



Converting Plasma Assays to DBS Assays: Challenges and Comparison of Two Riluzole Assays in Different Matrices

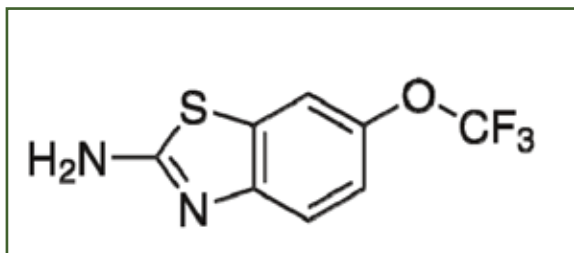
Authors

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Introduction

Riluzole (Figure 1) is a drug used to treat amyotrophic lateral sclerosis (also known as Lou Gehrig's disease). There are currently several publications describing methods for quantifying riluzole in plasma. In recent years, dried blood spot (DBS) analysis has become an appealing alternative to plasma analysis due to the advantages of minimal sample volume required, ease of handling, and reduced storage and shipment costs. However, due to the decreased sample size, DBS assays typically must overcome the challenge of reduced sensitivity. Our goal was to develop a dried blood spot method with the identical dynamic range and using the same LC/MS/MS platform as the previously validated plasma method. This presentation compares the performance of the two assays and discusses the practical considerations of handling DBS samples in the laboratory.

FIGURE 1: Chemical Structure of Riluzole



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



Plasma Method at a Glance

SAMPLE COLLECTION

Due to instability in whole blood, process to plasma at 1-8°C <30 minutes

SAMPLE PREPARATION

- Protein precipitation extraction
 - Aliquot Size: 50 µL plasma
 - Dynamic range: 5-2000 ng/mL

INSTRUMENTATION

- Mass Spec: Sciex API 5000™ (Positive ion mode)
- Source Type & Temp: APCI; 400 °C
- LC Column: XBridge™ C8, 2 x 50 mm
- LC program: Isocratic
- Mobile Phase: A: 0.1% FA in water; B: MeCN/MeOH

METHOD VALIDATION

- Fully validated for regulated analysis
- Passed all required tests
- Only stable in whole blood for up to 0.5 hrs
- Special handling required for sample collection due to whole blood instability

SAMPLE ANALYSIS AND ISR

- Supported multiple clinical studies
- All sample analysis runs acceptable – 100% pass rate
- ISR acceptable – 100% pass rate

DBS Method at a Glance

SAMPLE PREPARATION

- Liquid-Liquid Extraction
 - Sample collection: 25.0 µL whole blood on Whatman® DMPK-A
- DBS Sample Size: 5 mm punch (~8 µL whole blood)
- LLE Solvent: Ethyl acetate
- Dynamic Range: 5.0-2000 ng/mL

INSTRUMENTATION

- Mass Spec: Sciex API 5000™ (Positive ion mode)
- Source Type & Temp: ESI; 400 °C
- LC Column: XBridge™ C8, 2 x 50 mm
- LC program: Isocratic
- Mobile Phase: A: 0.1% FA in water; B: MeCN/MeOH



What are requirements for DBS validation?

Same as plasma validation:

- Three runs of precision and accuracy
- Selectivity and specificity, matrix effects
- Extract stability, reinjection reproducibility
- Whole blood collection stability

Different from plasma validation:

- F/T, bench-top matrix, or -20°C/-70°C LT matrix stability – Not required
- Hemolysis – Not required
- Recovery and ability to dilute – Alternate approach required
- Blood spot volume – Additional requirement
- RT whole blood spot drying stability - Additional requirement

Recovery:

- Need correction factor for the area ratio of sample disc/total sample spot.
- Experiment does not provide a true assessment of internal standard (IS) recovery because IS does not go through the “drying-on-card” process.

Ability to dilute:

- Extract DQCs in the same manner as undiluted QCs.
- Extract sufficient additional BLANK samples in the same manner.
- Use the final BLANK extracts as the diluent for the “post-extraction” dilution of sub-aliquots taken from DQCs extracts.

Blood spot volume:

- Determine the effect of different sized dried blood spots on quantitation.
- Prepare DBS at low and high volumes (~50% and ~200% of method volume)
- Analyze at n=6 and compare with control DBS (100% of method volume).

RT whole blood spot drying stability:

- Compare DBS samples dried at room temperature for 3 hrs and 21 hrs with time-zero samples.



