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Note

Sensitive measurements of warfarin by high-performance liquid chromatography with electrochemical detection

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Warfarin, 3-(1-phenyl-2-oxobutyl)-4-hydroxycoumarin, has found extensive use both as an effective oral anticoagulant and as a rodenticide. Because of its widespread clinical use, a highly sensitive and rapid method for its quantitation is desired. Several high-performance liquid chromatographic (HPLC) assays for warfarin have been reported [1–7]. Most notable are the reversed-phase procedure of Fasco and co-workers [1,5] and the normal-phase method of Lee et al. [3]. The method of Fasco and co-workers [1,5] utilizes ultraviolet absorption detection, offers high specificity but often lacks sufficient sensitivity. Lee et al. [3] used fluorescence detection, coupled with a post-column fluorescence enhancement, to achieve detection limits in the low nanogram range.

In recent years electrochemical detection (ED) following HPLC has been increasingly popular for the quantitation of electroactive analytes. Its principal advantages include high sensitivity and inherent specificity toward compounds undergoing redox reaction at the applied detector potential. In view of the redox behavior of warfarin, the following study was initiated for developing a highly sensitive and simple HPLC–ED procedure for trace measurements of this drug.

EXPERIMENTAL

Apparatus

The HPLC system (Bioanalytical Systems LC-303, West Lafayette, IN, U.S.A.) consisted of a dual-piston pump (PM-30A), a Rheodyne Model 7125 injector (20-μl loop), a Biophase ODS 5-μm reversed-phase column (25 cm$\times$4.6 mm, Bioanalytical Systems, Model MF6017), and an amperometric detector (Model

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LC-3) equipped with a thin-layer glassy carbon transducer. The reference electrode was an Ag/AgCl (Model RE-1, Bioanalytical Systems). A Bioanalytical Systems 3-cm Biophase 5-μm ODS guard cartridge was inserted between the injector and the analytical column. The system was operated at ambient temperature. Cyclic voltammetry was performed in a 10-ml voltammetric cell using a Princeton Applied Research Model 264A (Princeton, NJ, U.S.A.) voltammetric analyzer.

Materials and procedure

All aqueous solutions were prepared in double-distilled water. Stock solutions of warfarin (Sigma, St. Louis, MO, U.S.A.) were made up fresh each day by dissolution in ethanol. The mobile phase was 1.5% acetic acid (pH 4.7 with concentrated ammonium hydroxide)–acetonitrile (60:40), final pH 6.3. The solvents used in the preparation of the mobile phase were HPLC grade. The urine samples were obtained from healthy volunteers, filtered by passing through a 10–15 μm glass filter, and diluted (1:50) with the mobile phase solution. A pH 5.2 mobile phase was used in the urine assays. Amperometric detection was proceeded by applying the working potential +1.05 V and allowing transient currents to decay. Cyclic voltammetry was performed by scanning the potential between +0.2 and +1.2 V at 50 mV/s.

RESULTS AND DISCUSSION

Fig. 1 shows a cyclic voltammogram for warfarin over the anodic range of a stationary glassy carbon electrode, in the mobile phase used in chromatography. The compound exhibits a defined and irreversible anodic peak (at +0.93 V vs. Ag/AgCl), attributed to the oxidation of the hydroxy moiety. Similar peaks were

