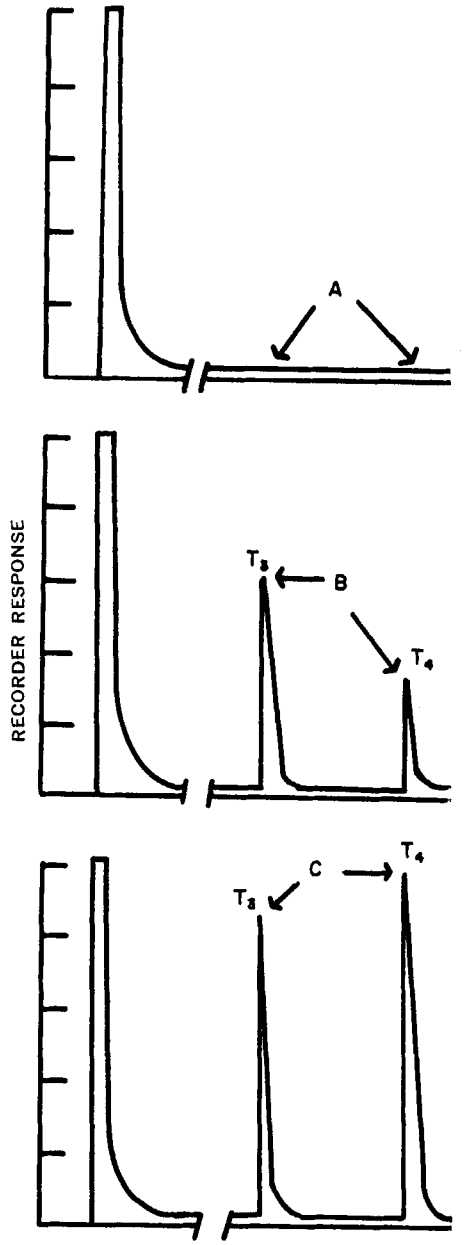


**Figure 2**—*Composite representation of recovery of a mixture of liothyronine ($T_3$) and thyroxine ($T_4$) from GLC columns containing 1.25 mg. (A), 0.125 mg. (B), or no (C) activated charcoal.*

support system previously described, a second with 1.25 mg. of activated charcoal[4] mixed with 50 mg. of this support and placed on top of the column on the injection site, and a third containing 0.125 mg. of activated charcoal mixed with 50 mg. of support and placed on top of the column. All columns were conditioned at 300° for at least 18 hr.

The peak heights obtained on injection of 4 μl. of the derivative mixture into the normal column 1, at an attenuation of $3 \times 10^{-9}$ amp., were comparable to those expected from previous new columns. No response was observed with the column containing 1.25 mg. of activated charcoal (Fig. 2).

These results appear to substantiate the reasoning that this amount of carbon on the column significantly reduces the ability to detect the iodoamino acids. It was determined that each injection of 3 μl. of hydrolyzed sample deposited at least 1.5 mg. of material on the column. After several such injections, carbon deposits could reach the levels used in the trials. Thus, it may be reasonable to assume that the presence of nonvolatile materials in the protein hydrolysates causes charring on the GLC column and could account for the incomplete recovery of iodoamino acids. This observation may be of some benefit to laboratories involved in this work.

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R. Bilous
E. Koehler
J. J. Windheuser
School of Pharmacy
University of Wisconsin
Madison, WI 53706

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## Assay of Warfarin

**Keyphrases** ☐ Warfarin—plasma levels, TLC and spectrophotometric assays ☐ Plasma levels—warfarin, assay ☐ TLC—assay, warfarin

*Sir:*

For the benefit of other investigators interested in the determination of the concentration of warfarin in plasma or stool samples, some comments are in order regarding the work of Welling *et al.* (1). Two of their findings are at variance with our observations.

First, these investigators were unable to detect the presence of metabolites of warfarin in the plasma. We have no experience with the TLC procedure described by Welling *et al.* (1). This procedure utilizes quenching, rather than fluorescence, as the marker for chromatographic loci of interest. In their hands, the same quenching loci were observed in blank plasma and in plasma from subjects receiving warfarin. Our work has been based on a procedure that was outlined in abstract form (2) and subsequently reported in detail (3). After TLC of several hundred extracts of plasma standards (plasma to which warfarin was added), we never observed loci with blue fluorescence with $R_f$ values corresponding to warfarin metabolites. On occasion, with any plasma samples, a clump of fluorescent material runs at the

solvent front, but we have not been able to resolve this into a discrete chromatographic locus. The metabolites that can be recovered from the plasma, the diastereoisomers of reduced warfarin and 7-hydroxy warfarin, were described at the June 1970 conference on Drug Metabolism in Man (4). Metabolites of warfarin were identified in human urine as well (5).

