



Quantitative Determination of Riluzole: Plasma with Protein Precipitation Extraction vs. Whole Blood (Dried Blood Spots) with Liquid-Liquid Extraction

Authors

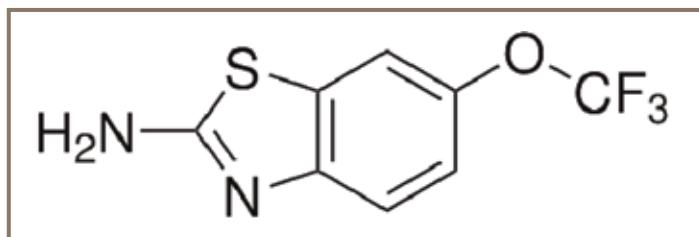
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Objectives

Riluzole (Figure 1) is a drug used to treat amyotrophic lateral sclerosis (also known as Lou Gehrig's disease). Two methods for the quantitation of riluzole in human matrices were developed using LC/MS/MS over a range of 5.00 ng/mL to 2000 ng/mL. One method utilized plasma samples and a protein precipitation extraction (PPE); the second method utilized a liquid-liquid extraction (LLE) from whole blood on dried blood spots (DBS). A stable-labeled internal standard was used in both methods. The advantages of DBS over traditional plasma analysis are minimal sample volume requirements and easy handling and storage; however, due to the minimal sample volume collected, DBS typically must overcome the challenge of low sensitivity. Our goal was to develop a DBS method using the identical dynamic range and LC/MS platform as the plasma method so that we are able to fully compare the performance of the two assays. Both methods were GLP validated and proved to be accurate, precise, and robust.

FIGURE 1. Chemical Structure of Riluzole

(MRM Transitions: Riluzole: 235 → 138; Riluzole-¹³C,¹⁵N₂: 238 → 141)





Method: Liquid-Liquid Extraction with Whole Blood on DBS

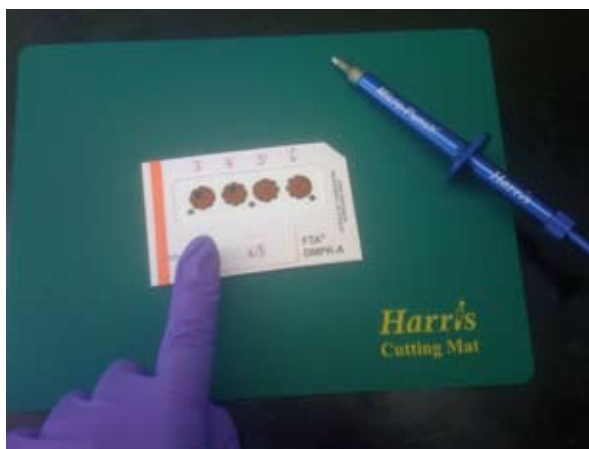
1. LIQUID-LIQUID EXTRACTION:

Sample Collection: 25.0 μ L on Whatman DMPK-A (Figure 2)
Cut Size: 5mm punch (~8 μ L whole blood)
DBS Extraction: Water/methanol containing Internal Standard
LLE Solvent: Ethyl acetate
Recon Solvent: Formic acid in water/acetonitrile

2. LC/MS/MS:

Mass Spec: AB Sciex API 5000
Ion Source: Electrospray Ionization (ESI); positive ion mode
Source Temperature: 500 °C
Column: Waters XBridge C8 50x2mm
Flow Rate: 0.50 mL/min
Mobile Phase: A: formic acid in water
B: acetonitrile and methanol
Needle Wash: 1: formic acid water/acetonitrile 2: formic acid in water
LC Program: Isocratic
Injection Volume: 10 μ L (~0.2 μ L of original whole blood sample)

FIGURE 2. Whole blood (DBS) sample on Whatman FTA DMPK-A card.





