Plasma Protein Binding of Warfarin: Methodological Considerations

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Abstract □ Recent theoretical work has suggested that radiochemical impurities can significantly alter the binding results for highly protein-bound drugs. We compared protein binding of warfarin by ultrafiltration and equilibrium dialysis with 98% radiochemically pure [^{14}C]warfarin. Ultrafiltration and equilibrium dialysis were performed at 37°C and pH 7.45 on the plasma of patients receiving chronic warfarin therapy. Binding to plasma from seven patients was measured in duplicate by both a nonspecific radioisotopic technique and a specific HPLC technique. The nonspecific technique gave percentage of free warfarin values of 1.84 ± 0.11 (mean ± *SD*) and 1.59 ± 0.14 for ultrafiltration and equilibrium dialysis, respectively. The HPLC procedure yielded a percentage of free warfarin by ultrafiltration of 0.969 ± 0.203 and a value of 0.690 ± 0.095 by equilibrium dialysis ($p < 0.05$). The HPLC procedure for protein binding was performed on plasma samples from 12 additional patients and yielded a percentage of free warfarin of 1.01 ± 0.69 by ultrafiltration and 0.44 ± 0.34 by equilibrium dialysis ($p < 0.05$). It can be concluded that radiochemical impurities may lead to significant overestimation of the percentage of free warfarin. Ultrafiltration yielded a higher percentage of free warfarin than did equilibrium dialysis, but the ability to distinguish binding differences among patients was similar.

Keyphrases □ Warfarin—methodological considerations, plasma protein binding □ Plasma protein binding—methodological considerations, warfarin, radiochemical impurities □ Radiochemical impurities—methodological considerations, plasma protein binding of warfarin

A number of factors may affect the results of protein binding studies. These factors include the administration of heparin, the type of blood collection tubes, the system used for determining binding (equilibrium dialysis, ultrafiltration), and the radiochemical purity of the radiolabeled compound. Bjornsson *et al.* (1) have recently emphasized the importance of radiochemical purity of radiolabeled drugs used for determining plasma protein binding. Theoretically, radiochemical impurities that are poorly bound to plasma proteins could lead to significant overestimations of the free concentration of drugs that are highly bound. Warfarin is an agent which is highly protein bound, and its binding could be susceptible to measurement errors related to the presence of radiochemical impurities. [^{14}C]Warfarin is ~98.5% radiochemically pure. Studies with [^{14}C]warfarin for binding without a purification procedure (2–4) have reported a significantly higher percentage of free warfarin than studies in which a TLC assay procedure was utilized (5, 6). Yacobi and Levy (7) have found that a twofold error in the estimation of the percentage of free warfarin was made when radiochemically impure warfarin was used to determine protein binding in rats. No data on human plasma are available concerning this issue.

The majority of studies evaluating warfarin binding have utilized equilibrium dialysis, and no information is available comparing ultrafiltration with equilibrium dialysis. In the present study, the influence of radiochemical impurity on warfarin binding in human plasma is evaluated, and plasma binding as determined by ultrafiltration and equilibrium dialysis is compared.

Table I—Comparison of Ultrafiltration and Equilibrium Dialysis Methods for Measuring Warfarin Protein Binding [a]

Procedure	Mean Unbound ± *SD*, %
Ultrafiltration	1.010 ± 0.69
Equilibrium dialysis	0.44 ± 0.34

[a] Free and total concentrations were determined by the chromatographic procedure described in the text ($n = 12$).

EXPERIMENTAL SECTION

Initial attempts were made to purify racemic [^{14}C]warfarin[1] (specific activity, 50 mCi/mmol) before binding studies were performed. The major impurity could be separated by HPLC. Collection and assay of sequential fractions of the eluant revealed that 98.5% of the eluted radioactivity corresponded to warfarin, and ~1.5% corresponded to a chromatographic peak with a somewhat longer retention time. However, an overall recovery of only 20–25% could be obtained by collecting the eluant fraction corresponding to warfarin and extracting into ether. A second method with a sample preparation column[2] did not separate the impurity from the parent compound. Thus, it was necessary to perform the binding studies with the 98.5% pure [^{14}C]warfarin. However, the radioactive warfarin was measured in plasma, ultrafiltrate, or dialysate by the specific chromatographic procedure described below.

Preliminary studies were performed to assess binding of [^{14}C]warfarin to the ultrafiltration device[3] and the effect of *in vitro* heparin on the binding of warfarin. Binding of [^{14}C]warfarin to the ultrafiltration device was tested between the concentrations of 0.02 μg/mL and 2 mg/mL. Less than 10% binding to the device was found with these concentrations. No difference in binding was found between heparinized (*in vitro*) and nonheparinized samples. The time to reach equilibrium with the equilibrium dialysis system[4] was 4 h.

Plasma samples from 19 outpatients treated chronically with warfarin were collected in evacuated blood collection tubes[5] containing 143 U of lithium heparin per tube. Total warfarin concentrations ranged from ~1.0 to 4.3 μg/mL. The coefficient of variation of the HPLC assay was 10.0%. All plasma samples were stored at −20°C until assayed.

Equilibrium dialysis—Aliquots of plasma (1 mL) were dialyzed against 1 mL of 0.1 M phosphate buffer (pH 7.45) with polytef cells separated by dialysis membranes[3]. Radiolabeled [^{14}C]warfarin (0.5 μCi) was added to each plasma sample. The cells were rotated in a water bath at 37°C for 4 h.