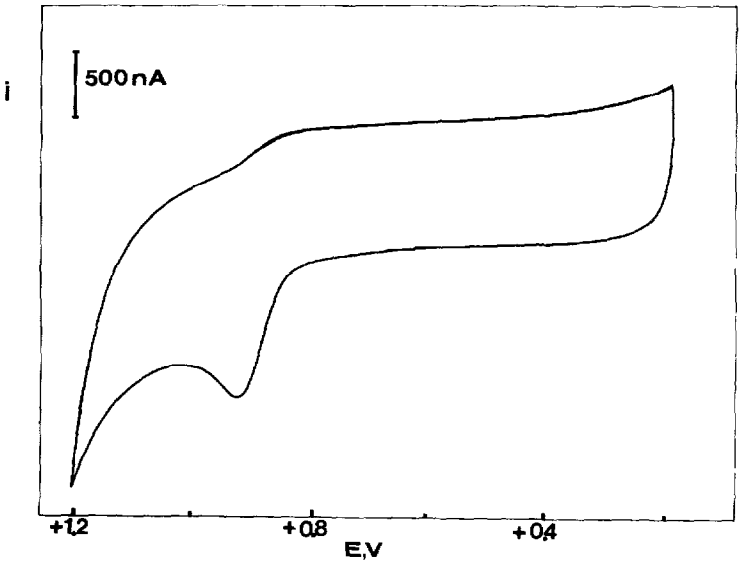

Fig. 1. Cyclic voltammogram for $1 \cdot 10^{-4}$ *M* warfarin at a glassy carbon electrode. Scan rate, 50 mV/s; electrolyte buffer: 1.5% acetic acid–acetonitrile (60:40), pH 6.3.

observed upon repetitive scans (not shown), indicating no interaction with the electrode surface. This anodic behavior of warfarin has been exploited recently in our laboratory for sensitive differential pulse voltammetric measurements of micromolar levels of the drug [8]. While warfarin also undergoes reduction at high cathodic potentials [9], oxidative HPLC–ED is preferred over the reductive one, because difficulties associated with deaeration of the mobile phase (and sample) and large noise levels are eliminated.

On the basis of the voltammetric information described above for warfarin, this compound represents a suitable candidate for HPLC–ED. To determine the optimum potential for amperometric detection of warfarin, a hydrodynamic voltammogram was generated by repetititve 200-ng injections of the drug, while changing the applied potential and using a flow-rate of 1.4 ml/min (Fig. 2). A well defined wave is observed, in agreement with the cyclic voltammetric data of Fig. 1. In order to reach the limiting current plateau, a high potential ($> +1.05$ V) must be applied. An applied potential of $+1.05$ V was used in most subsequent work.

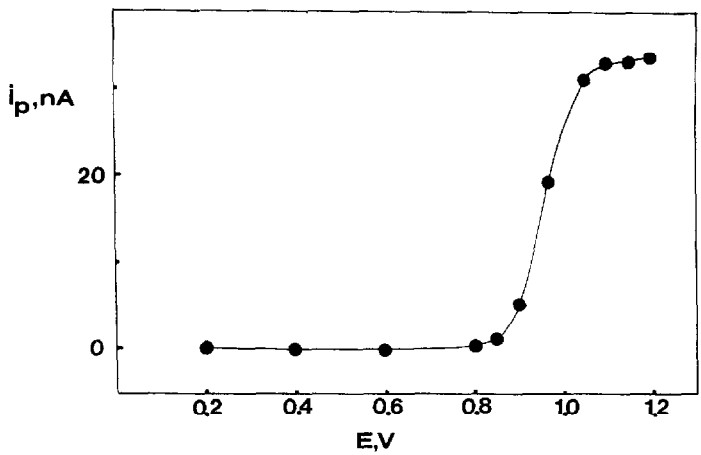

Fig. 2. Hydrodynamic voltammogram of repetitive 200-ng injections of warfarin. Flow-rate: 1.4 ml/min; mobile phase as solution described in Fig. 1.

Mobile phase pH plays a significant role in terms of both the amperometric response and the chromatography for warfarin (Fig. 3). Changes of the pH from 3.5 to 5.8 result in relatively similar retention times (ca. 12–14 min); a sharp decrease in retention time, down to 4 min, is observed by increasing the pH from 5.8 to 7.0. A gradual increase in peak current is observed by increasing the pH over the 3.5–5.8 range, with a sharper increase at higher pH values. A mobile phase pH of 6.3 was used in most subsequent work.

