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Determination of total and unbound warfarin and warfarin alcohols in human plasma by high performance liquid chromatography with fluorescence detection

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ABSTRACT

Two analytical procedures are presented for the determination of the total content and unbound fraction of both warfarin and warfarin alcohols in human plasma. Chromatographic separation was carried out in isocratic conditions at 25 °C on a C-18 reversed-phase column with a mobile phase consisting of a 70% buffer phosphate 25 mM at pH = 7, 25% methanol and 5% acetonitrile at a flow rate of 1.2 mL/min. Fluorescence detection was performed at 390 nm (excitation wavelength 310 nm). Neither method showed any detectable interference or matrix effect. Inter-day recovery of the total warfarin and warfarin alcohols at a concentration level of 1000 ng/mL was 89 ± 3% and 73 ± 3%, respectively, whereas for their unbound fraction (at a concentration level of 10 ng/mL) was 66 ± 8% and 90 ± 7%, respectively. The intra- and inter-day precision (assessed as relative standard deviation) was <10% for both methods. The limits of detection were 0.4 and 0.2 ng/mL for warfarin and warfarin alcohols, respectively. The methods were successfully applied to a pooled plasma sample obtained from 69 patients undergoing warfarin therapy.

1. Introduction

Warfarin [3-(α-acetonylbenzyl)-4-hydroxycoumarin, WAR], the most common anticoagulant drug, is prescribed for the treatment of many diseases and the prevention of thromboembolic events [1]. WAR is a weakly acidic drug (pK_a of 5.19 at $T = 25\,^{\circ}C$) with an enolic group (Fig. 1a) [2]. It has an asymmetric carbon center and is commercially available as a racemic mixture. The two enantiomers (R-WAR and S-WAR) have a different anticoagulant potency (S-WAR is 3–4 times more potent than R-WAR), metabolism and interaction with other drugs [3,4]. WAR is completely absorbed after oral administration ($F = 1$), is highly bound to site I of albumin (≈99%) with a high affinity ($K_d = 3.4 \pm 0.7\,\mu M$), has a small volume of distribution (0.11–0.18 L/kg), and low clearance (0.0005–0.0079 L/h/kg) [5–8]. Only the unbound fraction can carry out a therapeutic action and cross the biological membranes (e.g. salivary glands [9]). The drug is metabolized in the liver by cytochrome (CYP) P450 to inactive hydroxylated metabolites (OH-WAR) (major pathway) and by ketone reductases to warfarin alcohols (WAROHs, Fig. 1b) [10]. This reduction is more effectively catalyzed by nicotinamide adenine dinucleotide phosphate (NADPH) than by nicotinamide adenine dinucleotide (NADH [11]). This latter reduction generates a second chiral center (C-11) and makes two pairs of diastereoisomers possible, namely RS/SR-Warfarin alcohols and RR/SS-Warfarin alcohols [12,13]. After the administration of a single dose of WAR, S-7-hydroxywarfarin and RS-Warfarin alcohol are the two major metabolites observed in human plasma [14]. Gebauer reported that 7-OH-WAR is an inactive metabolite with a half maximal inhibitor concentration (IC_{50}) of 250 μM, whereas WAROHs show a low anticoagulant activity with IC_{50} of 12.5 μM (IC_{50} of WAR is 2.2 μM) [15].

Around 40 million patients are currently undergoing WAR therapy and this number has been increasing steadily [16]. Positive clinical outcomes during WAR therapy depend on maintaining the level of WAR within a narrow therapeutic range. This is challenging due to the large inter- and intra-individual variability in patient responses, as many factors (e.g. diet, liver disease, comorbidities, other drugs, and genetic variability) may interact with the therapy [17]. Furthermore, after about 60 years of clinical use, the anticoagulation mechanism of WAR has never been completely clarified and the possible role of WAROHs has received little attention in the literature. A few studies indicate that the anticoagulant activity of WAROHs may be an additional variable involved in

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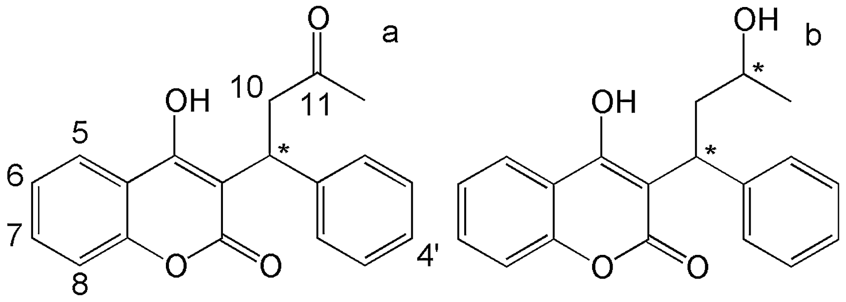

Fig. 1. Structures of WAR (a) and WAROHs (b) (* asymmetric center). Carbon 4′, 10, 6, 7, 8 are the hydroxylation sites of WAR by cytochrome P450.

the anticoagulant mechanism of WAR [13]. However, pharmacological data concerning these metabolites are scarce. Lewis et al. reported that the plasmatic concentrations of WAROHs after a single dose (100 mg) were steady for 20 days, and the long half-life may strengthen its potency and contribution to the overall anticoagulant activity. Unlike WAR, WAROHs are directly eliminated by renal excretion without any further transformation [13].

