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Determination of free and total warfarin concentrations in plasma using UPLC MS/MS and its application to a patient samples

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ABSTRACT: Warfarin is routinely monitored by assessing its pharmacologic effects on the international normalized ratio. However, having a patient with INR not responding to increasing warfarin dose mandates a direct measurement of warfarin concentrations (total and free) for better patient clinical management of warfarin therapy. Therefore, a new fully validated specific, precise and accurate ultra-performance liquid chromatography tandem mass spectrometry was developed for the determination of free and total warfarin in human plasma. Free warfarin was measured in plasma filtrate, prepared by ultrafiltration, and sample pretreatment involved protein precipitation with acetonitrile. Linear response ($r^2 \geq 0.99$) was observed over the studied range of free and total warfarin, with the lower limit of detection of 0.25 ng/mL. The intra- and inter-day precision (relative standard deviation) values were <10% and the accuracy (relative error) was ≤6.6 for free and total warfarin. There was no significant difference ($p > 0.05$) between inter- and intra-day studies for the free and total warfarin, which confirmed the reproducibility of the assay method. The mean extraction efficiency was 88.6–107.2% of free and total warfarin. The assay was sensitive to follow warfarin pharmacokinetics (free and total) in a patient with resistance to warfarin up to 24 h after a daily dose of warfarin. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: warfarin resistance; etoricoxib; UPLC MS/MS; free warfarin; ultrafiltrate

Introduction

Warfarin (a 4-hydroxycoumarin compound) is the most widely used oral anticoagulant to treat and prevent thromboembolism (Holford, 1986; Murray *et al.*, 1987; Parkash *et al.*, 2007). Warfarin is a narrow therapeutic index drug having a propensity for many drug–drug interactions which require conscientious monitoring of its concentrations by assessing its pharmacologic effects on the international normalized ratio (INR) to maintain it within therapeutic range. This range is usually between 2 and 3, as recommended by the expanded indications for oral anticoagulant therapy, particularly among older patients (Albers *et al.*, 2001; Ezekowitz and Falk, 2004). However, older patients are especially prone to high and low INRs affected by many drug and disease parameters (Hylek, 2001; Gage *et al.*, 2001). Achieving a stable INR on warfarin as soon as possible is important because it minimizes the duration of parenteral medication, which is necessary to obtain immediate anticoagulation. However, excessive or insufficient anticoagulation significantly increases the risk of bleeding and thromboembolic events, respectively (Cannegieter *et al.*, 1995; Samsa and Matchar, 2000; Levine *et al.*, 2001; Reynolds *et al.*, 2004).

Since adverse events are common during oral anticoagulation therapy (Linkins *et al.*, 2003), maximizing time in a therapeutic range is essential to improve outcomes. In fact, the response to warfarin administration is highly variable and requires close monitoring to ensure its safety and efficacy (Costa *et al.*, 2000; Wiedermann and Stockner, 2008).

Drugs are bound to various plasma proteins in different degrees and only unbound or free drug is pharmacologically active. Although free drug concentration can be estimated from total concentration, for strongly bound drugs, prediction of free level is not always possible. The drug is metabolized in the liver and kidneys, with the subsequent production of inactive metabolites that are excreted in the urine and stool. The half-life of warfarin ranges from 20 to 60 h (Majerus *et al.*, 1996).

There are various published HPLC assays for warfarin using UV or fluorescence detections (Ring and Bostick, 2000; Boppana *et al.*, 2002; Osman *et al.*, 2005; Locatelli *et al.*, 2005; Merli and Tzanis, 2009) most of these publications were focused on total chiral separation of warfarin. Recently, HPLC MS/MS was published for the determination of total warfarin but there was no mention of its application for free warfarin concentration in the clinical practice (Kwon *et al.*, 2009). Few LC-MS/MS (Naidong *et al.*, 2001;

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Abbreviations used: INR, international normalized ratio; MCA, multi channel analysis; QC, quality control.

