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Note

New method for the resolution of racemic warfarin and its analogues using low-pressure liquid chromatography

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Warfarin (Scheme 1, 1a) [3-α-(acetonylbenzyl)-4-hydroxycoumarin], which is an oral anticoagulant, is clinically administered as a racemic mixture. Studies have shown that *S*-warfarin is approximately five to six times more potent than *R*-warfarin in both the rat[1–3] and man[4]. It is therefore necessary to resolve racemic warfarin and its analogues in order to study their metabolism in detail.

Attempts made to form covalent diastereoisomers with 1-menthydrazine[5], 1-menthoxyacetylchloride[6], or 1-methylbromoacetate[7] gave unsatisfactory results. However, D-camphor-10-sulfonylchloride[8] gave an ester which apparently existed as a diastereo compound. Very recently isomers of warfarin in plasma have been determined using carbobenzyloxy-L-proline to form diastereomeric esters, which can be separated by high-performance liquid chromatography (HPLC)[9].

Warfarin is a weak acid (pK_a 4.8)[10] and it forms stable salts with alkaloids. Fractional crystallization of the diastereoisomeric salts formed with quinidine and quinine has been the most widely used method for the resolution of racemic warfarin[11]. However, this method is slow and gives poor yields. To overcome these disadvantages, we investigated some new approaches in the present work.

Although, several derivatives of l-menthol have been tried for the resolution of racemic warfarin[5–7], l-menthylchloroformate (2) has never been investigated as a resolving agent. Carbonates and carbamates derived from l-menthylchloroformate and racemic alcohols[12] or amines[13] have been used for analytical resolution of alcohols and amines by gas chromatography as well as in preparative resolutions by crystallization[14]. In the present work, warfarin and its analogues which have an enolic hydroxyl group were resolved by forming diastereomeric carbonates with l-menthylchloroformate (Schemes 1 and 2).

EXPERIMENTAL

Materials

l-Menthylchloroformate and Davisil silica gel, grade 710 (4–20 μm) were obtained from Aldrich (Milwaukee, WI, U.S.A.). (±)-Warfarin (Sigma, St. Louis, MO, U.S.A.) was recrystallized from acetone–water before use. HPLC grade heptane and ethylacetate were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). (±)-*p*-Chlorowarfarin and (±)-*p*-nitrowarfarin were synthesized by a modified pro-

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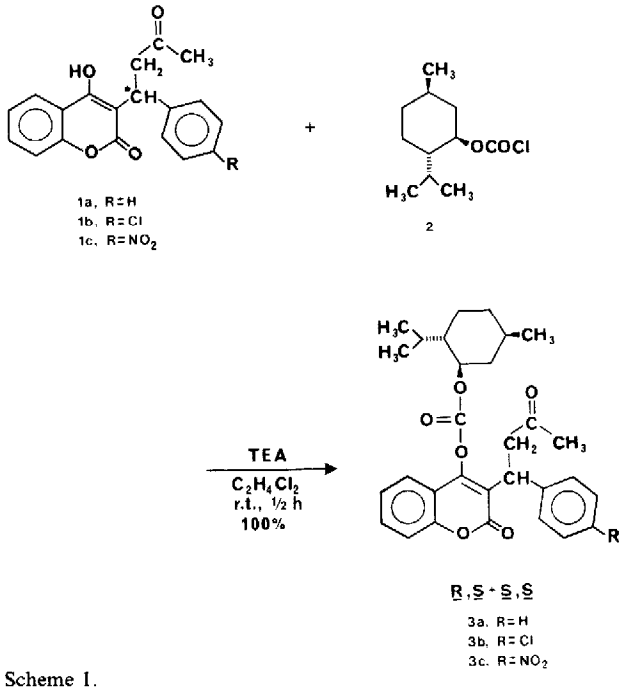
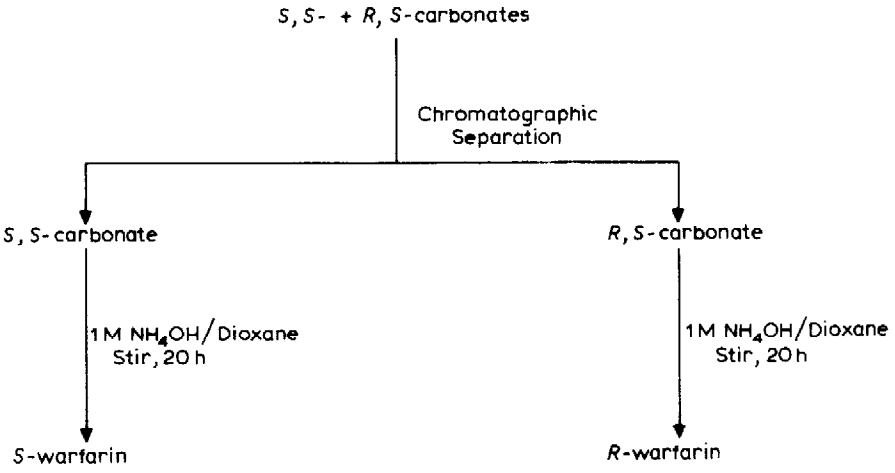


