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ANALYSIS OF WARFARIN IN PLASMA BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

An improved high-pressure liquid chromatography method for the estimation of warfarin in plasma was developed. Plasma was acidified and extracted with ethylene dichloride spiked with methylated warfarin [3-(α-acetonylbenzyl)-4-methoxycoumarin] as internal standard. The residue, redissolved in dioxane, was chromatographed on a reversed-phase column using a mobile phase of 40% dioxane in water (pH 4.2) on a high-pressure liquid chromatograph fitted with an UV absorbance detector. Recoveries from extraction, quantitated using tracer amounts of [^{14}C]warfarin and methylated [^{14}C]warfarin were 92.2 ± 3.16% and 82.33 ± 1.03%, respectively. The standard curve was linear between 0.625 and 5.0 μg/ml. Detection was sensitive to approximately 0.5 μg/ml and specific without the interference of normal plasma constituents and warfarin metabolites.

INTRODUCTION

Warfarin is an oral anticoagulant commonly used for the treatment of hematological disorders. Its measurement in biological fluids is of value both therapeutically and for pharmacokinetic studies. Analyses of warfarin by spectrophotometric[1], fluorometric[2,3], and thin-layer chromatographic (TLC)[4] methods have been reported. These methods often lack sensitivity or specificity, or both, and require large volumes of biological samples. Recently, Midha *et al.*[5] reported a sensitive and specific gas–liquid chromatographic (GLC) procedure for the determination of warfarin in plasma. However, their technique includes a lengthy extraction procedure and derivatization of the samples with diazomethane prior to chromatographic analysis.

The polar nature of warfarin makes high-pressure liquid chromatography (HPLC) a more suitable tool for its determination. HPLC often requires no sample derivative formation since the volatility of the sample is not a prerequisite to analysis. Also, the detection mechanism (*e.g.*, a variable-wavelength UV detector) affords possibilities for increased selectivity, which often results in a more direct and simple extraction procedure in the analysis.

Vesell and Shively[6] recently described an HPLC assay for warfarin in plasma using large-particle-size octadecylsilyl (ODS)-bonded silica polymers (Permaphase) as the stationary phase. With extraction and sample preparation being involved, these authors made no provision for use of an internal standard for the analysis. Also, no data were presented relating to extraction recoveries of samples, and quantitation at UV (254 nm) was found to have low sensitivity and sometimes suffered from the interference of endogeneous peaks in the plasma. In this report, we describe a more precise and specific HPLC analysis of warfarin in plasma in the presence of metabolites. A column comprised of micro-particle (10 μm) ODS-bonded silica gel was used, because it offered better peak capacity and resolution. Analysis was performed with the mobile phase dioxane–water (40:60), pH 4.2, at UV (305 nm) and methylated warfarin [3-(α-acetonylbenzyl)-4-methoxycoumarin] served as the internal standard.

MATERIALS AND METHODS

Liquid chromatography

A Varian-Aerograph Series 4100 liquid chromatograph equipped with a positive displacement pump capable of developing a pressure of 5000 p.s.i., a stop-flow injection port, a stainless-steel MicroPak™ CH-10 (10 μm silica gel) column, 25 cm × 2.2 mm I.D., and a variable-wavelength UV absorbance detector (Variascan) operated at 305 nm was utilized. The mobile phase was dioxane–water (40:60), pH 4.2. The mobile solvent was "degassed" by applying vacuum to the solvent reservoir for 2 min before use. The UV absorbance detector was set at 0.1 absorbance unit full scale (a.u.f.s.). All analyses were performed at ambient temperature.

Reagents and materials

Sodium warfarin was obtained from Merck Frosst Laboratories (Montreal, Canada). Sodium [^{14}C]warfarin (74 μCi/mg) was purchased from Amersham/Searle (Oakville, Ontario, Canada). Ammonium acetate was analytical grade supplied by BDH (Toronto, Canada). All solvents were reagent grade obtained from Fisher Scientific (Toronto, Canada), freshly distilled prior to use. Methylated warfarin was synthesized by reacting sodium warfarin with methyl iodide in N,N-dimethylformamide; preparative TLC purification (ethylene dichloride–acetone, 9:1) gave a pure compound, the mass spectrum of which was identical to that reported by Midha *et al.*[5]. Methylated [^{14}C]warfarin was similarly prepared from sodium [^{14}C]warfarin. 3-[α-(2-Hydroxypropyl)benzyl]-4-hydroxycoumarin was synthesized according to the procedure described by Trager *et al.*[7]. 6-Hydroxywarfarin and 7-hydroxywarfarin were kindly donated by Dr. K. K. Chan (University of Southern California, Calif., U.S.A.).

