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DETERMINATION OF WARFARIN IN HUMAN PLASMA BY USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY MASS SPECTROMETRY (HPLC-MS)

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Abstract

Background: Warfarin is a widely used as oral anticoagulant with broad within- and between individual dose requirements. Warfarin concentration can be monitored by measurement of its plasma concentration. However, this approach has not been applied in the routine clinical management of patients receiving warfarin therapy.

Objective: The aim of this study was to determine warfarin concentration in patients receiving warfarin by using high-performance liquid chromatography mass spectrometry (LC-MS) and to correlate between its level and the demographic data of patients included in the study.

Method: A total of 20 patients receiving warfarin for more than 1 yr were included. Warfarin plasma concentrations of the study subjects were determined by LC-MS. The potential effect of various factors on warfarin response was investigated by correlating these factors to Warfarin plasma concentration and INR.

Results: Demographic data shows that the age range of the study subjects was (25- 75 years) reflects the wide spread of cardiac disease among different age group. Data collected also shows that the majority of the patients are within the age group above 50 years. Dose range for patients was (14 - 42 mg/week). As the recommended maintenance dose for adults is 2 to 10 mg once a day, so all of study subjects were within the permitted range for adult's dose. Statistical analysis showed that no correlation between warfarin concentration in patients' plasma and INR level. The data also showed that no correlation was found between INR values and weekly dose (r^2=0.003).

Conclusion: There was no significant correlation between INR and warfarin plasma concentration. Also No correlation was found between INR values and study variables. Under such observations, it could be concluded that warfarin can be challenging to be managed due to its narrow therapeutic range, variable dose-response among different patients and possible common interactions with drugs, diet and other factors.

Introduction

Warfarin is an oral anticoagulant of the coumarin class. Warfarin is administered clinically as a racemic mixture of two enantiomers, (R)-warfarin and (S)-warfarin. (S)-Warfarin has more potent anticoagulant activity than (R)-warfarin and is metabolized mainly to (S)-7-hydroxywarfarin by CYP2C9. Warfarin is completely absorbed after oral administration and is highly bound to albumin in the plasma. The goal of anticoagulant therapy with warfarin is to administer the lowest effective dose of the drug to maintain the target international normalized ratio (INR)(1–4).

Warfarin toxicity is common and the management of warfarin overdose is usually complicated by patients using warfarin therapeutically, and thus knowledge of serum concentrations is most helpful when the drug in question requires individualized

dosing for optimal efficacy and more routine measures of therapeutic success are unavailable (5–8).

Warfarin in plasma samples can be determined by the use of different spectroscopic and analytical methods. Liquid chromatography mass spectrometry (LC/MS) instrument combines the advantages of sensitivity and selectivity of the HPLC and Mass spectrometry techniques(5,9,10).

Warfarin is subject to bewildering number and variety of drug interactions, producing increased or decreased clinical effect of warfarin itself or other drugs. Many of these effects are due to changes in protein binding or hepatic metabolism. The correlation of warfarin dosage or concentration with INR is very poor (4,11–13), and hence in order to adjust the dosage more objectively and accurately, concentration monitoring is necessary and helpful for patient management.

Methodology

This study adopted two parts; experimental study and a questionnaire.

(a)Experimental study

Blood samples were collected and 19 patients under warfarin and from 5 healthy subjects at the same time of their scheduled routinely INR measurement. The samples were centrifuged to separate plasma and were stored in ice container before analysis.

200 µl from each plasma sample was drawn by micropipette and placed in a centrifuge tube, 400 µl of 0.1 M acetonitrile was added to the sample and shaken instrumentally then 400 µl of 0.1 M formic acid was added and the mixture was shaken and centrifuged for 5min.

Preparation of standard solution and quality control samples

1 mg/mL stock standard solution of warfarin was prepared by dissolving the weighed tablet powder in acetonitrile and formic acid mixture and then diluted to prepare a 10 mg/mL standard solution. This solution was further diluted to prepare 0.5, 1, 2, 5, 10, 20, 50 and 100 ng/mL standard working solutions of warfarin.

