High-Pressure Liquid Chromatographic Analysis of Drugs in Biological Fluids I: Warfarin

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Abstract □ A rapid, sensitive, and specific high-pressure liquid chromatographic (HPLC) method for the quantitative analysis of warfarin in plasma is described. The method involves a single solvent extraction of warfarin and the internal standard from acidified plasma, followed by evaporation and HPLC analysis. The method is sufficiently sensitive and accurate for most pharmacokinetic studies; concentrations between 0.1 and 4.0 μg/ml can be measured with a coefficient of variation of 2–5%. Known metabolites of warfarin do not interfere with the analysis.

Keyphrases □ Warfarin—high-pressure liquid chromatographic analysis, human plasma □ High-pressure liquid chromatography—analysis, warfarin in human plasma □ Anticoagulants—warfarin, high-pressure liquid chromatographic analysis, human plasma

Several methods have been reported for the analysis of warfarin in biological fluids, but each has some disadvantage in specificity, sensitivity, reproducibility, or convenience. One reported method (1) involves solvent extraction of warfarin from plasma with dichloroethane, followed by a buffer wash and back-extraction of warfarin into dilute sodium hydroxide solution. Concentration is calculated by UV absorbance at two wavelengths. A modification of this method (2) is still time consuming, since it requires several quantitative transfers. A fluorometric method (3) is rapid but gives consistently higher values than the more commonly used spectrophotometric methods. Thus, its specificity is in doubt. A TLC method for the analysis of warfarin and its metabolites also was described (4), but it is time consuming, making the analysis of a large number of samples difficult.

More recently, two GLC methods were described for the analysis of warfarin. One method (5) involves a complex solvent extraction, evaporation, and derivatization before GLC analysis, but it offers no apparent benefits over other methods. The other GLC method (6) is sensitive and can detect as little as 0.02 μg of warfarin/ml of plasma. The procedure, however, is complex and includes solvent extraction, evaporation, TLC, derivatization, and GLC analysis. In view of the commonly encountered therapeutic levels of warfarin of 1.5 ± 0.5 μg/ml, the added sensitivity of this method will be required in only a few types of investigations.

A high-pressure liquid chromatographic (HPLC) analysis of warfarin was described previously (7), but this report gave few details. Moreover, an internal standard was not used, thus necessitating quantitative transfer and sampling techniques, with their inherent inconveniences.

The method described in this paper is specific, accurate, and convenient, and it has sufficient sensitivity to measure accurately concentrations of warfarin as low as one-tenth of the normally encountered therapeutic levels in humans.

Table I—Statistics of Calibration Curves and Reproducibility

Day	Concentration Range, μg/ml	Number of Samples	Mean PHR[a]/ Micrograms of Warfarin	*SD*	Coefficient of Variation, %
		Calibration Curve Data			
1	0.1–4.0	13	1.456	0.065	4.5
2	0.1–4.0	9	1.352	0.068	5.0
3	0.1–4.0	6	1.465	0.067	4.6
4	0.1–4.0	6	1.405	0.106	7.5
5	0.1–4.0	5	1.464	0.046	3.1
6	0.1–4.0	5	1.349	0.046	3.4
7	0.1–4.0	5	1.329	0.077	5.8
		Averages	1.403	0.068	4.8
		Reproducibility at a Given Concentration			
8	0.5	5	1.468	0.058	4.0
9	1.0	10	1.534	0.026	1.7
10	2.0	5	1.495	0.051	3.4

[a] PHR = peak height ratio of warfarin to internal standard.

EXPERIMENTAL

Reagents and Materials—Warfarin[1] and *p*-chlorowarfarin[2] [3-(α-acetonyl-*p*-chlorobenzyl)-4-hydroxycoumarin] were used as supplied. Stock solutions of warfarin (2.0 mg/ml) and the internal standard (1.2 mg/ml) were prepared in methanol[3] and stored in the dark at 4°. Appropriate dilutions of these stock solutions with water were made weekly to produce working standards of 1.2 μg/100 μl for the internal standard and 0.1–4.0 μg/100 μl for warfarin.

Sample Preparation—Plasma, 0.2–1 ml, was placed in a screw-capped[4] centrifuge tube, and 100 μl of internal standard solution (containing 1.2 μg of *p*-chlorowarfarin), 0.5 ml of 0.5 *N* aqueous sulfuric acid[5], and 5 ml of ether[5] were added. The samples were extracted by mixing, using gentle hand tilting of the tubes for 10 min or using any method of gentle mechanical agitation, followed by centrifugation at 2500 rpm for 10 min to separate the organic and aqueous phases.

