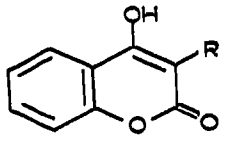
CHROM. 6016

Gas–liquid chromatography of coumarin anticoagulants, their trimethylsilyl ethers, acetates, trichloroacetates, and trifluoroacetates

The application of gas–liquid chromatography to the detection and separation of coumarin anticoagulants and their trimethylsilyl ethers was recently reported from this laboratory[1]. Since then other derivatives have also been tested for the 4-hydroxycoumarins of primary interest:



R = H for 4-hydroxycoumarin
R = $-CH(C_6H_5)CH_2CH_3$ for phenprocoumon
R = $-CH(C_6H_5)CH_2COCH_3$ for warfarin

The present paper reports the results obtained with 4-hydroxycoumarin, phenprocoumon, warfarin, and their trimethylsilyl ethers, acetates, trichloroacetates, and trifluoroacetates.

*Experimental**

Apparatus. A Varian Aerograph Model 1520 gas chromatograph with FID was used in this work. The 5 ft. × 1/8 in. aluminum column was packed with 5% SE-30 on (60–80 mesh) Chromosorb W/DMCS. Unless otherwise indicated, the nitrogen flow rate was 30 ml/min and the temperatures were 240° at the injector, 205° in the column oven, and 260° at the detector.

Materials. 4-Hydroxycoumarin and warfarin were generously supplied by the Wisconsin Alumni Research Foundation (Madison, Wisc.) and phenprocoumon by Hoffmann-La Roche (Grenzach/Baden).

Trimethylsilation. As previously described[1], the sample was dissolved in 100 μl anhydrous pyridine and reacted with 100 μl hexamethyldisilazane and 50 μl trimethylchlorosilane. After centrifugation the supernatant was injected.

Acetylation[2,3]. 2–10 mg of the respective pure compound was mixed with 1.0 ml pyridine–acetic anhydride (1:4). After heating the reaction mixture at 60° for 1 h, it was concentrated to approximately 200 μl, and 1–3-μl aliquots were injected. The trichloroacetates were prepared similarly using TCA anhydride. The trifluoroacetates formed more readily. The sample was dissolved in chloroform or acetone and treated with 5 μl pyridine and 500 μl TFA anhydride for 30 min at room temperature. Then the excess anhydride was removed with a stream of hot air while the glass vessel was heated in a 50–60° water-bath. 0.5–2.0-μl aliquots were then injected into the gas chromatograph.

In order to prepare the TFA derivative using only microgram quantities of samples, a modification of the method of WALLE AND EHRSSON[4] was used to assure

* Abbreviations: PPC = phenprocoumon; FID = flame ionization detector; ECD = electron capture detector; DMCS = dimethylchlorosilyl; TMS = trimethylsilyl; TCA = trichloroacetyl; TFA = trifluoroacetyl.

removal of the excess anhydride and any acid formed (which is necessary when using an ECD if maximum sensitivity is to be obtained). 250 μg of PPC were dissolved in 0.5 ml benzene and 10 μl anhydrous pyridine and 10–50 μl TFA anhydride were added. After 20 or 30 min the reaction mixture was shaken with 1.0 ml water for about 1 min and then centrifuged. 1 μl of the benzene layer was then injected into the gas chromatograph.

Results and discussion

Retention times for the free compounds and for the four derivatives of each, using the SE-30 column, are shown in Table I. Although both the free hydroxycoumarins and their derivatives gave suitable peaks in this system, tailing was greatly reduced by derivatization of the hydroxyl group. Compared to the underivatized compounds, their TMS ethers, acetates, and trichloroacetates have higher retention times. Only the TFA derivatives are characterized by a marked increase in volatility, and for this reason they are of special interest. Table II demonstrates the advantage of using the TFA derivative. Whereas peak broadening and tailing for PPC became intolerable below 180°, its TFA derivative showed only light tailing even at 160°.

TABLE I

RETENTION TIMES OF 4-HYDROXYCOUMARINS AND THEIR DERIVATIVES

Compound	*Retention time (min)*				
Derivative:	*Free*	*TMS*	*Acetyl*	*TCA*	*TFA*
4-Hydroxycoumarin	1.0	1.3	1.3	3.0	0.6
Phenprocoumon	6.2	9.6	11.7	22.0	3.5
Warfarin	13.0	14.3	16.6	39.0	9.0

TABLE II

TEMPERATURE DEPENDENCE OF THE RETENTION TIMES FOR PHENPROCOUMON AND ITS TFA DERIVATIVE

Temperature (°C)	t_R (*min*)	
	PPC	*PPC-TFA*
205	6.2	3.5
190	13.0	6.1
180	21.0	8.7
170	34.0	13.0
160	64.0	22.0

A 6-ft. column containing 10% UCC W-982 on Chromosorb W/DMCS also gave good results with the TMS and TFA derivatives of PPC, though the retention times were correspondingly higher — *e.g.* at 225° and at a nitrogen flow rate of 55 ml/min, these derivatives had retention times of 20 min and 7.1 min, respectively; at 225° and a nitrogen flow rate of 30 ml/min, the TFA derivative eluted after 12 min.

A 6-ft. column containing 3% XE-60 on Diatoport separated coumarin (2.7

min) and 4-hydroxycoumarin (7.9 min) adequately under standard conditions, but gave poor results with the anticoagulants, either in free or derivatized form.

A 6-ft. Porapak Q column was also tried, but only coumarin gave a reproducible sharp peak. Even this had a long retention time (8.1 min at 220° and a nitrogen flow rate of 30 ml/min). The various 4-hydroxycoumarins are less volatile and more polar and, thus, did not give good results with this column.

The methods described here should also be applicable to other 4-hydroxycoumarin anticoagulants (as has been demonstrated with the TMS derivatives[1]), their biological metabolites, and other related compounds. Hence this GLC system can be used for qualitative identification of these compounds, provided that their concentrations are sufficiently high, as in cases of poisoning or *in vitro* studies. The limited FID sensitivity for these compounds, however, prevents the direct application of this system for quantitative determinations in pharmacokinetic studies and routine clinical analyses, since it can only detect down to 1 μg (as TMS) or 100–300 ng (as TFA) of PPC per 1-μl injection.

Because of the extremely low plasma levels (*ca.* 1 μg/ml) of these compounds and the presence of interfering substances in plasma extracts, the increased sensitivity and selectivity of an ECD would be desirable. Theoretically, the free compounds themselves should be ECD sensitive, since they contain a conjugated carbonyl group, but the TFA derivatives should show up even better. Moreover, it is known that the ECD/FID response ratio is even higher for other fluorinated derivatives[4,5]. Consequently, our next studies will further explore the advantages of ECD for these compounds and their halogenated derivates.

I thank Prof. H. K. REMMER for his encouragement of this work and the Deutsche Forschungsgemeinschaft for financial support. For the use of the three (6-ft.) columns I thank Prof. H. UEHLEKE (Pharmakologisches Institut).

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Received February 7th, 1972

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