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Monitoring the Distribution of Warfarin in Blood Plasma

Annika M. Rosengren,[†,‡] Björn C. G. Karlsson,[†,‡] and Ian A. Nicholls*[,†,‡,§]

[†]Bioorganic and Biophysical Chemistry Laboratory, School of Natural Sciences, Linnæus University, SE-391 82 Kalmar, Sweden

[‡]SensiKal AB, Öltappargränd 10, SE-393 64 Kalmar, Sweden

[§]Department of Chemistry, Uppsala University, SE-751 23 Uppsala, Sweden

Supporting Information

ABSTRACT: Warfarin is an anticoagulant drug extensively used in the treatment and prevention of thrombotic disorders. Previous studies have shown that warfarin binds extensively to blood plasma proteins and that only a small fraction of the drug is unbound and thus available for therapeutic function. Both warfarin's narrow therapeutic window and the susceptibility of anticoagulant function to patient-dependent factors necessitate regular monitoring. In this study, we have shown that the lifetimes for each of the various bound and free forms of the drug in blood plasma can be quantified in situ by time-correlated single-photon counting fluorescence spectroscopy over the clinically significant concentration range. A relationship between the blood coagulation and the distribution of fluorescence lifetimes was observed. The in situ detection of clinically relevant concentrations of warfarin in its respective bound and unbound forms could provide a prognostic tool for use in patient treatment.

KEYWORDS: *warfarin, coagulation, plasma, TCSPC, fluorescence spectroscopy*

Warfarin has been used as an anticoagulant drug since the 1950s, though despite its long and widespread use, aspects of its biological function are not yet fully understood. Warfarin has a narrow therapeutic window, and the dose required for the maintenance of a therapeutic effect differs widely from patient to patient.[1] Traditionally, the anticoagulant effect of warfarin is measured by determining the clotting tendency of blood (prothrombin time). Because of the difficulties in maintaining patient coagulation response within the therapeutic window through management of warfarin dosing, regular monitoring is often required.

Efforts to correlate patient warfarin concentration and coagulation have thus far proved insufficient for development of a clinically viable diagnostic strategy.[2] This may be attributed to the complex mechanisms underlying warfarin's bioavailability, in particular that some 99% of warfarin in blood is bound to the Sudlow binding sites of human serum albumin (HSA) and that existing methods do not differentiate this from the remaining unbound 1% available for eliciting biological function.[3] In previous studies, liquid chromatography has been used when developing methods to determine the amount of free warfarin in patient blood.[4,5] However, this approach is time-consuming and necessitates several extraction steps.

A key factor steering warfarin's bioavailability is the inherent molecular-level diversity of its structure. We have previously through a series of both theoretical and spectroscopic studies investigated the intricate interplay between warfarin's various isomeric forms.[6−10] In particular, we have studied the impact of molecular environment on the isomeric distribution and the spectroscopic characteristics of warfarin, making it possible to develop a detection method that can discriminate between warfarin in its various isomeric states and environments, for example, bound to protein, polymeric synthetic receptors, and as a general molecular probe.[10−12] Here, the spectroscopic characteristics of warfarin in different environments have been used to develop a new detection technique where it is possible to measure directly on patient blood plasma and simultaneously determine the bound versus unbound amount of warfarin.

Blood was collected from two patients undergoing warfarin treatment, the blood was centrifuged, and the plasma was collected. Plasma from each patient was then divided, one portion used for coagulation measurements using the standard Owren PT test,[13] and the other portion was diluted with PBS buffer for later fluorescence spectroscopic measurements. A series of solutions containing pooled plasma were collected and prepared in the same way as described above, although with a varying amount (0−15 μM) of sodium warfarin. To allow for the added warfarin to bind to the proteins in plasma, all tubes were incubated at room temperature for 15 min.

A series of fluorescence spectroscopic measurements were performed on the spiked blood plasma samples using a clinical relevant concentration regime (0−15 μM).[2,14] The samples were analyzed using steady-state fluorescence spectroscopy, but because these measurements do not discriminate between the bound and the unbound warfarin, measurements determining the fluorescence lifetimes were also performed. Spectra from the steady-state fluorescence spectroscopic measurements are presented in in Figures S1 and S2 in the Supporting Information.

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The relation between the total amount of warfarin and the relative amounts of unbound and plasma protein bound is illustrated in Figure 1 and also in Table S1 in the Supporting Information. As the anticoagulant effect of warfarin treatment is based on the inhibition of the vitamin K epoxide reductase cycle, which results in a reduction in the concentration of important coagulation factors,[15,16] the prothrombin time was not measured in the spiked blood plasma samples. The relative amplitude of the short fluorescence lifetime originating from unbound warfarin decreases as the warfarin concentration increases, while the two relative amplitudes of the bound forms of warfarin increase as the warfarin concentration increases (Figure 1).

