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Assaying of Warfarin in Iranian Warfarin Resistance Patients Blood by HPLC

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Abstract: A simple and rapid HPLC method with UV detecting system has been used in determination of warfarin level in plasma of Iranian patients who received different doses of this drug. Six resistance (10-70 mg day⁻¹) and 5 sensitive patients (0.5-2.5 mg day⁻¹) were selected for this study. Range of warfarin level in plasma was between 0.93 and 22.8. After determination of warfarin level in warfarin sensitive and especially, warfarin resistance patients, we are going to find a relationship between this level and pharmacokinetic or pharmacogenetic factors. In the separate study which was done in our laboratory on the gene that is possibly responsible for warfarin resistance we did not find any mutation in our patient with high warfarin concentration in their blood.

Key words: HPLC, warfarin, pharmacokinetic, pharmacodynamic, warfarin resistance

INTRODUCTION

Warfarin is one of the most widespread oral anticoagulant drug employed (Hirsh *et al.*, 2003). However, its required dose is highly variable both inter-individually and inter-ethnically (Zhao *et al.*, 2004). 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 resistance patients. This sensitivity or resistance to warfarin is due to pharmacogenetic events in individuals. Warfarin sensitivity is due to the mutation in CYP2C9 gene (Gage *et al.*, 2004). This gene is a liver enzyme required for oxidation of a large number of drugs including warfarin (Rettie *et al.*, 1992). Very recently, a novel gene responsible, at least in part, for the activity of the Vitamin K Epoxide Reductase (VKOR) complex has been identified (Rost *et al.*, 2004; Li *et al.*, 2004). This gene is VKORC1 that is the subunit of VKOR and believed that its mutation is responsible for resistance to warfarin. VKOR is one of the component of the vitamin K cycle and the therapeutic target site of warfarin.

Because of this variability in warfarin's pharmacokinetic properties, physicians have to discover the proper maintenance dose by trial-and-error. Bleeding or other diverse events is the most serious complication of warfarin (Bogousslavsky and Regli, 1985; Landefeld and Beyth, 1993; Gullov *et al.*, 1994). So, much effort has been devoted to improve the safety of this drug by predicting warfarin dose in patients before treatment. Since different factors may influence the kinetic and

pharmacodynamic of warfarin, Prothrombin Time (PT) and International Normalized Ratio (INR) is not sufficient for monitoring the oral therapy of warfarin and different kinds of analytical methods have been developed to determine concentration of this drug in biological fluids (Helford, 1986; Chan and Woo, 1988). The aim of this study is to set up a HPLC technique to determine warfarin concentration in the plasma of Iranian patients who received different doses of warfarin. In this way, we can find whether resistance to warfarin is due to pharmacogenetic factors, or pharmacokinetic of this drug in blood.

MATERIALS AND METHODS

Chemicals and reagents: Warfarin standard was purchased from Sigma (St. Louis, Mo, USA), HPLC grade methanol, acetonitrile were obtained from E. Merck (Darmstadt, Germany). Deionized water was used for preparation and dilution of all solutions.

Apparatus: The HPLC system consisted of a HPLC pump, an injector, variable wavelength UV detector with an integrator all from waters and stainless steel reverse phase (C₁₈) column (4 mm ID, particle size 5 µm) (Knauer). In this system a mixture of filtrate and degassed 10 mM phosphate buffer (pH: 3.5), methanol and acetonitrile in 53:7:40 ratio was used as a mobile phase. We also adjust the flow rate on 1 mL min⁻¹, temperature on 25°C; UV detector on 270 nm, injection volume is 20 µL and detector range on 0.001 aufs.

Table 1: Patient's characteristics: 1-5: Warfarin sensitive, 6-11: Warfarin resistant

Patients	Age (year)	Sex*	Height (cm)	Weight (kg)	PT**	INR***	mg of warfarin per day
1	66	F	160	58	15.0	2.6	0.50
2	55	M	178	79	22.0	2.8	1.25
3	65	F	158	63	26.5	3.5	1.25
4	47	M	176	75	24.0	3.0	1.80
5	33	F	164	61	22.0	2.8	2.50
6	44	F	169	73	15.0	1.3	10.00
7	24	M	185	74	15.2	1.4	10.00
8	37	F	168	80	17.2	1.7	15.00
9	51	F	162	68	14.0	1.2	15.00
10	52	F	160	69	24.0	2.1	15.00
11	55	M	169	59	24.0	2.1	100.00

*: F: Female, M: Male, **: Prothrombin Time, ***: International Normalize Ratio

Patients: Iranian patients, who were prescribed oral warfarin either at high or low dose, were recruited from Imam Hospital (Tehran, Iran). Approximately 5 mL of blood sample were collected from volunteers in a tube containing 5 mg of EDTA. Plasma PT determination and complete clinical summary of all patients were obtained by clinical staff of the hospital (Table 1).

Procedure: The analyzed sample was prepared by precipitation method. In which, to 500 µL of plasma in a tube, 1 mL of methanol was added. The mixture was vortex for about 1 min to obtain a complete homogenous mixture. The tube was tightly closed and centrifuged for 10 min at 8000 rpm. Supernatant layer was separated and centrifuged two more time until we obtained a clear solution. Twenty microliter of this solution was injected into the liquid chromatograph.