Scheme 1.



Scheme 2.

cedure described by Starr and Haber[15]. All the other reagents were technical grade and were purified before use.

Apparatus

The liquid chromatograph consisted of a Beckman Model-110A pump (for analytical columns) or a FMI Model RP-SY-2CSY pump (for preparative columns), a pressure gauge (Alltech, Deerfield, IL, U.S.A.), a Rheodyne 7105 injector (Rheo-

dyne, Berkeley, CA, U.S.A.), a ISOC UA-5 UV detector coupled with a recorder (ISCO, Lincoln, NE, U.S.A.), a ISCO Model 2150 peak separator and a ISCO Model 1220 fracion collector. Michel-Miller glass columns (Ace Glass, Vineland, NJ, U.S.A.) of 130 × 22 mm, 300 × 22 mm and 600 × 50 mm I.D. were used. Mass spectra were recorded at 70 eV on a Finnigan Model 4000 mass spectrometer with Model 6000 data system.

Synthesis

Carbonates of (±)-warfarin. A solution of 2.31 g (10 mmol) of 1-menthylchloroformate in 10 ml of dichloromethane was added to a solution of 3.08 g (10 mmol) of (±)-warfarin in 15 ml dichloromethane and 1.5 ml of triethylamine. The reaction mixture was stirred at room temperature for 0.5 h. The crystalline precipitate of the amine salt was removed by filtration. The filtrate was washed first with diluted hydrochloric acid solution, then with water several times and dried with anhydrous sodium sulfate. Solvent was removed on a rotary evaporator and the solid product was dried. Yield 4.89 g (100%). The mass spectrum of the product has molecular ion peak at *m/e* 490 with abundant fragment peaks at *m/e* 308, 265, 187, 121 and 93. Analytically calculated for $C_{30}H_{34}O_6$: C, 73.43; H, 6.99. Found: C, 73.39; H, 7.03.

Carbonates of (±)-p-chlorowarfarin. This compound was prepared from 3.42 g (10 mmol) of (±)-*p*-chlorowarfarin and 2.13 g (10 mmol) of 1-menthylchloroformate in a manner similar to that used for the preparation of the carbonates of (±)-warfarin. Yield 5.20 g (100%). The mass spectrum of the product has molecular ion peak at *m/e* 524 with abundant fragments peaks at *m/e* 342, 299, 187, 121 and 93.

Carbonates of (±)-p-nitrowarfarin. This compound was prepared from 3.53 g (10 mmol) of (±)-*p*-nitrowarfarin and 2.31 g (10 mmol) of 1-menthylchloroformate in a manner similar to that described for the preparation of the carbonates of (±)-warfarin. Yield 5.28 g (100%). The mass spectrum of the product has molecular ion peak at *m/e* 535 with abundant fragments peaks at *m/e* 353, 310, 187, 121 and 93.

