Dried Blood Spots for Quantitative Bioanalysis and Their Stabilizing Effects

R6257

Melanie A. Rehder Silinski and Brenda L. Fletcher, RTI International, Research Triangle Park, NC

Purpose

Use of dried blood spots (DBS) for quantitative bioanalysis of drugs is gaining recognition for its advantages over traditional plasma sampling. Tiny sample volumes are required, and techniques for sample collection and preparation are faster and simpler. Blood-borne pathogens are inactivated by the card, and analytes, notably those that are sensitive to native enzymes in plasma, are believed to be stabilized, allowing safe sample handling, shipment, and storage at ambient conditions.

The goal of this work was to develop DBS methods for different compounds, and investigate the stabilizing effects of the DBS cards.

Methods

Fresh rat blood was spiked with selected analyte(s), spotted, and allowed to dry ≥ 2 hrs. A disc was punched from the spot, extracted, and analyzed by HPLC-UV or UPLC-MS/MS methods. Investigated analytes were lovastatin (undergoes rapid ester hydrolysis in plasma) and resveratrol sulfate and glucuronide metabolites (very broad chemical properties, and resveratrol undergoes *trans-cis* isomerization in UV light). Different extraction solvents were optimized for best analyte recovery with minimal matrix effects. Four different commercially-available cards were investigated. Lovastatin stability was determined with blood that was both treated (with sodium fluoride, NaF) and untreated upon collection; samples were extracted at 0, 2, 4, 6, and 22 hrs. Resveratrol stability was determined for spotted cards stored protected or exposed to UV light at 0, 2, 3, or 20 hrs, and 7 days.

Lovastatin (**Figure 1**), a natural product that is used for lowering cholesterol, is a prodrug which undergoes rapid hydrolysis in plasma by carboxyesterases. For accurate bioanalysis, it is critical to minimize the *ex vivo* hydrolysis for drugs (e.g. lovastatin) as well as potential metabolites (e.g. acyl-glucuronides). The effectiveness of the DBS cards for inactivating the native enzymes upon contact and under different drying conditions was investigated.

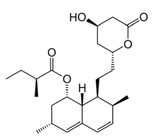


Figure 1. Structure of Lovastatin

Resveratrol (**Figure 2**) is a naturally-occurring phenolic phytoalexin found in grapes and red wine that is believed to play a role in the prevention of cancer, cardiovascular disease, and neurodegenerative diseases. Its predominant metabolites are the 3-sulfate and 3-O-β-D-glucuronide. Their broad differences in chemical properties presented a challenge for finding a suitable DBS extraction procedure for all three analytes. In addition, *trans-resveratrol* isomerizes to the *cis* form in UV light. While light can typically be controlled during bioanalysis, it was of interest to determine the effect of the DBS cards on UV stability.

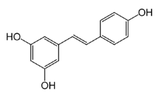


Figure 2. Structure of *Trans*-Resveratrol

Four different commercially-available cards were investigated during these studies:

- DMPK-A (GE Healthcare/Whatman) – smaller spot area; treated to denature proteins
- DMPK-B (GE Healthcare/Whatman) – treated to denature proteins
- BondElut (Agilent) – non-cellulose; reported to have more consistent spot size and better for MS
- Ahlstrom 226(PerkinElmer) – 100% pure cotton, no additives

Results (Lovastatin Method)

Various extraction solvents (combinations of formic acid, methanol, acetonitrile, and water) and techniques (vortex, sonication, etc.) were attempted. The best recoveries (~87% with a BondElut card) for lovastatin were achieved with 80:20 acetonitrile (ACN):water with vortex mixing. The procedure is described as follows:

Fresh rat blood (100 µL) was transferred into separate vials and spiked with 5 µL of lovastatin in ACN and gently mixed by hand. Using a pipet, 5 µL of the spiked blood was added to each card (without touching pipet tip to the card). The card was allowed to dry at least 2 hrs. A disc was punched from the center of the blood spot on the card and transferred to a vial. An aliquot (100 µL) of internal standard (IS; simvastatin in 80:20 ACN:water) plus 5 µL of DI water were added to each vial. Each vial was vortexed ~30 seconds, and centrifuged ~10 min at ~14,000 rpm. Each sample supernatant was transferred to a clean vial and analyzed by HPLC (below).

To determine linearity, a 5-point calibration curve was prepared for DBS from12.5 to 150 µg/mL.

