Table II lists the contributions of the electrostatic, polarization, dispersion, and repulsion energies to the total energy (Table I). The mentioned distance separates the phenyl ring carbon attached to X and the plane of the indole ring.

Relating the energies to the sweetness level (Table I) gives a correlation coefficient of $r = 0.887$. The interaction energy difference predicted between the two extreme cases is 2.3 kcal/mole, compared to the 3.0 kcal/mole predicted from the expression previously described.

DISCUSSION

The calculations reveal a fairly good correlation relating a biological activity with a single, relatively fundamental, molecular property. The property is a dynamic one, namely the total interaction energy with a model compound simulating the receptor feature. Accordingly, it can be concluded that the choice of receptor is fairly good under the circumstances.

The interaction energy difference calculated for the two extreme sweetness cases, 2.3 kcal/mole, is of the same magnitude as the energy difference predicted from the thermodynamic expression, 3.0 kcal/mole. The latter value presumes that all biological activity variation is due to differences in the energies of binding.

An interesting observation can be made relating to the value of the correlation and the extremes of binding energy predicted in the series. The correlation coefficient, $r = 0.887$, indicates that 79% of the variation has been accounted for in the relationship. By comparing the calculated interaction energy spread of 2.3 kcal/mole with the energy spread predicted from the thermodynamic expression of 3.0 kcal/mole, it can be said that the results imply that 77% of the biological activity ratio of the extreme cases is a result of interaction energies considered in the model.

The similarity of these values, derived from independent aspects of the study, indicates that the model is correctly simulating the part of the drug–receptor binding that can be ascribed to the calculated interaction energies. From Table II it is clear that the overwhelming contribution to those energies is, as predicted, due to dispersion forces. Electrostatic forces are relatively small.

The potential value of these results and of the general method in other cases lies in the possibility that a model can now be constructed that is isomorphic with the actual drug–receptor system. Such a model may be useful in explaining activity and designing new drugs.

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GLC Determination of Plasma Levels of Warfarin

K. K. MIDHAˣ, I. J. McGILVERAY, and J. K. COOPER

Abstract □ A novel method for the quantitative estimation of warfarin in plasma is described. Plasma containing warfarin to which a known amount of phenylbutazone is added as internal standard is acidified and extracted with ethylene dichloride. The drug and the internal standard are then back-extracted into alkali which, in turn, is acidified and reextracted with ethylene dichloride. The organic extract, after washing with phosphate buffer (pH 7.2), is evaporated and the evaporated extract is reacted with an ethereal solution of diazomethane (100 μl). The reacted mixture is evaporated and then dissolved in 25 μl of carbon disulfide. Aliquots (2–3 μl) are injected into a gas chromatograph equipped with a flame-ionization detector. The methyl derivatives of warfarin and the internal standard give sharp, well-separated, symmetrical peaks. The method is of sufficient sensitivity to determine plasma levels in humans after single doses (20 mg) of warfarin (sensitivity of 0.25 μg/ml).

Keyphrases □ Warfarin—GLC determination in plasma □ GLC—analysis, warfarin in plasma

Analysis of warfarin from biological fluids by spectrophotometric (1), fluorometric (2, 3), and TLC (4) methods have been described. The O'Reilly *et al.* (1) method, used by several investigators (3, 5–8), was modified by Welling *et al.* (9) to make it more sensitive. This modified method has been successfully employed to study the *in vivo* and *in vitro* availability of commercial warfarin tablets (10).

