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Study on warfarin plasma concentration and its correlation with international normalized ratio

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Abstract

A sensitive high-performance liquid chromatographic (HPLC) method was developed for warfarin determination in plasma of patients who undertook cardiac valve replacement and were on anticoagulation with warfarin. The method described proved to be accurate, sensitive, easy to perform, reproducible and specific for plasma warfarin measurement with relative standard deviation (R.S.D.) of <5.27% for inter-day and <6.89% for intra-day. The assay was linear in warfarin concentration ranges of 0.12–3 μg/ml ($r = 0.9995$) with mean recovery of 94.6%. The mean warfarin plasma concentration of 58 patients with heart valve replacement within 1 month of post operation was 567.6 ± 122.3 ng/ml. The anticoagulant effect of the drug was monitored by international normalized ratio (INR). The correlation of warfarin dosage and concentration with INR was analysed, and the coefficients were 0.21, $0.1 < p < 0.2$ and 0.30, $0.02 < p < 0.1$, respectively. The correlation of warfarin dosage or concentration with INR is very poor, and hence in order to adjust the dosage more objectively and accurately, concentration monitoring is necessary and helpful for the patient management. It is needed especially when the ideal INR is difficult to target.

Keywords: Warfarin; Anticoagulation; plasma concentration; INR; Therapeutic monitoring

1. Introduction

Warfarin is a Vitamin K antagonist which is the most widely used oral anticoagulant in clinics for the treatment of venous thromboembolism as well as for the prevention of systemic embolism in patients with atrial fibrillation and with prosthetic heart valves [1,2]. It exerts anticoagulant effect by interfering with the cyclic conversion of Vitamin K, thus, inhibiting the activation of the Vitamin K-dependent coagulation factors (II, VII, IX and X) and inhibitors (protein C and protein S). The dose response relationship of warfarin differs between healthy subjects and can vary to a much greater extent among sick patients [3]. It can be influenced by many factors such as pharmacokinetic 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 hemostatic response to given concentrations of warfarin) and technical factors including inaccuracies in laboratory testing and reporting, poor patient compliance, and poor communication between patient and physician [4–6]. Drug can influence the pharmacokinetics of warfarin by reducing its absorption from the intestine or by altering its metabolic clearance [3]. In addition, the therapeutic window of warfarin is very narrow. Exceeding the therapeutic window of these drugs triggers unwanted bleedings. In order to prevent the obvious adverse drug event, the dosage of warfarin must be adjusted accurately and the anticoagulant effect must be closely monitored to avoid over- or under-anticoagulation for the highly variable dose-response effect of warfarin.

Monitoring of warfarin in patients is usually done by their pharmacodynamic effects prothrombin time (PT) with results expressed as INR [7]. However, INR still has it's limitation in detecting the factors affecting the anticoagulants immediately, such as patient compliance, resistance to anticoagulant, drug interaction and food variety etc. In some clinic situations, measurement of the plasmatic levels of warfarin is essential in patients with an increased PT of unknown origin. Confirmation of the plasma concentration of warfarin can facilitate diagnosis and allows for the effective treatment of severe intoxication.

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Furthermore, plasma concentrations can also be helpful in distinguishing noncompliance from genuine anticoagulant resistance. Hence, a sensitive and specific routine analysis method for unambiguous identification and quantification of warfarin is strongly required.

Several analytical methods have been developed for the measurement of plasma anticoagulants, such as gas chromatography–mass spectrometry (GS–MS) [8,9], high performance liquid chromatography–mass spectrometry (HPLC–MS) [10,11] as well as HPLC [1,12–16]. GS–MS is a sufficiently sensitive and specific method for the detection and quantification of anticoagulants but requires time-consuming derivatisation procedure. The combination of HPLC with ESI–MS/MS leads to very short retention times and yields both high selectivity and sensitivity. However, the instrument is not available in many clinical laboratories. Several methods using HPLC have been reported in the literature for the quantification of warfarin enantiomers. On the whole, all methods have their own advantages but did not meet the demands in terms of speed, accuracy and cost-efficiency in our laboratory.

