GLC Determination of Warfarin in Human Plasma

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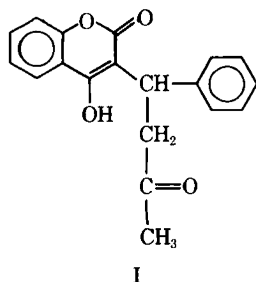
Abstract □ To evaluate the pharmacokinetics and potential drug interactions at relatively low doses (*i.e.*, 7.5 mg), a method for the determination of warfarin in human plasma was required. A sensitive and specific procedure was developed based on: (*a*) ethylene chloride extraction of the acidified specimen, (*b*) TLC of the ethylene chloride extract residue, (*c*) formation of the pentafluorobenzyl derivatives of the materials eluted from the thin-layer chromatogram, and (*d*) quantification of the pentafluorobenzyl derivatives by GLC, utilizing electron-capture detection. The lower level of assay detection sensitivity for measurement of intact warfarin in plasma is 0.02 μg/ml. Statistical analyses indicate a recovery of 98.8%, with a standard deviation of ±10.9%. IR and mass spectrometric analyses, in conjunction with GLC, confirmed that the intact drug was being measured and that no interferences from blank plasma were observed. The procedure was successfully applied to drug absorption studies in humans.

Keyphrases □ Warfarin—GLC determination in human plasma □ Anticoagulants—GLC determination of warfarin in human plasma □ GLC—analysis, warfarin in human plasma

The spectrophotometric method (1) for measurement of warfarin [3-(α-acetonylbenzyl)-4-hydroxycoumarin] (I) in human plasma has been utilized extensively (2–8) to study the absorption, distribution, and metabolism of this potent anticoagulant drug after single-dose oral administration of relatively large amounts (*i.e.*, 25–100 mg). Similarly, the fluorometric method (9) was employed to study the enantiomers of I in humans after single-dose oral administration of 15–100 mg (10). TLC, in conjunction with spectrophotometric or fluorometric techniques, has been used to determine or improve assay specificity for measurement of the intact drug and its metabolites in biological matrixes (6, 10–15).

Recently, the GLC behavior of several coumarin anticoagulants as well as their trimethylsilyl ethers, acetates, trichloroacetates, and trifluoroacetates was reported (16, 17), suggesting that derivatization of I with an electron-capturing group followed by GLC electron-capture detection might be useful for measuring relatively low drug concentrations in plasma specimens (*e.g.*, <1 μg/ml).

To study the pharmacokinetics and potential drug interactions with other therapeutic agents, after single- and multiple-dose oral administrations of relatively low amounts of I (*viz.*, 7.5 mg), a highly sensitive and specific GLC method for measurement of the intact drug in plasma was developed. Subsequent to these investigations, a high-pressure liquid chromatographic method was reported for measurement of I in human plasma (18). After single-dose oral administration of 0.75 mg drug/kg body weight, plasma I concentrations ranged from 1 to 10 μg/ml.



I

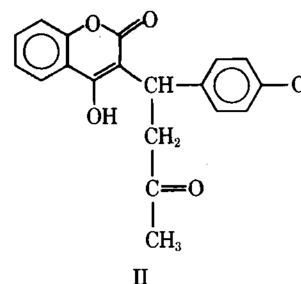
EXPERIMENTAL

Reagents and Materials—Warfarin (I) was used as supplied[1]. The 3-(α-acetonyl-*p*-chlorobenzyl)-4-hydroxycoumarin[2] (II), selected as an internal standard, was purified by chromatography on a column of silica gel 60[3] (4.8 × 35 cm) using a solvent system composed of 10% (v/v) acetone and ethylene chloride. Acetone, ethylene chloride, hexane, and methanol, distilled in glass, were used as supplied[4]. Stock solutions of I in 0.01 *N* aqueous sodium hydroxide (100 μg/ml), II in acetone (100 μg/ml), pentafluorobenzyl bromide[5] in acetone (0.1 ml/100 ml), aqueous sodium hydroxide (0.01 *N*), and aqueous sulfuric acid (3 *N*) were stored in glass containers. All other chemicals were analytical reagent grade. Phenyl methyl silicone fluid (OV-17) on 80–100-mesh Gas Chrom Q (1% w/w) was used as supplied[6].

