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Measurement of free concentrations of highly protein-bound warfarin in plasma by ultra performance liquid chromatography–tandem mass spectrometry and its correlation with the international normalized ratio

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ABSTRACT

Background: A novel method has been developed for the determination of free warfarin in human plasma by ultra performance liquid chromatography combined with tandem mass spectrometry (UPLC–MS/MS).
Methods: The plasma filtrate was prepared by a high-speed ultrafiltration (UF), and was then extracted by a liquid/liquid extraction (LLE) with ethyl acetate. The chromatographic separation was performed on an Acquity UPLC™ BEH C_{18} (2.1 mm × 50 mm, 1.7 μm, Waters) with a mobile phase consisting of 0.1% formic acid/acetonitrile (50:50, v/v). The analyses were carried out by multiple reaction monitoring (MRM) using the precursor-to-product combinations of *m/z* 307.3→161.2 for warfarin and *m/z* 347.2→161.3 for Cl-War.
Results: The lower limit of quantification (LLOQ) was 0.25 ng/ml and the assay exhibited a linear range of 0.25–16 ng/ml and gave a correlation coefficient (*r*) of ≥ 0.9999. Quality control samples (0.5, 2, 8 ng/ml) in 5 replicates from 3 different runs of analysis demonstrated intra-assay CVs of 3.0–10.2%, inter-assay CVs of 4.2–12.0%, and an overall accuracy of 85–115%.
Conclusions: The method can be applied to analyze the correlation of free concentration of warfarin and the international normalized ratio (INR).

1. Introduction

Warfarin, as a vitamin K antagonist, is the most commonly prescribed anticoagulant drug for the prophylaxis and treatment of venous and arterial thromboembolic disorders [1,2]. Because of a low therapeutic index, bleeding frequently complicates anticoagulation with warfarin. In order to ensure the effectiveness and safety of warfarin, the dosage must be adjusted accurately and frequently, which now is critically dependent on maintaining the prothrombin time (PT), expressed as the international normalized ratio (INR), within the therapeutic range [3]. However, INR still has its limitations in detecting factors such as patient compliance, resistance to anticoagulants, drug interaction and food variety and so on. Recently, more and more complications due to an unsuitable anticoagulant regimen have been reported.

Determination of the plasma concentration of warfarin can facilitate diagnosis and allows for the effective treatment of severe intoxication. Plasma concentration can also be helpful in distinguishing noncompliance from genuine anticoagulant resistance. Therefore, many studies have been performed to measure the plasma concentrations of warfarin and its correlation with INR [4,5]. It has been reported that the correlation of warfarin dosage or concentration with INR was very poor, although concentration monitoring has a role in research/development and may show value clinically.

Protein-bound drugs are too large to traverse the cell membrane, so in most cases only free unbound drugs are capable of entering cells and reaching the action site to generate a pharmacological response. Thus, the free drugs are an important determinant of pharmacodynamic activities. Pharmacokinetic (PK) parameters generated from free drug concentrations are often more clinically relevant than those derived from the total drug concentrations. The therapeutic ranges for most drugs have been established in terms of total drug concentration, but this may be sub-optimal for therapeutic monitoring of drugs that bind extensively to plasma proteins and may thus show inconsistent free fractions over the therapeutic range. Because it is highly protein-bound, the free concentration of warfarin was very low, and many reported methods cannot satisfy this LLOQ and no free concentration methods have been reported so far [6–9]. Therefore, in this study, a novel method has been developed for the determination of free warfarin in human plasma by UPLC–MS/MS. The chromatography time is 3 min, which is much shorter compared to the previous reports. Free concentration of warfarin in plasma was determined in 108 patients on oral anticoagulants after heart valve replacement, and the correlation of free concentration or dosage with INR was also evaluated.

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2. Materials and methods

2.1. Chemical reagents

Warfarin (purity >98%) was from the National Institute for Control of Pharmaceutical and Biological Products (China). Coumachlor (Cl-War, purity >98%) was from Sigma Chemical Co. (St. Louis, MO). High-performance liquid chromatography (HPLC) grade acetonitrile and methanol were from SK Chemicals (Korea). HPLC grade water was produced by a Milli-Q Reagent Water System (Millipore, MA, USA). Formic acid was from Pierce (Rockford, IL). Ethyl acetate and other chemicals and solvents used were analytical grade.

2.2. Preparation of standards

A stock solution of warfarin was prepared by dissolving the weighed reference compound in methanol to give a final concentration of 1 mg/ml. This solution was serially diluted with 50% methanol to achieve standard working solutions at concentrations of 2.5, 5, 10, 20, 40, 80 and 160 ng/ml for warfarin. A 100 ng/ml internal standard working solution was prepared by diluting the stock solution of Cl-War of 1 mg/ml with 50% methanol.

Each collected blood sample was immediately centrifuged at 1500 ×*g* for 5 min and plasma was transferred into a Nanosep 10K (Pall Life Sciences, Ann Arbor, MI). The device was filled with 1.0 ml of plasma, equilibrated for 1 h at 37 °C. Then the samples were placed in a temperature controlled centrifuge at 2400 ×*g* for 10 min. It was necessary to preheat the centrifuge to reach 37 °C by rotating it for at least 1 h at 3355 ×*g*. The collection device was weighed before and after ultrafiltration to determine the sample amount and the filtrate samples were kept frozen at −70 °C until use.