In this study, after optimising the chromatographic conditions, a sensitive and simple HPLC method was developed for the determination of plasma warfarin with naproxen as the internal standard. A single liquid–liquid phase extraction step with dichloromethane–*n*-hexane (9:1) was sufficient to eliminate interferences. The total HPLC run-time was relatively short (10 min). Warfarin plasma concentration was determined in 58 patients on oral anticoagulants after heart valve replacement by the method described, and the correlation of warfarin concentration or dosage with INR was also evaluated.

2. Materials and methods

2.1. Materials

Sodium warfarin (99.85%) was kindly provided by Shanghai Zhongxing medicine manufactory. It was dissolved in distilled water, which was then used to spike pooled normal plasma to final concentrations ranging from 0.12 to 3 μg/ml. Naproxen was used as the internal standard. It was purchased from National Institute for the Control of Pharmaceutical and Biological Products and was dissolved in distilled water to get a solution of 6.88 μg/ml. Dichloromethane, *n*-hexane, hydrochloric acid, methanol and ammonium acetate were purchased from Huamei medical reagent Ltd., Shanghai, China.

2.2. Patients population

From September 2001 to October 2002, 58 patients who undertook cardiac valve replacement and anticoagulation with warfarin were randomly selected for participation in this trial. Characteristics recorded included age, gender, weight, height, etc. To qualify for participation in the study, baseline renal function and liver function test, cardiac function examination were performed on all patients. Among the 58 investigated patients suffering from rheumatism cardiac disease, 33 patients were male, 25 patients were female, and 43 of them with the symptom of atrial fibrillation, three with the history of embolism, one with bacterial endocarditis. All of them were with normal function of renal and liver before operation. More than 85% of them were with III or IV grades cardiac function. From the 58 patients 33 had mitral valve replacement, nine had aortic valve replacement, 16 had mitral valve replacement accompanied with aortic valve replacement.

Patients were excluded if they had allergic reactions to warfarin. Other exclusion criteria were use of aspirin, barbitals, quinidine, and patients with abnormal liver and liver function test result, tendency of bleeding.

2.3. Study protocol

The Investigative and Research Board approved the study protocol at Qianfoshan Hospital. In this study all patients gave consent 1–2 days prior to surgery. Warfarin was used at the second day after operation. The warfarin dosage was recorded and patient complications were observed. All patients were evaluated for PT, INR and warfarin plasma concentration at the time of the day before operation, the 1–10th day, and every 5 day of the rest time until 1 month of post operation. On the other days, INR was tested additionally when it was needed. The targeted range of INR was 1.5–2.5. The anticoagulant effect was evaluated by the standards of prognosis, thrombus, bleeding and death according to the publication [17].

2.4. Plasma preparation

Blood was collected from patients on warfarin therapy, some of it was divided into vacuum tubes containing 129 mM trisodium citrate for PT and INR determination. The rest was centrifuged to get platelet-poor plasma, which was frozen and stored at −70 °C until testing.

2.5. INR determination

The INR was measured on fresh plasma by ACL200 automated coagulometer (Coulter, USA). The reagent used was PT-Fibrinogen HS 0008468210 (Instrumentation Laboratory Company-Lexington, MA 02421-3125, USA).

2.6. Plasma warfarin determination

The sample was centrifuged at 3000 rpm for 10 min. After separation, 600 μl plasma sample tested and 300 μl water (or 600 μl blank plasma and 300 μl warfarin standard solution) was applied to a cartridge containing 300 μl internal standard working solution, then, 300 μl 1 M hydrochloric acid was added. Warfarin in plasma was extracted with dichloromethane: *n*-hexane (9:1). The organic phase separated was evaporated to dryness under a stream of nitrogen at 50 °C. The residue was eventually dissolved in 150 μl mobile phase composed of methanol—50 mmol/l ammonium acetate buffer (pH 3.74) 67:33. And 20 μl of the solution was injected into the HPLC system (Waters). Separation was performed on a C18 column (Phenomenex, 5 μm, 150 mm × 4.6 mm) and peaks were

