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Pharmacokinetic Analysis of Warfarin in Iranian Warfarin Sensitive Patients

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Abstract: In this study, we investigated pharmacokinetic of warfarin in sensitive and control patients by HPLC. The analysis performed by HPLC consisted of a column (Perfectsil Target, 5 μ m, 125 \times 4.0 mm) and an isocratic mobile phase of methanol: acetonitrile: phosphate buffer (pH: 3.5) (7:55:38, v/v), flow rate: 1 mL min⁻¹ and UV detection at 270 nm. The assay was linear in warfarin concentration ranges of 0.1-10 μ g mL⁻¹ ($r^2 = 0.9975$) and with Relative Standard Deviation (RSD) of <8% for inter-day and <6% for intra-day. The mean warfarin blood concentration of 54 patients was 1.2 \pm 0.6 μ g mL⁻¹.

Key words: HPLC, warfarin, pharmacokinetics, warfarin sensitivity

INTRODUCTION

Individuals vary widely in their response to pharmacologic agents and such variability can have profound health effects. Little is known about drug-specific genetic differences, which lead to differential effects of commonly prescribed agents. Warfarin is the most commonly prescribed oral anticoagulant drug for the prophylaxis and treatment of venous and arterial thromboembolic disorders and is being prescribed to an increasing number of patients (Hirsh *et al.*, 1998, 2001). Following initiation of warfarin therapy, major bleeding episodes occur in approximately 12% of patients and death results in as many as 2% of patients (Levine *et al.*, 2001). Overall, the bleeding rate is 7.6 to 16.5 per hundred patient-years. Major or life-threatening bleedings occur at a rate of 1.3 to 2.7 per hundred patient-years. Although major bleeding can occur at therapeutic levels, the risk of bleeding rises with increasing intensity of anticoagulation (Van der Meer *et al.*, 1993; Cannegieter *et al.*, 1995). The effectiveness and safety of warfarin therapy is critically dependent on maintaining the prothrombin and time expressed as the International Normalized Ratio (INR), which is a ratio of the time required for the patient's blood to coagulate relative to a standardized coagulation time, within the desired therapeutic range (Sconce *et al.*, 2005). Identification of risk factors for the development of a high INR may identify patients at high risk of bleeding because the risk of bleeding increases with increasing intensity of anticoagulation (Taube *et al.*, 2000). The target INR level

will vary from case to case dependent upon the clinical indicators, but tends to be two-three in most conditions (Ansell *et al.*, 2001).

Thus, Warfarin treatment is problematic because the dose requirement for warfarin is highly variable, both inter-individually and inter-ethnically. Asian populations require a much lower maintenance dose than Caucasians and Hispanics (Takahashi *et al.*, 2003; Zhao *et al.*, 2004), for which the mechanisms remain elusive. The result of this variability is that some patients require less warfarin dose than others, warfarin sensitive patients and some of them need more warfarin dose, warfarin resistant patients. Variability in warfarin-dose requirement can be influenced by many factors such as pharmacokinetics factors (due to differences in absorption or metabolic clearance of warfarin induced by drug interaction or patient base situation such as gender), pharmacodynamic factors (due to differences in the homeostatic response to given concentrations of warfarin) (Modi *et al.*, 2005; Depr'e *et al.*, 2005) and pharmacogenetics factors (due to mutations in genes such as *CYP2C9*, *VKORC1*, etc).

In this study, after optimizing the chromatographic conditions, a rapid and simple HPLC method was developed for the determination of blood warfarin. A single precipitation method with cold acetonitrile was sufficient to eliminate interferences. The total HPLC run-time was relatively short (5 min). Warfarin plasma concentration was determined in 54 patients on warfarin therapy by the method described and the impact of warfarin concentration was investigated.

MATERIALS AND METHODS

Materials: Sodium warfarin (99.9%) was kindly provided by Pharmaceutical Division of Tehran University/Medical Sciences. HPLC grade methanol (PAI Panreac), acetonitrile and potassium dihydrogen phosphate (Merck, Darmstadt, Germany 2007).

Patients' population: Fifty-four treated patients with warfarin were recruited from Imam Hospital (Tehran-Iran) for participation in this trial. The study group clinically sensitive patients (19 patients) were selected and referred by participating centers according to the following criteria:

- Adult-patients who need less than 10.5 mg/week of warfarin to keep the INR in therapeutic range (INR = two-three) (Linder, 2001).
- Patients had been used usual dosage of warfarin (5-15 mg day⁻¹) but they had INR>4 even in seven days after cessation of warfarin or three days after receiving 3 mg day⁻¹ vitamin K1 (the criterion in Imam Hospital).