Stability was determined for DBS at 100 µg/mL with blood that was both treated (with NaF) and untreated upon collection. Three different cards were compared. Samples were extracted at time points 0, 2, 4, 6, and 22 hrs.

HPLC Parameters

HPLC: Waters Alliance HPLC
Software: Empower 2, Build 2154
Column: Waters Xbridge C18 (3.0×100 mm, 5 um)
Mobile Phase: A: Water + 0.05% Formic Acid; B: Acetonitrile
Gradient: 55 to 95% B in 4 min, then hold for 1 min
Flow Rate: 0.65 mL/min
Injection Volume: 5.0 µL
Detection: 240 nm

The lovastatin DBS method was linear over the range 12.5 to 150 ug/mL (**Figure 3**). Representative chromatograms for a DBS blank and standards are shown in **Figure 4**.

Figure 3. Calibration Curve for Lovastatin DBS Method

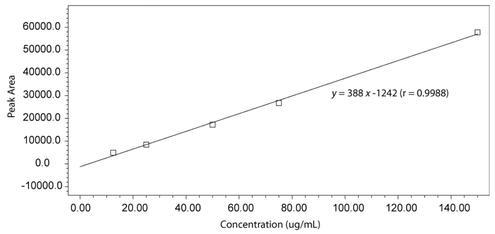
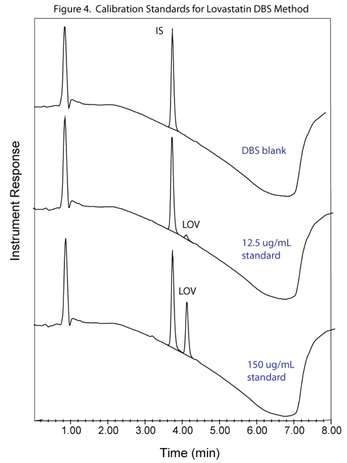


Figure 4. Calibration Standards for Lovastatin DBS Method



Results (Lovastatin Stability)

Representative chromatograms showing the conversion of lovastatin (LOV) to lovastatin hydroxy acid (LHA) are shown in **Figure 5**. After 4 hours in untreated blood, no lovastatin is present, but the hydrolysis is less pronounced when the blood contains sodium fluoride (NaF). When untreated blood is applied to one of the DBS cards (DMPK-A in this case) and allowed to dry for 4 hrs, the hydrolysis appears to be minimized.

Figure 5. Hydrolysis of Lovastatin after 4 hrs

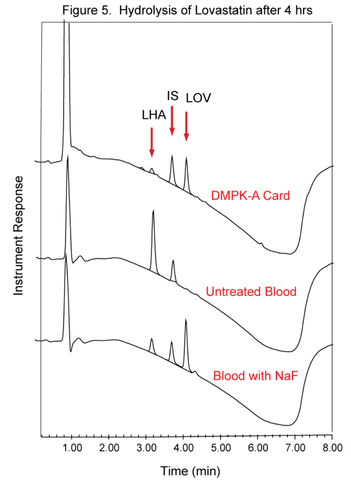


Figure 6 shows a comparison of lovastatin peak area ratio (LOV:IS) over time on three different DBS cards. No significant difference was observed; all three cards appeared to have the same stabilizing effect on lovastatin.

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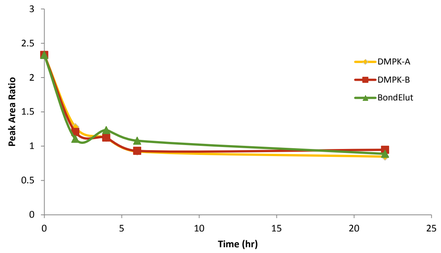
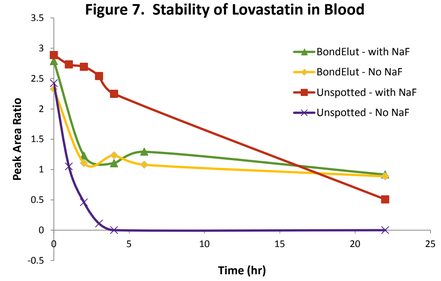
Figure 6. Stability of Lovastatin on Different DBS Cards



For lovastatin, a significant stabilizing effect was observed for unspotted blood treated with NaF vs. untreated (**Figure 7**). For spotted blood, no difference was observed for whether or not the blood was first treated with NaF. With the cards, there was an initial instability during drying, but by ~2-4 hrs lovastatin levels were stabilized. In comparison, lovastatin levels in unspotted, treated blood were still declining.