2.3. Sample preparation

Aliquots (200 μl) of human free plasma filtrate (HFPF), spiked with internal standard working solution (20 μl) and 0.1 mol/l acetic acid (20 μl), were vortex-mixed for 30 s and then extracted with ethyl acetate (1 ml) for 2 min using a vortex mixer. After centrifugation at 5000 ×*g* for 5 min, the upper organic phase (800 μl) was transferred into clean tubes and evaporated under nitrogen. The residue was then reconstituted in 100 μl 0.1% formic acid and methanol (1:1, v/v) immediately before analysis. After centrifugation at 14,000 ×*g* for 10 min at 4 °C the supernatant was transferred and 7.5 μl was used for analysis.

2.4. Instrumentation

The Ultra Performance™ liquid chromatography (UPLC) was performed on a Waters Acquity UPLC™ system, which was equipped with a binary solvent delivery manager, and a sample manager. Mass spectrometry (MS) was performed on a Waters Micromass® Quattro Premier™ tandem quadropole mass spectrometer. The LC/MS system control was by a Masslynx™ 4.1 with QuanLynx™ Application Manager.

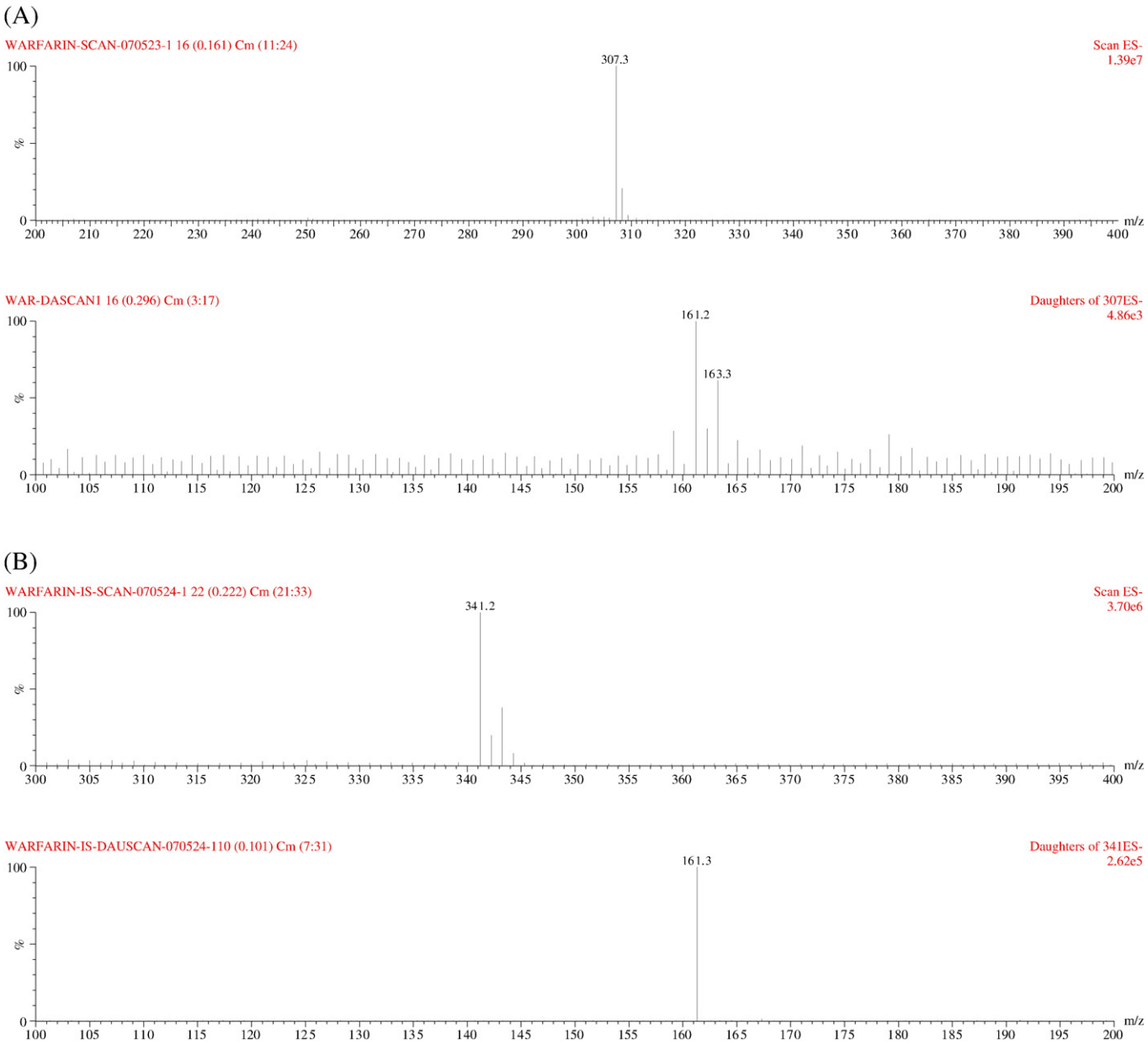

Fig. 1. Full scan and daughter scan product ion mass spectra of $[M–H]^-$ of warfarin (A) and Cl-War (B).