RESULTS AND DISCUSSION

High Performance Liquid Chromatography (HPLC) is one of the most popular methods for determination of concentration of coumarin type drugs like warfarin. Its facilitate analysis as well as increasing sensitivity and resolution are the most advantages of HPLC technique. In this way, several HPLC methods with UV detectors were described for determination of warfarin concentration in blood (De Veries and Volker, 1990; Lotfi *et al.*, 1996; Andalibi *et al.*, 1998). Therefore, we use this method for present study.

In this study, we examined six warfarin resistant patients, that their warfarin dose is more than 10 mg day⁻¹. We also examined five warfarin sensitive patients, as control group, that use less than 5 mg of warfarin per day. None of them use any kind of interfering drugs. Plasma sample of these individuals were prepared as described earlier.

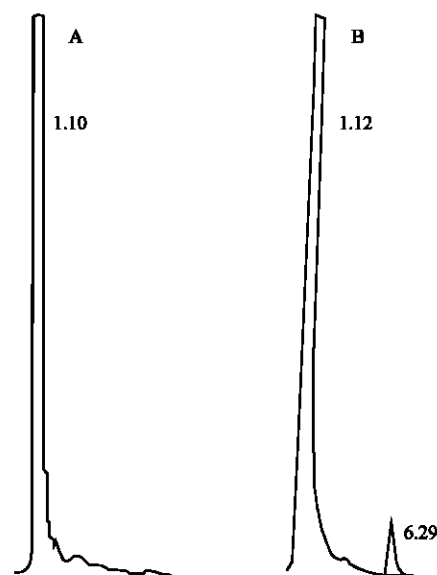


Fig. 1: Chromatograms obtained with the blank (A) and the standard at 5 µg mL⁻¹ warfarin concentration (B). The numbers above the peaks correspond to the retention time. X-axis is time (min.) and Y-axis is absorption

Chromatograph peaks of extracted blank plasma and blank Plasma spiked with warfarin were well separated (with 6.29 min retention time of warfarin) and didn't show any interference (Fig. 1). Five known warfarin concentration ranging from 0.5-25 µg L⁻¹ were analyzed. They yielded a straight calibration curve with r² = 0.9991 when peak height was plotted against the concentration of warfarin (Fig. 2). In this way warfarin concentration was determined for all of the patients and the results are shows in Table 2.

Andalibi *et al.* (1998) determined warfarin dosage requirement in Iranian patient. They found that their therapeutic level is 1193.81±300.35, i.e., they are more sensitive to warfarin than North American and European patients. Therefore, our five sensitive patients (control group), who have warfarin level between 0.8-1.18 mg L⁻¹, are in the normal therapeutic range. However, interesting results were shown in warfarin resistance patients. Three of them, which use 10-15 mg of warfarin per day, have blood warfarin level in therapeutic range too, but three other one (two of them need 15 mg and the third needs 100 mg of warfarin per day), showed blood warfarin level more than 2 mg L⁻¹.

These differences in required dose of warfarin resistance patients, suggest differences in pharmacokinetic of warfarin. In the first three patients, pharmacokinetic events are responsible for reduction of warfarin concentration in blood. Therefore, in order to

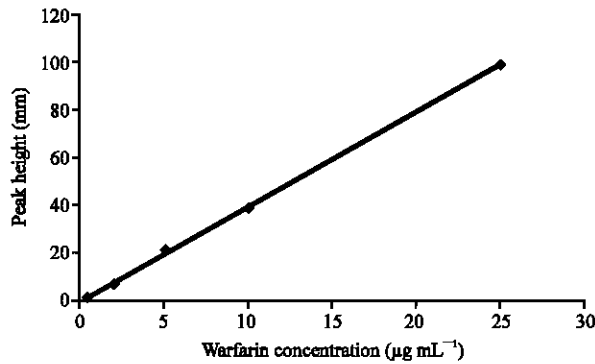


Fig. 2: Warfarin calibration curve; equation describing the relationship, $y = 3.9795x - 0.5594$; $r^2 = 0.9991$

Table 2: Blood warfarin concentration in individuals

Patients	mg of warfarin per day	Warfarin concentration in plasma
1	0.50	0.93
2	1.25	1.35
3	1.25	1.19
4	1.80	1.09
5	2.50	1.19
6	10.00	1.25
7	10.00	1.35
8	15.00	1.09
9	15.00	3.07
10	15.00	3.07
11	100.00	22.80

reach its therapeutic range, they need more warfarin dose. However, in the other three patients increasing warfarin dose, increase its concentration in blood. Therefore, they have normal warfarin metabolization and other reasons such as low receptor activity, or pharmacogenetic effects are responsible for their high required warfarin dose. The last is may be due to mutation in VCORC1 gene, which is located on the short arm of chromosome 16 and now has been identified as therapeutic target site of warfarin (Rost *et al.*, 2004; Li *et al.*, 2004).

Further work in our laboratory to determine the exact role of pharmacogenetic factors in warfarin resistance Iranian patients shows that these patients have no mutation in their VKORC1 gene. We suggest that other genes may responsible for warfarin resistance in Iranian patients.

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