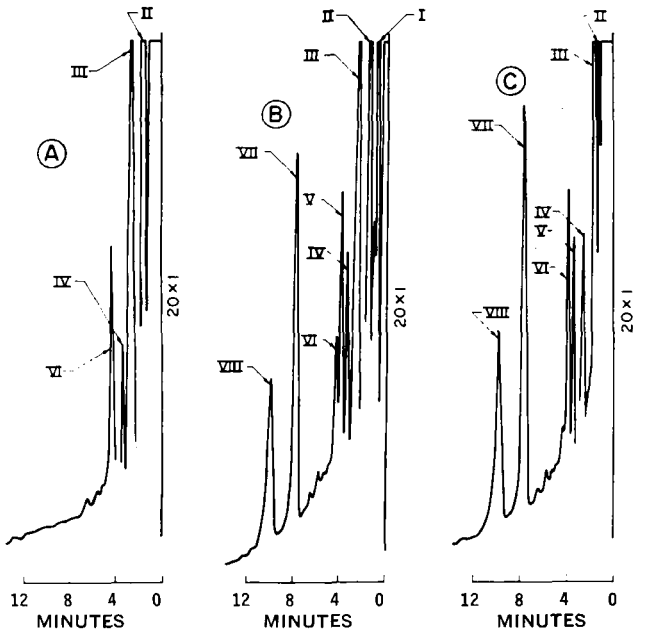


Figure 1—*Gas chromatogram of human plasma. Key: A, control plasma; B, plasma containing 1.25 μg/ml of warfarin and 5.0 μg/ml of phenylbutazone; and C, plasma from a human volunteer who has been given 20 mg of sodium warfarin. Peaks V and VII are peaks of methylated phenylbutazone, and peak VIII is methylated warfarin. Peaks I–IV and VI are due to endogenous materials in plasma.*

These methods are either tedious (4) or require large amounts of plasma samples (9). To study the bioavailability of commercial warfarin formulations on the Canadian market, a specific and sensitive GLC method for measuring plasma levels of warfarin following single doses has been developed.

EXPERIMENTAL

Reagents—Ethylene dichloride[1] and ether[2] were glass-distilled prior to use. Stock solutions containing 100 μg/ml of warfarin[3] (I) were prepared weekly in distilled water and stored in the dark in a refrigerator. Appropriate dilutions (0.25–8.00 μg/ml) were made daily before use. Aqueous solutions of the internal standard phenylbutazone[4] (II), containing 100 μg/ml, were prepared as reported previously (11) and diluted daily to 10 μg/ml with phosphate buffer[5] (pH 7.2). Spectral grade carbon disulfide[5], 1 *N* NaOH, and 3 and 5 *N* HCl were employed. An ethereal solution of diazomethane was synthesized from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide[6]. Warfarin metabolites[7], namely 3-[α-(2-hydroxypropyl)-benzyl]-4-hydroxycoumarin (two diastereoisomers), 6-hydroxywarfarin, and 7-hydroxywarfarin, were used.

Plasma Level Study—Sodium warfarin[8], 20 mg, was administered separately to three healthy male volunteers weighing 71, 82, and 89 kg. Blood samples, 10 ml, were withdrawn from the cubital vein, using heparinized evacuated tubes[9], at 10 appropriate time intervals after dosing. To compare the UV procedure (9) with the GLC method, 40 ml of blood was obtained from Volunteer 1 (71 kg) at 1, 5, 8, and 24 hr after drug administration. The blood samples were centrifuged and the plasma was transferred to another tube before storing at −10°.

[1] Caledon Laboratories Ltd., Georgetown, Ontario, Canada.
[2] Mallinckrodt Chemical Works Ltd., Montreal, Quebec, Canada.
[3] Warfarin Sodium, Warner-Lambert, Scarborough, Ontario, Canada.
[4] Butazolidin, Geigy Pharmaceuticals, Montreal, Quebec, Canada.
[5] Fisher Scientific Co. Ltd., Montreal, Quebec, Canada.
[6] Diazald, Aldrich Chemical Co., Milwaukee, WI 53233
[7] Donated by Dr. K. K. Chan, School of Pharmacy and Medicine, University of Southern California, Los Angeles, Calif.
[8] Coumadin Tablets, Endo Drugs Ltd., Montreal, Quebec, Canada.
[9] Vacutainers, Becton Dickinson Co., Mississauga, Ontario, Canada.