Second, these authors (1) contend that an assay of warfarin based on chromatographic isolation of unchanged drug offers no advantage over methods relying solely on differential extraction. The interested reader should make reference to our formal report on the assay of warfarin (3). The most pertinent aspects of this paper noted consistent (five of five subjects) differences when data from a fluorimetric assay (6) or a spectrophotometric assay (7) were used to determine apparent plasma clearance rates. When the spectrophotometric method was compared to the TLC method as we described it, the former indicated slower clearance rates in two of four subjects: $T_{1/2}$ of 57.5 hr. *versus* 43.1 hr. and $T_{1/2}$ of 36.2 hr. *versus* 30.5 hr. were determined by the least-squares best fit line relating log warfarin concentration to time.

Since we have no experience with the TLC assay of Welling *et al.* (1), it is impossible to assess adequately the possible sources of error. In common with these investigators, we noted that outdated blood bank plasma gives higher blank values in the spectrophotometric assay than does freshly drawn citrated plasma. For this reason, we prepare all plasma ourselves. The high blank values may have contributed to the discrepancy in their results. In Table I of their report (1), the slope of the line relating net absorbance to warfarin concentration is 0.182. Blank plasma in their hands resulted in a net absorbance of 0.216 ± 0.056 or 0.261 ± 0.024, the equivalent of 1.2 ± 0.3 or 1.4 ± 0.1 mcg. "warfarin"/ml. plasma. Since warfarin has an apparent volume of distribution of 8–9 l. calculations from the 25-mg. dose would indicate that the maximum plasma concentration of their samples would be about 3 mcg./ml. at $T_0$. Subsequent values would be much less. Thus, these investigators were working at plasma levels at most only 2–3 times their blank. In our studies, we employed a dose of 1.5 mg. warfarin/kg. body weight, which produced $T_0$ concentrations of about 12 mcg./ml. Our values for blank plasma were the equivalent of 0.15 mcg./ml. plasma.

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RICHARD J. LEWIS
Veterans Administration Hospital
San Francisco, CA 94121
and
Department of Medicine
University of California
San Francisco, CA 94122

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## Assay of Warfarin: A Rebuttal

**Keyphrases** ☐ Warfarin—small, single oral dosage plasma levels, TLC and spectrophotometric assays ☐ Plasma levels—warfarin, small oral dosage ☐ TLC—assay, warfarin

*Sir:*

Dr. Lewis' communication (1) refers to the warfarin report of Welling *et al.* (2) and his own published reports (3, 4). Concerning the application of our methods, Dr. Lewis and I are not nearly so far apart as his communication tends to indicate. We clearly indicated that our results were obtained following oral administration of 10- and 25-mg. single doses of sodium warfarin. We clearly stated that our results may not apply after administration of large single doses nor after multiple doses of sodium warfarin. However, under the conditions of our studies, metabolites of warfarin were not present in detectable amounts in plasma. Furthermore, the modified O'Reilly assay and TLC did give equivalent results, as shown in Fig. 1 of our paper (2). I have no doubt that following large single doses, such as 1.5 mg./kg. as administered by Lewis and Trager (5), or when assaying plasma samples obtained after multiple doses of warfarin, warfarin metabolites are detectable as shown by Lewis *et al.* (3, 4).

The TLC procedure that we used was almost identical to the method used by Lewis *et al.* (3, 4). The adsorbent in both cases was silica gel, but they used Eastman No. 6061 silica gel G TLC sheets, while we used our own 20 × 20-cm. plates with a 250-$\mu$ thickness of silica gel GF 254. The solvent was 9:1 (v/v) 1,2-dichloroethene–acetone in both cases. Our silica gel contained a fluorescent indicator, while theirs did not. However, both we and they reported essentially the same $R_f$ value (0.50–0.54) for unchanged warfarin. We reported clean separations of the 4′-OH, 6-OH, and 7-OH metabolites of warfarin, with $R_f$ values of 0.22, 0.18, and 0.23, respectively. Lewis and Ilnicki (3), following an unspecified dose of warfarin, reported separation of two metabolites from plasma ($R_f$ 0.23 and 0.19). Lewis *et al.* (4) displayed a photograph showing separation of a mixture of diasterisomeric warfarin alcohols from warfarin on their plates. The evidence suggests strongly that after 10- and 25-mg. doses of warfarin, metabolites are not detectable in plasma as we concluded, whereas after higher doses the metabolites are obviously detectable as Lewis *et al.* (4) showed. The discrepancy appears not to lie in any basic difference in the TLC procedures employed but rather in the doses administered. The reason why our blank plasma samples gave spots on the thin-layer plates, whereas Lewis *et al.* (4) never observed such spots, is unknown. The difference may or may not be due to the fluorescent indicator.

Two other comments made by Lewis (1) deserve special attention. Lewis stated: "When the spectrophotometric method was compared to the TLC method as we described it, the former indicated slower clearance rates in two of four subjects: $T_{1/2}$ of 57.5 hr. *versus* 43.1 hr. and $T_{1/2}$ of 36.2 hr. *versus* 30.5 hr. were determined