Decomposition of the carbonates. To a solution of 3.0 mmol of separated carbonate in 10 ml dioxane, 15 ml of 1 *M* NH_4OH solution was added and the mixture was stirred for 20 h. The solution was then extracted with 3 × 15 ml of ethyl ether. The aqueous extract was diluted to 50 ml with distilled water and acidified to pH 4.0 with 0.5 *M* hydrochloric acid solution to yield the pure enantiomers. The precipitate obtained was recrystallized from acetone–water.

Liquid chromatography

Columns were slurry packed by the method described by the manufacturer of the columns, Ace Glass. Analytical separations of carbonates were carried out on a glass column (300 × 22 mm I.D.) with a glass guard column (130 × 22 mm I.D.) packed with Davisil silica gel (4–20 μm) using heptane–ethylacetate (85:15) for elution. The flow-rate of the mobile phase wa either 4 ml/min at 85 p.s.i. or 5 ml/min at 105 p.s.i. Detection was by UV-absorbance at 310 nm.

Preparative separations were carried out on a glass column (600 × 50 mm I.D.) packed with Davisil silica gel (4–20 μm) using the same solvent system and detection used for the analytical separation. The flow-rate of the mobile phase was 14 ml/min at 90 p.s.i.

RESULTS AND DISCUSSION

Treatment of racemic warfarin (1a), *p*-chlorowarfarin (1b) and *p*-nitrowarfarin (1c) with 1-menthylchloroformate gave a mixture diastereoisomeric carbonates (3a–c) in 100% yield. The carbonates were then separated by a low pressure liquid chromatographic system on Davisil silica gel (4–20 μm) using heptane–ethylacetate (85:15) for development. The pure diastereoisomers were then decomposed with 1 *M* ammonium hydroxide to yield *S*- and *R*-enantiomers of 1a–c.

Fig. 1 shows the chromatograms obtained for the separation of the carbonates of (±)-warfarin, (±)-*p*-chlorowarfarin and (±)-*p*-nitrowarfarin on an analytical glass column (22 × 300 mm) with a glass guard column (130 × 22 mm I.D.) packed with Davisil silica gel. Table I shows the retention times and the resolution (R_s) of the mixtures of carbonates, 3a–c. The resolution of carbonates of (±)-warfarin and

