Rapid Fluorometric Assay for Plasma Warfarin

Milton Corn and Robert Berberich*

Acetone alters the ultraviolet absorption characteristics of warfarin (3-(α-acetonylbenzyl)-4-hydroxycoumarin) and increases the intensity of fluorescent emission. Fluorescence of warfarin in acetone is markedly decreased by acidification. These phenomena provide the basis for a rapid, simple assay for plasma warfarin.

The determination of warfarin (3-(α-acetonylbenzyl)-4-hydroxycoumarin) concentration in plasma by the spectrophotometric method of O'Reilly et al. (1) requires several time-consuming extraction procedures. This paper describes a simple, rapid fluorometric assay for this determination.

Methods

As in all fluorometric procedures, glassware, reagents, and specimens must be kept free of fluorescent contamination. Glassware must be cleaned with an inorganic detergent and rinsed with distilled water. Parafilm should be used to seal tubes for mixing procedures. Ten milliliters of venous blood was collected into tubes containing 0.1 ml. sodium citrate (38 gm./100 ml. water), or 20 mg. ethylenediaminetetraacetic acid (EDTA), or 14 mg. sodium oxalate. The latter two anticoagulants are added as solutions and then the solutions are dried. Plasma was separated by centrifugation at 1500 g for 10 min. and either analyzed within several hours or stored at -20° for subsequent analysis.

Plasma (0.2 ml.) was well mixed with 4 ml. reagent-grade acetone† (Fisher Scientific Company) and centrifuged at 1500 g for 3 min.; 3 ml. of the supernatant was transferred to a 10 × 75 glass tube (disposable),

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†Lower grades of acetone frequently display high fluorescence, which interferes with the assay.

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and its fluorescence was measured and designated F1. Then 0.05 ml.
0.1 N HCl was added to the tube and the fluorescence was again mea-
sured and designated F2. A Turner Model 110 filter fluorometer was
used for these measurements. The excitation filter had a peak at 365 m\(\mu\r
(Turner Filter 760), and the emission filter had a peak at 415 m\(\mu\r
(Turner Filters 2a and 47b). An acetone blank was used to provide a
stable reference point. A standard warfarin plasma, prepared by
adding 0.1 ml. warfarin (50 \(\mu g./ml.\) water) to 0.9 ml. warfarin-free
plasma, was carried through the procedure once daily. The decrease in
fluorescence (measured in arbitrary units) caused by the addition of
acid was used to determine warfarin concentration by the expression:

\[
\text{\(\mu g.\) warfarin/ml. plasma} = \frac{5(F1 \text{ unkn.} - F2 \text{ unkn.})}{F1 \text{ std.} - F2 \text{ std.}}
\]

Parallel spectrophotometric assays for plasma warfarin were per-
formed by the method of O'Reilly et al. (1). Absorption spectrums for
warfarin in water and in acetone were determined in a Beckman DU
spectrophotometer. Fluorescent emission spectrums were determined
in an Amineo Bowman spectrophotofluorometer.

Results

The absorption peak of warfarin in acetone occurs at a longer wave
length than that of warfarin in water (Fig. 1). Acetone also alters the
fluorescent emission spectrum and greatly increases the intensity of
emission (Fig. 2).

Addition of increments of warfarin to plasma caused a linear in-
crease in the fluorescence of acetone extracts of the plasma. However,
the variation in native fluorescence found among individual samples
of plasma prior to addition of warfarin made it difficult to relate a given
amount of fluorescence to a standard warfarin curve. To eliminate the
error introduced by such variation, warfarin concentration of plasma
was related to the decrease in fluorescence caused by addition of acid
to acetone extracts of plasma. Small amounts of acid abolished fluo-
rescence due to warfarin but had only minimal effect on native fluo-
rescence. The decrease in fluorescence caused by acidification was linearly
related to warfarin concentration (Fig. 3). The choice of anticoagulant
(citrate, oxalate, or EDTA) mixed with the specimen did not influence
the results. Addition of 0.05 ml. NaOH 0.1 N to the acidified acetone
extracts only partially restored the fluorescence present prior to
acidification.

