GLC Determination of Warfarin in Human Plasma

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Abstract □ A specific and quantitative GLC method for warfarin in human plasma is described. The procedure uses papaverine as the internal standard and involves a dichloroethane extraction of the acidified specimen. The organic extract is evaporated, and the evaporated extract is dissolved in 50 μl of chloroform. Aliquots of 2–3 μl are injected into a gas chromatograph equipped with a flame-ionization detector. The sensitivity of the method is such that 0.3 μg of intact warfarin can be detected in 1 ml of plasma. Statistical analyses indicate a recovery of 97.26 ± 1.89% *SD*. The procedure was successfully applied to plasma drug level studies in humans.

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

It has been more than 30 years since the oral anticoagulant warfarin was first used medically (1). Because of the numerous disease states in which warfarin is potentially useful, drug interactions appear to occur more frequently with it than with any other pharmacological class of drugs (2).

Warfarin measurement in human blood facilitated many pharmacokinetic and bioavailability studies. Analysis of warfarin in human plasma by spectrophotometric (3–10), fluorometric (11, 12), and TLC combined with spectrophotometric or fluorometric (13–19) techniques were described. Recently, GLC methods using warfarin methyl or trimethylsilyl ester, acetate, trichloroacetate, trifluoroacetate, and pentafluorobenzyl derivatives were reported (20–23). A high-pressure liquid chromatographic procedure also was used for the quantitative estimation of warfarin in human plasma (24). These methods are complex and lengthy or require large amounts of plasma samples.

This paper describes a relatively simple, easily reproducible, sensitive, and specific GLC method that eliminates the need for derivatization or special instrumentation.

EXPERIMENTAL

Reagents and Materials—Warfarin[1] and papaverine[2] were used as supplied. Spectral grade chloroform[3], analytical reagent grade sulfuric acid[4] (1 *N*), dichloroethane[4], and vinyl methyl silicone[5] on 80–100-mesh Gas Chrom WHP[6] were employed.

Instrumentation—A gas chromatograph[7] equipped with a flame-ionization detector was used. The column was a spiral glass tube (1.8 m × 0.25 cm i.d.) packed with 3.8% (w/w) vinyl methyl silicone on 80–100-mesh Gas Chrom WHP. The column was conditioned by being maintained at 275° for 12 hr with helium gas at a flow rate of 15 ml/min.

The injection port, detector, and oven temperatures were maintained isothermally at 300, 300, and 270°, respectively; helium as a carrier gas was maintained at a flow rate of 60 ml/min. Hydrogen and compressed air flow rates were adjusted to give maximum response. Chromatograms were recorded on a 1-mv recorder[8].

Table I—Recovery of Warfarin after *In Vitro* Addition to 5 ml of Human Plasma

Warfarin Added, μg	Warfarin Added per Milliliter of Blank Plasma, μg	Warfarin Recovered per Milliliter of Blank Plasma, μg	Average Recovery[a]	*SD*, %
1.50	0.300	0.2907	96.90	±0.678
3.0	0.600	0.5844	97.40	±0.919
5.0	1.00	0.9953	99.53	±0.674
10.0	2.00	1.9443	97.21	±0.680
		Mean	97.26	±1.89

[a] Mean of five samples.

Determination of Warfarin in Human Plasma—Throughout the procedure, scrupulously clean glassware was used; the glassware had been washed successively with hydrochloric acid, water, and ethanol, carefully dried, rinsed with dichloroethane, and then dried.

A 2-ml plasma sample (spiked or from dosed volunteers) was acidified with a few drops of 1 *N* H_2SO_4 to pH 1. The sample was then extracted with 3 × 5 ml of dichloroethane. The combined dichloroethane extracts were filtered through a dichloroethane-wetted glass wool pledget into a centrifuge tube containing 5.0 ml of a 5-μg/ml solution of papaverine in dichloroethane as the internal standard. The organic solvent was evaporated to dryness under a gentle stream of dry nitrogen over a steam bath.

The walls of the centrifuge tube were washed down with 1 ml of dichloroethane and evaporated to dryness with nitrogen over a steam bath. The tube was allowed to cool to room temperature, and the residue was dissolved in 50 μl of chloroform by mixing for 30 sec. A 2–3-μl aliquot was injected into the gas chromatograph.

