HPLC-MS/MS Assay for Dried Blood Spot Analysis: Exploring Sensitivity, Reproducibility and Automation

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Abstract

Dried blood spot (DBS) technology combined with the analytical capability of modern mass spectrometers (LC/MS/MS) has recently emerged as an important method for quantitative analysis of small molecules. DBS-LC/MS/MS is an attractive alternative to the conventional plasma based assays used to study the toxicokinetics of exposures or pharmacokinetic profiling of a new drug. Advantages of DBS include: small sample volume, reduced number of study animals, sample collection ease, reduced sample shipping and storage costs, and analyte matrix stability. Thus, DBS is attractive for pharmaceutical drug development and toxicology. Small sample sizes (15-20 µL) challenge DBS assays to meet low sensitivity requirements in sub-ng/mL quantitation range. However, drug analytes with high ionization efficiency allow assay optimization to achieve a lower limit of quantitation (LLOQ) in the pg/mL range. Here, we report a HPLC-MS/MS assay with a linear concentration range of 50-5000 pg/mL that has been validated for the quantitative bioanalysis of desipramine (Des) in DBS samples (DBSs) prepared from K_2EDTA human whole blood. Des was spiked in blood and DBSs were generated on DMPK cards using 20 µL of blood. A 3 mm punch was used to extract Des with 100 µL of 40% methanol in 0.1% formic acid. Two different approaches to incorporate the internal standard (IS), Des-d_3, were evaluated during validation. First, a conventional approach involved the inclusion of IS in the extraction solvent. In a novel approach, DMPK cards were spotted with IS prepared in MeOH and were allowed to dry prior to blood spotting. The DBSs were extracted with 40% methanol in 0.1% formic acid. Both approaches yielded data with great precision and accuracy (within ±15%). This novel approach attempts to address potential irregularities in the extraction step, but it showed a slightly greater variation in the IS signal. Manual and robotic blood spotting of QC samples were also directly compared during validation. We conclude DBS shows great promise for pharmacologic and toxicokinetic studies.

Introduction

Dried blood spot (DBS) has long been the preferred technique for the screening of newborn metabolic defects. The requirement of a very small sample volume has made DBS ideal for pediatric studies and with more recent development of sensitive mass spectrometers, it has gained significant interest as a potentially powerful tool for quantitative analysis of small molecules for pharmaceutical drug development and toxicology.

Small sample volume (10-20 µL) and the drying of the collected samples give DBS several advantages over the traditional plasma method. Small sample volume translates into reduced number of animals per study, ability to collect serial time points from a single animal, and ultimately, a reduction in the cost of a given animal study. For clinical studies, small sample volume and simple non-invasive sample collection techniques, like the finger prick, can potentially improve subject recruitment and retention. Once dry, the collected samples can be stored and shipped at ambient atmospheric conditions, which dramatically reduces processing, refrigeration and shipping costs. Certain coated sample collection cards, such as DMPK A and B cards, inactivate the enzymes present in the blood, which in turn results in an improved analyte stability on DBS cards compared to that in plasma. In addition, these coated cards can deactivate virus and reduce health hazards.

Bioanalytical processing and analysis of DBS is very comparable to the analysis of a plasma sample. Certain steps of the analysis process can be automated to achieve high throughput. Overall, DBS is an attractive technique for both preclinical (animal) samples and clinical (human) samples.

Despite its potential, certain aspects of DBS technology need further examination. Small sample size required for DBS analysis is a significant advantage that comes with the bioanalytical challenge of developing a DBS assay to meet low sensitivity requirements in the sub-ng/mL quantitation range. Furthermore, as sample is directly collected and dried on a collection card, when and how to incorporate the internal standard (IS) for bioanalysis remains a subject of concern. Lastly, while certain steps during the method validation can be automated to improve efficiency and throughput, automated procedures also require cross validation against a manual approach that closely represents the sampling conditions and procedures. Here we have investigated sensitivity, reproducibility and automation aspects of DBS bioanalysis using two different approaches to incorporate the internal standard (IS). Performance of two different sample collection card types was examined during the investigation of this DBS assay.

Methods

Sample Preparation

Analyte: Desipramine

Internal Standard: Desipramine-d_3

Standards and quality control samples were prepared by spiking desipramine in K_2EDTA human whole blood and gently mixing for 30 mins.