Instrumentation

Chromatographic measurements were carried out using a HPLC system (LC-MS 2020 – Shimadzu Corporation, Kyoto, Japan) equipped with, MS detector. Column used was C_{18} (150 mm × 4 mm, 3 um particle size). A mixture of formic acid (0.1% in water) and acetonitrile (40:60, v/v) at a flow rate of 1 ml min^{-1} and room temperature was used as mobile phase.

(b)Questionnaire-Based study

The study was carried out from March 2019 to August 2020 in Sudan Heart Center in Khartoum state. Adult patients who were receiving warfarin for at least 1 yr were included. The demographic and clinical data collected were age, gender, weight, age, weekly warfarin dose, duration and INR value. All participants filled out informed consent forms before participating. For the plasma warfarin assay, a single blood sample was obtained at least 12 hr after the administration of the last dose of warfarin.

Statistical analysis

Microsoft Office Excel 2016 was used to draw the diagrams and calculate r^2 values. Statistical analysis was done by SPSS (version 21, Chicago, IL) software.

Results

The chromatograms of a control sample and a patient's plasma sample are shown in Figure.1-2, respectively. Sharp and symmetric peaks were obtained at retention time of 1.1 minutes (control) and 4.5 minutes (patient).

Figure 1 chromatograms of control plasma sample.

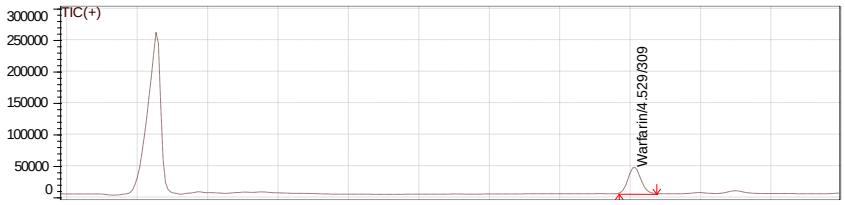

Figure 2 chromatograms of patient plasma sample

The calibration curve obtained for the different warfarin concentration is shown in Figure 3 . The regression line equation is y= (2391.7+ 982764.8 x) , R^2 = (0.999).

