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Note

Comparison of high-performance liquid chromatography and a spectrophotometric technique for determining plasma warfarin

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The advent of new methods for assaying drug levels in various body fluids gives the physician a mechanism for prescribing exact dosage and thus obtaining a more desirable therapeutic:toxicity ratio. Warfarin, which interferes with the function of the four vitamin K dependent clotting factors, is often used to treat thromboembolic disorders [1]. The one-stage prothrombin time can be used to monitor the effect of this drug. However, studies have indicated that many patients will either be under- and/or overtreated with this drug, resulting in serious complications [2, 3]. Reasons for this problem include drug interactions [4, 5] age [6] and discrepancies in results from the coagulation assays used to monitor warfarin's effect [7]. An easy, accurate method of assaying plasma warfarin levels could provide better control.

This study is a portion of a Veterans Association Cooperative Study in which warfarin is used in a random fashion as adjuvant to standard neoplastic regimens. Human experimentation committees at each of the participating institutions approved the study. In determining plasma warfarin levels using the spectrophotometric assay [8] we noted sporadic results in our first group of patients. For this reason a more reliable assay was required. Many techniques were available including thin-layer chromatography (TLC) [9] fluorometry [10], gas—liquid chromatography (GLC) [11, 12] and high-performance liquid chromatography (HPLC) [13—15]. We compared HPLC and the original spectrophotometric assay, a study which has not previously been reported. In our study, HPLC was found to be extremely sensitive, easily performed, and required only small volumes of plasma. These factors led us to conclude that in the clinical evaluation of plasma warfarin levels, HPLC is the procedure of choice.

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MATERIALS AND METHODS

Citrated plasma (Vacutainer®; Becton-Dickinson, Rutherford, N.J., U.S.A.) was obtained from each individual prior to and after at least one week of warfarin therapy. The same lot number of warfarin (supplied by Endo Labs., Garden City, N.J., U.S.A.) was given to all patients. Specimens were sent frozen to the central laboratory for analysis.

The spectrophotometric assay, which requires 2.0 ml of plasma for each assay, was performed as described by O'Reilly et al. [16].

The HPLC assay of Bjornsson et al. [17] was modified as described below. An internal standard of *p*-chlorowarfarin 3-(α-acetonyl-*p*-chloro-benzyl)-4-hydroxycoumarin (Aldrich, Milwaukee, Wisc., U.S.A.) was added at a final concentration of 2.0 μg/ml to 0.5—1.0 ml of plasma. The ether extracts were evaporated in glass Concentratubes® (Laboratory Research, Los Angeles, Calif., U.S.A.) using a Myer N-Evap (Organomotion Assoc., Shrewsbury, Mass., U.S.A.) at 40°.

After the specimens were completely evaporated, 0.25 ml of methanol (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.) was used to dissolve the residues. A 20-μl aliquot was injected into the chromatographic column, a 30 cm × 4 mm I.D. μBondapak C_{18} (Waters Assoc., Milford, Mass., U.S.A.). Other equipment included a Waters Model 6000 A pump and a U6K injector. A Model 440 absorbance detector set at 313 nm with a dual channel recorder (0.02 a.u.f.s.) was used to examine the column eluate. The eluent was methanol—acetic acid—water (75:0.5:25) at a flow-rate of 1 ml/min. All solutions were filtered through a 0.50 μm Fluoropore FH 47 cm filter using a hydrosol stainless filter holder (Millipore, Bedford, Mass., U.S.A.).

RESULTS

In the HPLC system, warfarin has a retention time of 5.5 min. and that of the internal standard *p*-chlorowarfarin, 6.7 min. (Fig. 1). This relationship was constant from day-to-day, although the retention time of the individual compounds at times varied. Fig. 2 represents the results obtained when crystalline warfarin was added to pooled control plasma from 0.3 μg/ml to 5 μg/ml prior to extraction. Each point and standard deviation is based on eight separate determinations. The extraction efficiency of each compound was greater than 95%. Peak heights were determined by triangulation and plasma concentrations of warfarin by peak-height ratios.

A similar experiment using the spectrophotometric assay demonstrated that this technique was inaccurate below 0.6 μg/ml. However, the correlation coefficient (0.95) for the standard curve was similar to that observed in the HPLC assay.

Fig. 3 shows the results of the two different methods of measuring plasma warfarin. For patient 14 only the post warfarin specimen was received. Values obtained on the pre-warfarin plasmas revealed that, except for patient 6, the spectrophotometric assay demonstrated significant levels of warfarin. In patient 9, the pretreatment value was higher than that obtained on the post-warfarin treatment plasma. Patient 8 had a small amount of warfarin in his

