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Single step separation of plasma from whole blood without the need for centrifugation applied to the quantitative analysis of warfarin

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Introduction

Dried plasma sample collection and storage from whole blood without the need for centrifugation separation and refrigeration opens new opportunities in blood sampling strategies for quantitative LC/MS/MS bioanalysis. Plasma samples were generated by gravity filtration of a whole blood sample through a laminated membrane stack allowing plasma to be collected, dried, transported and analysed by LC/MS/MS. This novel plasma separation card (PSC) technology was applied to the quantitative LC/MS/MS analysis of warfarin, in blood samples. Warfarin is a coumarin anticoagulant vitamin-K antagonist used for the treatment of thrombosis and thromboembolism. As a result of vitamin-K recycling being inhibited, hepatic synthesis is in-turn inhibited for blood clotting factors as well as anticoagulant proteins. Whilst the measurement of warfarin activity in patients is normally measured by prothrombin time by international normalized ratio (INR) in some cases the quantitation of plasma warfarin concentration is needed to confirm patient compliance, resistance to the anticoagulant drug, or diet related issues. In this preliminary evaluation, warfarin concentration was measured by LC/MS/MS to evaluate if PSC technology could complement INR when sampling patient blood.

Materials and Methods

Sample preparation

Warfarin standard was dissolved in water containing 50% ethanol + 0.1% formic acid, spiked (60uL) to whole human blood (1mL) and mixed gently. 50uL of spiked blood was deposited onto the PSC. After 3 minutes, the primary filtration overlay was removed followed by 15 minutes air drying at room temperature. The plasma sample disc was prepared directly for analysis after drying. LC/MS/MS sample preparation involved vortexing the sample disk in 40uL methanol, followed by centrifugation 16,000g 5 min. 20uL supernatant was added directly to the LCMS/MS sample vial already containing 80uL water (2uL analysed). Control plasma comparison was prepared by centrifuging remaining blood at 1000g for 10min. 2.5uL supernatant plasma was taken, 40uL methanol added, and prepared as PSC samples. LCMS/MS sample injection volume, 2uL.

LC-MS/MS analysis

Warfarin was measured by MRM, positive negative switching mode (15msec).

LC/MS/MS System	: Nexera UHPLC system + LCMS-8040 Shimadzu Corporation
Flow rate	: 0.4mL/min (0-7.75min), 0.5mL/min (7.5-14min), 0.4mL/min (15min)
Mobile phase	: A= Water + 0.1% formic acid B= Methanol + 0.1% formic acid
Gradient	: 20% B (0-0.5 min), 100% B (8-12 min), 20% B (12.01-15 min)
Analytical column	: Phenomenex Kinetex XB C18 100 x 2.1mm 1.7um 100A
Column temperature	: 50°C
Ionisation	: Electrospray, positive, negative switching mode
Desolvation line	: 250°C
Drying/Nebulising gas	: 10L/min, 2L/min
Heating block	: 400°C

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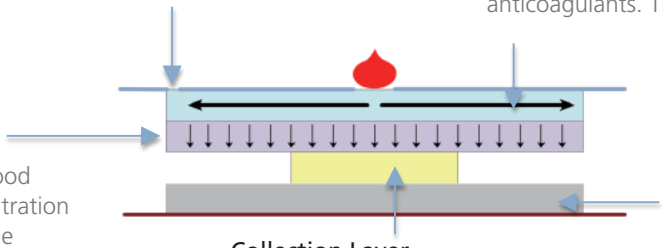
Design of plasma separator technology

Control Spot:
[Determines whether enough blood was placed on the card].

Spreading Layer
[Lateral spreading layer rapidly spreads blood so it will enter the filtration layer as a front while adding buffers and anticoagulants. The lateral spreading rate is 150um/sec].