Zuo *et al.*, 2010) methods have been developed for the determination of warfarin enantiomers in human plasma. Recently, a group has measured the free warfarin concentration to correlate it to INR using Micromass® Quattro Premier UPLC MS/MS (Huang *et al.*, 2008) with a negative ionization mode. Liquid–liquid extraction was used; however, there was no report of the values of the found free concentration/fraction of warfarin of the studied patients for comparison. To our knowledge, there is no published data of warfarin concentrations (total and free) using UPLC MS/MS which could be applied in clinical management of patients on warfarin therapy, especially if warfarin resistance is expected. This study was initiated by the need to determine the free and total concentrations of warfarin in those patients not responding to therapy. Therefore the aim of this work was to develop reliable, specific and sensitive ultra-performance liquid chromatography tandem mass spectrometry for the quantitation of the free and total fractions of warfarin in human plasma. The reported method was applied to a patient with warfarin resistance to present its feasibility.

Experimental

Chemicals and reagents

Warfarin was purchased from Aldrich (Milwaukee, WI, USA) and etoricoxib was kindly supplied by local pharmaceutical firm as standard certified samples. An HPLC-grade formic acid was purchased from Sigma–Aldrich (St Louis, MO, USA). All other reagents and chemicals were analytical grade, and were used as received.

Chromatographic system and conditions

The analysis was carried out on a Waters Acquity UPLC™ system with cooling autosampler and column oven. An Acquity UPLC™ BEH C_{18} column (50 × 2.1 mm, 1.7 µm (Waters Corp, Milford, MA, USA) was employed for separation with the column temperature maintained at 40°C. The gradient elution for UPLC analysis consisted of two solvent compositions: solvent A, 0.2% formic acid and 1% acetonitrile in water; and solvent B, 0.2% formic acid in acetonitrile. The gradient began with 80.0% eluent A and changed slowly to 70% A in 1.5 min and then changed linearly to 35% A for another 1.5 min, and remained for a further 0.8 min before changing back to 80% A. Throughout the UPLC process the flow rate was set at 0.3 mL/min and the run time was 4 min.

A Waters TQD ™ tandem quadropole mass spectrometer (Waters Corp., Milford, MA, USA) equipped with an electrospray ionization (ESI) interface was used for analytical detection. The ESI source was set in positive ionization mode. Quantification was performed using MRM of the transitions of *m/z* 309.2 → 251.2 for warfarin and *m/z* 359.1 → 280.3 for IS, with a scan time of 0.10 s per transition. The optimal MS parameters were as follows: capillary voltage 3.0 kV, cone voltage 28 V, source temperature 120°C and desolvation temperature 300°C. Nitrogen was used as the desolvation and cone gas with a flow rate of 500 and 60 mL/h, respectively. Argon was used as the collision gas at a pressure of approximately 0.3 Pa. The optimized collision energy for warfarin and IS was 30 and 50 eV respectively. All data collected in MCA (multi channel analysis) mode were acquired and processed using MassLynx™ V 4.1 software with QuanLynx™ V 4.1 program (Waters Corp., Milford, MA, USA).

Ultrafiltrate preparation

For free warfarin 1 mL of plasma sample was subjected to ultrafiltration using Ultrafree-MC (low binding cellulose 5000 normal molecular weight limit (NMWL); Nihon Millipore, Japan) units by centrifugation at 20,000 rpm for 30 min at 37°C to provide about 200 µL of the ultrafiltrate. The filtrate (200 µL) was spiked as described below.