Table I—Recovery of Warfarin and Phenylbutazone from Plasma

Micrograms Added to 1 ml Plasma	*n*	Mean Micrograms Recovered	Mean Percent Recovery	*SD* of Percent Recovery
Recovery of Phenylbutazone				
1.25	3	0.70	55.40	1.25
2.50	3	1.40	55.73	1.61
Mean = 55.57 ± 1.30%				
Recovery of Warfarin				
1.25	3	1.18	94.67	0.92
2.50	3	2.36	94.27	0.92
Mean = 94.47 ± 0.85%				

Table II—Estimation of Warfarin Added to Plasma by GLC

Added Warfarin, μg	*n*	Mean Peak Height Ratio Warfarin/Phenylbutazone	*SD*	*CV*
0.25	8	0.09	0.005	5.52
0.50	7	0.16	0.008	5.00
1.00	8	0.34	0.010	3.07
2.00	7	0.70	0.019	2.73
4.00	8	1.37	0.016	1.17
8.00	7	2.75	0.039	1.40
Mean *CV* = 3.15				
$y = mx$ where $m = 0.344 \pm 0.002$				
$r^2 = 1.000$				

Extraction of Warfarin—To a 2-ml plasma sample ("spiked" or from dosed volunteers) in a glass-stoppered centrifuge tube[10] (42 ml) are added 1 ml of II (5.0 μg/ml), 2 ml of 3 *N* HCl, and 20 ml of ethylene dichloride. The samples are stoppered and extracted by shaking at an angle of 30° for 10 min at 180 cpm on a flat-bed shaker[11]. After centrifugation at 3000 rpm for 10 min, the aqueous layer is removed by aspiration and 18 ml of the ethylene dichloride layer is transferred into another glass-stoppered centrifuge tube (42 ml) containing 10 ml of 1 *N* NaOH.

The extraction is repeated (shake 15 min, centrifuge 10 min) and 9 ml of the sodium hydroxide is transferred to another glass-stoppered tube (42 ml) containing 3 ml of 5 *N* HCl. The tube is swirled gently and the extraction is carried out with another 20 ml of ethylene dichloride for 20 min. The aqueous layer is removed by aspiration and the extraction is repeated for 5 min with 5 ml of phosphate buffer (pH 7.2).

After discarding the phosphate layer by aspiration, 18 ml of ethylene dichloride is transferred to a glass-stoppered centrifuge tube[10] (50 ml). The ethylene dichloride extract is evaporated[12] under a stream of dry nitrogen at 85° to a volume of approximately 4 ml. A crystal of anhydrous calcium chloride[5] is added and the tube is swirled gently. The extract is transferred quantitatively into a custom-made evaporating tube (12) containing a small anti-bumping granule[13] and evaporated as before to dryness.

The tube is allowed to cool to room temperature and the residue is dissolved in 100 μl of ethereal diazomethane by mixing[14] for 30 sec. Then the tube is left to stand for 10 min and the reaction mixture is then evaporated under a stream of dry nitrogen at room temperature. The evaporated extract is redissolved in 25 μl of car-

[10] Wilkens-Anderson Co., Chicago, Ill.
[11] Eberbach Corp., Ann Arbor, Mich.
[12] Thermolyne Dri-Bath, Fisher Scientific Co. Ltd., Montreal, Quebec, Canada.
[13] British Drug House, Toronto, Ontario, Canada.
[14] Vortex Genie Mixer, Fisher Scientific Co. Ltd., Montreal, Quebec, Canada.