The lower aqueous phase was frozen by immersing the tube in a dry ice–acetone bath, and the organic phase was poured off into another tube having an elongated cone at its base of approximately 50-μl capacity. The organic solvent was evaporated to dryness in a water bath at 40–45°. The residue was dissolved in 25 μl of methanol[3] by washing the sides of the lowest part of the tube and its elongated cone several times with the aid of a 25-μl syringe. Then 15–20 μl of the methanol solution was injected into the chromatograph.

Chromatography—A high-pressure liquid chromatograph[6] fitted with a 25-cm long, 6.3-mm o.d., 2.2-mm i.d. reversed-phase column[7] was used. The detector was a variable wavelength spectrophotometer[8] with a 10-μl flowcell. Absorbance was measured at 308 nm, with 0.1 absorbance unit full scale and 2-nm slit width. One pump of the dual-pump gradient elution chromatograph contained methanol[3], and the other contained 0.5% acetic acid in distilled water. An isocratic 1:1 mixture of the two solvents was used. Although it was convenient to use two pumps operating under isocratic conditions, a 1:1 mixture of the two solvents in a single pump can be used.

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Aldrich Chemical Co., Milwaukee, Wis.
[3] Spectroquality, MC & B Manufacturing Chemists, Norwood, Ohio.
[4] Lined with Teflon (du Pont).
[5] Analytical reagent, Mallinckrodt Chemical Works, St. Louis, Mo.
[6] Model 8500, Varian, Palo Alto, Calif.
[7] MicroPak CH-10, Varian, Palo Alto, Calif.
[8] Variscan, Varian, Palo Alto, Calif.

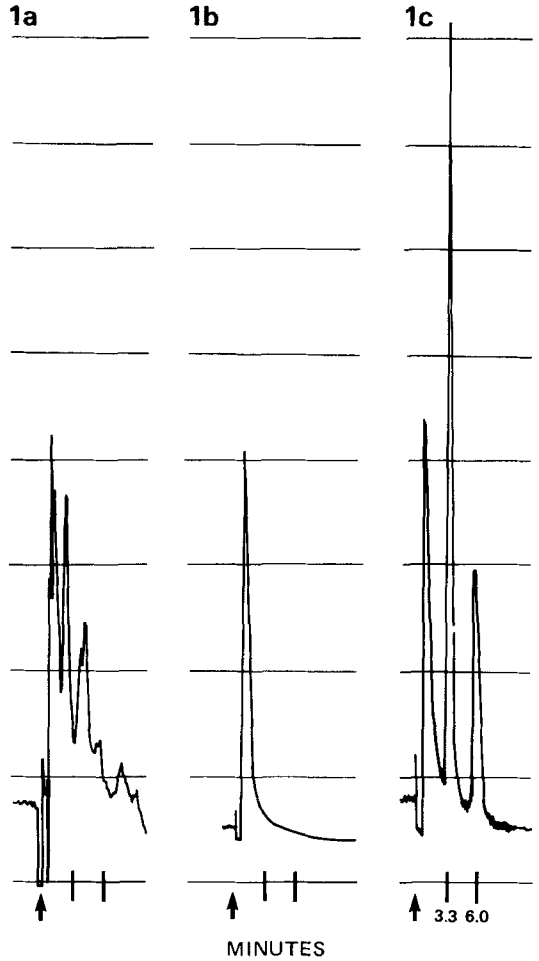

Figure 1—(a) *Chromatogram obtained when 1 ml of control plasma is extracted and absorbance is measured at 280 nm.* (b) *Chromatogram of the same control plasma with absorbance measured at 308 nm.* (c) *Typical chromatogram of plasma containing warfarin (2.0 μg/ml) and the internal standard. The marks on the chromatograms of control plasma correspond to the retention times of warfarin (3.3 min) and the internal standard (6.0 min). To improve visual clarity, only one tracing of the dual-pen recorder is shown.*

The flow rate of the solvent mixture was 60 ml/hr, with a column input pressure of 390–420 kg/cm^2 (374–408 atm). Chromatograms were recorded on a dual-pen recorder[9] with 0–50- and 0–100-mv spans.