Fig. 4 illustrates the sensitivity and linearity obtained employing HPLC–ED. The five chromatographic peaks are part of a series of seven successive injections of warfarin solutions of increasing amount (10–70 ng). The peak response increased linearly with increasing amounts of the drug; the slope of the resulting calibration plot (also shown) corresponded to a sensitivity of 0.16 nA/ng (correlation coefficient, 0.999; intercept, 0.10 nA). The detectability is illustrated in Fig. 5 that shows a chromatogram for an injection of 2.5 ng warfarin (flow-rate,

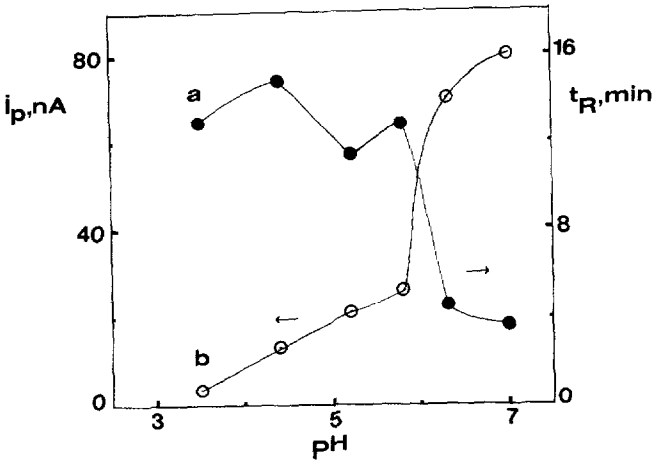

Fig. 3. Dependences of the retention time (a) and peak current (b) on the pH of the mobile phase. Injections of 200 ng warfarin; operating potential, +1.2 V; flow-rate and mobile phase as in Fig. 1.

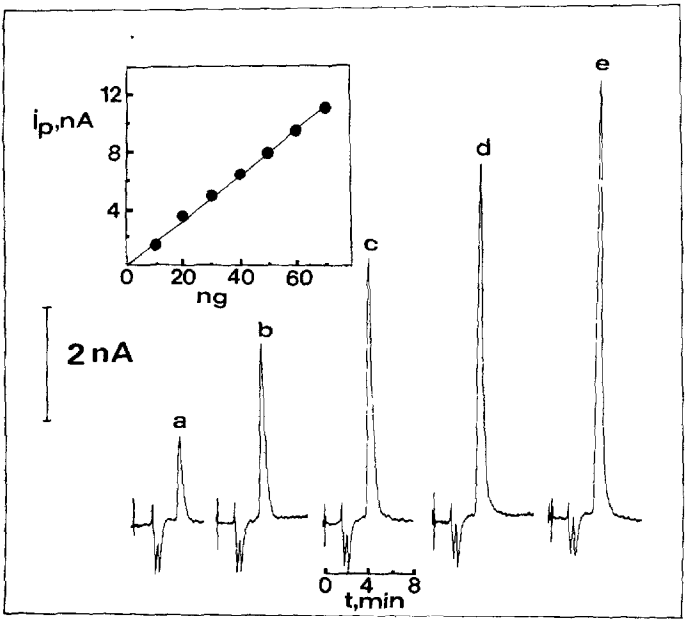

Fig. 4. Chromatograms for five injections (a–e) of increasing increments of 10–70 ng warfarin. Operating potential, +1.05 V; other conditions as in Fig. 2. Inset: calibration plot over the 10–70 ng range.

1.4 ml/min). Based on a signal-to-noise ratio of 3, these data correspond to a detection limit of 0.8 ng (20-μl injection). These data indicate the suitability of the method for trace measurements of warfarin. The precision of the results was estimated by six repeated injections of a 40-ng warfarin solution (conditions as in Fig. 2). The mean peak response found was 5.38 nA with a range of 5.28–5.52 nA and a relative standard deviation of 1.6%. The reproducible data indicate the absence of electrode poisoning (due to adsorption of warfarin).

To test the specificity of the method, a range of electroactive compounds that

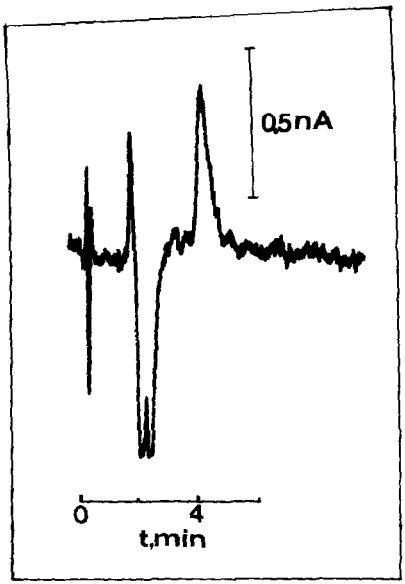

Fig. 5. Chromatogram for an injection of 2.5 ng warfarn. Operating potential, +1.05 V; other conditions as in Fig. 2.