Preparation of warfarin standards and quality control samples

Stock standard solutions of warfarin and etoricoxib (IS) were prepared in methanol at a concentration of 0.5 mg/mL and stored in 4 mL amber glass vials at −20°C. Different working standard solutions of warfarin (0.01–25 µg/mL) and the IS (1 µg/mL) were prepared by dilution of the abovementioned stock solutions in water–acetonitrile 50:50 (v/v) containing 0.1% formic acid and were kept refrigerated in amber vials at −20°C. The solutions of warfarin and the IS were stable for at least 3 months, under the described conditions. The plasma calibrations standards were prepared ($n = 6$) at a concentration range of 1–100 ng/mL for the free warfarin concentrations and 100–5000 ng/mL for total warfarin concentrations by spiking appropriate aliquots of warfarin working solutions and 20 µL of the IS to 200 µL of blank human plasma. Low, medium and high concentration quality control (QC) samples at concentrations of 5, 50, 100 ng/mL and 100, 1000, 5000 ng/mL for free and total warfarin, respectively, with 100 ng/mL for IS were prepared. The spiked samples were then treated as described in the plasma sample preparation section below.

Plasma sample preparation

Plasma samples collected from six healthy volunteers were subjected to ultrafiltration. The plasma and the ultrafiltrate samples were stored at −20°C until analysis. Frozen plasma or ultrafiltrate samples were thawed at room temperature and subjected to protein precipitation as follows. Samples aliquot of 200 µL (or a calibration standard or a QC sample) and 20 µL of IS working solution were added to a 1.8 mL Eppendorf tube and the mixture was vortexed for 10 s. Then, 800 µL of acetonitrile was added and the mixture was vortexed for 1 min followed by centrifugation at 20,000 rpm for 5 min at 10°C. The supernatant was transferred into a clean glass tube and evaporated to dryness under a gentle nitrogen stream. The residue was reconstituted in 100 µL of mobile phase, vortexed for 1 min, centrifuged at 3000 rpm for 5 min, transferred into a plastic autosampler vial with pre-slit septum (Waters, USA), and 1 µL was injected into the UPLC MS/MS system.

Ion suppression study

The absence of ion suppression was demonstrated by the method of Matuszewski *et al.* (1998). Six different batches of drug-free human plasma or ultrafiltrate were extracted without any drug or IS added as described above. The extracts were reconstituted with warfarin at three nominal concentrations for free (5, 50, 100 ng/mL) and total 100, 1000, 5000 ng/mL (low, medium, high) warfarin. The peak areas of the samples were compared with that of the unextracted reference standard solutions containing the equivalent nominal amount of warfarin in the mobile phase ($n = 6$). The mean area ratio (reconstituted extracts/reference solutions) was 0.98 for warfarin with RSD < 4%. Thus, no ion suppression was observed.

Method validation

The method was validated for selectivity, linearity, precision, accuracy, carry over, extraction recovery and stability according to the *United States Pharmacopoeia XXXIII* (United States Pharmacopeia Convention, 2003) and FDA guidelines (US Food and Drug Administration, 2001) for validation of bioanalytical methods. The selectivity of an analytical method is its ability to measure accurately an analyte in the presence of endogenous compounds. Therefore, six randomly selected blank, drug-free, plasma samples obtained from six different healthy volunteers were analyzed according to the procedure described above. The corresponding chromatograms were tested for possible interferences at the retention times of warfarin and the IS.

The specificity of the method was investigated by comparing the chromatogram of blank plasma spiked with standard solutions with the

samples collected from subjects after administration of warfarin. The intra-day precision and accuracy were determined within one day by analyzing six replicates of the QC samples at concentrations of 5, 50 and 100 ng/mL for free warfarin concentration and 100, 1000 and 5000 ng/mL for total warfarin concentration. The inter-day precision and accuracy were determined on three separate days. The intra- and inter-day precisions were defined as the relative standard deviation (RSD). The accuracy is presented as the percentage relative error [(measured concentration – nominal spiked concentration)/nominal spiked concentration] × 100.

Extraction efficiency was determined for the QC samples at the three concentration levels (low, medium, high) compared with those of post-extraction spiked blank plasma samples for free and total warfarin concentrations. The absolute extraction recoveries were calculated by comparing the peak area of the samples with that of the unextracted standard solutions containing the equivalent amount of warfarin ($n = 6$).