Method: Protein Precipitation Extraction with Plasma

1. PROTEIN PRECIPITATION EXTRACTION:

Aliquot Size: 50 μ L plasma
Precipitation Solvent: Formic acid in acetonitrile
Diluents: HPLC grade water
Final Extract: Formic acid in water/acetonitrile

2. LC/MS/MS:

Mass Spec: AB Sciex API 5000
Ion Source: Atmospheric Pressure Chemical Ionization (APCI); positive ion mode
Source Temperature: 400 °C
Column: Waters XBridge C8 50x2mm
Flow Rate: 0.50 mL/min
Mobile Phase: A: formic acid in water
B: acetonitrile and methanol
Needle Wash: 1: formic acid water/acetonitrile
2: formic acid in water
LC Program: Isocratic
Injection volume: 20 μ L (~1.2 μ L of original plasma sample)



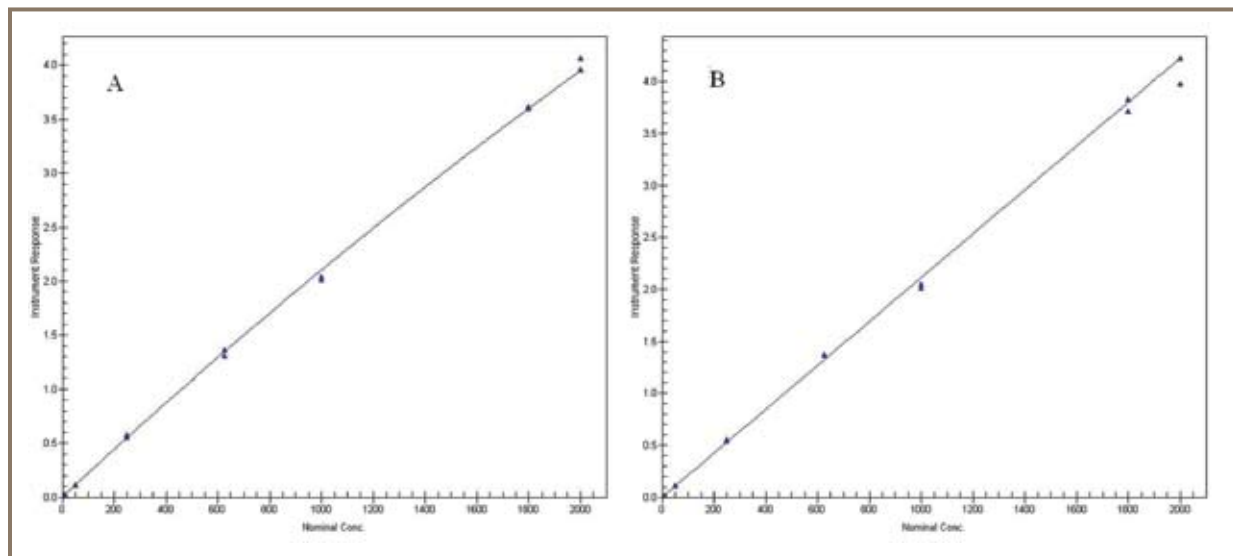
Results and Discussion

- Representative calibration curves (Table 1 and Figure 3) were quadratic for the DBS method ($R^2 > 0.998$) using a weighted $1/x^2$ regression and were linear for the plasma method ($R^2 > 0.997$) using a weighted $1/x^2$ regression.

TABLE 1. Accuracy and Precision of Riluzole Calibration Standards for (Top) Whole Blood DBS and (Bottom) Plasma.

Nominal concentration (ng/mL)		5.00	10.0	50.0	250	625	1000	1800	2000
DBS Method	Precision (%)	8.1	5.6	0.9	3.3	1.4	3.1	1.9	2.2
	Accuracy (%)	99.6	101	98.8	102	99.2	99.6	98.3	102
Plasma Method	Precision (%)	7.2	6.2	6.2	2.7	2.9	2.7	1.9	3.3
	Accuracy (%)	99.8	100	101	103	101	97.3	98.9	99.0
n		6	6	6	6	6	6	6	6

FIGURE 3. Calibration curves for (A) Whole Blood DBS; Quadratic $1/x^2$ and (B) Plasma; Linear $1/x^2$.

