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Original article

Estimation of plasma levels of warfarin and 7-hydroxy warfarin by high performance liquid chromatography in patients receiving warfarin therapy

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ABSTRACT

Warfarin is one of the most commonly prescribed oral anticoagulant for prevention of thromboembolic events. The effect of this drug is measured by monitoring prothrombin time expressed as International Normalized Ratio (INR). In some cases, however, the measurement of plasma concentration of warfarin was emphasized. In the present study, reversed phase high performance liquid chromatography (HPLC) was used to estimate the plasma drug levels. A total of 185 patients were enrolled in this study. Five milliliter of venous blood was collected using sodium EDTA tubes for pharmacokinetic analysis. Solid phase extraction was used to recover the warfarin and it's metabolite from plasma using isopropanol and potassium phosphate buffer (40:60) mobile phase. Warfarin, 7-hydroxy warfarin and carbamazepine (internal standard) were separated on a C18 column and had the retention time 3.6 min, 2.9 min and 5.9 min, respectively. The assay was linear in warfarin concentration ranges of 0.1–5 μg/ml. The extraction recovery was found to be ≈85%. The mean plasma concentrations of warfarin and 7-hydroxy warfarin were found to be 3.47 ± 1.87 (SD) μg/ml, 1.25 ± 0.81 (SD) μg/ml, respectively. Through the present study the plasma concentrations of warfarin, 7-hydroxy warfarin and their metabolic ratio was determined. The assay was sensitive to follow warfarin pharmacokinetics in a patient with warfarin therapy for 3 months and above.

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1. Introduction

Warfarin is an oral anticoagulant used in various cardiovascular and cerebrovascular disorders such as venous thromboembolism, pulmonary embolism, atrial fibrillation, valvular heart disease and coronary heart diseases.[1–3] It is a drug with a narrow therapeutic index and warrants careful monitoring of the patient. Bleeding can occur as an adverse drug reaction due to increased activity of the drug at a rate of 1.3–2.7 per 100 patient-years during the anticoagulation therapy.[4,5] In order to ensure the effectiveness and safety of oral anticoagulants, the dose must be adjusted accurately and frequently. The effectiveness and safety of oral anticoagulation therapy is critically dependent on prothrombin time (PT) expressed as the International Normalized Ratio (INR), which is the time required for the blood to coagulate relative to the standardized coagulation time, within the desired therapeutic range.[6]

Currently, estimation of INR is the mainstay of monitoring warfarin therapy and it estimates its pharmacodynamic effect. However, there are several clinical studies which warrant estimation of plasma levels of warfarin and its metabolites. Further estimation of plasma drug levels may be beneficial in evaluation of drug resistance or compliance in patients.[7] Several analytical methods have been developed to determine the warfarin concentration in plasma.[8–11]

Previous studies have been performed to measure the plasma concentrations of warfarin and its correlation with INR. However, the methods have been reported that the correlation of plasma concentrations of warfarin and warfarin mean daily dose with INR was very poor.[11,12] In the present study we aimed to develop a simple, rapid, cost effective, sensitive and specific method to determine the plasma levels of warfarin and 7-hydroxy warfarin in patients who are on treatment with warfarin.

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2. Materials and methods

2.1. Study setting

The study was conducted in the out-patient clinics of cardiology and cardio thoracic and vascular surgery at Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER) hospital, Pondicherry. Institute Ethics Committee approval was obtained prior to initiation of the study. The study was approved by Institute Ethics Committee of JIPMER and the study was conducted according to declaration of Helsinki. All the study participants were explained about the study and written informed consent was obtained.

Patients receiving warfarin maintenance therapy for 3 months and above, having a stable INR of the range 2–3.5 were included in the study. The study patients belonged to the age group of 18–65 years and of either gender. The study excluded patients with liver or renal dysfunction, concomitant drug interacting with warfarin, pregnant and lactating women, smokers and alcoholics. Patients' demographics such as age, gender, height, weight, body mass index, duration of warfarin therapy, concomitant illness status were obtained.

2.2. Plasma warfarin determination

2.2.1. Sample collection and preparation

Five ml of venous blood was collected into sodium Ethylene Diamine Tetra Acetic acid (EDTA) tubes from all patients, 12 h after the last dose of warfarin. The plasma was separated by centrifugation of blood samples at 3000 rpm for 10 min and stored at −70 °C until analysis was done.

2.2.2. Materials and reagents for HPLC assay

Pure powders of warfarin, 7-hydroxy warfarin and internal standard carbamazepine were obtained from Sigma–Aldrich, St. Louis, USA. All organic solvents used were of HPLC grade and purchased from Merck specialties Pvt Ltd, Mumbai, India. Potassium di hydrogen orthophosphate and di potassium hydrogen orthophosphate were obtained from S.D. fine-chem Ltd, Mumbai, India. The C18 solid phase extraction cartridges (100 mg, 3 ml) were obtained from Varian, Inc., Agilent Technologies, USA.