The control group (35 adult patients) was selected randomly from patients taking warfarin for various cases and showed normal response to warfarin.

Characteristics recorded included age, gender, weight, height, etc. To qualify for participation in the study, clotting factors FIX, FVI, FVII, FV, FII measured in order to assurance from correct function liver and clotting factors were performed on all patients. Among the 54 investigated patients, 25 patients were male, 29 patients were female and some of them with the symptom of arterial fibrillation or history of embolism, cardiac disease, replacement heart valves, etc.

Patient blood samples were collected into tubes containing EDTA. The samples were kept at -70°C unless they were analyzed in the same day.

Standard preparation: Stock solution of warfarin was prepared at 1 mg mL⁻¹ in methanol. Working solution was diluted from the stock solution to 100 µg mL⁻¹ with distilled water. The concentrations for the standard curve were freshly prepared before the analysis. For the standard curve, blood samples were spiked with warfarin working solution to make final concentrations of 0.1-10 µg mL⁻¹.

Sample preparation and extraction: One thousand microliters of cold acetonitrile (20 min in -20°C) was added

to 500 µL spiked or patient blood. The samples were rigorously vortexed in 5 min and centrifuged at 8000 g for 10 min to separate the precipitant. The upper phase was transferred into 1.5 mL tubes and centrifuged another time with the above conditions. Then 100 µL distilled water was added to 200 µL from supernatant and 50 µL of it was injected.

Chromatography: The chromatographic system consisted of Waters 600 pump, Waters 486 UV detector, reversed phase C18 column (Perfectsil Target ODS-3, 5 µm, 125×4.0 mm) and an isocratic mobile phase of methanol: acetonitrile: phosphate buffer (pH: 3.5) (7:55:38, v/v). The flow rate was 1 mL min⁻¹. Data analysis and valley-to-valley integration was performed with Autochoro 2000 1.0 (Younglin Instrument, Korea). The absorbance was analyzed at 270 nm, sensitivity of 0.001 AUFS (Absorbance Units Full Scale) with a total run-time of 5 min.

Method validation: Blood was spiked with known amounts of warfarin in triplicates (n = three). The linearity of the method was established and was then used for analysis of patient samples. The limit of detection (LOD) was defined as the concentration corresponding to a signal to noise ratio of three: one. The limit of quantification (LOQ) was defined as 10×LOD. Recovery was calculated from blood spiked with warfarin at three levels (0.5, 1 and 5.0 µg mL⁻¹) (n = three at each level) in compare to similar aqueous spiked samples. Intra-day and inter-day assay variations were calculated from blood spiked with warfarin at three levels (0.5, 1 and 5.0 µg mL⁻¹) (n = three at each level) run in the same day and in three different days, respectively.

RESULTS AND DISCUSSION

Plasma or blood warfarin measurement is considered helpful to the management of prescription of the drug for patients. The most often used methods for plasma warfarin concentration determination so far described are based on extraction of warfarin from plasma followed by reversed-phase HPLC and the extraction is the critical step, which requires careful conditions to ensure good recovery of warfarin before sample injection. In this way, several HPLC methods with UV detectors were described for determination of warfarin concentration in plasma (Andalibi *et al.*, 1998; Lombardi *et al.*, 2003; Sun *et al.*, 2006).

In this study, we developed a HPLC method for quantification of warfarin in blood and investigated the

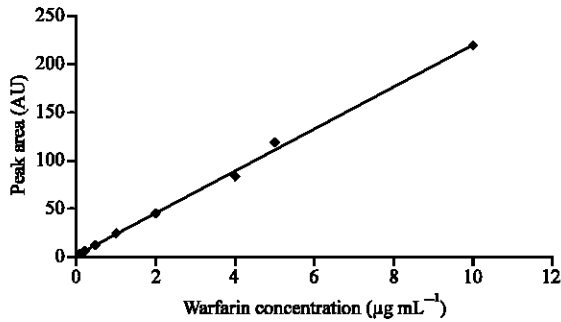


Fig. 1: Warfarin calibration curve. AU, absorbance unit. Equation describing the relationship: $Y = 21.843x + 2.0104$; $r^2 = 0.9975$

Table 1: Blood warfarin concentration in sensitive and normal patients

Concentration ($\mu\text{g mL}^{-1}$)	Sensitive	Normal	SUM (%)
<900	8	8	16 (29.6)
900-1500	5	11	16 (29.6)
1500<	6	16	22 (40.7)
SUM	19	35	54 (100.0)

blood warfarin concentration for the patients. The method has several advantages. It is sensitive, reliable, rapid, cost-efficient and easy to perform and the most important characterization is single precipitation method with cold acetonitrile was sufficient to eliminate interferences.