detected with a double wavelength absorbance UV detector (Waters 2487) at 308 nm. The eluent was applied at a flow rate of 1.2 ml/min (isocratic conditions). Chromatograms of drug free human plasma showed no interfering peaks with retention times similar to those of warfarin and the internal standard. Peak areas for warfarin and internal standards were measured. Linear responses in warfarin/internal standard peak area ratios were evaluated for warfarin concentration ranging from 0.12 to 3 μg/ml. A set of standards was run everyday (in duplicate) and the concentration of warfarin was calculated.

3. Results

3.1. The results of plasma warfarin determination study

Chromatograms corresponding to blank plasma, plasma spiked with warfarin and internal standard are shown in Fig. 1.

The HPLC method developed for warfarin determination in plasma is sensitive, precise and accurate. Linear response of warfarin/internal standard peak area ratios versus warfarin concentration ranging from 0.12 to 3 μg/ml is excellent ($Y = 0.7919X + 0.02409$, $r = 0.9995$). The mean recovery, estimated by measuring warfarin concentration in three different assays for a drug-free plasma sample pooled from healthy subjects and spiked with warfarin at the final concentration of 0.10, 0.50, 3.00 μg/ml is 94.6%. The precision and reproducibility of the assay, which was estimated as relatively standard deviation is <5.27% for inter-day and <6.89% for intra-day at the concentration of 0.10, 0.50, 3.00 μg/ml.

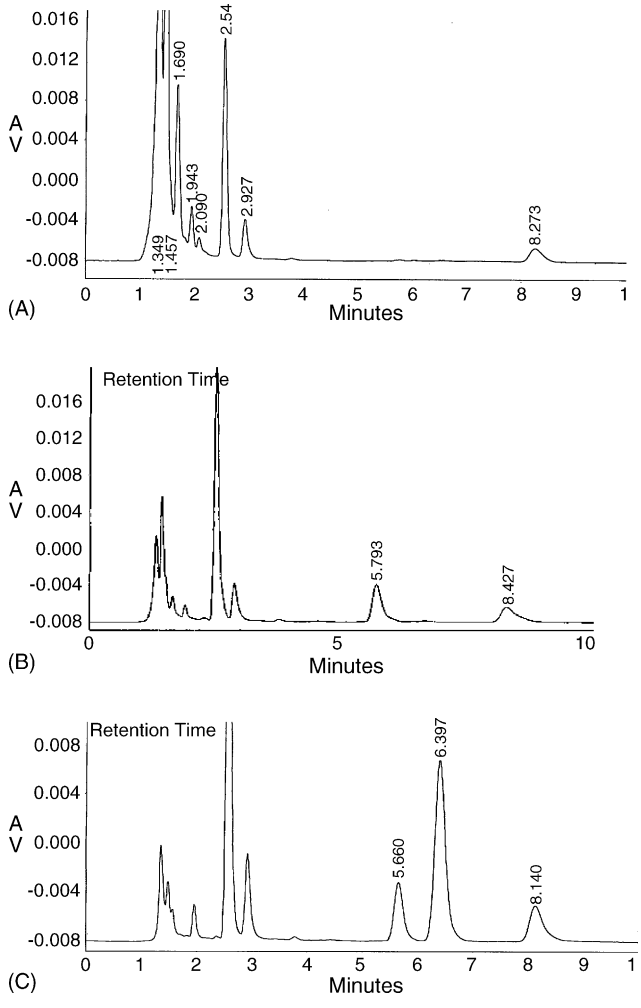

Fig. 1. Chromatograms obtained with the blank plasma (A), plasma spiked with naproxen (RT = 5.793) (B), plasma spiked with naproxen (RT = 5.660) and warfarin (RT = 6.397) in plasma (C). AV, absorbance units. The number above the peak identifies the corresponding peak retention time.