The nominal value of warfarin concentration (ng/mL) in plasma was plotted as a function of the peak area ratio obtained of warfarin and the IS. The day curve was accepted, if the relative standard deviation (RSD) was ≤ 10% for all the tested concentrations (low, medium and high) free or total. The limit of detection (LOD) was defined as the lowest concentration of the analyte resulting in a signal-to-noise ratio of 3:1. The lower limit of quantitation (LLOQ) was defined as the lowest drug concentration which can be determined with a R.S.D. ≤ 20% and an accuracy of 100 ± 20% on a day-to-day basis. Accuracy and precision at the LLOQ were estimated.

The robustness of the method was determined, by using two different Acquity UPLC™ BEH C_{18} columns (50 × 2.1 mm, 1.7 µm). The study was developed using two different warfarin concentrations ($n = 6$).

Freeze–thaw stability of the plasma samples was evaluated by exposing quality control samples to three freeze (−20°C) and thaw (room temperature) cycles before sample preparation. The stability of the samples in autosampler was evaluated by analyzing extracted quality control samples after being placed in the auto-sampler at ambient for 24 and 72 h.

Clinical applications on patient blood samples analyses

A 52-year-old Saudi woman was on chronic warfarin therapy with a p.o. dose of 20 mg (INR range 2). Her other medications included bisoprolol, symbicort, lasix, levothyroxin, simvistatin, alfacalcidol and calcium carbonate. In addition, aspirin 81 mg twice daily was given in the emergency room for 2 days. After cholesystectomy her INR readings were low despite increasing her daily warfarin to 30 mg and the addition of enoxaparin 60 mg twice daily to her therapy. Her thyroid-stimulating hormone, urine, electrolytes and complete blood count analysis were within the normal limits. There was no change in any of the other mentioned medication dosages. The patient denied any diet indiscretions, nonprescription (OTC) or herbal preparations. The patient signed a written consent form approved by King Khalid University Hospital Ethics Committee. Blood samples were collected at 0, 0.5, 2, 4, 5.75, 7, 9 and 24 h after the fourth 30 mg daily dose to measure her free and total warfarin concentrations.

Data and statistical analysis

All results were expressed as the mean ± SD of six replicates. The results were calculated by linear regression without weighting, using the equation: $y = a + bx$, where y is the area under the peak (AUP) ratio of the drug to the internal standard, a is the intercept, b is the slope and x is the concentration of warfarin. The RSD% was calculated for all values.

Pharmacokinetic parameters were estimated using model-independent methods (Gibaldi and Perrier, 1982). The terminal elimination rate constant (λ_n) was estimated by linear regression analysis of the terminal portion of the log–linear blood concentration–time profile of a drug. The terminal elimination half-life ($t_{1/2}$) was calculated from the terminal elimination rate constant using the formula $t_{1/2} = 0.693/\lambda_n$. The mean peak drug concentration (C_{max}) and the time to reach C_{max} (T_{max}) were derived directly from the individual blood levels. The area under each drug concentration time curve ($AUC_{0\text{-}\tau}$, mg/mL h) from dosing to the end of the dosing interval (τ) was calculated by the linear trapezoidal rule. The apparent oral clearance (Cl/F) was calculated from dose/$AUC_{0-\tau}$. The free warfarin fraction (F_u) at each concentration time point was calculated by $F_u = C_u/C$ where, C_u is the warfarin free concentration and C is the warfarin total concentration (free + bound).

The Student t-test was used to examine the concentration difference at each day, and one-way analysis of variance (ANOVA) was employed to evaluate the reproducibility of the assay using SPSS Statistics 17. The level of confidence was 95%.