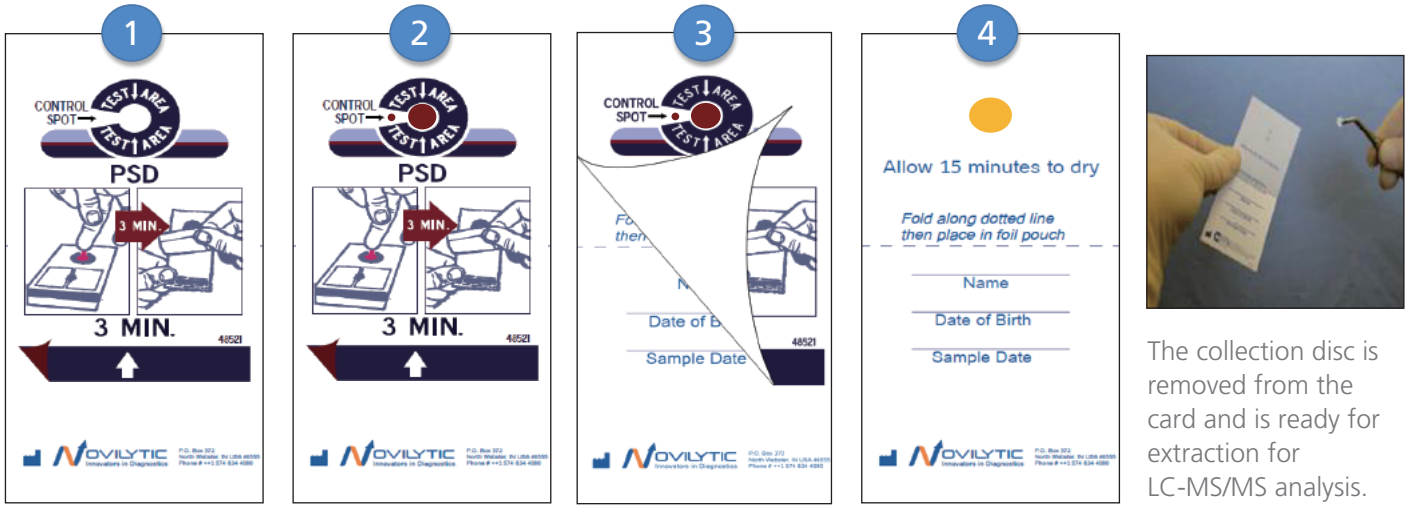


Filtration Layer
[Filtration layer captures blood cells by a combination of filtration and adsorption. The average linear vertical migration rate is approximately 1um/sec].

Isolation Screen
[Precludes lateral wicking along the card surface].

Collection Layer
[Loads with a specific aliquot of plasma onto a 6.35mm disc]. Although flow through the filtration membrane is unlikely to be constant throughout the plasma extraction process, the average loading rate of the Collection Disc was 13 nL/sec. This corresponds to a volumetric flow rate into the Collection Disc of 400 pL/mm^2/sec.

Plasma separation workflow



A NoviPlex card is removed from foil packaging.

Approximately 50uL of whole blood is added to the test area.

After 3 minutes, the top layer is completely removed (peeled back).

The collection disc contains 2.5uL of plasma. Card is air dried for 15 minutes.

The collection disc is removed from the card and is ready for extraction for LC-MS/MS analysis.

Figure 1. Noviplex workflow.

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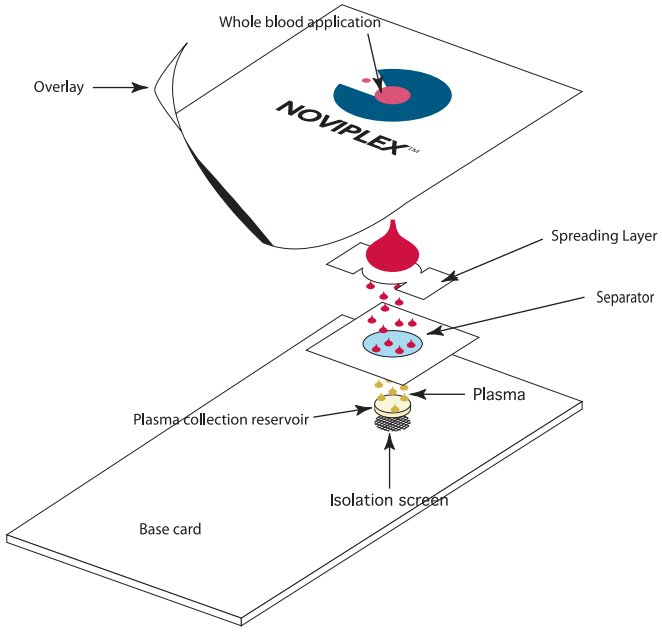

Figure 2. Applying a blood sample, either as a finger prick or by accurately measuring the blood volume, to the laminated membrane stack retains red cells and allows a plasma sample to be collected. The red cells are retained by a combination of adsorption and filtration whilst plasma advances through the membrane stack by capillary action. After approximately three minutes the plasma Collection Disc was saturated with an aliquot of plasma and was ready for LC/MS/MS analysis.