Extraction and analysis procedure

Extraction and analysis were performed in a minimum of light. To 1–2 ml of plasma from patients receiving warfarin or outdated blood bank plasma spiked with warfarin were added 1 ml phosphate buffer (pH 7.2), 2 ml 3 *N* HCl, and 25 ml ethylene dichloride containing 5.0 μg of methylated warfarin as internal standard. The extraction mixture was agitated in a horizontal shaker for 10 min and centrifuged at 750 *g*. Approximately 24 ml of the organic phase were removed and evaporated

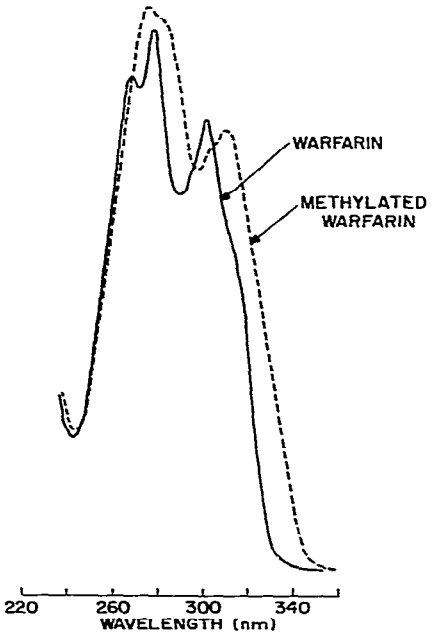
to dryness. The residue was redissolved in 50 μl of dioxane and aliquots of 6–10 μl were injected into the liquid chromatograph.

Evaluation of extraction efficiency

Drug-free plasma was spiked with [^{14}C]warfarin to give concentrations of 1.25, 2.5, and 5.0 μg/ml and extracted with ethylene dichloride containing methylated [^{14}C]warfarin (5 μg) as previously described. The resultant residue was redissolved in 100 μl of dioxane and the radioactivity of a 20-μl aliquot was determined. The remaining sample was spotted on a TLC plate (Anasil G; Analabs, North Haven, Conn., U.S.A.) and chromatographed using a mixture of ethylene dichloride–acetone (9:1). The peaks —[^{14}C]warfarin, R_F 0.3; methylated [^{14}C]warfarin, R_F 0.5— were quantitated by scraping off 0.5-cm-wide strips of silica and counting in a liquid scintillation counter.

RESULTS AND DISCUSSION

Methylated warfarin was found to be a suitable internal standard for the analysis. It is structurally similar to and has approximately the same UV absorption maxima (λ_{max}) and molar extinction coefficients (ε) as warfarin (Fig. 1). Fig. 2 illustrates the HPLC separation of warfarin (R_t = 3.2 min) and methylated warfarin (R_t = 5.0 min) using the system described above. The internal standard is well resolved from the warfarin peak and at the same time elutes close to it.



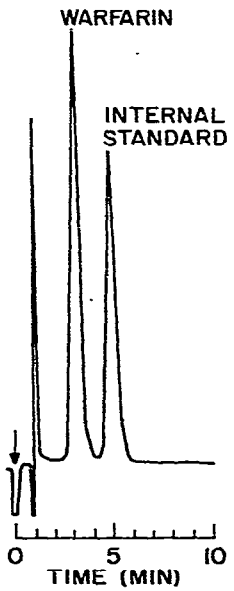


Fig. 1. UV absorption spectra of warfarin and methylated warfarin in dioxane–water (40:60), pH 4.2.

Fig. 2. Chromatogram of a standard sample of warfarin and the internal standard. Column, 25 cm × 2.2 mm I.D., packed with MicroPak™ CH-10; mobile phase, dioxane–water (40:60), pH 4.2; flow-rate, 50 ml/h at 2000 p.s.i.

For the preparation of samples, aliquots of plasma were treated with 3 *N* hydrochloric acid to release the protein-bound warfarin, and partitioned by agitating for 15 min with ethylene dichloride containing a known amount of the internal standard in a horizontal shaker. Vortexing of sample leads to emulsion formation and erratic recoveries. The sample residue was redissolved in dioxane for injection into the liquid chromatograph since warfarin and the internal standard are both readily soluble in this solvent and no diffusion of the compounds was observed when injected into the mobile phase.