Plasma Level Studies—All subjects[9] were between the ages of 21 and 50 years and ranged in body weight from 65 to 90 kg and in height from

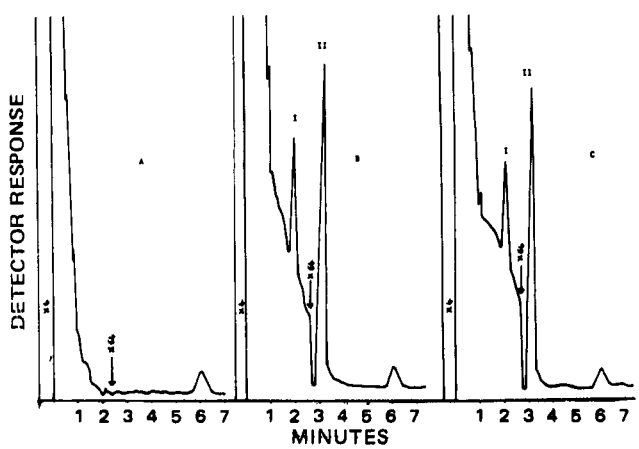

Figure 1—*Chromatograms of human plasma extracts. Key: A, control plasma; B, plasma containing 3 μg of warfarin/ml and 30 μg of papaverine/ml; and C, plasma from a human volunteer who had been given 15 mg of warfarin sodium, 2nd-hr plasma sample. Peaks I and II are peaks of warfarin and papaverine, respectively.*

[1] USP reference standard.
[2] Endo Laboratories, Garden City, N.Y.
[3] Burdick & Jackson Laboratories, Muskegon, Mich.
[4] J. T. Baker Chemical Co., Phillipsburg, N.J.
[5] UCW-98, Applied Science Laboratories, State College, Pa.
[6] Applied Science Laboratories, State College, Pa.
[7] Model 3920, Perkin-Elmer, Norwalk, Conn.
[8] Model 690, Perkin-Elmer, Norwalk, Conn.
[9] Informed written consent was obtained from each of the normal, healthy, male Caucasian volunteers prior to participation.

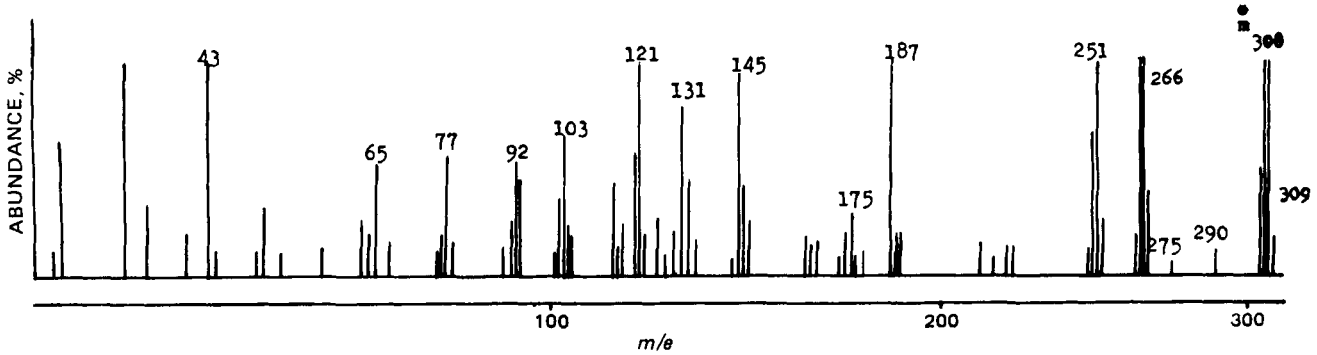

Figure 2—*GLC–mass spectrum of warfarin (normalized).*

1.5 to 1.9 m. Each subject had a drug history taken, and a physical examination was given which included measurement of blood pressure, pulse, and respiration rates. Before and after the study, adequate clinical pathological screening including hematology, blood chemistry, and urinalysis was performed. Seven subjects were selected who had all parameters in the normal range.