Figure 7. Stability of Lovastatin in Blood



Results (Resveratrol Method)

For resveratrol and its metabolites, broad differences in chemical properties presented a challenge for finding one suitable comprehensive extraction procedure. Recoveries varied significantly for the different analytes, cards, extraction solvents, and techniques. The best recoveries (50-60%) for resveratrol (RES) and its metabolites (GLUC and SULF) were achieved with 50:50 methanol : mobile phase A (see below) with vortex mixing using the Agilent BondElut cards. Recoveries with the Ahlstrom cards were 30-45%, and 11-15% with the DMPK-B cards. The procedure is described as follows:

Fresh rat blood (100 µL) was spiked with 5 µL of a mixture containing RES, GLUC, and SULF in water and gently mixed by hand. The spiked blood was spotted to the DBS card. The card was allowed to dry at least 2 hrs. A disc was punched from the center of the blood spot on the card and transferred to a vial. An aliquot (100 µL) of extraction solvent [50:50 methanol : mobile phase A (below)] was added to each vial. The vials were vortexed ~30 seconds, and centrifuged ~10 min at ~14,000 rpm. Each sample supernatant was transferred to a clean vial and analyzed by UPLC-MS/MS (below). An example chromatogram is shown in **Figure 8**.

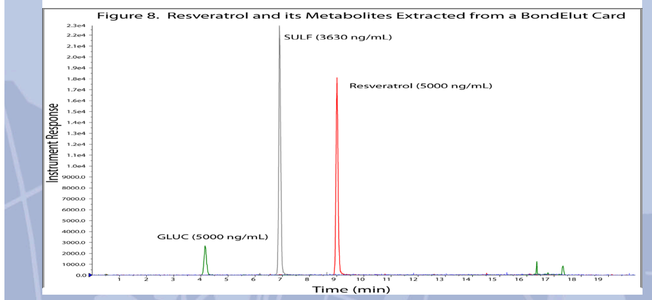
Light/dark stability was determined at ~5 µg/mL for each analyte using the BondElut , DMPK-B, and Ahlstrom cards. Cards were stored either continuously protected or exposed to UV light, and samples were extracted at time points 0, 2, 3, or 20 hrs, and 7 days. No significant differences were observed; *trans-cis* isomerization was not observed in blood.

UPLC-MS/MS Parameters

UPLC-MS/MS: Waters Acquity UPLC; Applied Biosystems 4000 QTRAP MS/MS
Software: Analyst 1.4.2
Column: Waters Acquity HSS T3 (2.1×50 mm, 1.8 um) with HSS T3 (2.1×5 mm, 1.8 um) guard
Mobile Phase: A: 5 mM ammonium acetate in water with 2% 2-propanol; B: Methanol with 2% 2-propanol
Gradient: 15% B for 2 min, ramp to 65% B from 2 to 15 min, ramp to 95% B in 0.5 min; then hold for 1 min
Flow Rate: 0.3 mL/min
Injection Volume: 2.0 µL
Ionization Mode: ESI, negative ion mode
Curtain Gas: Nitrogen, 15 psi
Collision Gas: High
IonSpray Voltage: - 3700 V
Source Temperature: 600 °C
Interface Heater: On
Ion Source Gases: Nitrogen, 60 psi (1); 40 psi (2)
Entrance Potential: -10 V

	RES	GLUC	SULF
MRM Transitions (m/z):	227→185	403 → 227	307 → 227
Dwell Time (msec):	150	150	150
Declustering Potential (V):	-65	-55	-55
Collision Energy (V):	-26	-34	-34
Collision Cell Exit Potential(V):	-15	-11	-11

Figure 8. Resveratrol and its Metabolites Extracted from a BondElut Card



Conclusions

Significant differences in extraction recoveries were observed across different compounds, cards and extraction solvents, indicating that these variables are critical for DBS method development.

DBS cards were shown to stabilize ester hydrolysis, with long-term effects more pronounced than for treatment with a stabilizer. There was a short term instability (during drying of the blood spot), but by ~2-4 hours and up to 22 hours the levels of lovastatin remained unchanged. For comparison, treating unspotted blood with a stabilizer such as sodium fluoride slowed hydrolysis, but lovastatin levels continued to decline even after 22 hours.

Acknowledgement

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