Results and Discussion

METHOD DEVELOPMENT

- During initial method validation in human plasma, it was determined that riluzole was only stable in whole blood for ~0.5 hours (Tables 1 and 2). Prior to conducting full scale method development in DBS, a quick DBS stability experiment was performed in which DBS QCs dried at RT for 3 hr and 24 hr were compared with a time-zero DBS sample. The results indicated that riluzole was stable on the Whatman® DMPK-A card (Table 3).
- During early MD for DBS assay, it was also identified that there was not enough sensitivity by simply utilizing the plasma method's extraction and instrument conditions. A rough calculation showed that the amount of riluzole "on column" for a plasma LLOQ sample was ~12.5 fg compared with ~1.25 fg "on column" for a DBS LLOQ sample.
- In order to recover the sensitivity lost due to the smaller DBS sample volume, the extraction method was changed to LLE. The cleaner extract then allowed the ionization source to be changed from APCI to ESI, which was roughly 8 times more sensitive than APCI for riluzole, with no impact from commonly occurring matrix effects.

TABLE 1. Whole Blood Collection Stability for Plasma Assay Validation

Results are expressed as instrument response (i.e., analyte area/IS area)

| Run Number | Whole Blood QC 0 Hour (Control) | Whole Blood QC 1 Hour (Room Temp) | Whole Blood QC 2 Hours (Room Temp) | Whole Blood QC 1 Hour (1 to 8 °C) | Whole Blood QC 2 Hours (1 to 8 °C) |
|--------------|---------------------------------------|---|--|---|---|
| 5 | 0.450921 | 0.40287 | 0.321772 | 0.358055 | 0.328975 |
| | 0.483276 | 0.379169 | 0.329824 | 0.41262 | 0.336279 |
| | 0.441525 | 0.386329 | 0.307562 | 0.394763 | 0.324009 |
| | 0.463044 | 0.358362 | 0.326154 | 0.403048 | 0.327866 |
| | 0.459147 | 0.399422 | 0.325952 | 0.37422 | 0.328375 |
| | 0.438626 | 0.381141 | 0.332501 | 0.364084 | 0.331293 |
| Mean | 0.456 | 0.385 | 0.324 | 0.384 | 0.329 |
| S.D. | 0.016 | 0.016 | 0.009 | 0.022 | 0.004 |
| %C.V. | 3.6 | 4.2 | 2.7 | 5.8 | 1.2 |
| %Theoretical | 100 | 84.3 | 71.0 | 84.3 | 72.2 |
| % Bias | 0.0 | -15.7 | -29.0 | -15.7 | -27.8 |
| n | 6 | 6 | 6 | 6 | 6 |



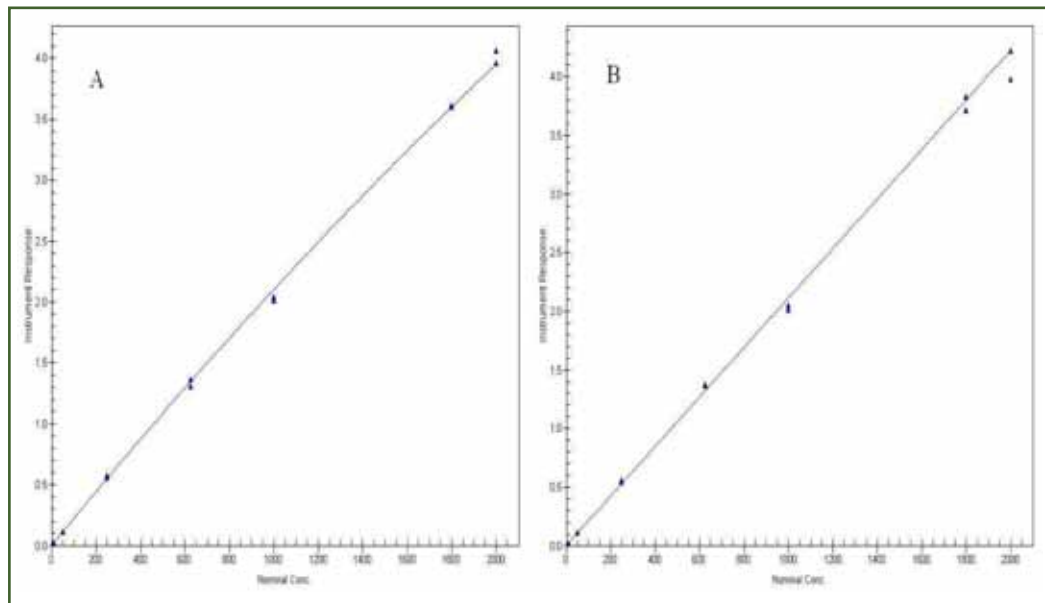
Results and Discussion (continued)