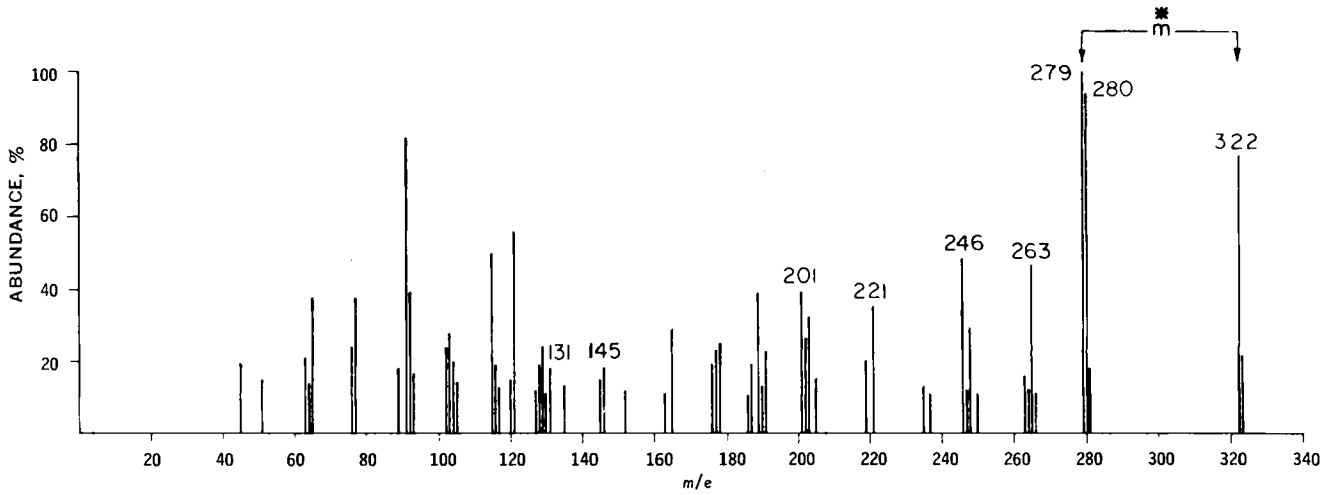


Figure 2—*GLC–mass spectrum of methylated warfarin.*

bon disulfide by mixing[14] for 30 sec and injected (2–3 μl) into the gas chromatograph[15].

GLC—A gas chromatograph[15] equipped with a flame-ionization detector was employed. The column was a spiral glass tubing[15], 1.8 m (6 ft) × 0.25 cm (0.10 in.) i.d., silanized with a 5% solution of trimethylchlorosilane[16] and hexamethyldisilazane[16] (1:1) in ether. It was packed with 5.0% phenyl methyl dimethyl silicone[17] coated on acid-washed, dimethylchlorosilane-treated, 80–100-mesh, high performance flux-calcined diatomite support[18].

The column was conditioned by injecting 25 μl of a mixture of *N',O*-bis(trimethylsilyl)acetamide, trimethylsilyl diethylamine, and hexamethyldisilazane[19] and maintaining the column at 300° for 18 hr with low nitrogen flow. The injection port, detector, and oven temperatures were 280, 270, and 260°, respectively. Nitrogen, as a carrier gas, was maintained at a flow rate of 63 ml/min. Hydrogen and compressed air flow rates were adjusted to give maximum response.

Calculations—Peak height ratios were calculated by dividing the height of the peak due to warfarin (9.8 min) by that of phenylbutazone (7.7 min). Calibration curves were constructed from the results of spiked control plasma samples by plotting the peak height ratios against the concentration of warfarin (micrograms per milliliter of plasma).

RESULTS AND DISCUSSION

Methylation of warfarin with diazomethane gave one peak on GLC analysis with a retention time of 9.8 min (peak VIII, Fig. 1B). Methylation of phenylbutazone with diazomethane gave two peaks on GLC with retention times of 3.9 and 7.7 min (peaks V and VII, Fig. 1B). The heights of the respective peaks were in the ratio of 0.7:1.0. This ratio was independent of oven temperature and sample size. The second peak (VII), which was well separated from the peaks of endogenous material in plasma, was used for quantification.

To establish the structure of the compound giving rise to the peak (VIII, Fig. 1B) from diazomethane treatment of warfarin, eluates from the GLC column were fed directly into the mass spectrometer[20] and the mass spectrum of VIII was recorded. The mass spectrum (Fig. 2) showed a molecular ion at *m/e* 322 and abundant ions at *m/e* 280, 279, 265, 263, 246, 235, 221, 219, 203, 202, 201, 145, and 131. Structures I–XIV have been postulated for these ions. These fragmentations are in agreement with some of those proposed by Trager *et al.* (13) and suggest that the methylated derivative of warfarin has the structure 3-(α-acetonylbenzyl)-4-methoxycoumarin. The combined GLC–mass spectral evidence was obtained for the major peak (VII, retention time of 7.7 min) of the internal standard in the present study and was consistent (molecular ion at *m/e* 322 and other diagnostic ions at *m/e* 77, 183, 266, and 279) with the structure 1,2-diphenyl-3-methoxy-4-*n*-butyl-5-oxopyrazoline (14).