Recovery of warfarin from plasma by acetone extraction was ap-
proximately 95%, as determined by comparing results obtained when
identical quantities of warfarin were added to plasma and to water. Continued exposure of the acetone extract containing warfarin to the ultraviolet light source of the fluorometer caused gradual diminution of fluorescence; the decrease was not detectable within the first 3 minutes and caused no practical difficulty. There was no advantage to determining the fluorescence at other temperatures since the fluorescence at 15° and 37° was similar to that obtained at 23°. Storage of plasma containing added warfarin at room temperature for 3 hr., at 4° for 48 hr., or at -20° for 1 month did not alter the apparent warfarin content. The acetone extracts of plasma which contained warfarin did not alter their fluorescence if they were stored for several hours at 4° in parafilm-covered tubes.

**Fig. 1. Absorption**
spectra obtained when warfarin is dissolved in water and in acetone.

**Fig. 2. Fluorescent**
spectra obtained when warfarin is dissolved in water and in acetone.
Eight replications of a warfarin assay of a plasma sample to which warfarin had been added to a final concentration of 5 \( \mu \text{g./ml.} \) yielded a mean value of 54 fluorescence units with standard deviation of \( \pm 0.54 \) units.

**Fig. 3.** Relationship between warfarin content of plasma and decrease in fluorescence caused by adding HCl to acetone extracts of plasma.

**Fig. 4.** Comparison of results obtained when 65 plasma samples obtained from patients receiving warfarin were analyzed by both spectrophotometric and fluorometric procedures.

When plasma samples obtained from patients receiving warfarin were assayed both by the fluorometric and the spectrophotometric procedures, the results were similar although the fluorometric method tended to give slightly higher values (Fig. 4).
The presence of jaundice or uremia in the donor or of slight hemolysis in the specimen did not affect the fluorometric assay. Analysis of plasma samples obtained from a number of patients receiving drugs other than warfarin did not reveal any drugs which interfered with the assay. The following list, not intended to be exhaustive, is based on analyses of plasma obtained from 1 or more patients receiving the listed agents.

<table>
<thead>
<tr>
<th>Aminophylline</th>
<th>Ferrous sulfate</th>
<th>Phytonadione</th>
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<tbody>
<tr>
<td>Bishydroxycoumarin</td>
<td>Guanethidine</td>
<td>Prednisolone</td>
</tr>
<tr>
<td>Chlordiazepoxide</td>
<td>Insulin</td>
<td>Prochlorperazine</td>
</tr>
<tr>
<td>Digoxin</td>
<td>Kanamycin</td>
<td>Thiamine</td>
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<tr>
<td>Diphenylhydrantoin</td>
<td>Nitrofurantoin</td>
<td>Thiomerin</td>
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<tr>
<td>Erythromycin</td>
<td>Phenobarbital</td>
<td>Tolbutamide</td>
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**Discussion**

Acetone, in addition to its deproteinizing effect on plasma, has an unexplained auxochromic influence on warfarin that obviates the need for quartz glassware in the subsequent analysis and greatly increases the intensity of fluorescent emission. The ability of HCl to decrease fluorescence of warfarin in acetone presumably involves more than the conversion of the salt to the acid form since the process is largely irreversible.

The filters used, although not optimal for the known absorption and emission peaks, provide sufficient sensitivity for rapid, reasonably precise assays in a filter fluorometer. A technician can analyze several dozen plasma samples in an hour.

It is not known whether the fluorometric assay, like the spectrophotometric, also measures a warfarin metabolite which has been found in urine (1). The fluorometric assay is not suitable without modification for use with urine specimens because most urine specimens are highly fluorescent. However, since the metabolite is not found in plasma (1) the fluorometric assay applied to plasma presumably is measuring only unchanged warfarin.

Occasionally warfarin is to be measured in plasma from patients receiving other drugs. None of the other drugs tested, including bishydroxycoumarin, appeared to influence the assay; further experience, however, may reveal interference due to variations in drug metabolism or to drugs not tested by us.

Compared to the spectrophotometric method, fluorometric assay requires less manipulation, is faster, and has no troublesome residual
blank. Although both methods provide a linear response to increments of warfarin added to plasma in vitro, it is not known which method more accurately measures plasma warfarin levels after oral or parenteral administration of this agent. At present we prefer the fluorometric assay because of its relative technical simplicity.

References