TABLE 2. Repeated Blood Collection Stability for Shorter Duration

Results are expressed as instrument response (i.e., analyte area/IS area)

| Run Number | Whole Blood QC 0 Hour (Control) | Whole Blood QC 30 min (Room Temp) | Whole Blood QC 30 min (1-8 C) |
|--------------|---------------------------------------|---|-------------------------------------|
| 7 | 0.49623 | 0.479369 | 0.484502 |
| | 0.497228 | 0.46897 | 0.476263 |
| | 0.477894 | 0.491851 | 0.474554 |
| | 0.475452 | 0.484579 | 0.476682 |
| | 0.483943 | 0.474074 | 0.461894 |
| | 0.48066 | 0.469745 | 0.456251 |
| Mean | 0.485 | 0.478 | 0.472 |
| S.D. | 0.009 | 0.009 | 0.011 |
| %C.V. | 1.9 | 1.9 | 2.2 |
| %Theoretical | 100 | 109.0 | 107.5 |
| % Bias | 0.0 | 9.0 | 7.5 |
| n | 6 | 6 | 6 |

FIGURE 2: Representative Calibration Curves (A: DBS; B: Plasma)





Results and Discussion (continued)

COMPARISON OF METHOD VALIDATION IN PLASMA AND DBS

- Representative calibration curves (Figure 2) were quadratic for the DBS method and linear for the plasma method.
- Representative chromatograms for both methods are shown in Figures 3 and 4. The sensitivity of each method was similar on a Sciex API 5000™ (DBS method/ESI source vs. plasma method/APCI source).
- The accuracy and precision of standards and quality control samples analyzed by the two methods are shown in Tables 4 and 5, with comparable results.
- Both methods were evaluated for interference with common over-the-counter (OTC) medications, for method selectivity and for determination of matrix factor. All tests in both matrices met acceptance criteria (data not presented).
- Extraction recovery with the DBS method varied between ~45% and 60.0% for riluzole but was ~70% for the internal standard. The latter is understandably higher because the internal standard is added during the extraction and does not undergo the “drying-on-card” process in the same fashion as the analyte. Extraction recoveries for the plasma method were more consistent at ~60% to 65% (Figure 5).
- To ensure the ruggedness of the sample collection and spotting process, a blood spot volume test was conducted by spotting 15.0 μL and 50.0 μL of Low and High QCs (n=6 for each) onto the DMPK-A card. The accuracy and precision were both less than 5.0% at each level and showed no significant difference with control QCs spotted at 25 μL as specified in the method.
- The normal storage conditions for DBS cards are: ambient temperature, inside a paper bag, inside a storage drawer. No additional special handling requirements to “desiccate” or “protect from light” were required. Three month long-term matrix stability in DBS was established.



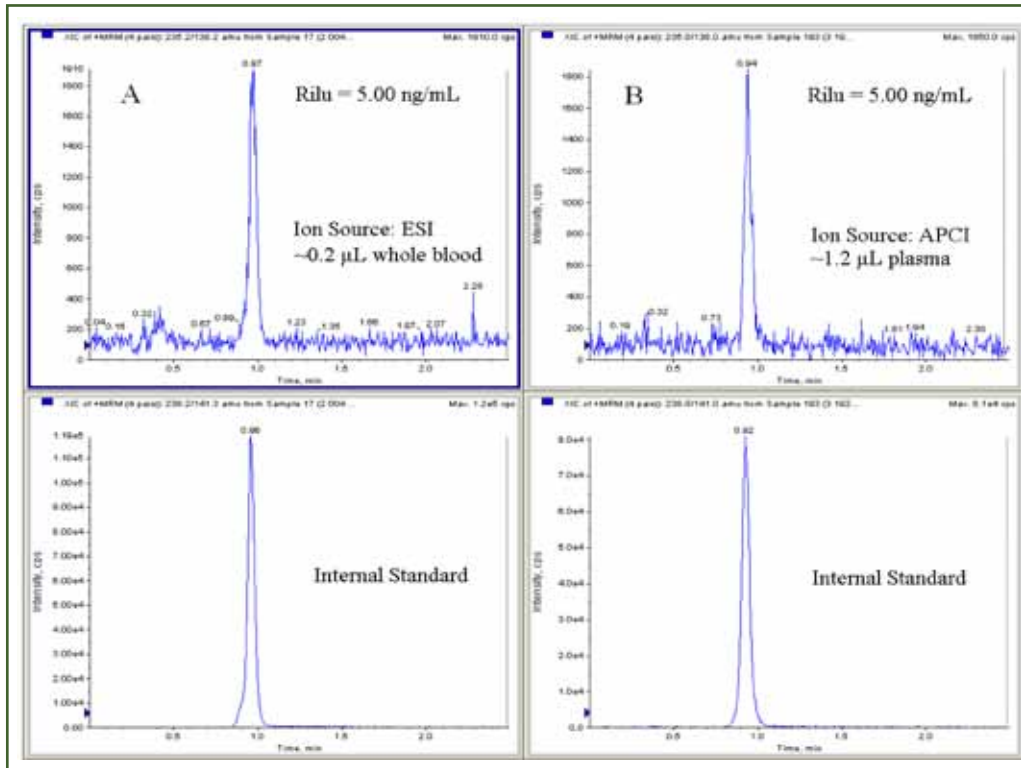
Results and Discussion (continued)