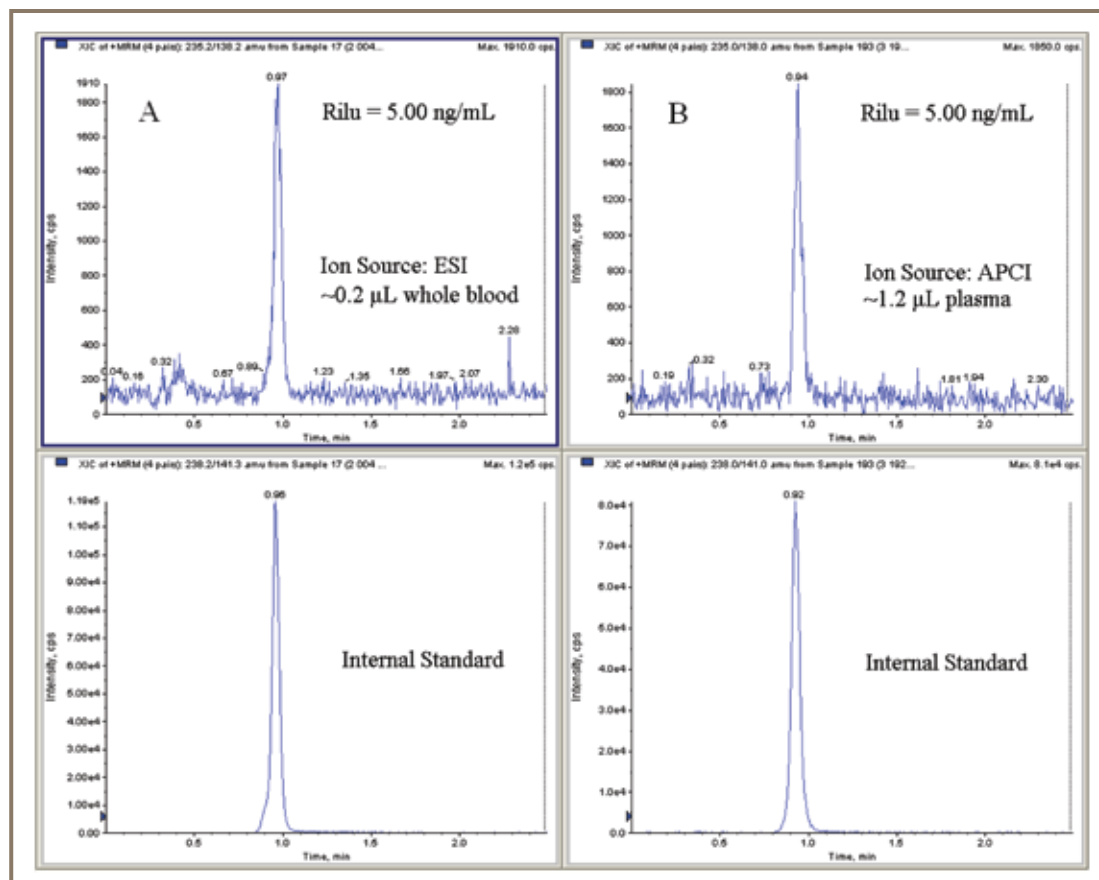




Results and Discussion continued

- Representative chromatograms for both methods are shown in Figure 4 (LLOQ at 5.00 ng/mL) and Figure 5 (ULOQ at 2000 ng/mL). The sensitivity of each method was similar on a Sciex API 5000; however, the DBS method used an ESI source while the plasma method used an APCI source. Comparatively, ESI had roughly 8 times the sensitivity of APCI for riluzole, which corresponds well to the ratio of original sample contained in each injection (0.2 μ L of whole blood vs. 1.2 μ L of plasma).