Results and discussion

Chromatography and selectivity

UPLC MS/MS chromatograms are shown in Fig. 1 for (A) a blank human plasma sample (no warfarin/IS), (B and C) MRM transitions of etoricoxib (IS) and free warfarin, respectively, and (D and E) MRM transitions of IS and total warfarin, respectively, after 24 h of oral administration of 30 mg warfarin in a Saudi patient. The retention times were approximately 1.4 and 3.2 min for etoricoxib and warfarin, respectively. The peaks of etoricoxib and warfarin were well separated without any significant interference from any endogenous substance or given drugs at the retention times of warfarin or the IS within the 4 min run time. During the 3 months of validation, there was no significance change in the observed retention times of warfarin or the IS (RSD < 1.0%).

UPLC MS/MS validation

Excellent linear relationships ($r^2 > 0.99$) were demonstrated between AUP ratio of warfarin to the IS in the ultrafiltrate or plasma over the concentration ranges of 1.0–100 and 100–5000 ng/mL for free and total warfarin, respectively. The mean linear regression equation of the peak area ratios (y) vs drug concentrations (x) of free warfarin was $y = -0.072 + 0.006x$ and for total warfarin $y = 0.062 + 0.002x$ with a mean correlation of 0.997 ± 0.001 and 0.993 ± 0.003, respectively. With an injection volume of 1 µL, the LLOQ of this assay was 0.25 ng/mL in human plasma with the corresponding RSD of 9.6% in the ultrafiltrate. The LOD was 1 ng/mL at a signal-to-noise ratio of >5.

The reproducibility of the assay was evaluated by comparing the linear regressions of three standard plots prepared at three different days over a 3-month period. The mean correlation coefficient was >0.992 with the RSD of the slopes of the three lines 7.8 and 9.7% for the free and total warfarin, respectively. Analysis of variance of the data indicated no significant difference ($p > 0.05$) in the slopes, intra- and inter-day, of the calibration curves. The results confirmed the reproducibility of the assay method.

The mean percentage recovery of warfarin (free and total) was 89.8 ± 8.0% with an RSD of ≤ 9%. There was no significant difference in the extraction efficacy of the present assay over the range of concentrations studied. The accuracy and precision results are shown in Table 1. The maximum acceptable limit for precision and accuracy was set at 10%. The intra-run and inter-run precision (RSD) was ≤ 10% and accuracy as relative error. was 4.8 ± 1.7 and 4.0 ± 1.2 for free and total warfarin, respectively.

Warfarin and IS were both stable in processed samples held in the autosampler at 10°C for at least 72 h with mean calculated