Instrumentation—A two-speed reciprocating shaker[7] was used for shaking the samples in the horizontal position. A mixer[8] was used to aid in preparing the samples and eluting the zones removed from thin-layer chromatograms. GLC measurements were made with a chromatograph[9] equipped with a nickel-63 electron-capture detector and a −0.1–1.0-mv dual-pen recorder[10]. The detector was operated at 14.5 v in the dc mode. All cylinders of gases (*i.e.*, nitrogen) used for chromatography were fitted with filters containing molecular sieve 4A.

TLC—All analytical chromatography was conducted on thin layers (250 μm) of silica gel F_{254}[11], ascendingly developed in a solvent system composed of 10% (v/v) acetone and ethylene chloride. The separated materials were visualized by irradiation of the plates with a short wavelength (254 nm) UV lamp. Under these conditions, I and II have the same R_f value of 0.40.

GLC—All chromatography was conducted using U-shaped glass columns (0.61 m × 3 mm i.d.) packed with 1% (w/w) OV-17 on 80–100-mesh Gas Chrom Q. All newly prepared columns were preconditioned at 275° for 1 hr without carrier gas flow and for 16 hr with a carrier gas flow of 10 ml/min. During analysis, the column, injection port, and electron-capture detector were maintained iso-



II

[1] S. B. Penick & Co., Chicago, Ill.
[2] Aldrich Chemical Co., Milwaukee, Wis.
[3] EM Laboratories, Inc., Elmsford, N.Y.
[4] Burdick & Jackson Labs, Inc., Muskegon, Mich.
[5] Pierce Chemical Co., Rockford, Ill.
[6] Applied Science Labs. Inc., State College, Pa.
[7] Eberbach & Sons, Ann Arbor, Mich.
[8] Vortex model K-500, Scientific Industries, Queens Village, N.Y.
[9] Tracor model MT-220, Tracor, Inc., Austin, Tex.
[10] Westronics, Inc., Fort Worth, Tex.
[11] Brinkmann Instruments, Westbury, N.Y.

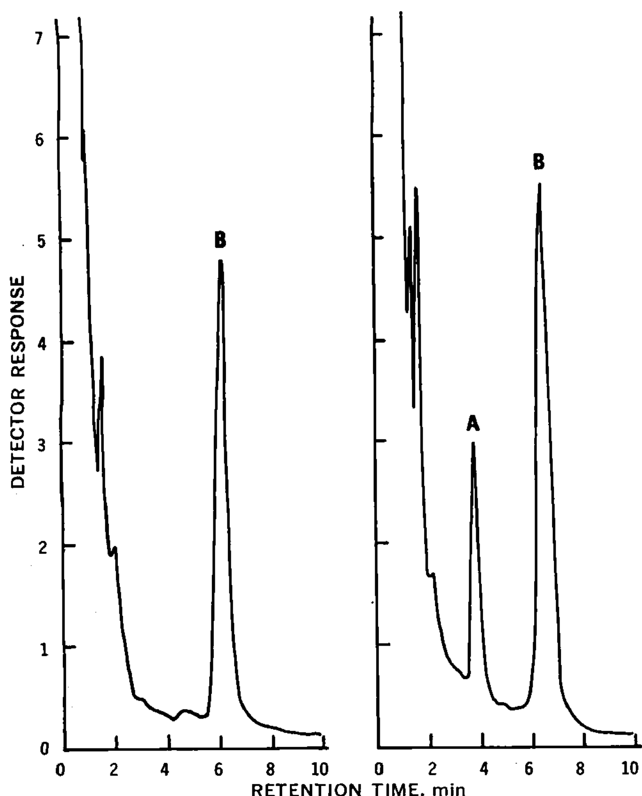


Figure 1—*Gas-liquid chromatograms of human plasma extracts. Left: normal plasma specimen. Right: plasma specimen from subject at 2 hr after single-dose oral administration of 7.5 mg warfarin. Key: A, pentafluorobenzyl derivative of warfarin; and B, II-pentafluorobenzyl derivative.*

thermally at 240, 275, and 285°, respectively. Flow rates of nitrogen gas, used as carrier and purge, were maintained at 75 and 30 ml/min, respectively. Under these conditions, the pentafluorobenzyl derivatives of I and II had retention times of 3.6 and 6.1 min, respectively (Fig. 1).