- 4 sets of blood spots were generated on two different types of sample collection cards
  1) DMPK-B card (GE Healthcare)
  2) DMPK-B card with IS spotted and dried prior to blood spotting
  3) Ahlstrom 226 card (ID Biological Systems)
  4) Ahlstrom 226 card with IS spotted and dried prior to blood spotting
- 20 µL of blood was used to generate each blood spot
- IS spotted collection cards were generated by spotting 10 µL of 20,000 pg/mL IS, desipramine-d_3, and drying at RT for overnight
- Robotic spotting of blood and IS was achieved using Hamilton Star Liquid Handling System
- A 3 mm disc was punched out for sample extraction
- DBS from IS free DMPK-B and Ahlstrom 226 cards were extracted using 100 µL of 1,000 pg/mL IS solution prepared in 40% methanol with 0.1% formic acid
- DBS samples from IS spotted cards were extracted with 100 µL of 40% methanol with 0.1% formic acid
- Extraction was achieved by vortexing the samples for 30 secs
- Extracts were centrifuged at 16,000 RCF for 4 mins and transferred into autosampler vial for LC-MS/MS analysis

Sample Analysis

•Column: Phenomenex Luna C-18

•Elution: Gradient flow, Mobile Phase A: Water with 0.01% FA, Mobile Phase B: MeOH

•Detector: Applied Biosystems/MDS Sciex API 5000 MS/MS

Data Analysis

•Weighing: 1/(concentration^2)

•Chromatographic Peak Integrations: Applied Biosystems/MDS Sciex Analyst 1.4.2

Results

Robotic and Manual Spotting

Hand Spotted DMPK Cards

Robot Spotted DMPK Cards

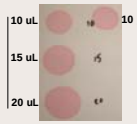
IS Spotting Followed by Blood Spotting Using Robot

Front View

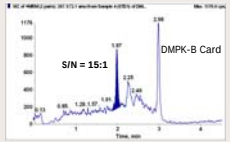
MeOH diffusion

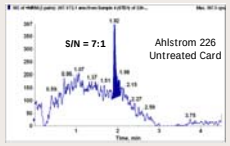
Back View

MeOH was spiked with Methyl Red and spotted on the DMPK cards to mimic the spotting of the IS prepared in MeOH. Once dry, 20 µL of blood was spotted on top of the MeOH spot.



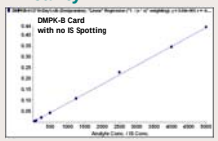
Method Sensitivity





- **LLOQ 50 pg/mL**
- **DMPK-B card gave higher method sensitivity and improved S/N ratio than Ahlstrom 226 card**
- **Signal baseline was elevated for extracts from both types of cards**
- **Less matrix peaks were observed for Ahlstrom 226 card**
- **Both cards gave similar quantitative results**

Linearity



Regression Statistic Summary

	Number of Curves	Mean Slope	Mean Intercept	Mean R^2	LLOQ (pg/mL)	ULOQ (pg/mL)
DMPK-B	3	8.01E-05	0.000737	0.995	50	5000
DMPK-B with Spotted IS	3	2.10E-04	0.000420	0.992	50	5000
Ahlstrom 226	3	7.66E-05	0.000836	0.996	50	5000
Ahlstrom 226 with Spotted IS	3	3.01E-04	0.004863	0.997	50	5000

Calibration Standard Performance

DMPK-B	Std 1 (pg/mL) 50	Std 2 (pg/mL) 100	Std 3 (pg/mL) 250	Std 4 (pg/mL) 500	Std 5 (pg/mL) 1250	Std 6 (pg/mL) 2500	Std 7 (pg/mL) 4000	Std 8 (pg/mL) 5000
Inter-run Mean	50.9	97.8	246	492	1173	2717	3960	5113
Inter-run SD	2.23	11.4	10.2	9.8	66.6	66.6	44	97
Inter-run %CV	4.4	11.7	4.2	2.0	5.7	2.5	1.1	1.9
Inter-run %Bias	1.7	-2.2	-1.7	-1.6	-6.1	8.7	-1.0	2.3
n	3	3	3	3	3	3	3	3

ISWS Spotted DMPK-B	Std 1 (pg/mL) 50	Std 2 (pg/mL) 100	Std 3 (pg/mL) 250	Std 4 (pg/mL) 500	Std 5 (pg/mL) 1250	Std 6 (pg/mL) 2500	Std 7 (pg/mL) 4000	Std 8 (pg/mL) 5000
Inter-run Mean	50.8	97.4	239	533	1247	2343	4433	4737
Inter-run SD	1.62	3.00	27.0	23.4	65.1	37.9	146	302
Inter-run %CV	3.2	3.1	11.3	4.4	5.2	1.6	3.3	6.4
Inter-run %Bias	1.7	-2.6	-4.4	6.5	-0.3	-6.3	10.8	-5.3
n	3	3	3	3	3	3	3	3

