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Monitoring of warfarin therapy: Preliminary results from a longitudinal pilot study

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ABSTRACT

The aim of this study was to investigate the relationship between warfarin dosage, international normalized ratio, plasma and oral fluid concentrations of warfarin, and its metabolites, namely RR/SS- and RS/SR-warfarin alcohols. Nine patients on long-term warfarin therapy (4 with stable and 5 with unstable international normalized ratio values) were longitudinally monitored for over two months by recording warfarin dosage and measuring international normalized ratio, warfarin and warfarin alcohols concentrations in oral fluid and in plasma.
At equivalent dose (20–30 mg week^{-1}), the international normalized ratio was in the range 2.0–2.5 and 1.5–3.1 for stable and unstable patients, respectively. Moreover, stable patients showed nearly double total and unbound plasma warfarin concentration, and nearly triple oral fluid warfarin concentrations compared to unstable patients. Correlations between warfarin dosage and total plasma concentration of warfarin ($r = 0.65$, $p < 0.01$) or RS/SR-warfarin alcohols ($r = 0.66$, $p < 0.01$), as well as between stimulated oral fluid and total plasma concentrations of warfarin ($r = 0.72$, $p < 0.01$) and RS/SR-warfarin alcohols ($r = 0.95$, $p < 0.01$) suggest that the relative changes of the oral fluid concentrations of these species may provide clinically useful information for monitoring individual patients. Follow-up data revealed that even in the absence of changes of warfarin dose, the oscillations of plasma and oral fluid of WAR and RS/SR-warfarin alcohols parallel oscillations of international normalized ratio. Due to the long delay of its biological action, monitoring the plasma concentration of warfarin might help to predict variations of international normalized ratio and prevent the risk of thrombotic or haemorrhagic events. The information collected suggests that non-invasive monitoring of warfarin in oral fluid might represent a suitable tool for this purpose.

1. Introduction

Vitamin K is necessary to convert inactive precursors of vitamin K-dependent coagulation factors to active zymogens. The vitamin K antagonists, such as warfarin (WAR), are the most widely diffused oral anticoagulant drugs [1]. Warfarin is thought to interfere with clotting factors by inhibiting the *C*1 subunit of the vitamin K epoxide reductases (VKORC1) enzyme complex, thereby reducing the regeneration of vitamin K1 epoxide. In blood, WAR is highly bound to albumin (≈99%) with a high affinity ($K_d = 3.4 \pm 0.7$ μM). It is mainly metabolized in the liver by cytochrome (CYP) P450 to inactive hydroxylated metabolites (OH-WAR) and by ketone reductases to warfarin alcohols (RR/SS- and RS/SR-warfarin alcohols) [2]. These latter metabolites show a limited anticoagulant activity, with a half maximal inhibitor concentration (IC_{50}) of 12.5 mM, six-times lower than WAR [3], but their possible role in the anticoagulation process has received little attention up to now.

Despite its effectiveness, the WAR treatment has several shortcomings related to many factors that may interfere with the anticoagulant therapy by impairing metabolic pathways or increasing the pharmacologically active fraction due to the displacement of WAR from serum albumin [4–8]. The large inter- and intra-individual variability in patients' responses makes WAR therapy difficult to control, and this increases the risk of bleeding or thrombosis [9]. Such events occur in approximately 12% of patients, with a higher probability during the first two weeks of therapy than during maintenance [9]. A complex and expensive service has been set-up in most countries to monitor WAR effects by measuring the prothrombin time (PT) in blood expressed in terms of international normalized ratio (INR) and limit risks for the patients [10].

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Warfarin shortcomings have prompted the development of direct oral anticoagulants (DOACs), which target key coagulation factors (e.g. factors Xa and IIa) instead of vitamin K [11]. Compared to WAR, DOACs have a more rapid onset of action (time to peak concentration: 2–4 h instead of 72–96 h) and shorter half-lives (5–17 h instead of 40 h). Moreover, they are administered at fixed doses, and do not require a routine coagulation monitoring [12–15]. Nevertheless, the new anticoagulant drugs also have drawbacks: a) they are more expensive compared to the traditional vitamin K antagonists, b) patients quickly lose the anticoagulant effect and are unprotected from thrombosis if a single DOACs dose is missed, c) specific antidote (i.e. Idarucizumab) is currently available only for Dabigatran and d) DOACs are unsuitable for treating patients with mechanical heart valves.

For these reasons, WAR is not going to disappear from the scene. Several pilot studies have been conducted to verify if the safety and effectiveness of the WAR therapy may be improved by the knowledge of the plasma concentrations of WAR [16–19]. All these cross-sectional studies failed to find a significant correlation between the measured plasma concentration of WAR and INR. Such result is not surprising in view of the large inter-individual variability in the dose-effect ratio [4–8]. However, our group hypothesized that a correlation between unbound plasma WAR and INR might be found based on measurements of WAR concentrations in oral fluid (OF) [20].

The perspective of a minimally invasive monitoring of WAR therapy is particularly appealing because large meta-analysis studies have shown that DOACs are less safe than WAR in terms of haemorrhagic or thrombotic incidents [21–23]. As a consequence, vitamin K inhibitors will continue to be used in future due to the limited indications of DOACs and inherent restrictions such as poor renal function, and for this reason any improvement of WAR therapy would reduce risks and costs of the anticoagulant therapy in a large number of patients.