Synthesis of Standard Materials—Place 100 mg of I in a suitable reaction flask containing a mechanical stirrer and reflux condenser. Add 10 ml of acetone, 0.5 ml of undiluted pentafluorobenzyl bromide, and 1 g of anhydrous potassium carbonate and heat in a water bath at 60° for 5 hr. Evaporate to dryness with a gentle stream of nitrogen gas. Add 2 ml of water and 5 ml of cyclohexane. Shake for 10 min and allow the phases to separate. Transfer the cyclohexane to a 15-ml glass-stoppered centrifuge tube. Repeat the extraction with three additional 5-ml portions of cyclohexane and evaporate the pooled cyclohexane extracts to dryness with nitrogen gas.

Reconstitute the cyclohexane extract residue in 10 ml of 10%

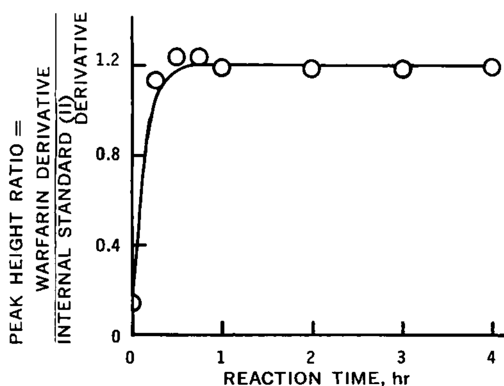


Figure 2—*Effect of reaction time on formation of warfarin pentafluorobenzyl derivative.*

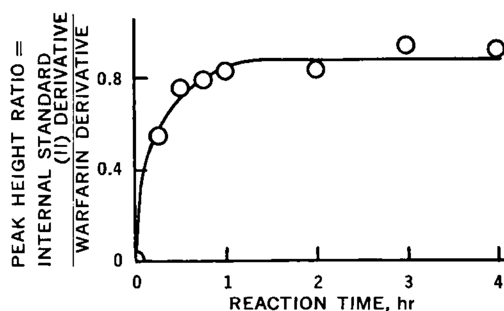


Figure 3—*Effect of reaction time on formation of internal standard pentafluorobenzyl derivative.*

(v/v) acetone in ethylene chloride. Apply the solution to a silica gel column (4.8 × 35 cm) and develop at a flow rate of 3 ml/min with solvent composed of 10% (v/v) acetone in ethylene chloride. Collect 10-ml fractions and monitor each fraction by TLC (*vide supra*). Pool fractions 22–31, remove the solvent under reduced pressure (15 mm), and recrystallize from methanol–ether and water. The pentafluorobenzyl derivative of I is isolated as white crystalline material, mp 107–108°.

Anal.—Calc. for $C_{26}H_{17}F_5O_4$: C, 63.93; H, 3.52; F, 19.45. Found: C, 63.60; H, 3.77; F, 19.68.

Synthesize standard material for identification of the II-pentafluorobenzyl derivative, utilizing the same general reaction conditions as described for I. Recrystallize from methanol–ether and water. The pentafluorobenzyl derivative of II is isolated as white crystals, mp 137–138°.

Anal.—Calc. for $C_{26}H_{16}ClF_5O_4$: C, 59.72; H, 3.09; Cl, 6.78; F, 18.17. Found: C, 60.25; H, 3.37; Cl, 6.58; F, 17.56.