Attempts to prepare trimethylsilyl ethers with hexamethyldisi-

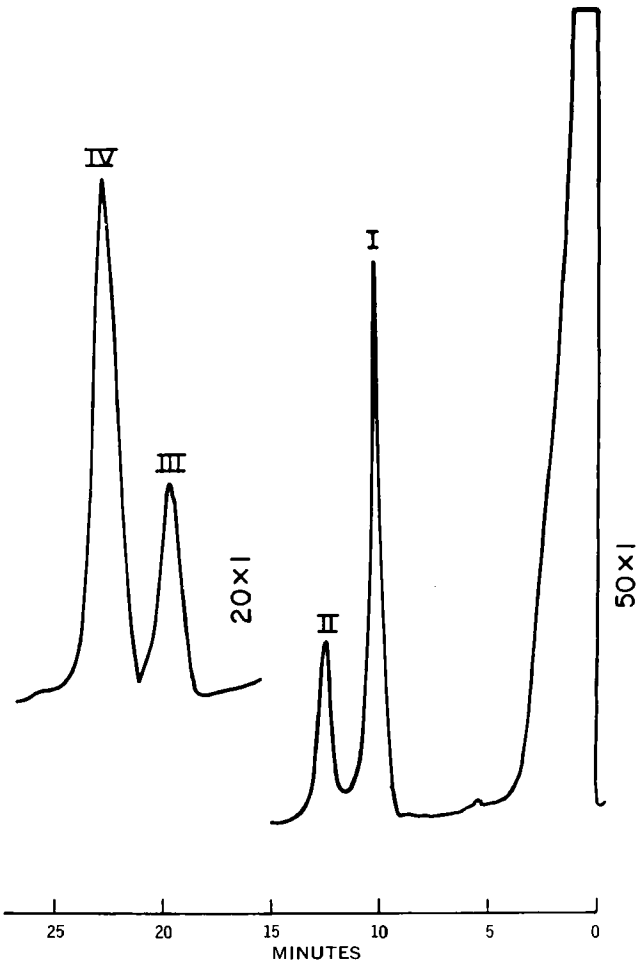
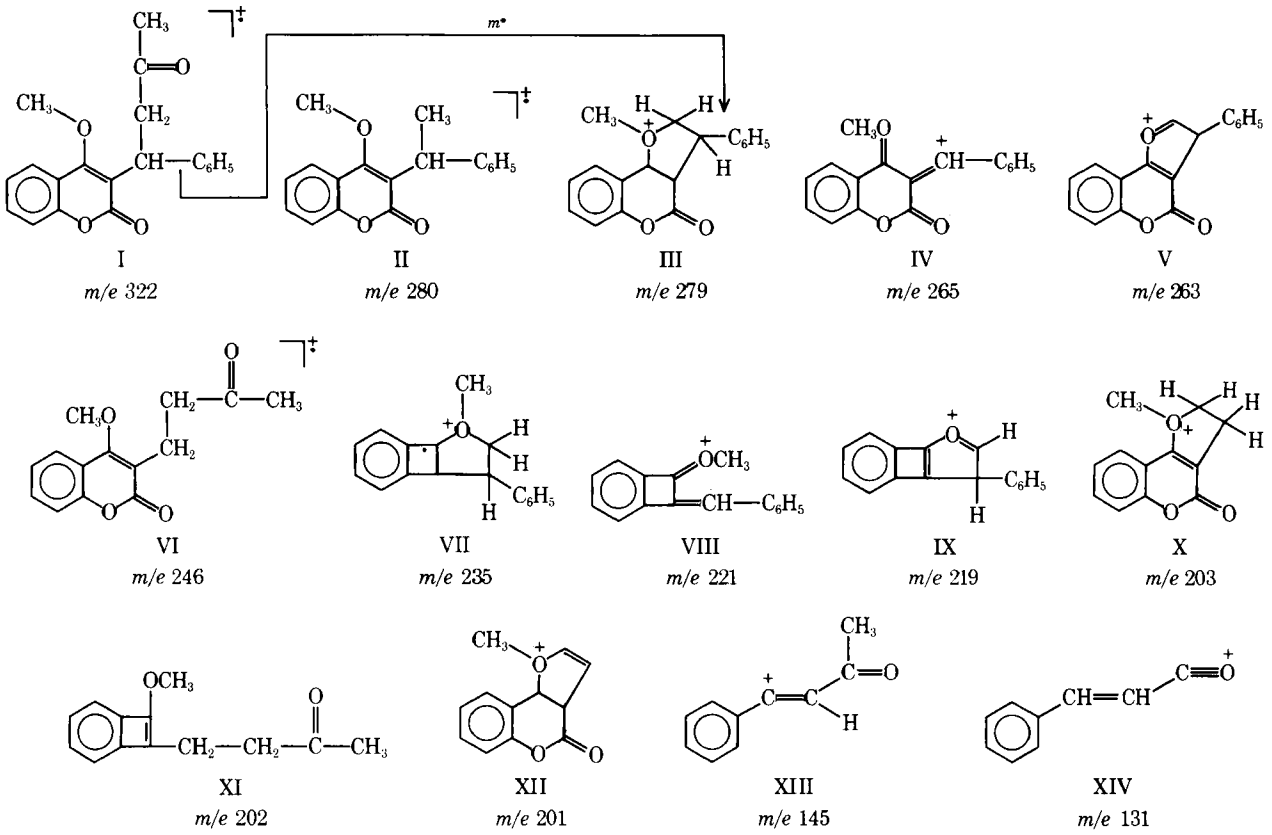