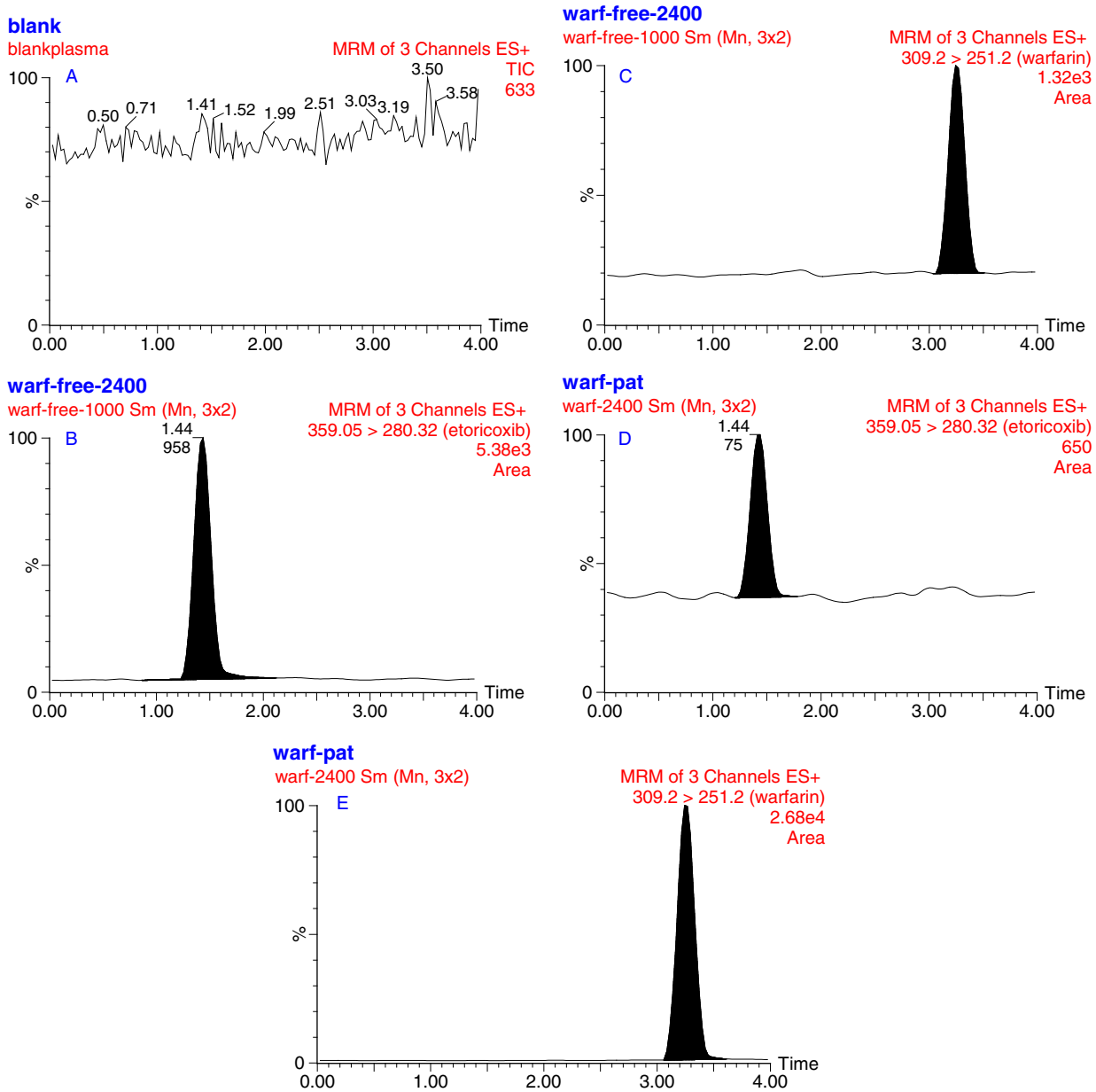

Figure 1. UPLC MS/MS Extracted chromatograms; (A) a blank human plasma sample (no warfarin/IS); (B and C) MRM transitions of etoricoxib (IS) and free warfarin, respectively; and (D and E) MRM transitions of IS and total warfarin, respectively, after 24 h of oral administration of 30 mg warfarin in a Saudi patient.

values within 8.7% of the nominal concentration. There was no evidence of sample carry-over from run to run. In addition, the matrix effect assessed by spiking samples post-processing showed <10% difference from spiked injection solvent.

Clinical applications on patient blood samples analyses

Subjects requiring 9 mg or more are classed as warfarin-resistant, which may be due to several factors (either pharmacokinetic, pharmacodynamic) or poor compliance (Routledge *et al.*, 1998). Kulkarni *et al.* (2008) reported that the pharmacokinetic parameters along with INR seem to correlate ($r^2 = 0.65$) with the total concentration and the weekly doses of warfarin in patients on long-term anticoagulation. These parameters may therefore be useful for predicting warfarin doses. It was also mentioned that there was an extremely poor correlation between the plasma total warfarin and its metabolite (7-hydroxywarfarin) ratio and the weekly dose of warfarin ($r^2 = 0.02$). Huang *et al.* (2008) mentioned that there is a correlation between free warfarin concentration and INR ($r = 0.207$), or weekly warfarin dosage of patients on oral anticoagulation ($r = 0.378$). None of the above-mentioned researchers reported the values of the free warfarin concentration or F_u to compare with our finding.