Calibration and Accuracy—Calibration curves were constructed by adding known amounts of warfarin and internal standard to control plasma. The peak height ratio of warfarin to the internal standard was plotted against the amount of warfarin added. To calibrate the method and to determine its accuracy for each batch of unknown samples, standards of 0.1, 0.5, 1.0, 2.0, and 4.0 μg of warfarin were added to 1-ml samples of control plasma, which were assayed concurrently with the unknown samples. The peak height ratio of each standard was divided by the amount of warfarin added to give normalized peak height ratios. The mean normalized peak height ratio was used to calculate the amount of warfarin in unknown samples, and the standard deviation of the normalized peak height ratios was used to determine the accuracy of the method over the range of warfarin standards employed.

Reproducibility of the method was also studied by carrying replicate plasma samples containing 0.5, 1.0, and 2.0 μg of warfarin through the procedure. The effect of sample size on the method was investigated by adding 1 μg of warfarin to tubes containing different volumes of plasma, 0.2–2 ml, which were then assayed for warfarin. The volumes of internal standard solution, sulfuric acid, and ether were constant.

Plasma Level Study—A normal human male volunteer (weight 63.0 kg) was given 15 mg of warfarin sodium in aqueous solution after fasting for approximately 12 hr. Samples of venous blood (3 ml) were withdrawn

[9] Varian A-25, Varian, Palo Alto, Calif.

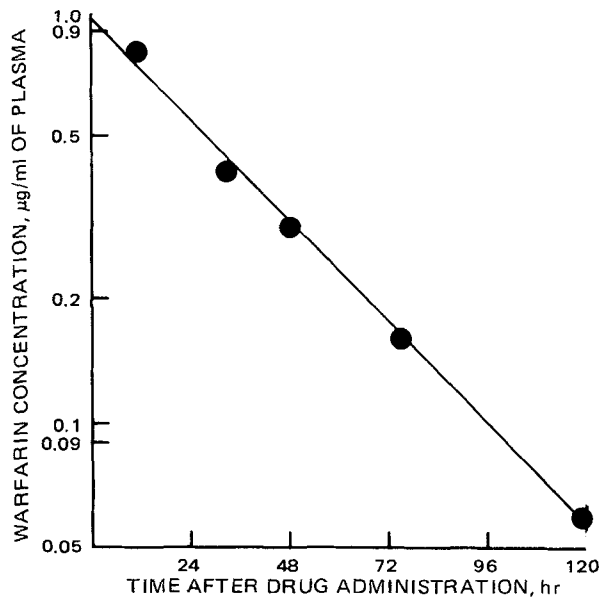

Figure 2—*Semilog plot of plasma warfarin levels in a male volunteer who received 15 mg of warfarin orally.*

by means of heparinized containers[10] prior to, and at intervals over 120 hr after, drug administration. The blood samples were centrifuged, and the plasma was transferred to glass containers; these containers were stored at −15° until analyzed.

RESULTS AND DISCUSSION

In preliminary work, ^{14}C-warfarin[11] was satisfactorily extracted from plasma acidified with dilute sulfuric acid by toluene, ether, isopentyl acetate, dichloroethane, and ethyl acetate. These solvents extracted 90–95% of warfarin from the plasma using a solvent to plasma ratio of 3:1. Although dichloroethane is used frequently, ether was selected for the extraction of warfarin from plasma for the following reasons. Ether has a lower specific gravity than water and has a low freezing point; thus it can be separated easily from the aqueous phase by freezing. It is readily evaporated and, because of its lower polarity, it is less likely to extract interfering compounds than dichloroethane.

Initially, absorbance was measured at 280 nm with a sensitive fixed-wavelength detector. At that wavelength, however, peaks interfering with both warfarin and the internal standard were present on chromatograms from control plasma (Fig. 1*a*). Extractions with various solvents and back-extraction of warfarin into 2.5 *N* sodium hydroxide solution failed to eliminate these peaks. The use of a UV absorbance detector at 308 nm eliminated these interfering peaks from the chromatograms and enabled the use of the simple extraction procedure described earlier. A chromatogram of control plasma sample (1 ml) obtained under these conditions is shown in Fig. 1*b*.

Although the use of a variable wavelength detector resulted in a loss in sensitivity compared to that possible with the fixed-wavelength detector due to differences in the energy output of the lamps, good reproducibility and accuracy were still attainable with 0.1 μg of warfarin. This lower limit is sufficient for most clinical and pharmacokinetic purposes. When using the chromatographic conditions previously described, the peaks corresponding to warfarin and the internal standard had retention times of 3.3 and 6.0 min, respectively. A typical chromatogram of a plasma sample containing 2.0 μg of warfarin/ml is shown in Fig. 1*c*.