Fig. 3 illustrates the standard curve for the assay. Results were obtained from four determinations of each warfarin concentration. The peak height ratio of warfarin and internal standard was used as an index for quantitation, and a linear response was obtained with warfarin concentrations ranging from 0.625–5.00 μg/ml. The overall coefficient of variation was 3.4%.

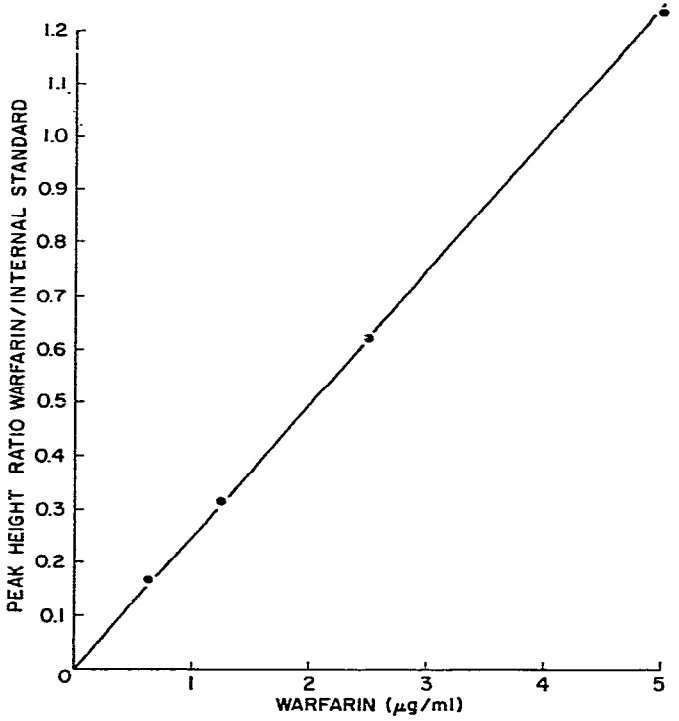


Fig. 3. Standard curve for warfarin.

Next, recoveries were studied using tracer amounts of [^{14}C]warfarin and methylated [^{14}C]warfarin. A known amount of [^{14}C]warfarin was added to plasma at concentrations of 1.25, 2.5, and 5.0 μg/ml and extracted with ethylene dichloride containing ^{14}C-labelled internal standard. The mean recoveries of warfarin and internal standard ranged from 88.0–98.4% and 81.2–84.4%, respectively (Table I). A proportional increase in the mean recovered ^{14}C ratio of warfarin and the internal standard *versus* warfarin concentration was observed, indicating that the internal standard employed has the same partition characteristics as warfarin over the range studied.

TABLE I

RECOVERY OF [^{14}C]WARFARIN AND METHYLATED [^{14}C]WARFARIN FROM PLASMA

Micrograms [^{14}C]warfarin added to 1 ml plasma	*n*	*Mean micrograms [^{14}C]warfarin recovered ± S D (% recovery)*	*Micrograms methylated [^{14}C]warfarin added to 1 ml plasma*	*n*	*Mean micrograms methylated [^{14}C]warfarin recovered ± S D (% recovery)*	*Mean recovered ^{14}C ratio of warfarin and methylated warfarin*
1.25	3	1.23 ± 0.02 (98 4)	5.00	3	4.22 ± 0.07 (84.4)	0.29
2.50	3	2.20 ± 0.10 (88.0)	5.00	3	4.07 ± 0.03 (81.4)	0.54
5.00	3	4.51 ± 0.24 (90.2)	5.00	3	4.06 ± 0.43 (81.2)	1.11

Analysis of samples was performed at UV (305 nm) rather than at maximum sensitivity at UV (280 nm) (Fig. 1), because of interference from extraneous compounds in the plasma when monitored below 300 nm. By monitoring above 300 nm, these extraneous compounds gave no UV absorption peaks. This demonstrates the versatility of the UV detector compared to GLC's most commonly employed flame ionization detector, which forms an almost universal detection system[8]. The HPLC–UV detection mechanism may selectively detect only molecules with appreciable absorption at a certain wavelength and consequently a more direct and simple extraction procedure can often be derived for the analysis.