The international normalized ratio (INR) is the primary assay used in monitoring WAR therapy [18]. When a patient is started on WAR therapy, INR monitoring should be performed on a daily basis until the INR is within the therapeutic range and should be performed two or three times a week for one to two weeks [19]. Therapeutic monitoring is essential in some situations to assess anticoagulant affecting factors, especially when the INR is difficult to target. Knowledge of the plasma concentration of WAR and WAROHs is valuable for clinical decisions and enables severe intoxication due to interference with other drugs and food to be treated effectively [18].

Sensitive methods to measure WAR concentrations and its hydroxylated metabolites in biological samples have been proposed [19–21], but to our knowledge no study has been published on analytical methods for the determination of the unbound fraction of WAROHs in human plasma and other biological fluids. Most of the recently reported methods for measuring WAR in biological samples use high performance liquid chromatography (HPLC) with mass spectrometric detection (MS) [22,23]. These methods have a high sensitivity, but are too expensive for routine analyses in a clinical laboratory.

WAR has the advantage of a native fluorescence if excited at 310 nm, which can be successfully exploited for detection at low concentrations [24].

This work illustrates an HPLC method with fluorescence detection (FLD) to determine the total content and unbound fraction of WAR and WAROHs in human plasma. These methods are not expensive, show a good recovery and sensitivity, and are thus suitable for use in pharmacokinetic studies and for therapy monitoring. The proposed methods are currently being used in a clinical trial, whose results will be published in a future paper.

2. Materials and methods

2.1. Chemicals and reagents

Racemic WAR, i.e. 3-(α-acetonylbenzyl)-4-hydroxycoumarin sodium salt (purity ≥98.0%), sodium borohydride (purity ≥98.0%), sulfuric acid (purity ≥99.5%), ethanol (purity ≥99.5%) and deuterated methanol were purchased from Sigma Aldrich (Milan, Italy). Sodium phosphate monobasic (purity ≥99.0%), potassium phosphate dibasic (purity ≥99.0%), dichloromethane (purity ≥99.5%), hexane (purity ≥95.0%), formic acid (purity ≥99.5%), acetonitrile and methanol were purchased from Sigma Aldrich at HPLC grade. HPLC grade water was produced by a Milli-Q Reagent Water System (Millipore, USA). The thromboplastin reagent (HemosIL RecombiPlasTin 2G), thromboplastin diluent (HemosIL RecombiPlasTin 2G Diluent), calibration plasma samples, normal control assay, low and high abnormal control assays used both for INR measurements and quality control were supplied by the Instrumentation Laboratory (Milan, Italy).

2.2. Synthesis of warfarin alcohols

WAROHs were obtained in our laboratory by reduction of a racemic WAR standard solution with sodium borohydride [25]. WAR (a): ^{1}H NMR (CD$_3$OD, δ/ppm): 7.98–7.94 (1H, m), 7.67–7.61 (1H, m), 7.41–7.18 (7H, m), 4.12 (1H, dd, J = 0.7 Hz, CH at C-9), 2.22 (2H, ddd, J = 30.0, 14.0 and 6.9 Hz, CH$_2$ at C-10), 1.74 (3H, s). ^{13}C NMR (CD$_3$OD, δ/ppm): 207.1 (1C, carbonyl at C-11), 163.0, 159.9, 152.7, 143.7, 131.7, 128.1, 127.8, 127.1, 126.8, 125.9, 125.7, 124.0, 123.9, 122.7, 99.5, 42.8, 26.4, 24.6. ESI-QTOF-MS: m/z 309.1 [M+H]$^+$.

WAROHs (b): ^{1}H NMR (CD$_3$OD, δ/ppm): 7.99–7.95 (1H, m), 7.60–7.50 (1H, m), 7.50–7.14 (7H, m), 4.70–4.63 (1H, m, CH at C-11), 3.71–3.68 (1H, m, CH at C-9), 2.37 (2H, m, CH$_2$ at C-10), 1.27–1.19 (3H, m). ^{13}C NMR (CD$_3$OD, δ/ppm): 161.2, 159.9, 152.7, 142.9, 131.6, 127.9, 127.6, 127.5, 125.8, 125.6, 125.2, 123.7, 122.9, 122.6, 116.0, 65.8 (1C, hydroxyl at C-11), 40.2, 22.8, 22.4. ESI-QTOF-MS: m/z 311.1 [M+H]$^+$.

Carbonyl reduction at C-11 generates a second chiral carbon, which makes four stereoisomers (RR, RS, SR, and SS) possible. These can be seen as two pairs of diastereoisomers (RR-SS and RS-SR), with a different chromatographic behavior. Fig. 1 shows the structures of WAR (1a) and WAROHs (1b).

The HPLC separation of the reduced solution revealed that the reaction with sodium borohydride was completed and WAROHs were generated. The product of sodium borohydride reduction was confirmed by comparing the ^{1}H NMR and ^{13}C NMR spectra of WAR and WAROHs.

2.3. Plasma samples

Plasma samples were obtained from the local oral anticoagulant center with the permission of the Ethics Committee of the local hospital (AOUP, Pisa, Italy). Human whole blood samples were collected into vacuum tubes containing 109 mM (3.2%) sodium citrate (Vacutest Kima, Italy) and immediately centrifuged at 3000 rpm for 10 min to obtain platelet-poor plasma. The plasma sample was divided into two aliquots: the first was immediately used for the determination of INR, whereas the second was stored at −80 °C together with the nominally healthy volunteer samples until the WAR and WAROHs were measured.

2.4. Preparation of calibration standards and quality control samples

A phosphate buffer solution (PBS) (1 M, pH = 7.0) was prepared by dissolving 18.30 g of sodium phosphate monobasic and 49.20 g