Ultrafiltration—[^{14}C]Warfarin (1 μCi) was added to 1-mL plasma samples placed in the reservoir of the ultrafiltration device[3]. An initial 100-μL plasma sample was obtained for measurement of total radioactivity. The samples were placed in a centrifuge in an incubator maintained at 37°C. Samples were allowed to equilibrate for 15 min and were then centrifuged for 15 min at 1000×*g*.

Sample Analysis—For the dialysis experiments, 0.4 μg of phenprocoumon (the internal standard) and 10 μg of unlabeled warfarin (the carrier) were added to 250-μL samples of plasma and dialysate. For the ultrafiltration experiments, similar quantities of internal standard and carrier were added to 100-μL samples of plasma and ultrafiltrate. Each sample was then processed

[1] Amersham Corp., Arlington Heights, Ill.
[2] Sep-Pak; Waters Associates, Milford, Mass.
[3] MPS-1; Amicon Corp., Danvers, Mass.
[4] Spectrum Medical Industries, Los Angeles, Calif.
[5] Texfuno Medical Corp., Elkton, Md.

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and described by Fasco *et al.* (8). Each sample was extracted with 5 mL of ether, and the ether was evaporated to dryness at room temperature under a stream of nitrogen. The residue was diluted in 100 μL of mobile phase and injected onto a reverse-phase HPLC column[6]. The mobile phase was 40% acetonitrile in 1.5% acetic acid and was adjusted to pH 4.7 with ammonium hydroxide. The flow rate was 2 mL/min[7], and detection was at 309 nm[8].

The effluent which corresponded to the warfarin peak was collected in a liquid scintillation vial, mixed with 15 mL of scintillation fluid[9], and counted for 10 min in a liquid scintillation counter[10]. The percentage of [^{14}C]warfarin that was unbound was calculated as follows:

$$\% \text{ unbound} = (0.93A/B)100\% \qquad \text{(Eq. 1)}$$

where A is the net counts in dialysate or ultrafiltrate per internal standard peak height for dialysate or ultrafiltrate and B is the net counts in plasma per internal standard peak height for plasma. The correction factor of 0.93 was used to correct for the fact that plasma is ~93% water, and only this portion was available for the distribution of free drug.

Plasma samples of 7 of the 19 patients were used to determine the apparent percentage of free warfarin if the extraction and chromatography procedures were omitted. Aliquots of plasma and dialysate or filtrate were directly placed in scintillation fluid and counted. The percent unbound was calculated as follows:

$$\% \text{ unbound} = 100\% \frac{0.93 \text{ (net counts in dialysate or filtrate)}}{\text{net counts in plasma}} \qquad \text{(Eq. 2)}$$

For these seven patients, all procedures were performed in duplicate.

RESULTS AND DISCUSSION

Table I shows the mean binding values by ultrafiltration and equilibrium dialysis when the HPLC procedure was used. Ultrafiltration yielded a higher percentage of unbound warfarin than did equilibrium dialysis ($p < 0.05$).

Table II demonstrates the effect of the radiochemical impurities on warfarin plasma protein binding and compares the results obtained between ultrafiltration and equilibrium dialysis in seven additional patients. A significant difference in the percentage of unbound warfarin was found between the direct counting and the chromatographic procedures ($p < 0.05$). The samples counted directly yielded unbound values approximately twice those given by the more specific chromatographic procedure. These results are similar to those found by Yacobi and Levy (7) in rat plasma.

An important consideration in evaluating binding procedures is the relative ability of each to assess differences in the percentage of unbound warfarin between patients. This was evaluated in the present study by using the Spearman rank correlation coefficient (Rs). In the 12 patients in which equilibrium dialysis and ultrafiltration were performed singly and samples were assayed chromatographically, the Rs was 0.7 ($p < 0.05$). For the seven patients in which binding was determined in duplicate, these procedures yielded an Rs value of 0.81 ($p < 0.05$). An Rs of 0.63 ($p < 0.05$) was found between unchromatographed and chromatographed samples from the ultrafiltration procedure, and an Rs of 0.929 ($p < 0.05$) was found between unchromatographed and chromatographed samples from the equilibrium dialysis procedure. These results suggest that the same relative binding results may be obtained for equilibrium dialysis whether or not a cleanup procedure is used. Further study of a group of patients with greater differences in warfarin binding is needed.

This study demonstrates that radiochemical impurities can significantly alter the percentage of unbound warfarin as determined by ultrafiltration or equilibrium dialysis, and that equilibrium dialysis and ultrafiltration are similar in their ability to discriminate warfarin binding differences between patients.

Table II—Effect of Radiochemical Impurity on Protein Binding of Warfarin [a]

Procedure	Mean Unbound ± *SD*, %
Ultrafiltration (counted directly)	1.84 ± 0.11
Equilibrium dialysis (counted directly)	1.59 ± 0.14
Ultrafiltration (extracted, chromatographed)	0.97 ± 0.20
Equilibrium dialysis (extracted, chromatographed)	0.69 ± 0.10

[a] Comparisons in seven patients before and after HPLC cleanup procedure. All samples were performed in duplicate.

[6] μ-Bondapak C_{18}; Waters Associates, Milford, Mass.
[7] Model M-6000A; Waters Associates, Milford, Mass.
[8] Varichrom U-V detector; Varian Instrument Division, Palo Alto, Calif.
[9] Aquasol-2; New England Nuclear Corp., Boston, Mass.
[10] Beckman LS 7500; Beckman Instrument Co., Irvine, Calif.

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