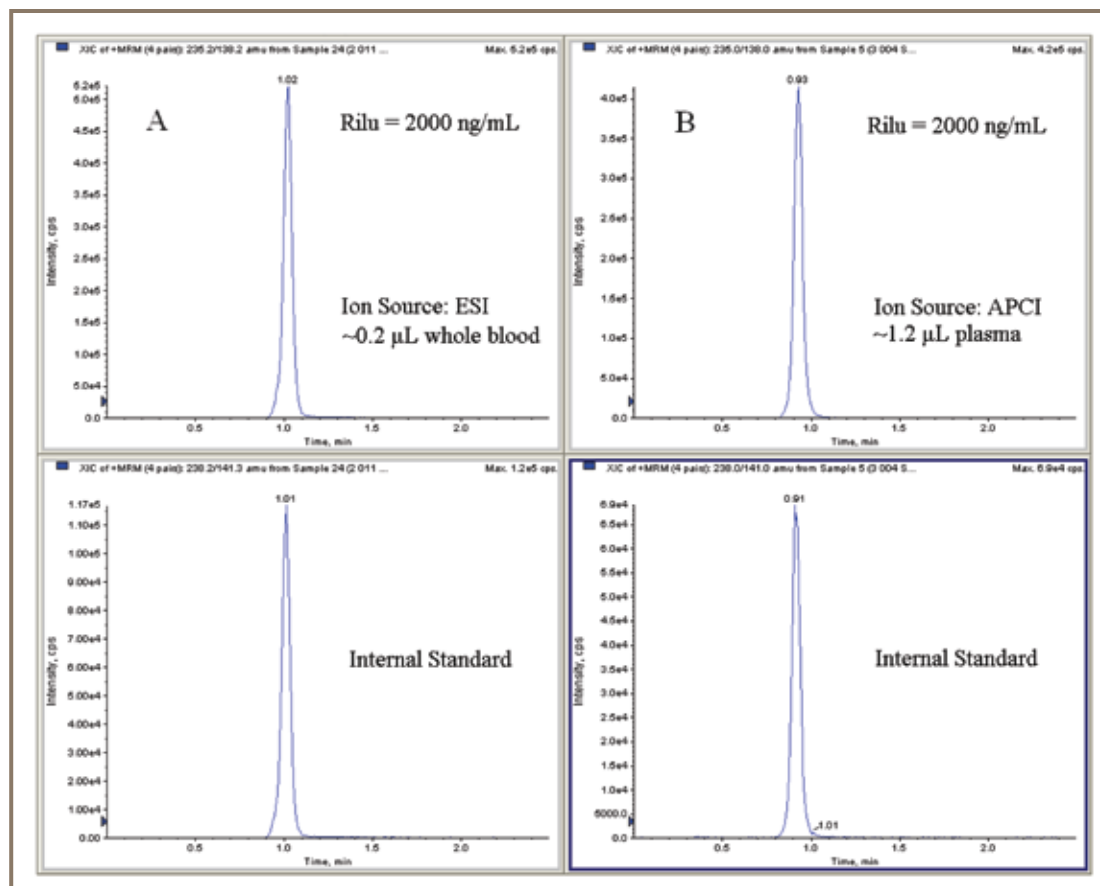
FIGURE 4. Representative LC/MS/MS chromatograms of LLOQ at 5.00 ng/mL for (A) Whole Blood DBS and (B) Plasma.





Results and Discussion continued

FIGURE 5. Representative LC/MS/MS chromatograms of ULOQ at 2000 ng/mL for (A) Whole Blood DBS and (B) Plasma.



- The initial DBS method was developed using acidified acetonitrile to extract riluzole from DBS card. The extraction recovery was good, but low concentration samples were adversely affected by non-phospholipid matrix effects when analyzed by ESI. Plasma samples were similarly affected when precipitated with acidified acetonitrile and analyzed by ESI. The problem was solved by utilizing a liquid-liquid extraction for the DBS method and switching to an APCI source for the plasma method.



Results and Discussion continued

- The relative accuracy and precision of quality control samples analyzed by the two methods is shown in Figure 6 and Table 2, respectively. Results reflect QCs analyzed at LLOQ, Low, Medium, High concentrations over three validation runs (n=6 each run).

FIGURE 6. Accuracy for Riluzole in Quality Control Samples in Whole Blood DBS and Plasma (n=18).