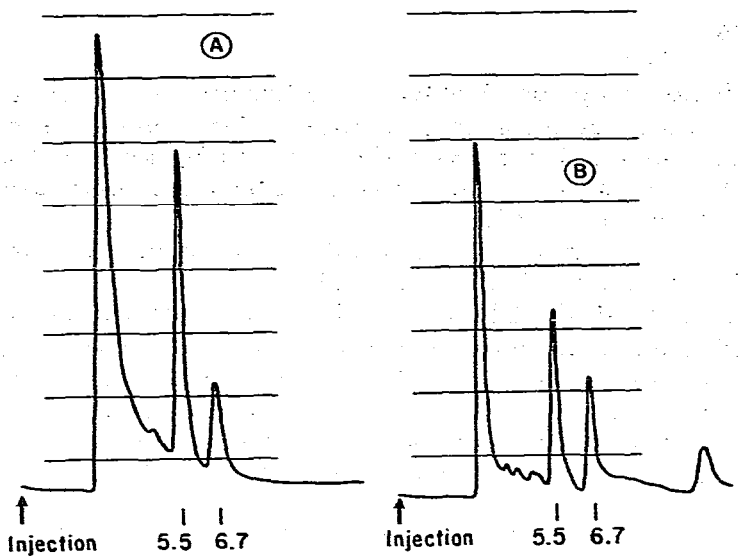
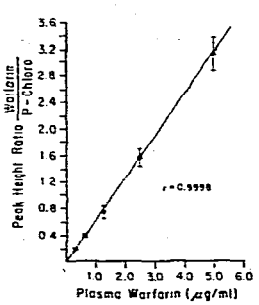


Fig. 1. HPLC of plasma extracts. (A) Normal plasma to which 1.25 μg/ml of crystalline warfarin and 2.0 μg/ml of *p*-chlorowarfarin were added prior to ether extraction. (B) A patient's plasma containing warfarin to which the internal standard has been added. Retention time for warfarin is 5.5 min. The *p*-chlorowarfarin elutes at 6.7 min. Additional peaks can be seen in the patient's sample clearly separated from warfarin.



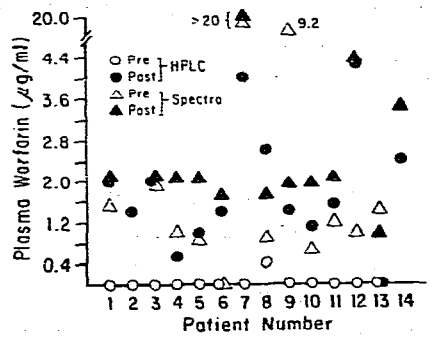


Fig. 2. Standard curve constructed by adding varying concentrations of crystalline warfarin and 2.0 μg/ml of *p*-chlorowarfarin to normal plasma prior to ether extraction and HPLC. Elution conditions are given in the text.

Fig. 3. Comparison of plasma warfarin values determined by HPLC and spectrophotometry.

pre-treatment specimen by HPLC. However, there is doubt whether plasma was actually obtained prior to beginning warfarin therapy. Patient 13 although scheduled to receive warfarin was never started on therapy. The pre- and post-HPLC values in this patient were zero, while the spectrophotometric assay suggested that this individual did have warfarin in his plasma. Only three post-warfarin specimens and one pre-warfarin specimen gave similar results by both techniques.

DISCUSSION

The use of methods which determine the presence of drugs and their metabolites in biological fluids must be accurate in the ideal as well as in the practical setting. We compared two methods for measuring plasma warfarin; HPLC and spectrophotometry. The HPLC technique was far superior. It is specific for warfarin. The chromatographic pattern for the crystalline drug and that of extracted warfarin-containing plasma were similar. The separation of the substances extracted from plasma by chromatography excludes any materials which interfere with the detection of warfarin, a process that is not part of the spectrophotometric technique. We reviewed the medical records of our patients in an attempt to determine whether any were taking medications which might account for the wide fluctuations in the spectrophotometric assay. Every individual was receiving so many drugs that it was not possible to make any correlations.

The use of the internal standard in HPLC standardizes the extraction between specimens, which cannot be done with the spectrophotometric assay. The spectrophotometric assay is inaccurate below 0.6 μg/ml, while HPLC can easily detect 0.3 μg/ml of warfarin in plasma. Another advantage of the HPLC method is that both the metabolites of warfarin, and warfarin can be detected [18].

The biological effect of warfarin, which is to interfere with the proper synthesis of the vitamin K dependent coagulation factors, is easily assessed using the one-stage prothrombin time. In the clinical setting only about one half of those on this drug achieve the appropriate level of anticoagulation. O'Reilly and Aggeler [19] in an extensive review cite many factors contributing to this problem. However, to date, correlation of plasma warfarin level and prothrombin time has not been achieved. Using the HPLC method described it is possible to examine the relationship between the biologic effect of warfarin and its concentration in plasma without interference by other drugs. It is anticipated that the HPLC method will not only assist in evaluating patients who have abnormal responses to warfarin, but in addition, the pharmacology of this compound and its metabolites can be more specifically investigated.

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NOTE ADDED IN PROOF

Methyl cyanide—acetic acid/water [46:54 (1.5%, pH 5.0)] can be used, as the eluent. In this situation warfarin has a retention time of 4.5 min and the internal standard 5.8 min.

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