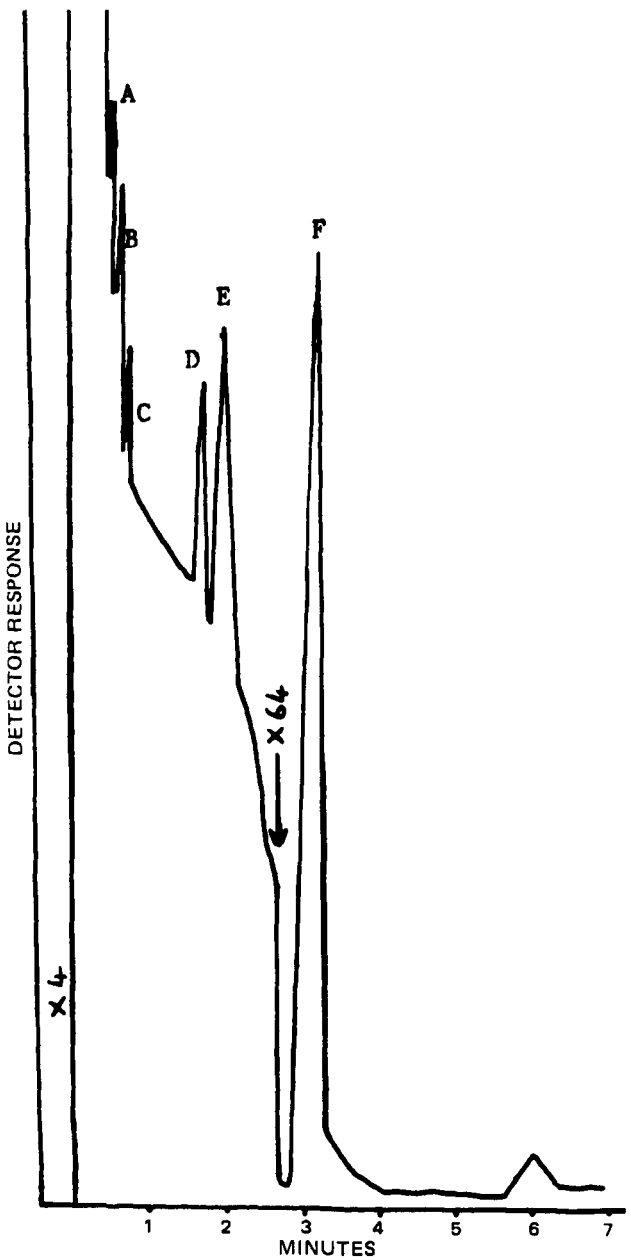

Figure 3—*Chromatogram of warfarin and its degradation and metabolic products. Key: A, 5-hydroxywarfarin; B, 7-hydroxywarfarin; C, 8-hydroxywarfarin; D, 3-(o-hydroxyphenyl)-5-phenyl-2-cyclohexen-1-one; E, warfarin; and F, papaverine.*

All subjects received no medication for 7 days preceding initiation of the studies and only the prescribed medication during the study. Subjects were fasted overnight with water *ad libitum* (at least 12 hr predosing) and for 4 hr after drug administration. Then a standard light meal was eaten. Each subject swallowed three whole 5-mg crystalline warfarin sodium[10] tablets with 200–500 ml of water.

Blood samples of 30 ml were withdrawn from a forearm vein into heparinized evacuated tubes[11] at zero time and at 1, 2, 4, 6, 8, 12, 48, 72, and 96 hr after dosing. The blood samples were centrifuged[12] at 2000 rpm for 10 min, and the plasma was transferred to another tube before being stored at −20°.

Calculations—Peak height ratios were calculated by dividing the height of the peak due to warfarin by that due to papaverine. Calibration curves were constructed from the results of spiked control plasma samples by plotting the peak height ratios against the warfarin concentration, expressed as micrograms of warfarin per milliliter of plasma. Values of unknown concentrations of warfarin in plasma specimens, obtained in the same manner, were then read directly from the graph or calculated from the slope of a two-point standard curve.

RESULTS AND DISCUSSION

Under the assay conditions described, a linear relationship between detector response and concentration was obtained for warfarin over the range of 0.3–2 μg/ml of plasma with a correlation coefficient of 0.9944. Regression analysis showed that the regression equation was $y = 0.338x - 0.081$ with a standard error of the estimate of y on x of 0.021 and standard errors of the estimate of the intercept and slope of 0.107 and 0.675, respectively. The retention times of warfarin and papaverine were 2 and 3.1 min, respectively (Fig. 1).

Several plasma samples taken at the predetermined intervals were pooled, extracted, and chromatographed using the described method. The material obtained within the warfarin peak (retention time of 2 min) was trapped and analyzed by mass spectrometry[13]. The mass spectrum of the collected warfarin peak was identical with that obtained from a USP reference standard (Fig. 2). The mass spectrum showed a molecular ion at *m/e* 308 and abundant ions at *m/e* 43, 65, 77, 92, 103, 121, 131, 145, 175, 187, 249, 251, 265, 266, 275, and 290.