Assay Procedure—*Preparation of Plasma Standards*—Pipet 0.1-ml aliquots of the II stock solution, diluted to 20 μg/ml, into a series of 15-ml glass-stoppered centrifuge tubes. Evaporate to dryness with a gentle stream of nitrogen gas. Pipet 0.5-ml aliquots of I stock solution, diluted to 0.2, 0.4, 0.8, 1.2, 1.6, and 2.0 μg/ml, into successive centrifuge tubes containing the internal standard. Add 1 ml of control plasma to each centrifuge tube and mix well. Prepare an appropriate blank. Extract all standards in the same manner as described later for the plasma specimens.

Preparation of Samples—Pipet 0.1-ml aliquots of the II stock solution (20 μg/ml) into a series of centrifuge tubes and evaporate to dryness with a gentle stream of nitrogen gas. Add 0.5 ml of aqueous sodium hydroxide (0.01 *N*) and 1 ml of plasma and mix well. Add 0.5 ml of 3 *N* aqueous sulfuric acid and 10 ml of ethylene chloride and shake in the horizontal position for 10 min. Centrifuge for 10 min at 2000 rpm and aspirate off the aqueous layer. Transfer an 8-ml aliquot of the ethylene chloride layer to a fresh glass-stoppered centrifuge tube and evaporate to dryness with a gentle stream of nitrogen gas. Wash down the walls of the centrifuge tube with 1 ml of ethylene chloride and evaporate to dryness with nitrogen.

Reconstitute the ethylene chloride extract residues from the plasma standards and samples in 100 μl of ethylene chloride. To-

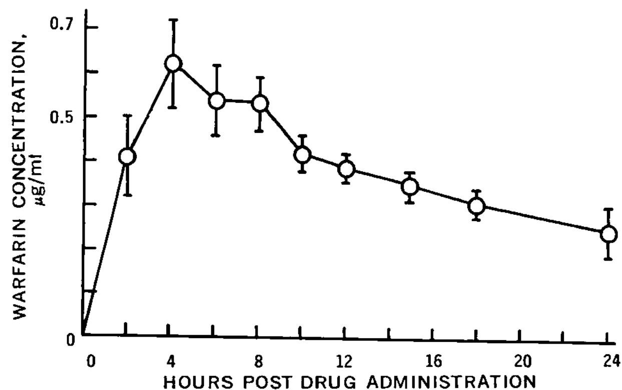


Figure 4—*Mean* (±SEM) *plasma concentrations of warfarin* versus *time in humans* (n = 6) *after single-dose oral administration of 7.5 mg of drug as compressed tablets.*

gether with appropriate standards, spot all of each sample on thin layers of silica gel F_{254} (*vide supra*). Ascendingly develop each plate for a distance of 165 mm, air dry all chromatograms thoroughly at room temperature (24°), and visualize the zones by irradiation with a 254-nm UV lamp. Scrape the zones corresponding to I into separate glass-stoppered centrifuge tubes. Add 2 ml methanol to each tube, shake for 10 min in a horizontal position, and centrifuge for 10 min at 2000 rpm. Transfer 1 ml of the methanol layer to a fresh glass-stoppered centrifuge tube and evaporate to dryness with nitrogen gas.

Add 25 mg anhydrous potassium carbonate and a 0.5-ml aliquot of the pentafluorobenzyl bromide stock solution to each centrifuge tube. Stopper tightly and heat in a water bath at 60° for 1 hr. Evaporate to dryness with nitrogen gas. Add 1 ml of water and 2 ml of hexane, shake for 10 min, and allow the phases to separate. Inject a 1-μl aliquot of the hexane layer for analysis into the chromatograph.

Calculations—The peak heights for I- and II-pentafluorobenzyl derivatives are measured. Peak height ratios are obtained by dividing the peak height of the I-pentafluorobenzyl derivative by the peak height of the II-pentafluorobenzyl derivative. Calibration curves from known concentrations of I in plasma are prepared by plotting peak height ratios *versus* I concentration, expressed as micrograms per milliliter. Values for unknown concentrations of I in plasma specimens, obtained in the same manner, are then read directly from the graph or calculated from the slope of the standard curve.