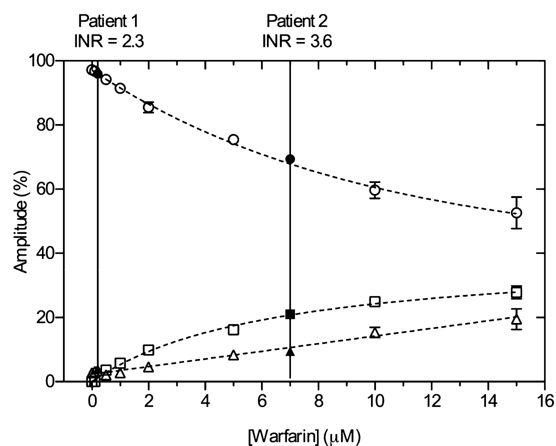

Figure 1. TCSPC fluorescence spectroscopic measurements on warfarin-spiked blood plasma (0−15 μM) and from blood plasma from patients on warfarin treatment (filled symbols). The graph illustrates the relative amplitudes of the different fluorescence lifetimes, 0.1 (circle), 1.5 (square), and 3.5 ns (triangle), at different amounts of warfarin. Data, from three replicates, are presented as means ± standard errors.

Second, time-correlated single-photon counting (TCSPC) fluorescence spectroscopic measurements were performed on samples from two patients undergoing warfarin therapy (decay profiles presented in Figure S3 in the Supporting Information). The patient samples were also analyzed to determine the international normalized ratio (INR), a measure of the coagulation rate of blood, where INR > 1 indicates a longer prothrombin time of the patient blood as compared to a control.[17] Results from TCSPC fluorescence spectroscopic measurements on the patient blood plasma samples imply that a higher INR value is correlated to a lower relative amount of unbound warfarin (the corresponding data using amplitude derived concentrations are shown in Figure S4 in the Supporting Information). While further study is required to assess the general significance of this observation, the insight gained with this strategy suggests the possibility of using this method to differentiate between the unbound and the bound warfarin and to use these results to forecast the anticoagulant effect of warfarin. Moreover, this technique should be useful for improving our understanding of warfarin's bioavailability.

In summary, we here present a method for discriminating between bound and unbound warfarin in blood plasma based upon TCSPC fluorescence spectroscopy. The applicability of the method to the clinically significant concentration range was demonstrated by relating the amplitudes of the observed fluorescence lifetimes and coagulation.

■ EXPERIMENTAL PROCEDURE

Blood was collected from two patients undergoing warfarin treatment using Vacutainer plastic tubes (2.7 mL) with sodium citrate buffer from Becton Dickinson and Co. (Plymouth, United Kingdom). This study was performed in accordance with institutional regulations. The blood was centrifuged (3000*g*, 20 min, room temperature), and the plasma was collected. All equipment that came in contact with blood and plasma was furnished with a Corline heparin surface according to recommendations by the manufacturer (Corline AB, Uppsala, Sweden). Plasma from each patient was then divided, one portion used for coagulation measurements using the standard Owren PT test,[13] and 5 mL was diluted with PBS buffer [15 mL (0.05 M $NaHPO_4/Na_2PO_4$, 0.01 M NaCl, pH 7.3)] for later fluorescence spectroscopic measurements. A series of solutions containing pooled plasma collected and prepared in the same way as described above, although with a varying amount (0−15 μM) of sodium warfarin (Sigma Aldrich, St. Louis, MO), were prepared. To allow for the added warfarin to bind to the proteins in plasma, all tubes were incubated at room temperature for 15 min on a rocking table and then stored at −80 °C until use.

All plasma samples were analyzed using steady-state fluorescence spectroscopy, and experimental details are presented in the Supporting Information. Plasma samples were then analyzed by TCSPC fluorescence spectroscopy using an instrument (IBH 5000M, Jobin Yvon IBH Ltd., Glasgow, United Kingdom) equipped with IBH reconvolution software. The decay times with associated amplitudes were obtained using excitation pulses (334 nm, 1.0 MHz repetition rates) from a light emitting diode (NanoLED-340, HORIBA Jobin Yvon IBH). A single grating monochromator (model 5000 M IBH) with a spectral bandwidth set to 32 nm was used. The data were collected in 4048 channels, and the time calibration was 13.4 ps/channel. The fluorescence emission was detected with an IBH TBX-04 photon detection module. The full width at half-maximum of the instrumental response function was typically around 794 ps, which was measured with a suspension of silica particles (Ludox TMA-34 Sigma-Aldrich) dissolved in deionized water. Experiments were monitored at 370 nm. Time-resolved fluorescence data were analyzed using IBH DAS6 decay analysis software.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedure and spectra from the steady-state fluorescence spectroscopic measurements together with detailed data from the time-resolved fluorescence spectroscopic measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: ian.nicholls@lnu.se.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare the following competing financial interest(s): The authors are part owners of a start-up company that holds a patent describing an invention that is partly reflected in the results presented in this paper.

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■ ABBREVIATIONS

TCSPC, time-correlated single-photon counting; HSA, human serum albumin; INR, international normalized ratio

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