The accuracy and precision of the GLC assay procedure are demonstrated in Table I. The quantitative lower limit of detection sensitivity for warfarin in human plasma was 0.3 μg/ml. The average recovery of warfarin from spiked plasma was 97.26 ± 1.89%. The resolution factor between warfarin and internal standard peaks was 4.6 (25). The degradation product, 3-(*o*-hydroxyphenyl)-5-phenyl-2-cyclohexen-1-one[2], and three metabolic products of warfarin (16, 23, 24), 5-hydroxywarfarin[14], 7-hydroxywarfarin[14], and 8-hydroxywarfarin[14], do not interfere with the assay since they gave retention times of 1.65, 0.6, 0.7, and 0.75 min, respectively, under the assay conditions described (Fig. 3).

The GLC procedure was compared with a UV spectrophotometric assay (14). Results from measurements of plasma warfarin levels in seven normal human subjects after single-dose oral administration of crystalline warfarin sodium, 15 mg, are shown in Fig. 4. A peak mean level of warfarin (1.80 μg/ml) was observed at 1.0 hr after oral drug administration, fol-

[10] Coumadin, Endo Laboratories, Garden City, N.Y.
[11] Becton, Dickinson and Co., Rutherford, N.J.
[12] Model IEC-HN-S, Damon IEC Division, Needham Heights, Mass.
[13] Model 1015, Finnigan Corp., Sunnyvale, Calif.
[14] Dr. C. Schroder, WARF Institute, Madison, Wis.

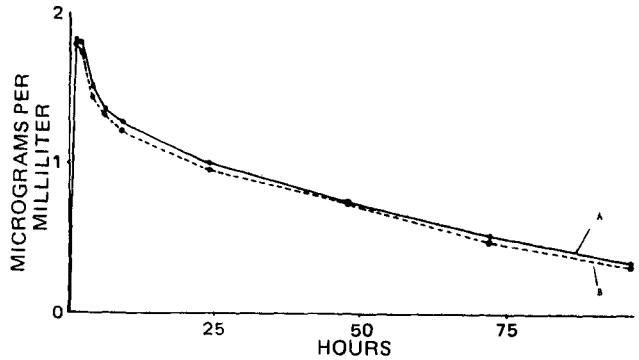

Figure 4—*Mean plasma warfarin concentrations in seven volunteers following a single oral dose of 15 mg of crystalline warfarin sodium. Key: A, UV method; and B, GLC method.*

lowed by a smooth, gradual decline from 24 to 96 hr, indicating slow drug disappearance from peripheral circulation. The estimated areas under the plasma concentration–time curves from 0 to 96 hr were 73.58 and 75.86 μg/ml × hr while the elimination half-lives were 50.9 and 51.4 hr as determined by the GLC and UV methods, respectively.

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Determination of Time Course of Tablet Disintegration II: Method Using Continuous Functions

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Abstract □ An analysis of the disintegration–dissolution sequence of drug release from a tablet leads to a mathematical expression relating disintegration to the dissolution profile of the tablet and the dissolution rate of the primary drug particles in the tablet. The equation describing the disintegration of an acetaminophen tablet is determined to demonstrate the application of the theory.

Keyphrases □ Disintegration, tablet—related to dissolution profile of tablet and dissolution rate of primary drug particles □ Dissolution—profile of tablet and rate of primary drug particles related to tablet disintegration

A method involving numerical analysis was described recently (1) that accounts for both the disintegration and dissolution aspects of drug release from a tablet. If the dissolution characteristics of the primary drug particles in the tablet are known, it is possible to determine the time course of the disintegration process from tablet dissolution data. This disintegration–dissolution analysis requires a numerical data analysis over many small time intervals. The purpose of the present paper is to extend this method to provide a model based on continuum mathematics.

THEORETICAL

The principle of the disintegration–dissolution analysis described previously (1) is to consider the processes of disintegration and dissolution to be discrete events over many small, equal time intervals. At any arbitrary interval, the fraction of drug in the tablet that has dissolved is considered to be the sum of the amounts dissolved from all disintegrated fractions, calculated using the cube-root equation for the time periods between the disintegration intervals and the arbitrary interval. The mathematical expression given that describes this phenomenon is:

$$M_n = \sum_{i=0}^{n} w_i\{1 - [1 - K(t_n - t_i)]^3\} \qquad \text{(Eq. 1)}$$

where M_n is the fraction of drug in the tablet dissolved after n time intervals and corresponds to time t_n. The fraction disintegrated at the ith interval is w_i, and K is the cube-root law dissolution constant. The term