3.2. The results of plasma warfarin concentration and INR in patients with heart valve replacement

The results determined for the 58 patients tested showed that warfarin concentration increased gradually within the 5 days of post operation and there was no big fluctuation after 5 days as shown in Fig. 2. The concentration range was 567.6 ± 122.3 ng/ml.

The result of PT and INR showed that there was no obvious difference displayed for PT and INR within 48 h after warfarin administration compared with those of before warfarin administration ($p > 0.05$). Significance difference was observed after 48 h of administration ($p < 0.05$), and more significant difference was revealed after 72 h of administration ($p < 0.001$). The INRs for most of the patients samples tested are in the range of 1.5–2.5 (data not shown).

3.3. The correlation of warfarin dosage or concentration with INR

Relativity analysis showed that there is no significant correlation between warfarin concentration or dosage and INR as

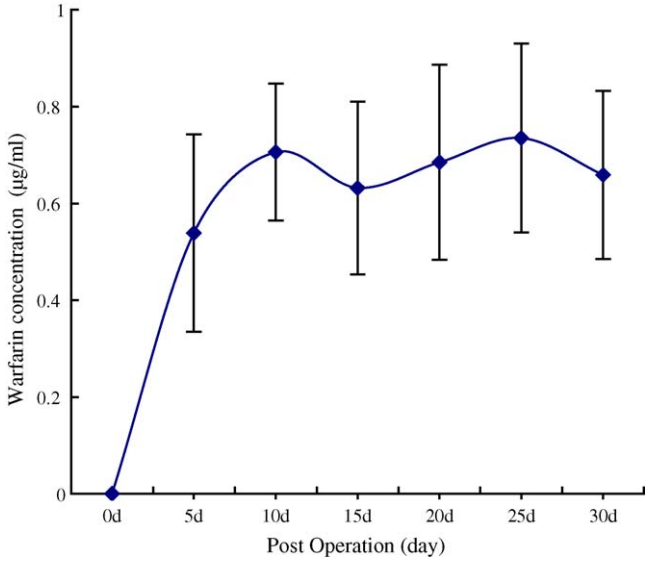

Fig. 2. Plasma-concentration-time curve of warfarin determined from samples of 58 patients with heart valve replacement. The patients administered warfarin once daily from the second day of post operation. The plasma samples were collected at the time point designed and the concentration was determined using the HPLC method developed. Data was expressed as mean ± 95% confidence interval.

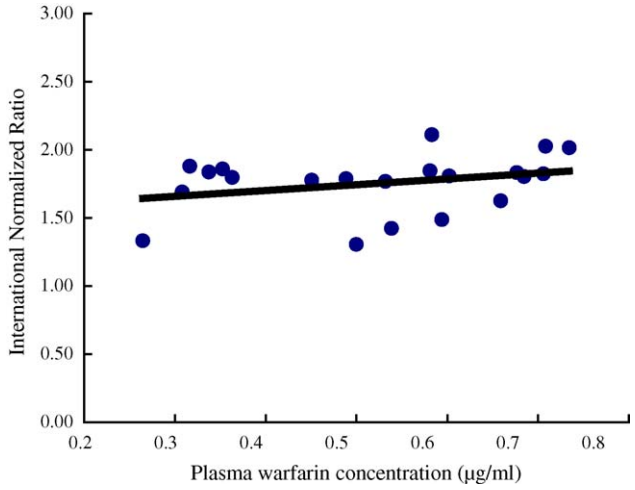

Fig. 3. Correlation between plasmatic warfarin concentration and international normalized ratio ($r = 0.30$, $0.02 < p < 0.05$). The INR was tested for the 58 patients on oral anticoagulation after heart valve replacement at the same time of sample collection for concentration measurement.