The specificity of the technique from the interference of warfarin metabolites was also investigated. Fig. 4 illustrates the HPLC separation of warfarin from its three metabolites, namely 6-hydroxywarfarin, 7-hydroxywarfarin and 3[α-(2-hydroxy-

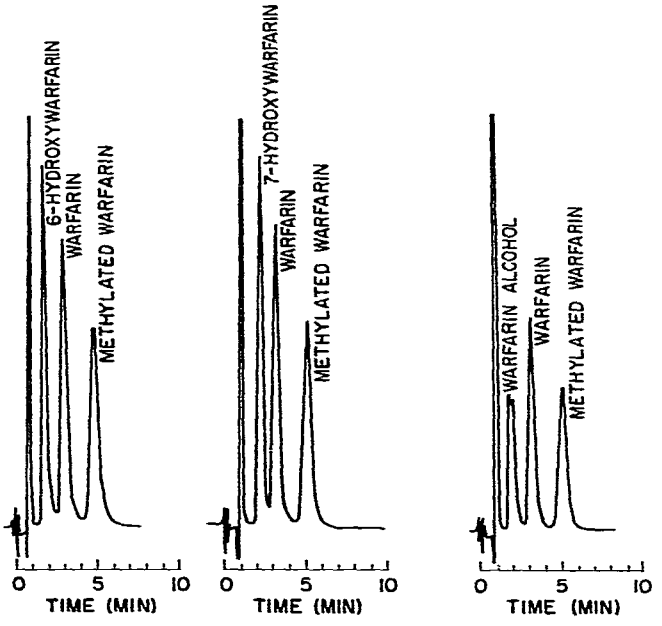
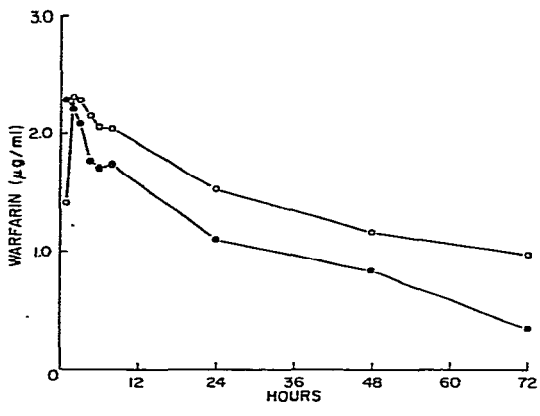


Fig. 4. Chromatographic separation of warfarin and the internal standard from (a) 6-hydroxywarfarin, (b) 7-hydroxywarfarin, and (c) warfarin alcohol. Column, 25 cm × 2.2 mm I.D., packed with MicroPak™ CH-10; mobile phase, dioxane–water (40:60), pH 4.2; flow-rate, 50 ml/h at 2000 p.s.i.

propyl)benzyl]-4-hydroxycoumarin (warfarin alcohol). No interference by the metabolites of warfarin was observed since they have a shorter retention time (R_t = approx. 2 min) and are well separated from warfarin.

This HPLC method has been applied to the analysis of plasma warfarin concentrations in humans. Fig. 5 illustrates the blood profiles of two human volunteers who had ingested a 20-mg dose (p.o.) of sodium warfarin. Using this technique, as low as 0.5 μg/ml of warfarin in plasma could be analyzed.



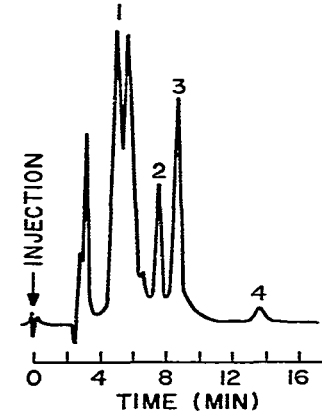


Fig. 5. Plasma warfarin levels in two human volunteers following a single oral dose of 20 mg sodium warfarin.

Fig. 6. Chromatogram of warfarin and metabolites. Column, 25 cm × 2.8 mm I.D., packed with 10-μm silanized LiChrosorb SI-60; mobile phase, 0.1 *M* ammonium acetate in acetonitrile–water (30:70), pH 5; flow-rate, 30 ml/h at 1600 p.s.i. 1 = Warfarin alcohol; 2 = 6-hydroxywarfarin; 3 = 7-hydroxywarfarin; 4 = warfarin.

Application of reversed-phase HPLC for the separation of warfarin metabolites is illustrated in Fig. 6. By employing a mobile phase of 0.1 *M* ammonium acetate in acetonitrile–water (30:70), pH 5, warfarin and its three metabolites can be simultaneously resolved. With the incorporation of a highly sensitive UV detection device, this technique could be useful for analyzing warfarin metabolites in biological fluids.

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