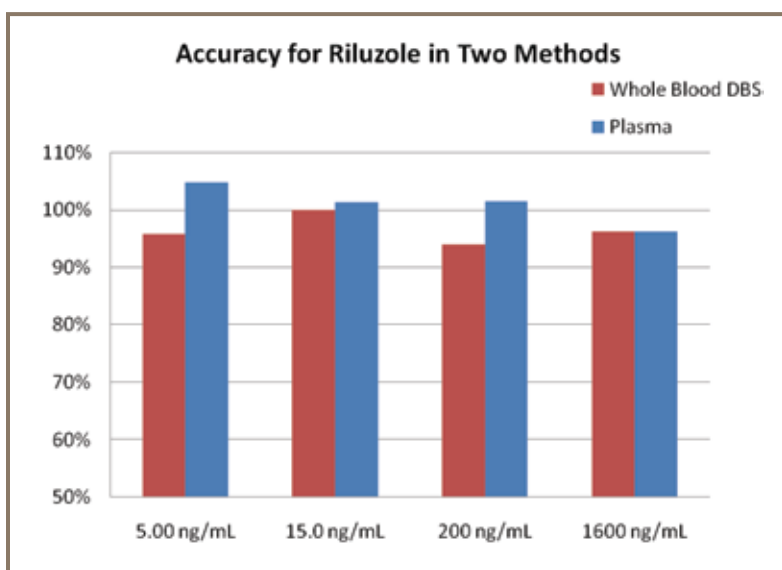


TABLE 2. Precision for Riluzole Quality Control Samples in (Top) Whole Blood DBS and (Bottom) Plasma.

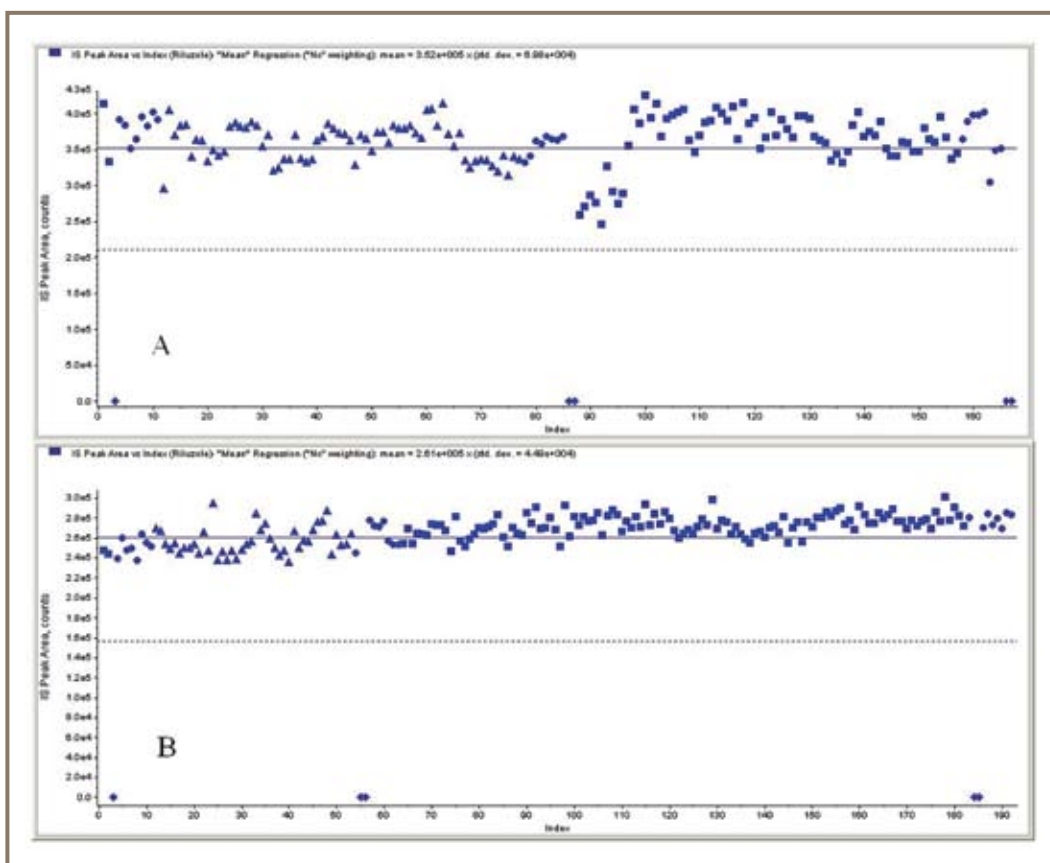
Nominal concentration (ng/mL)		LLOQ 5.00	Low 15.0	Medium 200	High 1600
DBS Method	Between Run Precision (%)	3.2	2.4	4.0	3.6
	Within Run Precision (%)	6.6	3.6	2.3	2.8
	Total Variation (%)	7.3	4.3	4.6	4.5
Plasma Method	Between Run Precision (%)	7.3	2.4	2.5	1.6
	Within Run Precision (%)	10.0	4.1	3.5	2.4
	Total Variation (%)	12.4	4.8	4.3	2.8
	n	18	18	18	18
	Number of Runs	3	3	3	3