Drug Administration to Humans—Informed written consent was obtained from each of six normal human male volunteers prior to participation in the study. All subjects were between the ages of 27 and 51 years and ranged in body weight from 64.1 to 91.8 kg and in height from 1.63 to 1.85 m. All subjects fasted for 16 hr prior to drug administration. Each subject received three tablets (each tablet containing 2.5 mg of I) and 8 fl oz of water. Food was withheld for an additional 2 hr. Blood specimens (10 ml) were withdrawn into heparinized syringes at predetermined time intervals from 0 to 24 hr after drug administration. The plasma was harvested and stored at −18°.

RESULTS AND DISCUSSION

Synthesis and Identification of Pentafluorobenzyl Derivatives—The utility of pentafluorobenzyl bromide as a reagent for the microdetermination of phenols, mercaptans, and organic acids in water (19, 20), as well as a potent nonsteroidal anti-inflammatory agent in human plasma (21), has been reported. A series of samples containing known amounts of I and II were prepared to determine optimal reaction times for the formation of the pentafluorobenzyl derivatives. In studies with I, known amounts of the II-pentafluorobenzyl derivative were added as internal standard. Similarly, in studies with II, known amounts of the I-pentafluorobenzyl derivative were added as internal standard. The results indicated that derivative formation for both compounds was completed within 1 hr (Figs. 2 and 3). In the presence of water and hexane, the derivatives were stable for at least 96 hr.

Synthesis of standard materials showed that the pentafluorobenzyl derivatives of I and II were crystalline at room temperature. GLC, using a solid sample injector and flame-ionization detector, indicated that the pentafluorobenzyl derivatives of I and II submitted for elemental analysis were greater than 99 and 98% pure, respectively. IR and mass spectrometric analyses supported the proposed structures. GLC, in conjunction with mass spectrometry, confirmed that the pentafluorobenzyl derivatives of I and II chromatographed as the intact molecules.

Assay Sensitivity and Specificity—At a sensitivity of 1.6×10^{-11} amp/mv, 0.52 ng of I as its pentafluorobenzyl derivative produced a full-scale response. However, under the assay conditions, the lower limit of detection sensitivity for I in extracts of human plasma is 0.02 μg/ml of the original sample aliquot. This value is based on a sample signal equivalent to 2% of full-scale response. Under the assay conditions described, a linear relationship between detector response and concentration is obtained for I over the range of 0–1 μg/ml. Quantification of plasma specimens from drug-treated human subjects, using GLC in conjunction with mass spectrometry, showed that the material responding to the assay was identical to the known I-pentafluorobenzyl derivative.

Recovery Experiments—Plasma was added to known amounts of I and II contained in centrifuge tubes. The samples were thoroughly mixed and extracted with ethylene chloride. All extract residues were chromatographed on thin layers of silica gel F_{254}, and the zones corresponding to I and II were eluted. The pentafluorobenzyl derivatives were prepared and analyzed *via* GLC. The results indicated that recoveries of I (98.8 ± 10.9%) and II (95.6 ± 3.7%) from plasma were essentially quantitative as compared to an unextracted standard curve of the derivatives.

Plasma Levels of Warfarin in Humans—Results from measurement of plasma I concentrations in six normal human subjects, after single-dose oral administration of 7.5 mg of drug (as three 2.5-mg tablets), demonstrated the utility of the analytical methodology (Fig. 4). A peak mean (±*SEM*) level of I (0.62 ± 0.10 μg/ml) was observed at 4 hr after drug administration. Substantial amounts of intact drug (0.25 ± 0.06 μg/ml) were found in the 24-hr plasma specimens, indicating slow drug disappearance from peripheral circulation. The combined results from these investigations showed that the GLC method could be used for: (*a*) evaluating pharmacokinetics, and (*b*) evaluating potential drug interactions after oral administration of relatively low doses of I (*viz.*, 7.5 mg) to humans.

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