2.2.3. Preparation of standard

The standard solution of warfarin, 7-hydroxy warfarin and internal standard carbamazepine were prepared at a concentration of 1 mg/ml in methanol. A series of six standard solutions of warfarin and 7-hydroxy warfarin were prepared in drug free human plasma at concentrations of 0.1, 0.5, 1.0, 2.5 and 5.0 μg/ml. Plasma standards and quality control (QC) samples were aliquoted, stored and treated the same way as patient blood samples. The solutions for the standard curve were prepared fresh before the analysis. For estimation of drug recovery, six analytes standard water solutions were prepared by diluting the standard stock solution in MilliQ water to serve as 100% control.

2.3. Extraction procedure

Solid phase extraction was used to extract the drug from plasma samples. Ten microliters of 1 mg/ml internal standard (carbamazepine) was added to 1 ml standard, QC and patient plasma samples. C18 cartridges were used for extraction. The cartridges were conditioned briefly by using 2 ml of 1% methanol (pH 2.8 adjusted with orthophosphoric acid) before adding plasma. The plasma samples were rigorously vortexed and added to the conditioned columns. The warfarin and 7-hydroxy warfarin retained in the column were eluted with 2 ml of acetonitrile. The organic phase was transferred to fresh glass tubes and evaporated to dryness under nitrogen gas in a water bath at 60 °C in sample evaporator. The samples were reconstituted with 200 μl of MilliQ water. Fifty microliter of the resultant solution was injected into HPLC.

2.4. Chromatography

The mobile phase consisted of (40:60) isopropanol and potassium phosphate buffer (di potassium hydrogen phosphate; pH 7.0 adjusted with potassium di hydrogen orthophosphate). The HPLC system consisted of a Shimadzu LC-10AD *VP* solvent delivery module, Shimadzu SPD-10A *VP* UV–VIS detector and a 100 μl injection loop. The flow rate was maintained at 1 ml/min. The analytes were detected at 308 nm, with absorbance set at 0.005 Aufs. Separation was performed on a C18 column (Phenomenex, 150 × 4.6 mm, 5 μm). Warfarin, 7-hydroxy warfarin and carbamazepine had the retention times 3.6 min, 2.9 min and 5.9 min, respectively. All the chromatograms were analyzed by using the software CLASS-VP version 6.14 SP2.

To evaluate accuracy, precision and reproducibility of the method, we have estimated quality control samples containing warfarin and 7-hydroxy warfarin at the concentration ranges 0.1–5.0 μg/ml. To determine intra-day assay accuracy and precision, we performed six replicates at each of 2.5 and 5 μg/ml. Inter-day accuracy and precision were determined over a period of 15 days using the same concentration range. The standard was analyzed on the same day of sample analysis to calculate the concentrations of warfarin and 7-hydroxy warfarin. The limit of detection was established by serial extraction of plasma samples containing decreasing concentrations of warfarin and 7-hydroxy warfarin.

2.5. Statistical analysis

GraphPad Instat® version 3.06 (San Diego, USA) and IBM® SPSS® Statistics version 19.0 (SPSS Inc., Chicago, IL, USA) were used for statistical analysis. The patients were assigned into three different groups based on their maintenance dose (≥2.5 mg – low dose group, ≤2.5 mg to 8.5 mg ≥ – intermediate dose group and ≥8.5 mg – high dose group). The average mean plasma concentration of warfarin, 7-OH warfarin and their metabolic ratio were calculated. Warfarin metabolic ratio (MR) was compared by Mann–Whitney *U* test. Correlation of peak area between plasma levels of warfarin and

Table 1
Demographic parameters and plasma concentration of warfarin and 7-hydroxy warfarin in study participants.

Parameters	All patients ($n = 178$)
Age (Years) (Mean ± SD)	40.3 ± 9.6
Gender	
Male (%)	64 (36.0)
Female (%)	114 (64.0)
BMI (Kg/m^2)	24.7 ± 31.7
BSA (m^2)	1.53 ± 0.17
Duration of therapy (Years)	9.6 ± 10.4
Average daily dose of warfarin (mg ± SD)	4.88 ± 1.63
Mean plasma warfarin (μg/ml ± SD)	3.47 ± 1.87
Mean plasma 7-hydroxy warfarin (μg/ml ± SD)	1.25 ± 0.81
Indications for warfarin	
Rheumatic Heart Disease (Mitral stenosis) (%)	116 (65.2)
Atrial fibrillation (%)	12 (6.8)
Deep vein thrombosis (%)	10 (5.6)
Coronary Heart Disease (%)	10 (5.6)
Mitral valve replacement (%)	7 (3.9)
Pulmonary embolism (%)	7 (3.9)
Coronary artery disease (%)	6 (3.4)
Aortic valve replacement (%)	5 (2.8)
Cardiomyopathy (%)	5 (2.8)

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