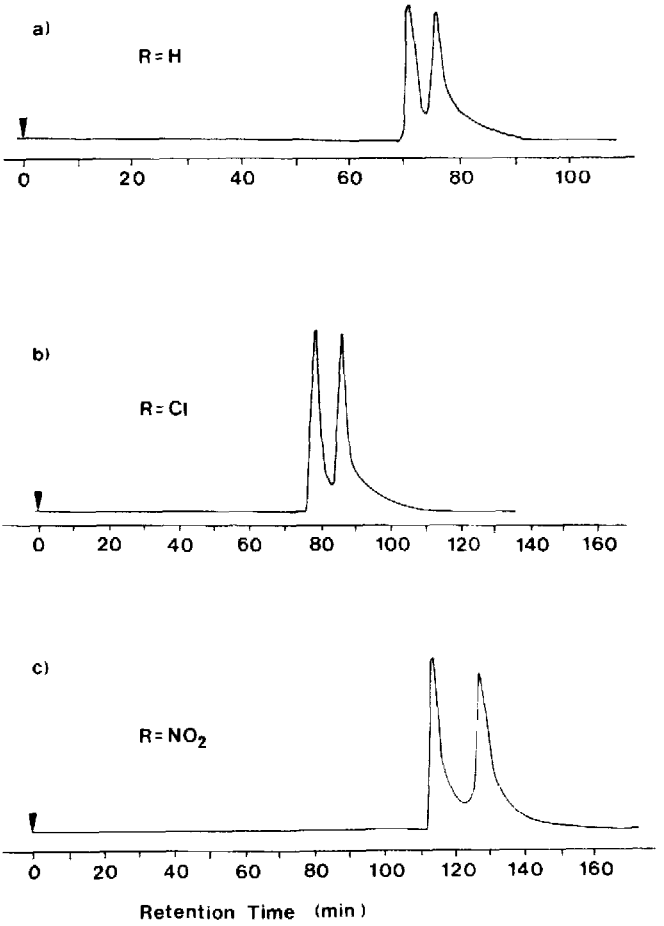


Fig. 1. Separation of the diastereoisomeric carbonates of (±)-warfarin (a), (±)-*p*-chlorowarfarin (b), and (±)-*p*-nitrowarfarin (c). Analytical column, 300 × 22 mm I.D.; guard column, 130 × 22 mm I.D.; stationary phase, Davisil silica gel (4–20 μm); mobile phase, heptane–ethyl acetate (85:15); flow-rate, 4 ml/min at 85 p.s.i. for (a) and (b) and 5 ml/min at 105 p.s.i. for (c); detection is by UV absorption at 310 nm.

TABLE I

RETENTION TIMES AND RESOLUTION OF CARBONATES ON A LOW-PRESSURE LIQUID CHROMATOGRAPHIC COLUMN

Carbonates of	*Retention time (min)*		*Resolution (R_s)*
	Peak 1	*Peak 2*	
(±)-Warfarin	72	76	1.23
(±)-*p*-Chlorowarfarin	79	88	1.64
(±)-*p*-Nitrowarfarin	114	127	1.85

(±)-*p*-chlorowarfarin could be improved to 1.40 and 1.77 respectively by changing the mobile phase to heptane–ethylacetate (90:10).

Fig. 2 shows the separation of the carbonates of (±)-warfarin (76 mg) on a preparative glass column (600 × 50 mm I.D.) packed with Davisil silica gel. The retention times for this separation were 177 and 192 min with a resolution (R_s) of 1.30. Sample sizes up to 250 mg can be resolved in one injection on this column although the diastereoisomeric purity of the second carbonate is slightly reduced by tailing of the first band. In the case of overlapping of bands, one might collect the second band in multiple fractions and recycle the middle fractions. By carefully determining the total elution time for the bands, one can make successive injections of samples and separate about 1.5–2.0 g of sample in one day. This makes the method much faster compared to the classical method using fractional crystallization[15], which takes about 2–3 weeks for the resolution of 308 g racemic warfarin to yield 70 g (45%) *S*-warfarin and 43 g (28%) *R*-warfarin. In the present method, enantiomers of racemic warfarin and its analogues can be recovered almost quantitatively.

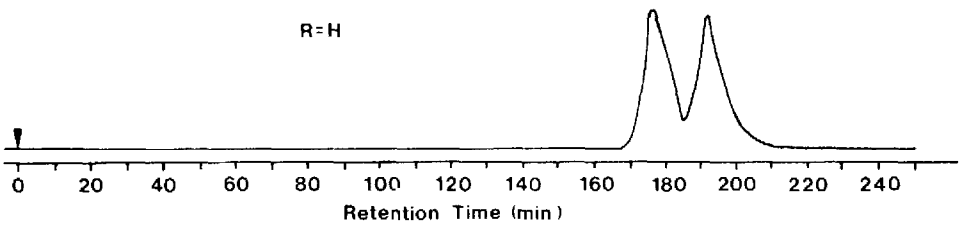


Fig. 2. Preparative separation of the diastereoisomeric carbonates (3a) of (±)-warfarin. Column, 600 × 50 mm I.D.; stationary phase, Davisil silica gel (4–20 μm); mobile phase, heptane–ethylacetate (85:15); flow-rate, 14 ml/min at 90 p.s.i. Detection is by UV absorption at 310 nm.

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