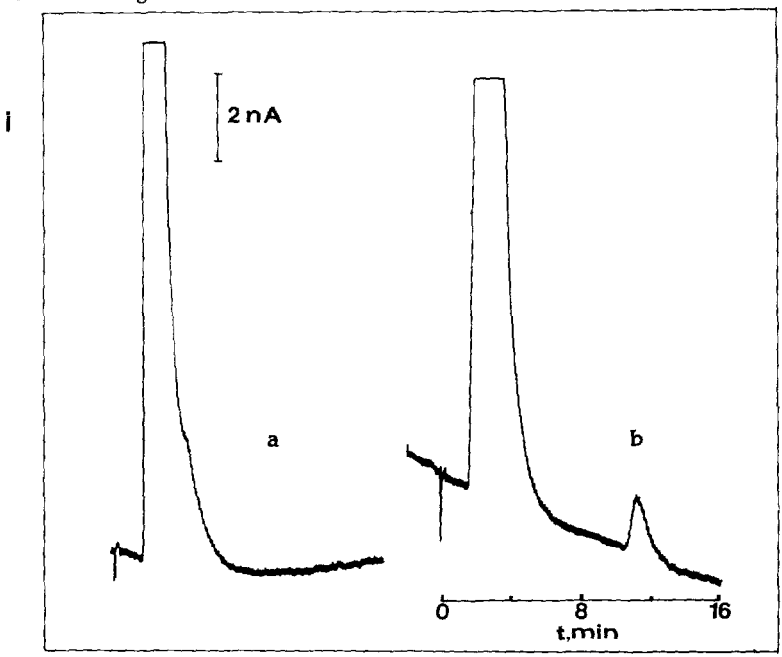

Fig. 6. Chromatograms obtained before (a) and after (b) spiking a urine sample with warfarin at 60 μg/ml and diluting 1:50 with the mobile phase (pH 5.2). Operating potential, +1.05 V; other conditions as in Fig. 2.

may be present in clinical samples (endogenous constituents and co-administered drugs) was screened. As a result of differences in the retention and/or redox behaviors the 40-ng warfarin peak was not affected by the presence of 40 ng ascor-

bic acid, uric acid, propranolol, perhexilene, metoprolol, desipramine or procainamide (conditions as in Fig. 2).

Fig. 6 illustrates the suitability of the present method for direct measurements of warfarin in body fluids. A urine sample, spiked with warfarin at the 60 μg/ml level and diluted 1:50 (24 ng injected), was used without any preliminary treatment, to yield the chromatogram shown in Fig. 6b. The warfarin peak (retention time 11 min) is not affected by other endogenous electroactive constituents that are eluted earlier. The absence of interferences is attributed to the selectivity of the electrochemical detection. A change of mobile phase pH to 5.2 was required to achieve the desired resolution. Doubling of the peak size was observed upon injecting a diluted urine sample containing 48 ng warfarin (not shown). Urine samples collected from different volunteers yielded response characteristics similar to those shown in Fig. 6. A similar (or slightly lower) mobile phase pH allows effective resolution of warfarin from its known, hydroxylated metabolites that elute substantially earlier [1]. These metabolites are expected to yield a defined amperometric response (attributed to the oxidation of their hydroxyl group), and thus to be measured simultaneously with the parent compound. Samples containing lower levels of warfarin or other body fluids (e.g. plasma) should be assayed following a commonly used clean-up procedure, extraction with diethyl ether [7], instead of the dilution step. To overcome variation in recovery, an internal standard would be needed. Direct assays are expected for analysis of pharmaceutical dosage forms. Overall, ED is shown to provide a highly sensitive, selective and simple approach for monitoring low levels of warfarin in chromatographic effluents.

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