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Note

Rapid, reliable and sensitive assay for warfarin using normal-phase high-performance liquid chromatography

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Warfarin [3-(α-acetonylbenzyl)-4-hydroxycoumarin] is one of the most popular oral anticoagulants and is frequently used in both clinical and basic research.

A number of techniques have been published to assess warfarin concentrations in biological fluids. These include thin-layer chromatography [1], gas—liquid chromatography [2, 3] and fluorimetric [4] methods. Many assays have involved reversed-phase high-performance liquid chromatography (HPLC) procedures [5—9] in which suitability for a given application is compromised by cost, number of clean-up steps required, or interferences from other water soluble sample components. One assay system using normal-phase HPLC has been described [10] but this involves a lengthy sample preparation procedure.

We describe here a rapid, reliable and sensitive assay for serum or plasma warfarin which uses normal-phase HPLC and is suitable for both clinical and analytical investigations.

EXPERIMENTAL

Apparatus and conditions

The chromatographic system was assembled using components from various manufacturers as follows: Model 110A solvent metering pump (Altex, Berkeley, CA, U.S.A.); Model CV-6-UHPa-N60 universal injection valve (Valco Instrument, Houston, TX, U.S.A.); Model 8200 ultraviolet absorption detector fitted with a 280-nm wavelength kit (Spectra-Physics, Santa Clara, CA, U.S.A.); Recordall strip chart recorder (Fisher Scientific, Whitby, Canada). To reduce background noise and improve stability the detector was modified by replacing the PTFE tubing in the heat exchanger with stainless-steel capillary tubing.

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The column (250 × 2.1 mm stainless steel) was packed with 5 μm silica gel (Partisil 5, Whatman, Clifton, NJ, U.S.A.) by a balanced density slurry method [11]. All connecting tubing was stainless steel and all fittings were zero-volume stainless steel (Crawford Fittings, Niagara Falls, Canada).

The mobile phase was hexane—ethanol (93.5:6.5, v/v) and was delivered at 1.5 ml/min producing a back pressure of 221 bar (3200 p.s.i.).

Disposable 1.5-ml plastic centrifuge tubes (Fisher Scientific) were used throughout.

Chemicals and reagents

HPLC grade hexane was purchased from Caledon (Georgetown, Canada) and absolute ethanol was obtained from Consolidated Alcohols (Toronto, Canada). Spectrophotometric grade 1,2-dichloroethane was purchased from Fisher Scientific. Drugs were obtained from the following suppliers: caffeine, acetaminophen, salicyclic acid, phenytoin (diphenylhydantoin), quinidine, Sigma (St. Louis, MO, U.S.A.); warfarin, Frosst (Pointe Claire, Canada); phenobarbital, Allen and Hanburys (Toronto, Canada). Serum and plasma were obtained from the blood bank at Kingston General Hospital.

Sample preparation

The sample or standard (0.2 ml) and 0.2 ml of dichloroethane—hydrochloric acid (99.5:0.5, v/v) were added to the centrifuge tubes. The tubes were capped, then shaken horizontally for 8 min and finally centrifuged for 2 min (Microcentrifuge Model 235, Fisher Scientific). A 20-μl aliquot of the organic (lower) phase was injected onto the column and peak heights were measured to determine standard curve or sample warfarin concentration.

The same procedure at a 1:1 (sample:extraction solvent) ratio was reliably applied to sample volumes as low as 0.05 ml.

RESULTS AND DISCUSSION

A typical chromatogram obtained by this procedure is illustrated in Fig. 1. As shown, samples can be easily injected every 2.5 min while preserving reliability of response. Interference from other serum or plasma components is negligible, and extraction efficiency from either medium is 96.3 ± 3.5 (S.D.)% for 1 μg/ml; 96.3 ± 1.7 (S.D.)% for 5 μg/ml; and 95.3 ± 2.5 (S.D.)% for 10 μg/ml.

Similarly, other drugs which are frequently encountered in patient serum samples do not interfere with warfarin measurements as shown in Table I.

The assay is linear above and below the normal therapeutic range of 1.0—10.0 μg/ml [12] and is described by the equation $y = 0.00205x$ where y = absorbance units and x = plasma concentration in μg/ml. Concentrations as low as 0.05 μg/ml can be easily and reliably detected and the standard deviation of the points within each day is less than 1%, indicating the high degree of reproducibility of this assay.

The ease of sample preparation relative to existing HPLC methods, high sensitivity and speed of assay (about 15 min total for duplicate samples) make this assay a significant improvement over previously published pro-

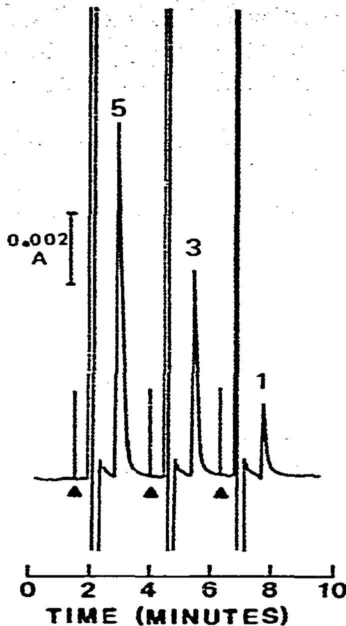

Fig. 1. Chromatogram of successive injections of 5, 5.0 μg/ml; 3, 3.0 μg/ml; and 1, 1.0 μg/ml warfarin extracted from plasma. ▲ indicates the points of injection.

TABLE I

ASSAY RETENTION TIMES FOR DRUGS COMMONLY FOUND IN HUMAN SERUM

Drug	Retention time (min)
Warfarin	1.5
Acetaminophen	6.6
Salicylic acid	2.9
Phenobarbital	1.2
Caffeine	N.D.*
Quinidine	N.D.
Phenytoin	N.D.

*N.D. = not detected under these conditions.

cedures and appropriate for both clinical and research oriented applications. Furthermore, normal-phase chromatography offers a significant advantage over reversed-phase assays by both decreasing the interference from biological sample components and reducing the cost of pre-packed columns and/or packing materials.

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