Ahlstrom 226	Std 1 (pg/mL) 50	Std 2 (pg/mL) 100	Std 3 (pg/mL) 250	Std 4 (pg/mL) 500	Std 5 (pg/mL) 1250	Std 6 (pg/mL) 2500	Std 7 (pg/mL) 4000	Std 8 (pg/mL) 5000
Inter-run Mean	52.3	90.9	250	497	1240	2460	4030	5340
Inter-run SD	0.46	2.87	14.7	9.0	72.1	36.1	200	150
Inter-run %CV	0.9	3.2	5.9	1.8	5.8	1.5	5.0	2.8
Inter-run %Bias	4.6	-9.1	0.0	-0.7	-0.8	-1.6	0.8	6.8
n	3	3	3	3	3	3	3	3

ISWS Spotted Ahlstrom 226	Std 1 (pg/mL) 50	Std 2 (pg/mL) 100	Std 3 (pg/mL) 250	Std 4 (pg/mL) 500	Std 5 (pg/mL) 1250	Std 6 (pg/mL) 2500	Std 7 (pg/mL) 4000	Std 8 (pg/mL) 5000
Inter-run Mean	51.9	93.4	246	494	1207	2597	4057	5203
Inter-run SD	1.25	2.74	12.8	27.0	15.3	135.8	78.0	121
Inter-run %CV	2.4	2.9	5.2	5.5	1.3	5.2	1.9	2.3
Inter-run %Bias	3.8	-6.6	-1.6	-1.3	-3.5	3.9	1.4	4.1
n	3	3	3	3	3	3	3	3

QC Sample Performance

DMPK-B	LLOQ QC (pg/mL) 50	Low QC (pg/mL) 150	Mid QC (pg/mL) 500	High QC (pg/mL) 3750	Dil QC (pg/mL) 2205
Inter-run Mean	51.3	150	518	3915	2325
Inter-run SD	4.15	12.1	43.4	272	119
Inter-run %CV	8.09	8.03	8.37	6.94	5.11
Inter-run %Bias	2.63	0.27	3.64	4.41	5.44
n	15	15	15	15	10

Three out of seventy QC samples failed to meet acceptance criteria of 15% accuracy.

ISWS Spotted DMPK-B	LLOQ QC (pg/mL) 50	Low QC (pg/mL) 150	Mid QC (pg/mL) 500	High QC (pg/mL) 3750	Dil QC (pg/mL) 1875
Inter-run Mean	50.2	149	458	3521	1857
Inter-run SD	3.82	7.87	38.1	287	69.0
Inter-run %CV	7.60	5.29	8.31	8.14	3.71
Inter-run %Bias	0.48	-0.76	-8.39	-6.10	-0.96
n	15	15	15	15	10

Four out of seventy QC samples failed to meet acceptance criteria of 15% accuracy.

Ahlstrom 226	LLOQ QC (pg/mL) 50	Low QC (pg/mL) 150	Mid QC (pg/mL) 500	High QC (pg/mL) 3750	Dil QC (pg/mL) 1875
Inter-run Mean	49.8	145	467	3559	1810
Inter-run SD	3.24	11.0	34.2	308	42.7
Inter-run %CV	6.50	7.59	7.34	8.67	2.36
Inter-run %Bias	-0.48	-3.42	-6.69	-5.08	-3.47
n	15	15	15	15	10

One out of seventy QC samples failed to meet acceptance criteria of 15% accuracy.

ISWS Spotted Ahlstrom 226	LLOQ QC (pg/mL) 50	Low QC (pg/mL) 150	Mid QC (pg/mL) 500	High QC (pg/mL) 3750	Dil QC (pg/mL) 1875
Inter-run Mean	48.0	143	492	3510	1996
Inter-run SD	4.14	9.03	30.1	310	93.7
Inter-run %CV	8.61	6.31	6.12	8.84	4.70
Inter-run %Bias	-3.91	-4.58	-1.56	-6.40	6.45
n	15	15	15	15	10

Five out of seventy QC samples failed to meet acceptance criteria of 15% accuracy.

Robotic and Manual QC Performance

DMPK-B

Sample Name	Expected Concentration	Number Of Values Used	Mean	Standard Deviation	%CV	%Bias
LLOQ QC Manual	50	5 of 5	49.6	3.23	6.51	-0.72
Low QC Manual	150	5 of 5	155	8.51	5.50	3.06
Mid QC Manual	500	5 of 5	533	30.5	5.72	6.56
High QC Manual	3750	5 of 5	4072	121	2.97	8.58
LLOQ-Robot	50	5 of 5	48.5	5.33	11.0	-3.09
Low-Robot	150	5 of 5	146	7.74	5.30	-2.56
Mid-Robot	500	5 of 5	517	30.4	5.89	3.35
High-Robot	3750	5 of 5	4042	70.0	1.73	7.78

All QC samples met acceptance criteria of 15% accuracy.

