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Note

High-performance liquid and thin-layer chromatography of coumarin anticoagulants and their degradation products

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Both thin-layer chromatography (TLC)[1–9] and gas–liquid chromatography (GLC)[10–14] have been widely used for the separation of anticoagulant mixtures and their detection in biological materials. This paper describes the high-performance liquid chromatography (HPLC) and the high-performance thin-layer chromatography (HPTLC) of six anticoagulants derived from 4-hydroxycoumarin. Solvent systems, adsorbents and packings were selected so as to permit the separation of degradation products that occur in solution, in the presence of some tablets vehicles or of the adsorbent itself and probably by metabolic transformation.

EXPERIMENTAL

Apparatus

A Waters Assoc. liquid chromatograph (6000 M solvent delivery system) was equipped with a 30 cm × 1/4 in. I.D. stainless-steel column pre-packed with either μ-Porasil or μ-Bondapak C_{18}, supplied by Waters Assoc. (Milford, Mass., U.S.A.), (mean particle size, 10 μm in both instances). Detection was performed at 313 nm using a Waters Assoc. Model 440 absorbance detector (sensitivity, 0.05 a.u.f.s.).

Materials

All of the solvents were of analytical-reagent grade and were twice distilled. HPTLC was performed on non-activated 10 × 10-cm plates coated with silica gel 60 F_{254} (Merck, Darmstadt, G.F.R.)*. Compounds were applied as a 0.75-μl volume of a 1% (w/v) solution in ethanol or tetrahydrofuran at 1.2 cm from the lower edge of the plate. The plates were developed to a distance of 8.6 cm from the point of application in a 20 × 10-cm chromatographic chamber without previous saturation. The solvent system was benzene–carbon tetrachloride–dioxan–acetic acid (50:40:10:1).

Further details of the solvent systems, sample sizes, flow-rates and pressures used in HPLC chromatography are summarized in Table I.

The columns were equilibrated with solvent system A, which was used alone

* Pre-coated plates for nanogram-scale TLC (pore size 60 Å).

TABLE I

EXPERIMENTAL CONDITIONS FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Parameter	*Operating conditions*		
	A	*B*	*C*
Packing	μ-Porasil	μ-Porasil	μ-Bondapak C_{18}
Solvent system	(A) Carbon tetrachloride–benzene–acetic acid (40:50:1) (B) Carbon tetrachloride–benzene–dioxan–acetic acid (40:50:5:1) (C) Carbon tetrachloride–benzene–dioxan–acetic acid (37:27:25:1)	(A) Carbon tetrachloride–benzene–dioxan (49.5:49.5:1.0)	(A) Ethanol (94%)–water containing 0.1% acetic acid (1:1) (B) Ethanol (94%)–water containing 0.1% acetic acid (3:2)
Flow-rate (constant)	2.0 ml/min	2.0 ml/min	1.4 ml/min
Pressure	*ca.* 2000 p.s.i.	*ca.* 1900 p.s.i.	*ca.* 5000 p.s.i.
Sample size	5 μl of a 0.01% (w/v) solution in methanol (saturated methanolic solution for bishydroxycoumarin)	5 μl of a 0.01–0.1% (w/v) solution in chloroform	5 μl of a 0.01% (w/v) solution in tetrahydrofuran
Chart speed	1 cm/min	1 cm/min	40 cm/h

or modified during chromatography. All of the separations were conducted at room temperature (20–22°).

RESULTS AND DISCUSSION

High-performance liquid chromatography

The best separation of the anticoagulants (Fig. 1) was obtained on the μ-Bondapak C_{18} column, but warfarin and acenocoumarin were not resolved.

More complex solvents systems were required on μ-Porasil (Fig. 2); under these conditions, warfarin and acenocoumarin were separated but it is assumed that ethyl biscoumacetate was decomposed.

An additional chromatography on μ-Porasil is helpful in studying the degradation products of the anticoagulants; a typical example (phenprocoumon) is shown in Fig. 3. However, the separation of the two isomeric compounds present in the degradation products of class II was achieved on μ-Bondapak C_{18} with methanol–water (7:3, v/v) as the solvent system (flow-rate 2.0 ml/min).

The addition of small amounts of acetic acid to the solvent systems helps to prevent tailing of the peaks, presumably because it suppresses ionization of the phenolic function.

The high resolving power and sensitive detection achieved with this method would be particularly advantageous for the detection and the determination of the metabolites of the anticoagulants.