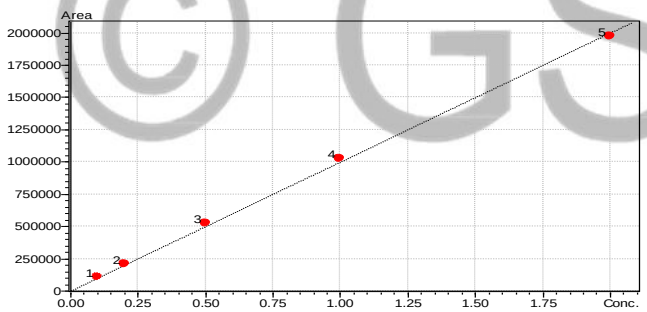

Figure3: SIM mode mass spectrum of warfarin

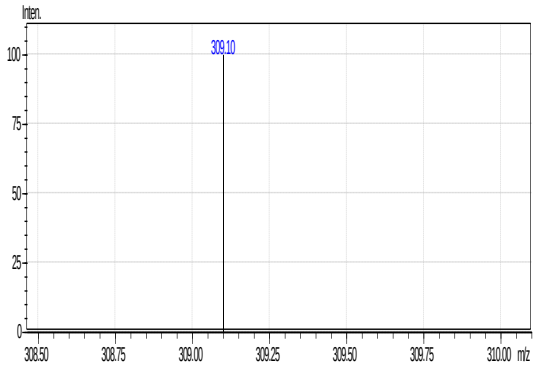

Figure 4: SIM mode mass spectrum of warfarin

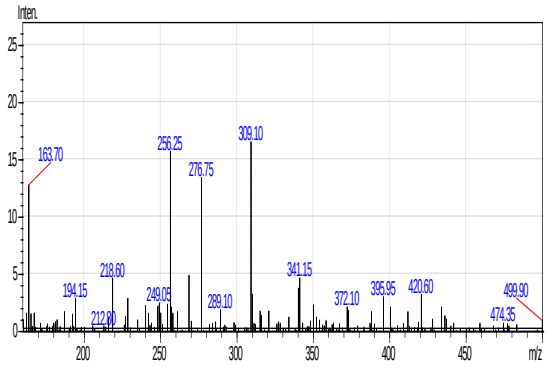

Figure 5: SIM mode mass spectrum of warfarin

The collected demographic data and the measured warfarin level and their respective INR are shown in table 1.

Table 1: The measured INR, concentrations levels of the patients along with their demographic data.

No.	Drug Conc.ppm	INR	weekly dose	duration	Weight	Age	Gender
1	1.19	1.7	21	5	60	67	f
2	1.76	2.3	35	5	73	73	m
3	1.25	2.1	28	1	85	60	f
4	0.91	1.7	23	13	74	54	f
5	1.27	2.9	23	13	72	55	f
6	0.94	2.4	14	1	59	50	f
7	1.35	1.7	28	14	73	72	f
8	1.30	2.3	21	3	68	38	f
9	1.56	1.9	35	5	70	51	f
10	0.83	2.8	28	6	73	53	m
11	1.14	2.7	21	13	81	75	f
12	1.43	3	28	13	75	43	f
13	0.83	1.9	14	1	68	46	f
14	1.55	2.3	35	17	70	49	m
15	1.04	2.3	25	14	63	38	f
16	1.22	2.8	35	2	68	44	f
17	1.10	1.6	42	16	81	57	m
18	1.14	3.2	28	1	67	32	m
19	1.09	2.6	25	2	80	55	f
20	2.43	>10	21	5	60	25	m

Correlation of different variables to plasma concentration and INR

The correlations among plasma warfarin concentrations, INR, warfarin daily and weekly dose, duration, age and weight were studied in the 19 patients.

Figure 6 reported INR versus warfarin concentration, no correlation was found (r^2=0.0, P= 1).

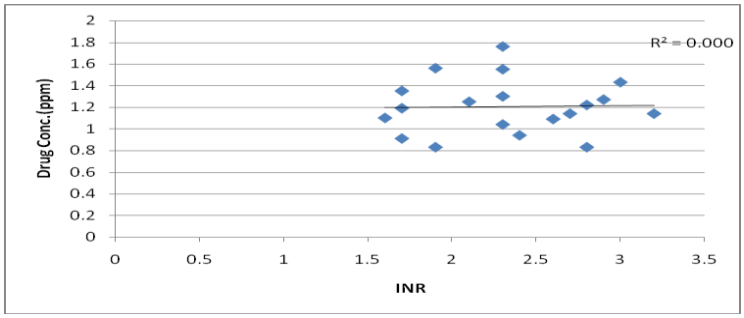

Figure 6: Plot of drug concentration (ppm)Vs.INR Vs.