Results

Comparison between plasma separation cards (PSC) and plasma

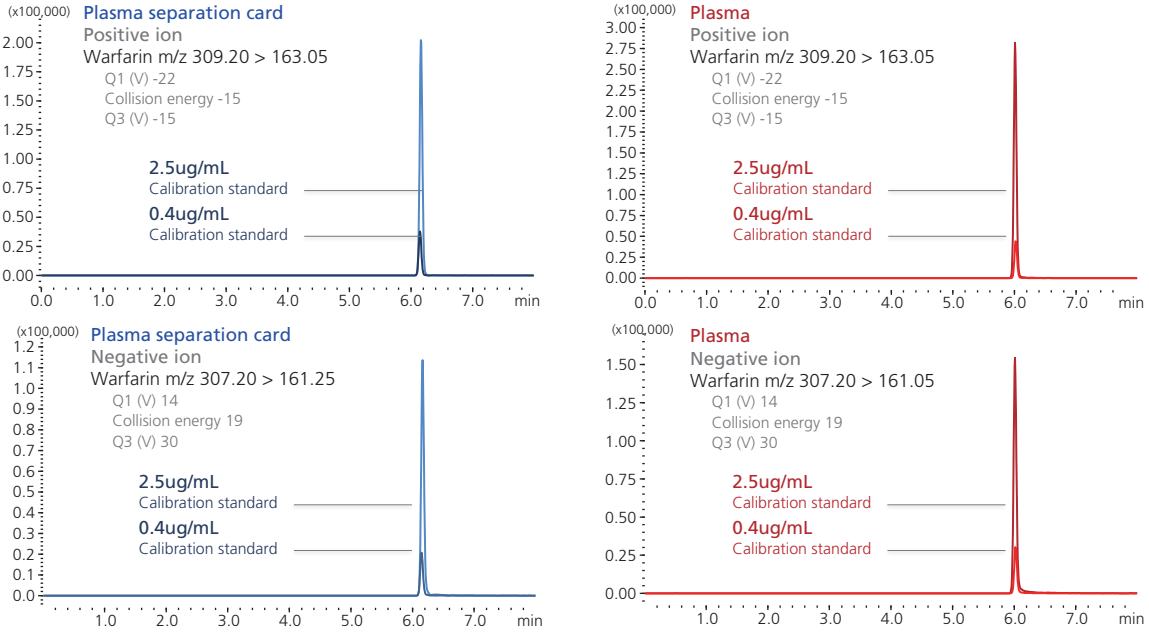

Figure 3. Comparison between the warfarin response in both positive and negative ion modes for warfarin calibration standards at 2.5ug/mL and 0.4ug/mL extracted from the plasma separation cards and a conventional plasma sample. There is a broad agreement in ion signal intensity between the 2 sample preparation techniques.

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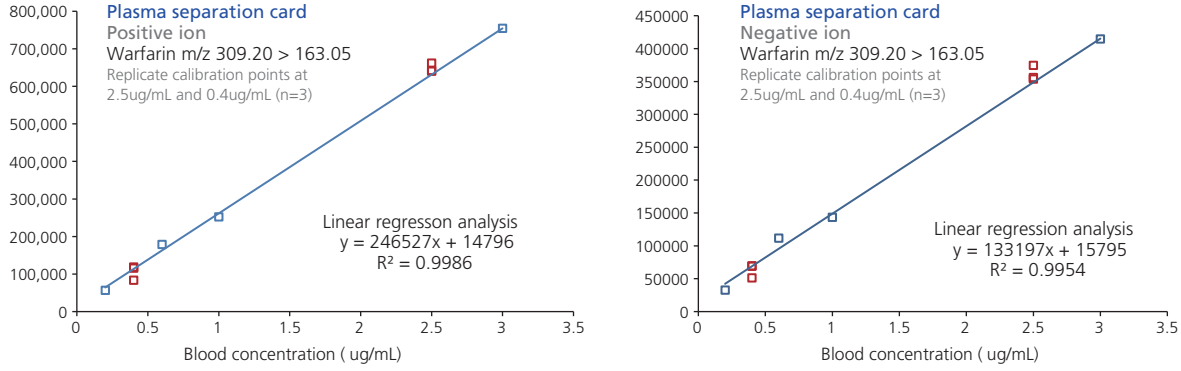

Figure 4. In both ion modes, the calibration curve was linear over the therapeutic range studied for warfarin extracted from PSC's (calibration range 0-3ug/mL, single point calibration standards at each level with the exception of replicate calibration points at 2.5ug/mL and 0.4ug/mL (n=3); r2>0.99 for PSC analysis [r2>0.99 for a conventional plasma extraction]).