Based on this background, we performed a longitudinal pilot study enrolling nine patients undergoing a long-term WAR therapy. Our purpose was to get preliminary experimental evidences about the relationship between WAR dosage, INR, plasma and OF concentration of WAR, RR/SS- and RS/SR-warfarin alcohols.

2. Materials and methods

2.1. Statement of ethics and data collection

Nine patients (5 males, 4 females) on long-term WAR therapy were monitored for over two months. During this period the drug has been regularly taken. Clinical variables such as age, sex, INR target range and duration of WAR therapy were collected at the enrolment by a patient interview. Possible patient-specific factors (e.g. new drugs administered) affecting the anticoagulant effect of WAR were also recorded at each sampling time.

A patient was considered stable when his INR values had remained within the target range (2.0–3.0) over (at least) three consecutive months before the first medical examination.

All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The pilot study was conducted at the Azienda Ospedaliero-Universitaria Pisana (Pisa, Italy) upon approval by the Ethical Committee of the Pisa Hospital. Informed consent was obtained from all individual participants included in the study.

2.2. Blood sample collection

Two venous blood samples were consecutively collected into vacuum tubes (Vacutest Kima, Padua, Italy) containing 109 mM (3.2%) sodium citrate at least 12 h after the administration of the last WAR dose. Blood samples were immediately centrifuged at room temperature for 10 min at 3000 rpm to obtain platelet-poor plasma. Plasma samples were divided in two aliquots and stored in polypropylene tubes at −80 °C until use. An aliquot of sample was used to measure the INR value, whereas the other was used to measure concentrations (both total and unbound fraction) of WAR, RR/SS- and RS/SR-warfarin alcohols.

2.3. Oral fluid sample collection

After blood collection, stimulated OF samples were collected in a quiet room between 7 and 10 AM by asking the patient to roll a Salivette polyester swab (Sarstedt, Nümbrecht, Germany) in mouth for 2 min. Patients were asked not to take any food or beverages within 1 h prior to OF collection. After sampling, OF pH was measured by two independent observers using a Pehanon narrow range ($6.0 < pH < 8.1$) pH paper strips (Macherey Nagel, Düren, Germany) with a resolution of 0.3 pH units. The OF flow rate (grams per minute) was calculated from the ratio between the weight difference of the sampling device before and after sampling and the collection time, considering the density of OF equal of 1 g mL^{-1}. The sampling procedure and pH measurements were always completed in <3 min. The OF was recovered by centrifugation of the swabs at 3000 rpm for 5 min at room temperature and then stored in a polypropylene tube at −80 °C until use.

2.4. Determination of international normalized ratio

Spectrophotometric INR measurements were carried out at a wavelength of 671 nm by an ACL TOP700 automatic system (Instrumentation Laboratory, Oragenburg, USA) equipped with an auto-sampler. The quality control procedure for INR measurements consisted in analysing three reference samples (normal, low and high INR levels) provided by the Instrumentation Laboratory at least once every 8 h. A RSD <1% for measurements performed on the same day ($n = 6$) and <3% for measurements performed on different days ($n = 6$) demonstrated the good inter- and intra-day precision of the method.

2.5. Determination of warfarin and warfarin alcohols in oral fluid and plasma

The concentration of warfarin and its metabolites (i.e., RR/SS- and RS/SR-warfarin alcohols) in OF and plasma samples were determined by a high-performance liquid chromatography (HPLC) (Jasco Europe, Lecco, Italy) equipped with an autosampler (AS 2055), a quaternary low-pressure gradient pump (PU 2089) and a fluorescence detector (FP 2020). The full details of the analytical procedures are reported elsewhere [24]. Briefly, an aliquot of OF (1 mL) or plasma (0.5 mL) was acidified with H_2SO_4 (2 mL, 0.5 M) and extracted with 4 mL of dichloromethane/hexane mixture (1:5, v/v). The resulting mixture was vortex-mixed for 30 s and then centrifuged at 5000 rpm for 5 min at room temperature. The supernatant was recovered, evaporated under nitrogen and reconstituted in 0.25 and 1 mL of phosphate buffer solution (PBS) 25 mM at pH = 7, for OF and plasma, respectively. An aliquot (25 μL) of the reconstituted sample was finally injected into the HPLC system. Warfarin, RR/SS- and RS/SR-warfarin alcohols were separated with a Poroshell EC-C-18 reversed-phase column (100 × 4.6 mm, 2.7 μm) (Agilent, Santa Clara, USA) connected to a TC-C-18 guard column (12.5 × 4.6 mm, 5 μm) (Agilent, Santa Clara, USA) using isocratic elution with 30% methanol and 70% phosphate buffer 25 mM at pH = 7 at a flow rate of 0.7 mL/min. Fluorescence detection was performed at excitation and emission wavelengths of 310 and 390 nm, respectively. The HPLC run time was 25 min.

Calibration curves, evaluated by the Deming regression analysis, in the range 300–970 nM and 3–97 nM were used for the quantification of WAR and its metabolite, in plasma and OF, respectively. The absence of a matrix effect was evaluated by comparing the slope of the calibration curves prepared in PBS and pooled OF and plasma samples. The

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