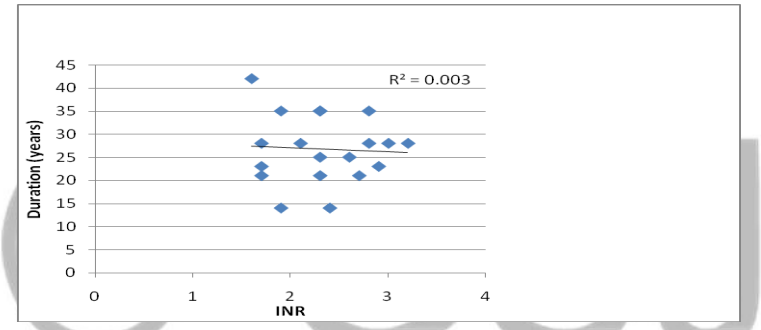

Figure 7: Plot of weekly dose Vs. INR

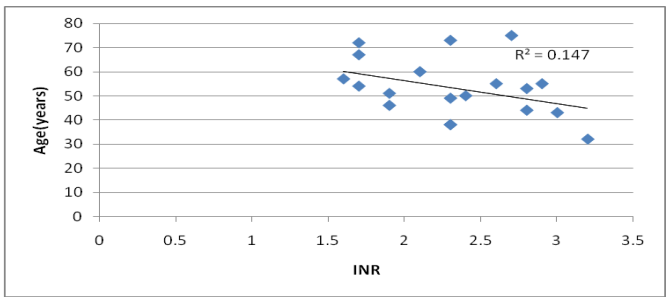

Figure 8: Plot of Age Vs INR

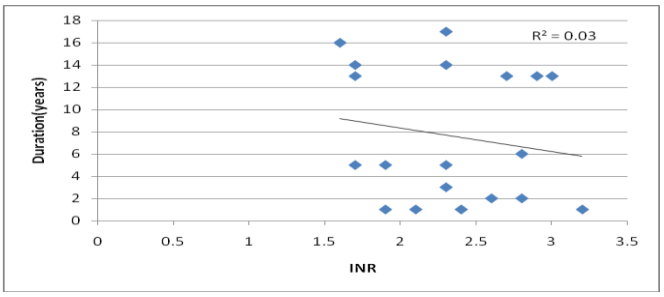

Figure 9: Plot of duration Vs. INR

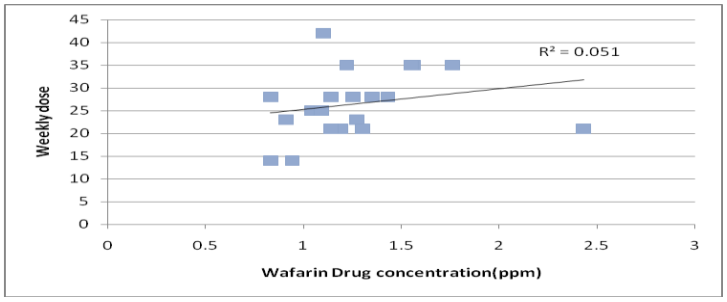

Figure 10:Plot of weekly dose Vs. warfarin plasma concentration (ppm)

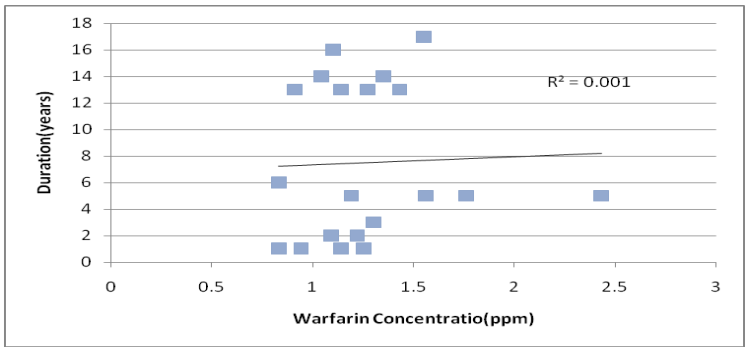

Figure 11:Plot of Age Vs. warfarin plasma concentration (ppm).

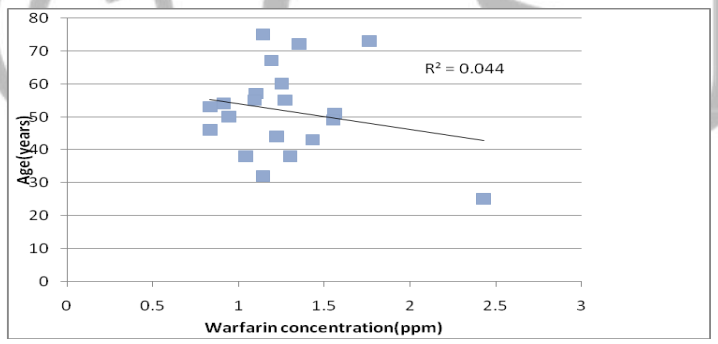

Figure 12: Plot of Age Vs. warfarin plasma concentration (ppm).