IS Spotted DMPK-B

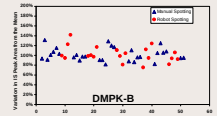
Sample Name	Expected Concentration	Number Of Values Used	Mean	Standard Deviation	%CV	%Bias
LLOQ QC Manual	50	5 of 5	50.6	3.11	6.15	1.22
Low QC Manual	150	5 of 5	153	10.0	6.57	1.79
Mid QC Manual	500	5 of 5	457	34.7	7.60	-8.57
High QC Manual	3750	5 of 5	3649	370	10.1	-2.69
LLOQ-Robot	50	5 of 5	49.3	1.80	3.64	-1.33
Low-Robot	150	5 of 5	139	7.28	5.24	-7.26
Mid-Robot	500	5 of 5	441	33.6	7.61	-11.8
High-Robot	3750	5 of 5	3207	66.0	2.06	-14.5

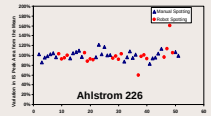
Five (3 robotic spotting and 2 manual spotting) out of forty QC samples failed to meet acceptance criteria of 15% accuracy.

IS and the blood spots were both generated either manually or using a robot.

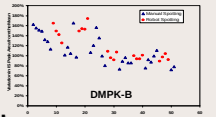
Internal Standard Performance

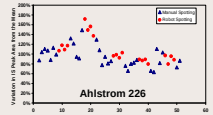
Samples Extracted with Extraction Solvent Containing IS





IS Spotted on the Sample Collection Card Prior to Blood Spotting





Discussion

Method

A LC-MS/MS method was successfully developed for the quantitative analysis of desipramine from dried blood spots with a linear calibration range of 50.0 pg/mL to 5000 pg/mL. A single step was used to extract analyte from DBS. The extracted sample was centrifuged and injected into a LC-MS/MS system for analysis. This one step process made this assay very efficient and sensitive. The method was linear, accurate, precise, selective, and rugged and met all the FDA suggested precision requirements for bioanalytical LC-MS/MS methods. (Analyte long term stability on DBS cards has not been evaluated at this point).

Sensitivity

A LLOQ of 50.0 pg/mL for desipramine was established for this method. Extracts from the DMPK-B cards gave best sensitivity, but also displayed more matrix peaks during LC-MS/MS analysis. Fewer matrix peaks were observed for extracts from the uncoated Ahlstrom 226 card, suggesting the coating on the DMPK-B cards as the potential source of the non-analyte peaks. Signal baseline for all analytical conditions was elevated, and this is potentially due to low molecular weight noise.

Reproducibility

The method was noted to be very reproducible. Quality control samples at all levels were found to be within acceptance criteria of 15% (precision and accuracy) and the inter-run precision and accuracy were also within 15%.

Internal Standard

Both approaches of incorporating IS in the bioanalysis gave satisfactory results as indicated by the inter- and intra-run precisions and accuracies of standards and QC samples. As expected, less variation in the IS signal was observed when the samples were extracted with solvent containing internal standard. Despite some variation, the DBS sample with IS pre-spotted on the sample collection cards gave excellent assay precision and accuracy. The fluctuation observed in the IS signal followed similar trend for both robotic and manual spotting. Based on this single observation it is difficult to assess full advantages of incorporating internal standard on the sample card as a pre-spot; however, this method appears to be very attractive as it will correct for variations in extractions and also serve as a control for samples that are poorly processed. With the former approach of using IS in the extraction solvent, one would always observe a consistent IS peak in the chromatogram regardless of ineffective or missed extraction, thus giving rise to a biased result.

Automation

Hamilton Star Liquid Handling System was successfully used to generate QC samples by spotting both blood and the IS standard on to the sample collection card. The robotically spotted QC samples were extracted and analyzed together with the manually spotted standard curve and QC samples. The precision and accuracy of the robotically spotted QC samples were found to be within 15%. Additionally, the variation in the IS signal for the robotically and manually spotted cards were very comparable. Further robotic development and optimization are currently underway, which include: 1) full automation of standards and QC preparation and DBS generation steps, 2) improved control over blood spot generation to reduce spotting artifacts (such as bubble formation), and 3) engineering of the paper size, rigidity and holder to fit the automated workflow. These developments and optimizations will certainly improve the accuracy and precision of the automated DBS method.

Conclusions

•DBS assays can be developed to meet low sensitivity requirements in the sub-ng/mL quantitation range. Success will largely depend on the ionization efficiency of the analyte and the identification of optimal extraction and analysis protocols.

•Coated sample collection cards have the potential to aid analyte stability and virus deactivation, but also give rise to unwanted chromatographic interference due to inherent chemical coatings.

•Internal standard prepared in organic solvent, such as methanol, can be used to spot the sample collection cards prior to blood spotting. This approach of IS incorporation potentially can correct and check for extraction deficiencies.

•Automated liquid handling systems can be used to generate DBS during method validation to improve the efficiency of a bioanalytical process. Robotically generated DBS samples were in close agreement with manually spotted DBS samples.

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