A typical calibration curve (peak area as absorbance unit vs. warfarin concentration) is shown in Fig. 1 with a good linearity ($r^2 = 0.9975$). Due to loop system, injection internal standard was not used. Chromatograms correspondent to the samples containing warfarin at $5 \mu\text{g mL}^{-1}$, the blank sample and a patient sample are shown in Fig. 2. The mean recovery, estimated by measuring warfarin concentration in three different assays for a drug-free blood sample pooled from healthy subjects and spiked with warfarin at the final concentration of 0.5, 1 and $5 \mu\text{g mL}^{-1}$ is 124.6%. The precision and reproducibility of the assay, which was estimated as relatively standard deviation is <8% for inter-day and <6% for intra-day at the concentration of 0.1-10 $\mu\text{g mL}^{-1}$. The limit of detection in human plasma was 20 ng mL^{-1} for warfarin. Limit of quantification defined was calculated to 200 ng mL^{-1} .

The results were determined for the 54 patients (19 sensitive and 35 normal) (Table 1). Present results show that there is variability in blood warfarin concentration in sensitive and normal patients, because sensitivity to warfarin is because of mutation in some genes like *CYP2C9* and *VKORC1* and the influence of these genes in individuals is different. Further work in our laboratory is going to determine the exact role of pharmacogenetic factors in warfarin sensitive Iranian patients in their *CYP2C9* and *VKORC1* genes.

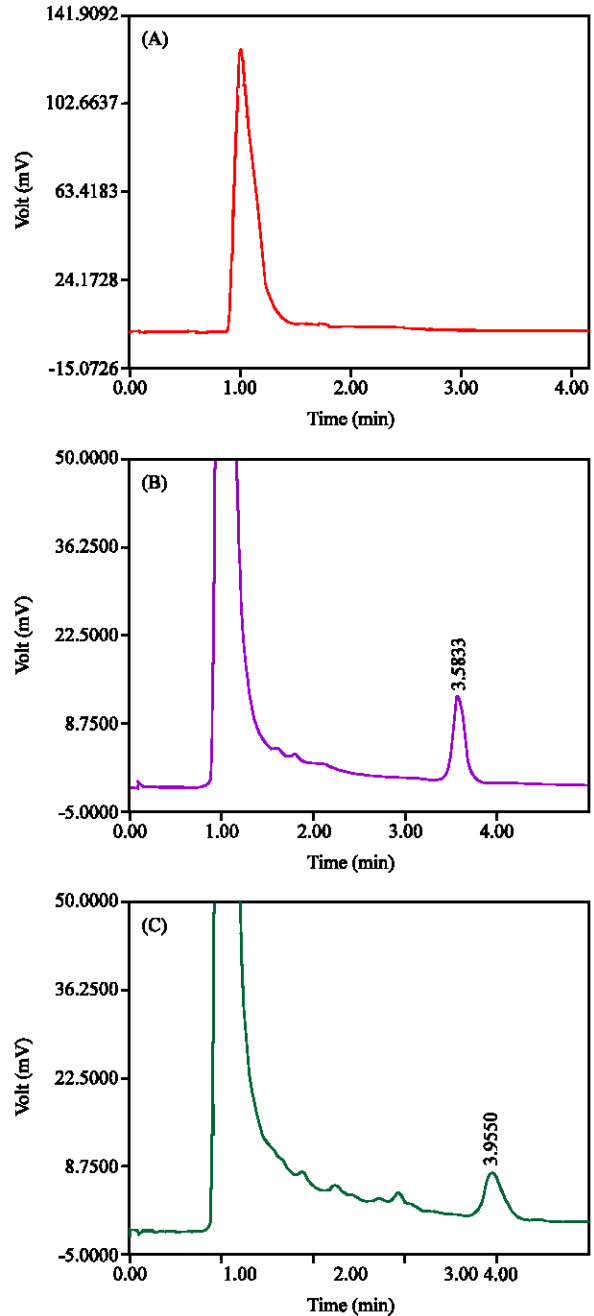


Fig. 2: Chromatograms obtained with (A) blank blood, (B) blood spiked with warfarin ($5 \mu\text{g mL}^{-1}$) (RT = 3.58), (C) patients' blood (RT = 3.95). The numbers above the peak are retention times (RT)

CONCLUSION

Present study developed a sensitive, reliable, rapid, cost-efficient and easy to perform HPLC method for assaying of warfarin in blood sample.

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