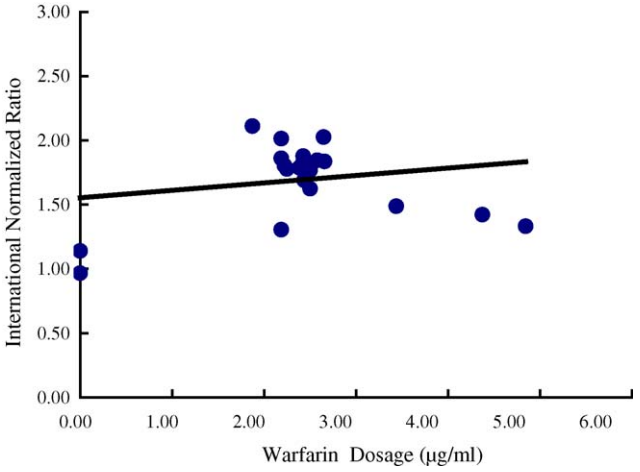

Fig. 4. Correlation between plasmatic warfarin dosage and international normalized ratio for 58 patients on oral anticoagulation ($r = 0.21$, $0.1 < p < 0.2$) after heart valve replacement.

shown in Figs. 3 and 4. The coefficient is 0.21, $0.1 < p < 0.2$ for warfarin dosage with INR, and 0.30, $0.02 < p < 0.1$ for warfarin concentration with INR.

4. Discussion

One purpose of this study was to develop a sensitive, rapid and stable method, free from interferences and sufficiently easy to perform in the hospital laboratory for warfarin plasma concentration determination. Another purpose was to determine the patients' plasma warfarin concentration of post heart valve replacement using the method developed and analysis the correlation of warfarin concentration and INR.

Great strides have been made in monitoring the anticoagulant effect of warfarin with the development of INR to standardize prothrombin time. But it still has limitation for the patients who are difficult to manage. Incorrect or inattentive management is dangerous because of its narrow therapeutic index [18]. Other monitoring approach is strongly needed in some situations. Plasma warfarin measurement is considered helpful to the management of complicated 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 as mentioned above, and the extraction is the crucial step, which requires careful conditions to ensure good recovery of warfarin before sample injection. In this study we developed a HPLC method for quantification of warfarin in plasma and investigated the plasma warfarin concentration for the patients who took heart valve replacement. The method has several advantages. It is sensitive, reliable, rapid, cost-efficient and easy to perform.

Correlation between the warfarin concentration or the dosage and the INR was also assessed. Interestingly, the correlation between concentration or the dosage and the INR was not good. This is not surprising. Interaction with other drugs, late effect response, resistance, dependence from diet, seasonal variation and age make the INR poorly dependent on warfarin concentration [3]. This strengthens the concept that INR measurement alone would be only of limited value for dose-adjustment in patients with complicated situations. In other words, we need to exclude the impact of other anticoagulation effect factors as mentioned above before dosing adjustment. If we make sure the warfarin concentration is not low when the INR is still not falling in the targeted range, other measures should be taken to find out the real impact factors other than to adjust the dosage directly. There is a danger of bleeding for the patient when the impact factors disappear. So warfarin plasma concentration measurement may be helpful in managing patients, especially to the patients with fluctuant INR and was very difficult to manage.

Further study should focus on the detailed mechanism of the poor correlation between warfarin concentration and INR to provide the clinical doctors a solution for the patient management who have achieved a given concentration but with an un-ideal INR.

5. Conclusion

The described method proved to be accurate, sensitive, rapid, easy to perform, reproducible and specific for plasma warfarin measurement. It does not require sophisticated equipment other than the regular HPLC apparatus that is available in most of the clinical laboratories. The mean warfarin plasma concentration of the 58 post operation patient with heart valve replacement was 567.6 ± 122.3 ng/ml within 1 month. There was no significant correlation between the dosage or concentration of warfarin with PT and INR. It revealed that the anticoagulant effect of warfarin could be affected by many factors. So although the INR is widely accepted as a golden standard for the monitoring of oral anticoagulation therapy, in order to adjust the dosage more objectively and accurately, concentration monitoring is essential in some situations for the confirmation of anticoagulant affecting factors, especially when the ideal INR is difficult to target.

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