TABLE 3. Riluzole DBS Room Temperature Drying Stability

Results are expressed as instrument response (i.e., analyte area/IS area)

| Run Number | DBS QC 0 Hour (Control) | DBS QC 3 Hr (Room Temp) | DBS QC 21 Hr (Room Temp) |
|--------------|-------------------------------|-------------------------------|--------------------------------|
| 10 | 3.622095 | 3.919494 | 3.843139 |
| | 3.462553 | 3.88919 | 3.747764 |
| | 3.493218 | 3.740795 | 3.586982 |
| | 3.520922 | 3.816635 | 3.789363 |
| | 3.562599 | 3.934428 | 4.003197 |
| | 3.578936 | 3.926496 | 3.805589 |
| Mean | 3.540 | 3.871 | 3.796 |
| S.D. | 0.059 | 0.077 | 0.135 |
| %C.V. | 1.7 | 2.0 | 3.6 |
| %Theoretical | 100 | 109.4 | 107.2 |
| % Bias | 0.0 | 9.4 | 7.2 |
| n | 6 | 6 | 6 |

FIGURE 3: Representative LLOQ at 5.00 ng/mL (A: DBS; B: Plasma)





Results and Discussion (continued)

FIGURE 4: Representative ULOQ at 2000 ng/mL (A: DBS; B: Plasma)