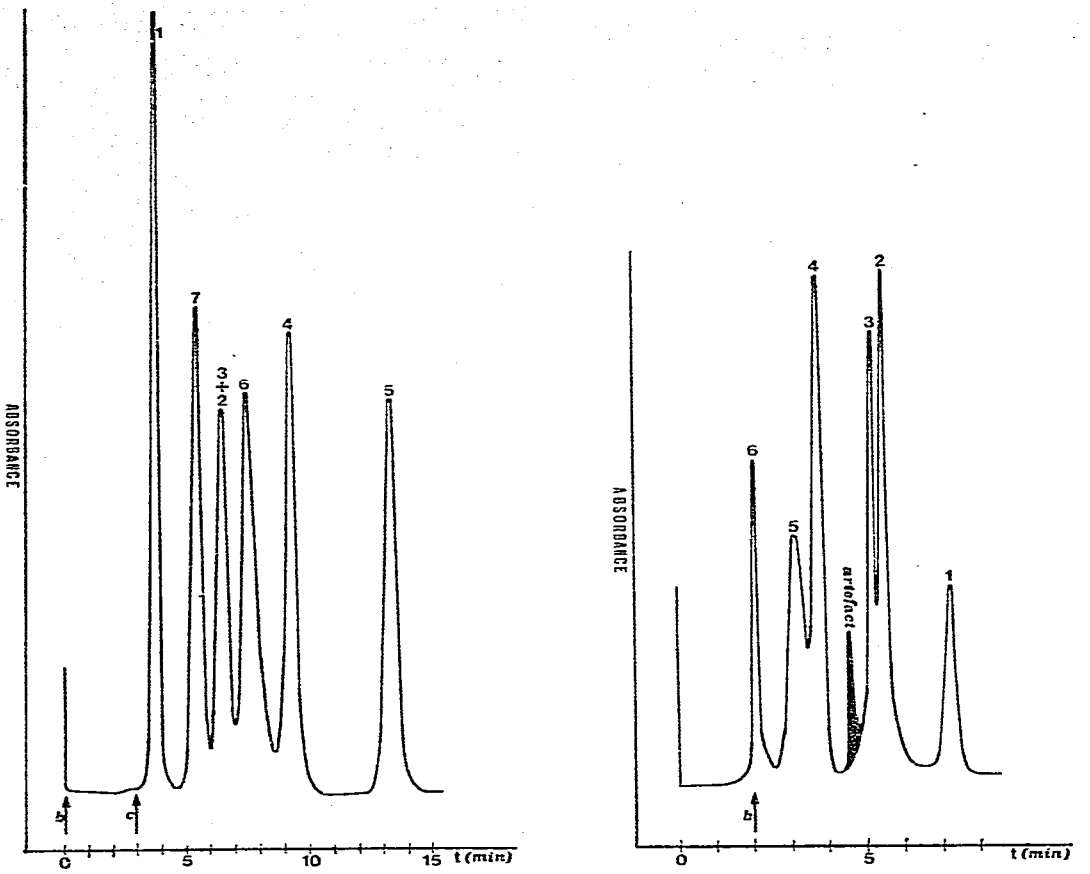


Fig. 1. High-performance liquid chromatogram of 4-hydroxycoumarin (1), acenocoumarin and warfarin (2 + 3), phenprocoumon (4), Clocoumarol (5), bishydroxycoumarin (6) and ethyl biscoumacetate (7). Operating conditions: C in Table I.

Fig. 2. High-performance liquid chromatogram of 4-hydroxycoumarin (1), acenocoumarin (2), warfarin (3), phenprocoumon (4), Clocoumarol (5) and bishydroxycoumarin (6). Operating conditions: A in Table I. The artefact is assigned to the modification in the solvent system.

High-performance thin-layer chromatography

The R_F values of the anticoagulants are presented in Table II. In order to obtain the degradation products, the compounds were applied on the silica gel layer 4 days before development; samples spotted just before development give reference chromatograms.

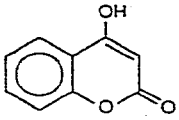
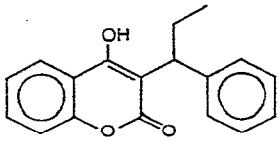
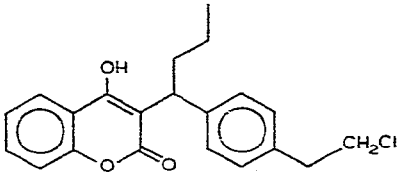
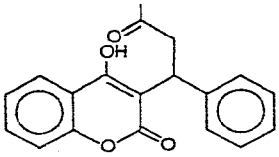
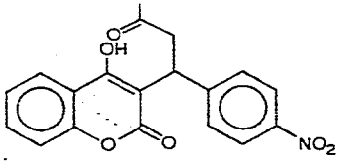
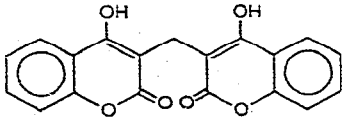
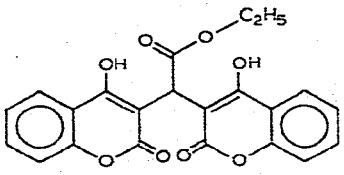
A degradation such as that shown in Fig. 4 has been reported previously by Welling *et al.*[7], Lau-Cam and Chu-Fong[8] and Pohl *et al.*[15]. The blue fluorescent spots observed by these workers correspond to degradation products of class III.