There has been some controversy about the specificity of the nonchromatographic methods for the quantitative determination of warfarin in plasma (2, 4, 7–9). Lack of specificity in such analyses arises from variable blank values and the measurement of drug metabolites. Although not specifically investigated by some authors, it is probable that the chromatographic methods measure only unchanged warfarin.

The effect of known metabolites of warfarin on the assay was investigated by adding 6-hydroxy- and 7-hydroxywarfarin[11] and the ste-

[10] Vacutainers, V.W.R. Scientific, San Francisco, Calif.

[11] Provided by Dr. C. H. Schroeder, WARF Institute, Inc., Madison, Wis.

Table II—Comparison of Published Methods for the Analysis of Warfarin in Plasma

Reference	Method	Sensitivity, μg/ml	Specificity[a]	Reproducibility (Coefficient of Variation), %
1	Spectrophotometric	Not given	Questionable	9.9[b] (duplicates of mean 7.1 μg/ml)[c]
2	Spectrophotometric	0.1	Questionable	2.2[b] (replicates at 5 and 10 μg/ml)
3	Fluorometric	Not given	Nonspecific	1.0[b] (replicates at 5 μg/ml)
4	TLC–fluorometric	0.25	Specific	1.6 (replicates between 2 and 7 μg/ml)
5	GC	0.25	Specific	3.15 (replicates between 0.25 and 8.0 μg/ml)
6	TLC–GC	0.02	Specific	Not given
7	HPLC	Not given	Specific	2.0 (Not specified)
This method	HPLC	0.1	Specific	3.0 (replicates at 0.5, 1.0, and 2.0 μg/ml)

[a] See *Discussion*. [b] Value not reported but calculated by present authors from published data. [c] Comments in parentheses indicate the range of concentrations over which estimates of reproducibility were made.

reoisomeric alcohol metabolites[12] (10) to plasma samples, which were then taken through the procedure. These four metabolites all had retention times of 2 min or less under the conditions described and did not interfere with measurement of the peaks corresponding to warfarin or the internal standard. Since no peaks interfere with warfarin or the internal standard in blank plasma and the known metabolites of warfarin are well separated from the two peaks of analytical interest, it may be concluded that the method is specific for warfarin. Furthermore, plasma samples obtained in 18 patients receiving 50 different drugs were handled in a manner identical to that for samples known to contain warfarin, except that an internal standard was not added. Chromatograms of these samples showed no peaks that would interfere with the peaks of warfarin or the internal standard[13].

The average normalized peak height ratios obtained from seven calibration curves containing five or more data points had a mean coefficient of variation of 4.8% over approximately 3 months (Table I). This estimate of accuracy covers the entire range of the assay procedure, from 0.1 to 4.0 μg of warfarin/sample. Reproducibility studies using replicates of five samples of 0.5 μg of warfarin, 10 samples of 1.0 μg of warfarin, and five samples of 2.0 μg of warfarin/ml of plasma gave coefficients of variation of 4.0, 1.7, and 3.4%, respectively (Table I). Although these estimates of reproducibility for this method are larger than some estimates shown in Table II for other methods, most estimates for other methods were made at plasma warfarin concentrations in the upper range of therapeutic values or above. In contrast, the coefficients of variation presented in this paper were calculated at concentrations more commonly encountered in patients. Since it may be necessary to take variable volumes of plasma for warfarin measurement, the effect of plasma volume on the peak height ratio of warfarin to the internal standard was examined. Between the range of 0.2 and 2 ml, the peak height ratios were independent of the volume of plasma.

Application of the method to plasma level determinations in a normal human male volunteer is demonstrated in Fig. 2. The data are well described by a one-compartment open model, with $r = 0.998$ for a plot of log warfarin concentration *versus* time. The half-life of 29.5 hr and the volume of distribution of 0.25 liter/kg [assuming complete absorption (11)] are within the range of previously reported values (11). After this relatively small dose, it was possible to monitor plasma concentrations for four half-lives.

The HPLC method described here for the quantitative determination of warfarin in plasma is simpler and more rapid than other published methods with similar sensitivity or specificity (Table II). By using the techniques described, 30–40 samples can easily be assayed in a day. The method is not only convenient, but it is specific; and its accuracy and sensitivity are satisfactory for most pharmacokinetic studies.

[12] Provided by Dr. W. F. Trager, School of Pharmacy, University of Washington, Seattle, Wash.

[13] A list of these drugs is available upon request from the authors.

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