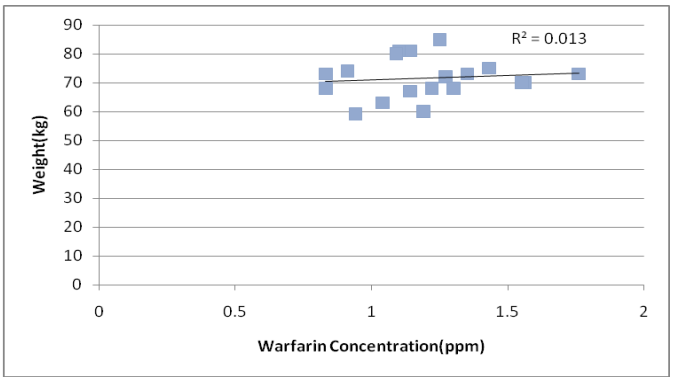

Figure 13: Plot of weight Vs. warfarin plasma concentration (ppm).

Discussion

HPLC-RP is a sensitive, accurate and precise tool for measurement of warfarin concentration in plasma. Sharp, symmetric chromatographic peaks with short retention times were obtained from the analysis (Fig1and 2, respectively). For quantitation of the drug concentration, a set of warfarin standard solutions were used and the obtained calibration curve is shown in Fig3.

To obtain the best analyte ionisation efficiency in mass spectrometer, screening of the main factors affecting drug ionisation was performed. The studied factors were: ionisation source type (electrospray (ESI) positive mode and atmospheric pressure chemical ionization (APCI) positive mode), the organic modifier used (methanol and acetonitrile), the aqueous phase used (Figure 4). The best analyte response was obtained using ESI positive mode and a mobile phase composed acetonitrile and 0.1% formic acid in water (60:40, V/V). In the acidic mobile phase warfarin easily accepts a proton generating the pseudo-molecular ion $[M+H]^+$ (m/z 309.1).

After collision induced dissociation in the mass spectrometer, this pseudo-molecular ion (m/z 309.1) produces fragments Figure 5. The fragment having m/z 163.7 was chosen for the quantification of warfarin.

The concentration of warfarin in blood plasma of patients was found to be in the range of (0.83 -2.43) well below the critical value of 10ppm (Table1).

Demographic data shows that the age range of the study subjects was (25- 75 years) a fact that reflects the wide spread of cardiac disease among different age group. Data collected also shows that the majority of the patients are within the age group above 50 years.

The study also shows that eight subjects from the sample population were above or below the therapeutic range which is 2-3. The duration range of warfarin usage in the study subjects was (1-17 years) and as the duration of warfarin therapy is indefinite in some medical cases; those with long term of treatment must periodically reassess risk-benefit ratio of continuing warfarin therapy.

Statistical analysis showed that no correlation between warfarin concentration in patients' plasma and INR level. ($r^2 = 0$) as shown Figure 6. The result is in line with other similar studies [(14-15)].

Figure 7 indicates that no correlation was found between INR values and weekly dose (r^2=0.003). This result reflects the wide variability of patient responses to the drug. which could be ascribed to factors such as gastrointestinal absorption, age, renal and hepatic function, lifestyle (in particular diet, alcohol consumption, smoking etc.)[(16-17)].

The data also shows that no correlation was found between INR values and age (r^2*=0.147*) as shown in Figure8.

Figure 9 indicates that no correlation was found between INR values and duration (r^2*=0.03*).

The plot of weekly dose vs. warfarin plasma concentration (Figure10) indicates no strong relation between the two variables (r^2=0.03). The study also shows that warfarin plasma concentration is independent of or duration or age or weight (Figure11, 12and 13, respectively).

Conclusion

There was no significant correlation between INR and warfarin plasma concentration. Also the study reveals that no correlation was found between INR values and weekly dose. No correlation was found between INR values and age or between warfarin plasma concentration and age.

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