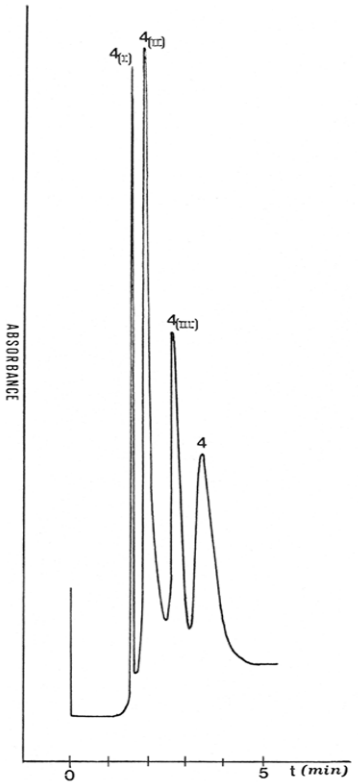
The solvent system used in our work was found to be the most suitable for the separation of the anticoagulants and their degradation products in a single step in less than 60 min. Acidic solvent systems prevent possible degradation during chromatography, although this eventuality can be ruled out if the solvents are peroxide-

TABLE II

CHEMICAL STRUCTURES OF THE ANTICOAGULANTS AND RESULTS OF THEIR HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

Operating conditions: see Experimental.

Compound	*Structure*	R_F *values*				
		Anti-coagulant	*Degradation products*			
			Not classified	*Class III*	*Class II*	*Class I*
4-Hydroxycoumarin		0.09		0.43	0.48	
Phenprocoumon (Marcoumar)		0.49	0.28	0.57	0.66, 0.74	0.79
Clocoumarol		0.53	0.32	0.60	0.71, 0.76	0.82
Warfarin (Coumadin)		0.28	0.17	0.37	0.50, 0.60	0.66
Acenocoumarin (Sintrom)		0.20	0.10	0.29	0.34	0.53
Bishydroxycoumarin (Dicumarol)		0.45				
Ethyl biscoumacetate (Tromexan)		0.09		0.25	0.35	0.52



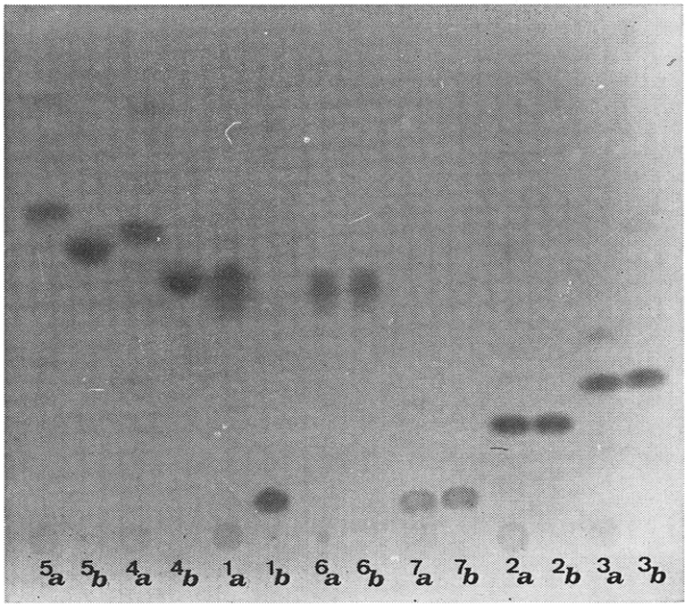


Fig. 3. High-performance liquid chromatogram of phenprocoumon (4) and of its degradation products $4_{(I)}$, $4_{(II)}$ and $4_{(III)}$. Operating conditions: B in Table I.

Fig. 4. Separation by HPTLC of Clocoumarol (5), phenprocoumon (4), 4-hydroxycoumarin (1), bishydroxycoumarin (6), ethyl biscoumacetate (7), acenocoumarin (2) and warfarin (3) and their degradation products. a, Samples applied 4 days before the development; b, samples applied just before the development. Operating conditions: see Experimental. UV detection at 254 nm.

free. The use of high-performance silica gel improves the separation of all of the compounds, but good results can also be obtained with silica gel of conventional particle size.

Compounds such as phenprocoumon and Clocoumarol undergo extensive degradation when allowed to remain on silica gel; warfarin and acenocoumarin are less sensitive, whereas bishydroxycoumarin, ethyl biscoumacetate and 4-hydroxycoumarin seem to undergo another degradation process. Most of the degradation products have been investigated in our laboratory and the results will be published later; these compounds are of great interest as regards their identification in pharmaceutical preparations, and they may also appear during biological transformations.

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