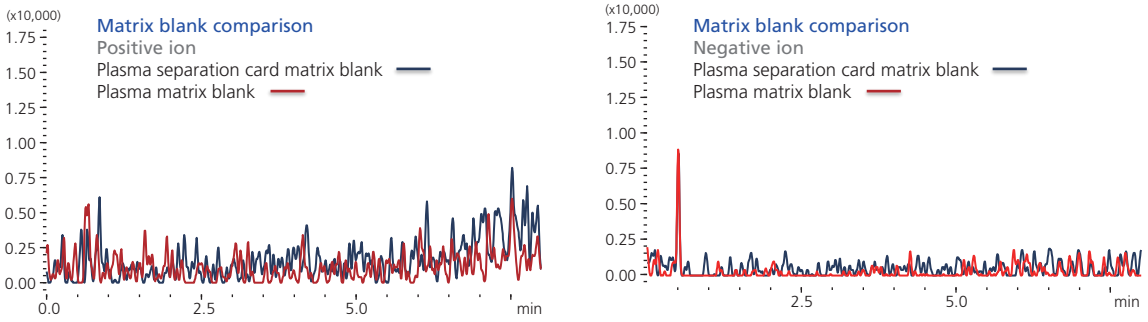

Figure 5. Matrix blank comparison. In both ion modes, the MRM chromatograms for PSC and plasma are comparable. Warfarin ion signals were not detected in the any PSC or plasma matrix blank.

Plasma separation card comparison

The drive to work with smaller sample volumes offers significant ethical and economical advantages in pharmaceutical and clinical workflows and dried blood spot sampling techniques have enabled a step change approach for many toxicokinetic and pharmacokinetic studies. However, the impressive growth of this technique in the quantitative analysis of small molecules has also discovered several limitations in the case of sample instability (some enzyme labile compounds, particularly prodrugs, analyte stability can be problematic), hematocrit effect and background interferences of DBS. DBS also shows noticeable effects on many lipids dependent on the sample collection process. To compare PSC to plasma lipid profiles the same blood sample extraction procedure applied for warfarin analysis was measured by a high mass accuracy system optimized for lipid profiling.

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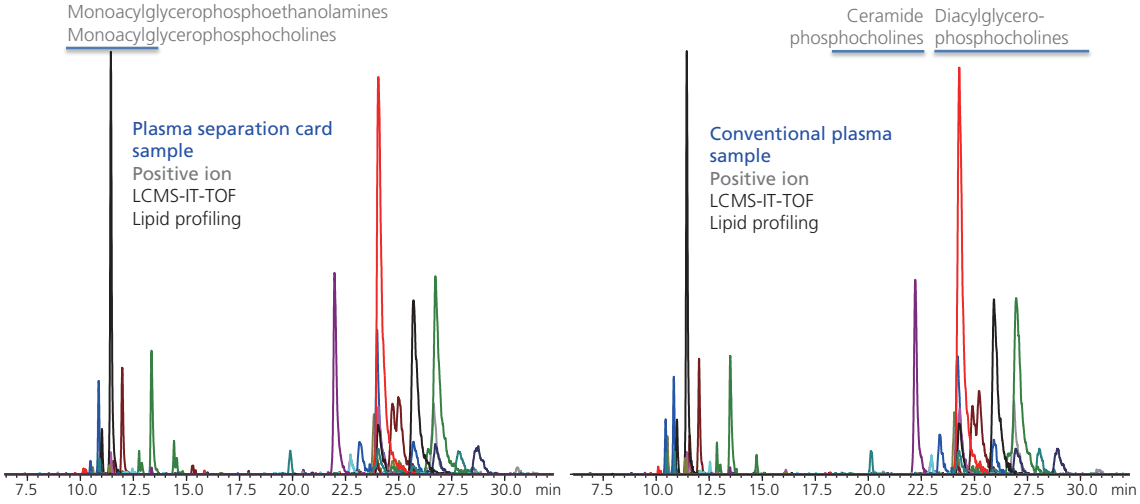

Figure 6. Lipid profiles from the same human blood sample extracted using a plasma separation card (left hand profile) compared to a conventional plasma samples (centrifugation). Both lipid profiles are comparable in terms of distribution and the number of lipids detected (the scaling has been normalized to the most intense lipid signal).

Conclusions

- In this limited study, plasma separation card (PSC) sampling delivered a quantitative analysis of warfarin spiked into human blood.
- PSC generated a linear calibration curve in both positive and negative ion modes (r2>0.99; n=5);
- The warfarin plasma results achieved by using the PSC technique were in broad agreement with conventional plasma sampling data.
- The plasma generated by the filtration process appears broadly similar to plasma derived from conventional centrifugation.
- Further work is required to consider the robustness and validation in a routine analysis.

References

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- Radwan, M.A., Bawazeer, G.A., Aloudah, N.M., Aluadeib, B.T., Aboul-Enein, H.Y. **(2012)** Determination of free and total warfarin concentrations in plasma using UPLC MS/MS and its application to patient samples. *Biochemical Chromatography*, **26**, 6-11

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