Figure 2 shows the time course of warfarin free and total of a Saudi patient using UPLS MS/MS. Table 2 represents the pharmacokinetic parameters of warfarin in this patient. It was noticed that the average warfarin free fraction (F_u) was

Table 1. Mean precision (RSD), accuracy (relative error) and recovery for determination of warfarin free and total in spiked human plasma samples ($n = 6$) using UPLC MS/MS

Nominal concentration (ng/mL)	Recovery (%)	Intra-day[a] precision		Inter-day[b] precision	
		Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
5[c]	85.9	5.3	6.5	6.6	7.8
50[c]	92.7	4.9	5.8	6.3	8.4
100[c]	93.4	2.3	3.2	3.4	6.7
100[d]	91.8	3.3	8.3	2.9	9.6
1000[d]	84.6	2.7	3.9	4.8	7.8
5000[d]	90.4	4.5	3.1	5.7	6.4

[a]The analyses were performed in the same day; RSD, relative standard deviation.
[b]The analyses were performed on three different days within 3 months.
[c]Free warfarin.
[d]Total warfarin.

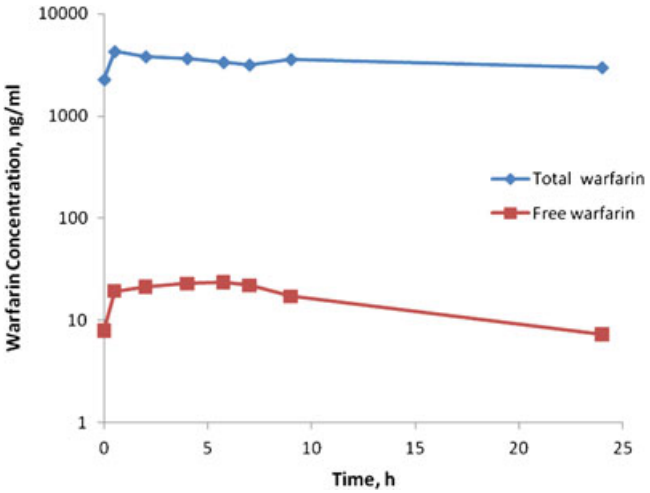

Figure 2. Warfarin plasma concentration (free and total) time profiles using UPLC MS/MS assay following a 30 mg daily doses of warfarin in a Saudi patient.

Table 2. Pharmacokinetic parameters of free and total warfarin after 30 mg dose in a Saudi patient using UPLC MS/MS

Parameters	Free	Total
C_{max}[a] (ng/mL)	23.7	4279.8
T_{max}[a] (h)	5.75	0.5
$AUC_{0-\tau}$ (mg h/L)	0.368	79.6
Cl/F (L/h)	81.5	0.377
$t_{1/2}$ (h)	12.2	56.5
F_u	0.0052 ± 0.0016	

[a]Calculated from the actual data.

0.0052 ± 0.0016, which was lower than most literature suggested for warfarin. This may explain in part warfarin resistance in this patient. It should be mentioned that, although the patient was taken different drugs, none of them interfere with warfarin detection using the MS/MS. Even though it seems that free warfarin concentrations should be monitored for better patient clinical management, more patient data is needed to confirm that, acquisition of which is currently underway.

Conclusion

In this report a specific and sensitive UPLC MS/MS method for the determination of total and free warfarin concentrations in human plasma was described. Chromatographic conditions have been optimized to be simple and rapid for the analysis of large numbers of plasma samples. The method was completely validated and it offers significant advantages over previously reported different methods. Therefore, the proposed method is useful for the application to both total and free warfarin pharmacokinetics and interactions. The results were used to generate profiles and to calculate the pharmacokinetic parameters of free and total warfarin. This assay will be utilized in further investigations of warfarin drug or food supplement interactions in a larger patient population.

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