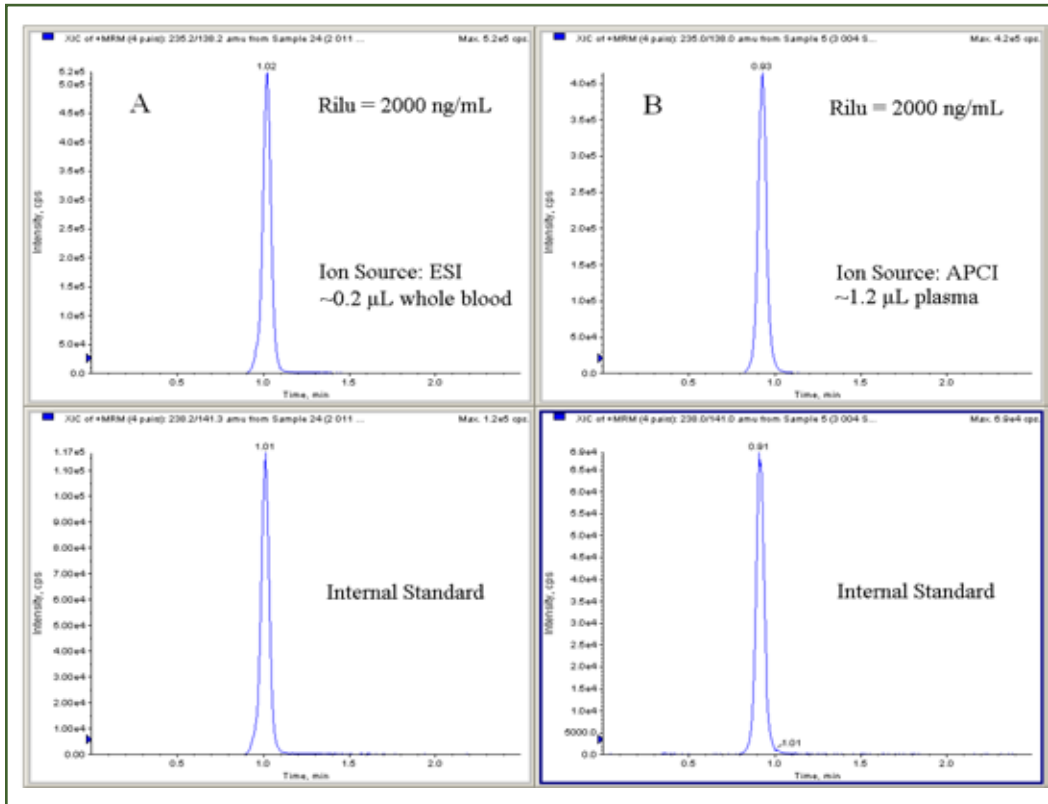
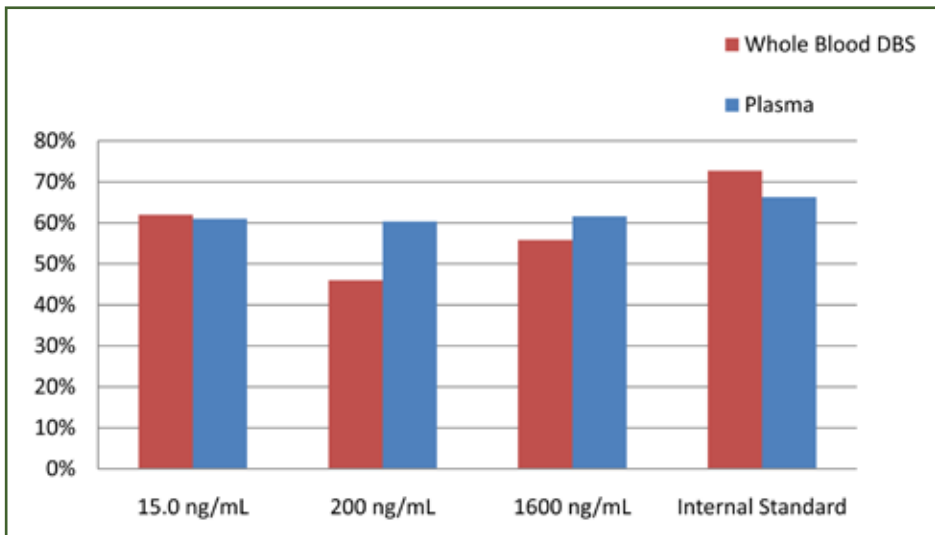


FIGURE 5: Extraction Recoveries (Plasma vs. DBS)





Results and Discussion (continued)

TABLE 4. Comparison of Standard Calibrators (Plasma vs. DBS)

| Nominal Concentration (ng/mL) | | 5 | 10 | 50 | 250 | 625 | 1000 | 1800 | 2000 |
|-------------------------------|-----------------|------|-----|------|-----|------|------|------|------|
| DBS Method | Precision (%CV) | 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 (%CV) | 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 |
| n | | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |

TABLE 5. Comparison of Analytical QC (Plasma vs. DBS)

| Nominal Concentration (ng/mL) | | LLOQ 5 | Low 15 | Medium 200 | High 1600 |
|-------------------------------|----------------------------|-----------|-----------|---------------|--------------|
| DBS Method | Between Run Precision (CV) | 3.2 | 2.4 | 4 | 3.6 |
| | Within Run Precision (CV) | 6.6 | 3.6 | 2.3 | 2.8 |
| | Accuracy (%) | 104.8 | 101.3 | 101.5 | 96.2 |
| Plasma Method | Between Run Precision (CV) | 7.3 | 2.4 | 2.5 | 1.6 |
| | Within Run Precision (CV) | 10 | 4.1 | 3.5 | 2.4 |
| | Accuracy (%) | 95.8 | 100 | 94 | 96.2 |
| n | | 18 | 18 | 18 | 18 |
| Number of Runs | | 3 | 3 | 3 | 3 |



Conclusion

- 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.
- 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.
- Factors that could potentially affect the data integrity for a DBS method (e.g. blood spot volume, sample handling, DBS card lot-to-lot variability, etc.) were investigated. These factors had no significant impact on the ability of the method to successfully quantify riluzole in human whole blood using dried blood spots.
- Two robust LC/MS/MS methods (one in whole blood DBS using liquid-liquid extraction and one in plasma using protein precipitation extraction) were developed and validated per GLP for riluzole with a dynamic range of 5.00 ng/mL – 2000 ng/mL.
- The DBS method was thoroughly tested during validation and exhibited results for accuracy, precision, sensitivity, selectivity, recovery and interference with OTC medications that were comparable in all respects to the traditional plasma method.

Acknowledgement

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