Figure 3—*Gas chromatogram of methylated warfarin metabolites. Key: peaks I and II, warfarin alcohols (two diastereoisomers); peak III, methylated 6-hydroxywarfarin; and peak IV, methylated 7-hydroxywarfarin.*

[15] Model F/11, Perkin-Elmer, Montreal, Quebec, Canada.
[16] Pierce Chemical Co., Rockford, Ill.
[17] OV-7, Chromatographic Specialties Ltd., Brockville, Ontario, Canada.
[18] Chromasorb W, Chromatographic Specialties Ltd., Brockville, Ontario, Canada.
[19] Silyl-8, Chromatographic Specialties Ltd., Brockville, Ontario, Canada.
[20] Perkin-Elmer model 900 gas chromatograph attached to a Hitachi Perkin-Elmer model RMSU mass spectrometer through a jet separator.



lazane in pyridine according to the method of Deckert (15) led to the breakdown of warfarin with no reproducibility. Silylation of warfarin with other silylating agents such as bis(trimethylsilyl)trifluoroacetamide[16], a mixture of trimethylsilylimidazole, bis(trimethylsilyl)acetamide, and trimethylchlorosilane[21], and a mixture of hexamethyldisilazane and trimethylchlorosilane[22] in solvents such as pyridine[16], dimethyl sulfoxide[16], acetonitrile[16], and dimethylformamide[16] led either to extensive breakdown of the drug or to formation of the trimethylsilyl derivative to which the flame-ionization detector was not sensitive enough for plasma level determinations following single doses of warfarin (20 mg).

Attempts to prepare the methoxime derivative of warfarin with methoxyamine hydrochloride[16] followed by silylation were successful. However, these derivatives did not significantly increase the sensitivity of the flame-ionization detector to warfarin. Flash-heater methylation of warfarin using trimethylanilinium hydroxide (0.2 *M*)[23] in methanol and subsequent GLC analysis gave symmetrical and well-defined peaks. However, on-column degradation of the derivative with time was observed. Since the degradation was not reproducible, this procedure of derivatization was not employed.