Results and Discussion continued

- The internal standard (riluzole-¹³C,¹⁵N₂) response observed for the plasma method was slightly more consistent than the IS response observed for the DBS method, but both were well within the acceptance criteria per Tandem Labs SOP (Figure 7).

FIGURE 7. Representative IS (Riluzole-¹³C,¹⁵N₂) response for (A) Whole Blood DBS and (B) Plasma



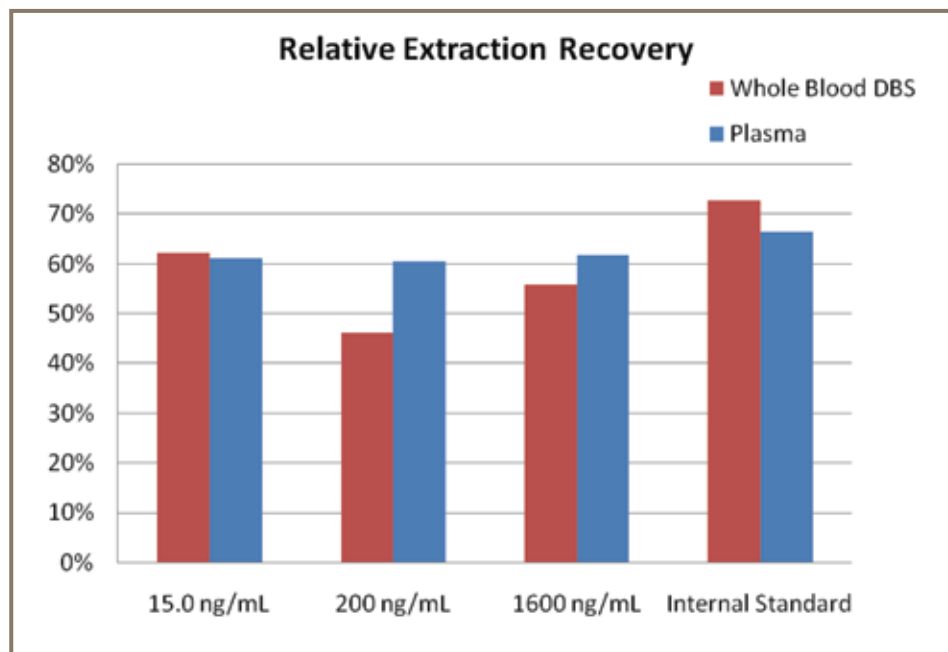
- Interference for common over-the-counter (OTC) medications at a level 20.0 µg/mL was tested at the Low QC level (15.0 ng/mL) for riluzole. The text mix included acetaminophen, ibuprofen, caffeine, chlorpheniramine maleate, naproxen, and R,R(-)-pseudoephedrine. No ion suppression or enhancement was observed by either method.
- Method selectivity was successfully demonstrated by both methods in control blanks and Low QCs prepared using six sources of human whole blood or plasma.



Results and Discussion continued

- Extraction recoveries for the DBS method were 62.0%, 46.0% and 55.8% for the Low, Medium and High QC's, respectively, and 72.7% for the internal standard. The latter is understandably higher because the internal standard is added during the extraction and does not go through the "drying-on-card" process in the same fashion as the analyte. Extraction recoveries for the plasma method were more consistent with recoveries of 61.0%, 60.3% and 61.6% for the Low, Medium and High QC's, respectively, and 66.3% for the internal standard (Figure 8).

