycoumarin; △, warfarin 3-(3'-oxo-1'-phenylbutyl)-4-hydrocycoumarin; ○ dicumarol 3,3'-methylene*bis*(4-hydroxycoumarin); X, 3-(ω-bromoacetyl)-4-hydroxycoumarin: ▲, 4,7-dihydroxycoumarin. A, Spot area vs. amount applied. B, Absorbance of stain eluates (5 ml of eluent per spot: $CHCl_3$ for all compounds except 4,7-dihydroxycoumarin which was eluted with acetone) vs. amount applied; measurements were made at the wavelengths of maximum absorbance as indicated in the graph.

* Two additional anticoagulants, phenprocoumon Marcumar[a] by Hoffmann — La Roche, Basle, Switzerland: 3-(1'-phenylpropyl)-4-hydroxycoumarin and ethyl biscoumacetate Tromexan[R] by Geigy, Basle, Switzerland: 3,3'-carbethoxymethlene-bis(4-hydroxycoumarin), too, had practically the same R_f value on chromatograms with either, layer thickness: 0.79—0.78 and 0.54—0.54, respectively. The minimum detectable mas of both compounds was assessed by fluorescence only and was one μg.

-eluate absorbances (at the wavelengths of maximum absorbance indicated) and the amounts applied. Again, this relationship is obeyed up to 30 μg, except, for the parent 4-hydroxycoumarin for which it only holds up to 20 μg.

In conclusion: the results obtained by using EBB to detect, identify, and quantitatively determine several derivatives of 4-hydroxycoumarin separated by TL chromatography are promising and the EBB treatment may turn out to be at least as satisfactory with other derivatives from the same series. This potentially useful method requires only basic equipment as may be found in any laboratory.

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SAŽETAK

Kvalitativni i kvantitativni aspekt uporabe echtblausalz B za određivanje 4-hidroksikumarina i njegovih biološki aktivnih derivata na tankoslojnom kromatogramu

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Za svrhe kvalitativne i kvantitativne analize 4-hidroksikumarina i nekih njegovih fiziološki aktivnih derivata kao što su antikoagulansi (warfarin, dikumarol, tromexan) i derivati s fungicidnim i insekticidnim djelovanjem (ω-brom-3-acetil i ω-brom-3-acetil-7-brom-4-hidroksikumarin) oni su bili razdvojeni tankoslojnom kromatografijom na silikagelu HF_{254} pri čemu je za detekciju uspješno primjenjen Echtblausalz B. Azo-spojevi kumarinskih derivata sa Echtblausalz B nakon eluiranja organskim otapalima pokazuju linearnu zavisnost apsorbancije o koncentraciji kromatografiranih supstancija od 10—30 μg. Na taj način dobivena je nova osjetljiva i brza metoda za kvantitativnu i kvalitativnu analizu derivata 4-hidroksikumarina.

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