Ethereal diazomethane was effective in the methylation of warfarin and other coumarin derivatives such as phenprocoumon[24] and acenocoumarol[25]—the later two being investigated as possible internal standards for the assay of warfarin. These derivatives gave sharp well-defined GLC peaks. Phenprocoumon could not be employed as an internal standard because a peak from endogenous material in the plasma interfered with the peak of methylated phenprocoumon on GLC. Acenocoumarol was discarded as a possible internal standard for the assay of warfarin because its methylated derivative had a much longer retention time than that of warfarin, thereby increasing the time of analysis. However, since methylated phenylbutazone gave a GLC peak with the desired retention time and had similar partition characteristics to warfarin, it was chosen as an internal standard for the assay of warfarin.

Several other liquid phases such as saturated hydrocarbon lubricant[26], methyl silicone gum[27], and phenyl methyl silicone fluid[28] were tested, but the peak of methylated warfarin was either not sharp or lacked the response as compared to that obtained on phenyl methyl dimethyl silicone[17]. Carbon disulfide employed as the injection solvent rendered a very small solvent peak which greatly improved the analysis. Plasma extracts, when left unreacted, were much less stable than those reacted immediately after extraction. The plasma extracts that had been reacted with diazomethane were stable up to 8 hr at room temperature.

Figure 1A shows a typical chromatogram obtained by processing

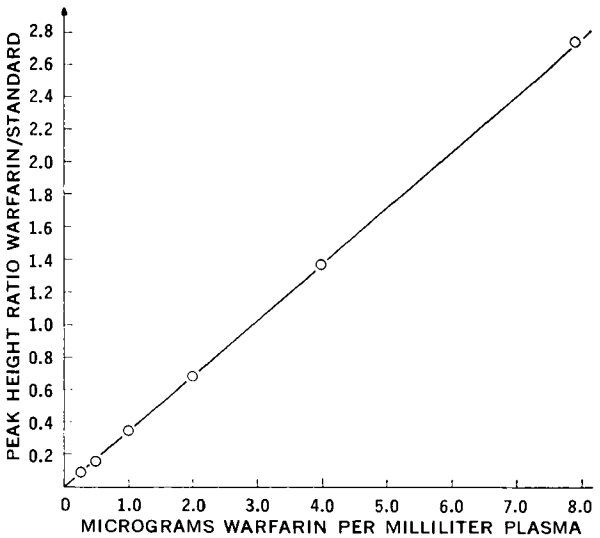


Figure 4—*Calibration curve of warfarin extracted from human plasma. Each point is a mean of seven determinations.*

[21] Tri-Sil TBT, Pierce Chemical Co., Rockford, Ill.
[22] Tri-Sil-Concentrate, Pierce Chemical Co., Rockford, Ill.
[23] Methelute, Pierce Chemical Co., Rockford, Ill.
[24] Marcumar, Hoffmann–La Roche Inc., Montreal, Quebec, Canada.
[25] Sintrom, Geigy Pharmaceuticals, Montreal, Quebec, Canada.

[26] Apiezon-L, Chromatographic Specialties Ltd., Brockville, Ontario, Canada.
[27] SE-30, Chromatographic Specialties Ltd., Brockville, Ontario, Canada.
[28] OV-17, Chromatographic Specialties Ltd., Brockville, Ontario, Canada.

Table III—Comparison of Modified O'Reilly UV Assay (9) and GLC Assay of Warfarin from Plasma of a Dosed Volunteer following a Single 20-mg Dose

Hours after Ingestion	UV Method		GLC Method	
	Concentration, μg	Mean	Concentration, μg	Mean
1	2.52	2.46	2.72	2.73
	2.40		2.74	
	2.13	2.12	2.11	2.12
5	2.10		2.13	
8	1.94	1.92	1.96	1.99
	1.89		2.02	
24	1.41	1.37	1.16	1.20
	1.32		1.23	

fresh blank plasma as described in the *Experimental* section. The extraneous peaks (I–IV and VI) were observed in chromatograms of all human plasma samples. A chromatogram obtained when the method was applied to spiked plasma containing 1.25 μg/ml of warfarin and 5.0 μg/ml of phenylbutazone is shown in Fig. 1B, where it is clear that the extraneous peaks (I–IV and VI) do not interfere with the peaks from the internal standard (VII, retention time of 7.7 min) or warfarin (VIII, retention time of 9.8 min).