FIGURE 8. Relative Extraction Recovery for Whole Blood on DBS with Liquid-Liquid Extraction and Plasma with Protein Precipitation Extraction.



- Matrix Factor is defined as the ratio of the average analyte response from extracted matrix samples (6 individual lots) to the analyte response in neat solution, and is a measurement of ionization suppression or enhancement for a method. The Matrix Factors for riluzole using the DBS method and the plasma method were 0.9 and 1.0, respectively, indicating minor ionization suppression for the DBS method, and no ionization suppression or enhancement for the plasma method.



Results and Discussion continued

- To ensure assay ruggedness for DBS, a blood spot volume test was conducted by spotting 15.0 μL and 50.0 μL of Low QC and High QC ($n=6$ for each) onto the DMPK-A card, and comparing the results against a standard curve spotted at 25.0 μL . The accuracy and precision results were both less than 5.0% at each level.
- The normal storage conditions for DBS are: ambient temperature, inside a paper bag, inside a storage drawer. No additional special handling requirements to “desiccate” or “protect from light” were required. Long-term matrix stability testing is currently in progress for the DBS method.

Advantages of DBS Method

- The requirement for a very small whole blood sample volume results in a less-invasive sample collection method for clinical studies (e.g., pin-prick, capillary tube). Likewise, for pre-clinical studies, it also reduces the overall number of animals per study and allows a full PK assessment from a single animal, resulting in more reliable data than studies which use the non-serial collection method.
- DBS samples can be stored and shipped at ambient temperature which greatly reduces the processing, storage and shipping costs associated with clinical and pre-clinical studies.
- DBS analysis is compatible with most current instrumentation in a typical bioanalytical lab, making it a relatively easy technique to adopt in most labs.

Limitations of DBS Method

- The amount of sample in each DBS “punch” is much lower than in traditional “wet” assays. Therefore, to achieve a level of sensitivity comparable to traditional “wet” methods, extensive optimization of the extraction process and utilization of high sensitivity instrument platforms are often needed for DBS methods.



Limitations of DBS Method continued

- DBS card “punching” is a more time consuming and laborious process compared to traditional aliquoting (e.g., pipetting) for normal “wet” assays. However, several automated techniques are currently under development for use with DBS applications, and this will likely become less of a concern as DBS techniques are more widely used throughout the pharmaceutical industry.
- Assays which require large dilution protocols to bring study samples into the appropriately validated range are not ideal for DBS methods. While the ability to dilute samples was successfully tested in our validation, the generation of large volumes of blank DBS “diluent” is limited by the nature of the DBS extraction and can be problematic if required on a large scale.
- Sample collection techniques at clinical and toxicology facilities may require substantial initial training in order to change the manner in which samples are collected for bioanalysis. While this problem is relatively easy to resolve in well-controlled toxicology (i.e. animal) facilities, the switch to DBS sampling techniques may be more difficult to overcome in latter phase clinical trials which are conducted at multiple sites.

Conclusion

- Two robust LC/MS/MS methods (in whole blood using DBS and liquid-liquid extraction and in plasma using protein precipitation extraction) were developed and validated per GLP for riluzole with a range of 5.00 ng/mL – 2000 ng/mL.
- Despite a much smaller sample volume, the lower limit of quantification was successfully obtained for the DBS method by adding a liquid-liquid extraction clean-up and changing the mass spectrometer’s ion source from APCI to ESI.
- The DBS method was thoroughly tested in the validation and exhibited results for accuracy, precision, sensitivity, selectivity, and recovery that were comparable to the traditional plasma method.
- Factors that could potentially adversely affect the data integrity for a DBS method such as blood spot volume, OTC medications, sample handling, and DBS card lot-to-lot variability were investigated. No significant effects were observed on the quantitation of the method.



Acknowledgement

- Dr. Troy Voelker, Dr. Olivia Uitto, and Stephanie Harrison (Tandem Labs, Salt Lake City, Utah) are gratefully acknowledged for their valuable input during the method development of this study.
- Julie Hilton and Dr. James Robbins (Whatman, Piscataway, New Jersey) are gratefully acknowledged for their discussion and technical support on FTA DMPK cards.