Figure 1C shows a chromatogram from a 2-ml plasma sample (25 hr after ingestion) from a volunteer who received 20 mg of sodium warfarin[8]. The time required for analysis is 12 min. The metabolites of warfarin, namely 3-[α-(2-hydroxypropyl)benzyl]-4-hydroxycoumarin (two diastereoisomers), 6-hydroxywarfarin, and 7-hydroxywarfarin (13), do not interfere with the assay since, on methylation with diazomethane (Fig. 3), they gave retention times of 10.5, 12.5, 19.6, and 22.7 min, respectively. No metabolites were detected in plasma samples of dosed volunteers (n = 10) following single doses of 20 mg of sodium warfarin[8], an observation that confirms the findings of Welling *et al.* (9).

The response of the flame-ionization detector to methylated warfarin was linear with concentration in the range of 0.25–8.00 μg/ml. The peak height ratio of the drug and the internal standard was used as the index of detector performance and overall efficiency of the analytical procedure. The overall recoveries of warfarin and phenylbutazone from plasma were of the order of 94.47 ± 0.85 and 55.57 ± 1.30%, respectively (Table I).

The accuracy and precision of the GLC assay are demonstrated in Table II. Results are based on at least seven determinations of each warfarin concentration, ranging from 0.25 to 8.00 μg/ml, which were treated as described under *Experimental*. The overall coefficient of variation was 3.15%. Figure 4 shows the calibration curve obtained by plotting the peak height ratio of methylated warfarin–phenylbutazone *versus* the concentration of warfarin. The plot is a straight line ($y = mx$) over the concentration range of 0.25–8.00 μg warfarin/ml plasma. A mean slope value of 0.344 ± 0.002 ($r^2 = 1$) was obtained.

The GLC procedure was compared with the modified O'Reilly assay (9). Routine analysis by both methods of duplicate plasma samples from a human volunteer (Volunteer 1, 71 kg) who was administered 20 mg of sodium warfarin[8] gave good agreement with an overall difference of 6.75% (Table III).

Application of the method to plasma level determinations is demonstrated in Fig. 5. A 20-mg dose of sodium warfarin[8] (2 × 10-mg tablets) was given to two healthy male volunteers (82 and 89 kg), and plasma was withdrawn at intervals over 96 hr and analyzed for the drug by the described GLC method.

The described GLC procedure is sensitive for the determination of warfarin. It offers the advantage that the plasma samples required are not large. It is specific since it clearly distinguishes the drug from its metabolites and therefore can be employed for single- as well as multiple-dose pharmacokinetic studies.

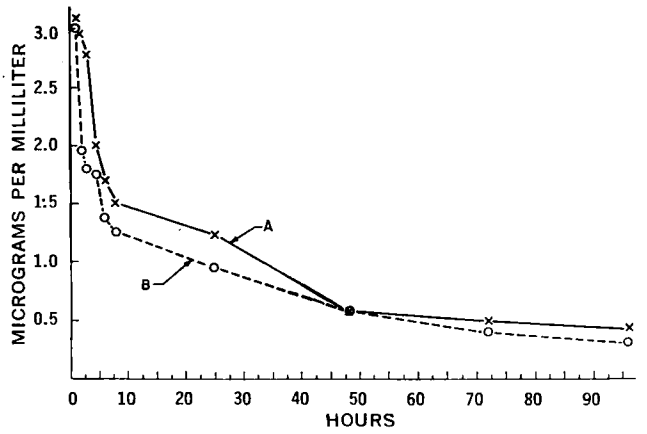


Figure 5—*Warfarin concentrations in two human volunteers following a single oral dose of 20 mg of sodium warfarin. Key: A, Volunteer 2 (82 kg); and B, Volunteer 3 (89 kg).*

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