ASSESSMENT OF ANALYTE LOSSES IN A HUMAN DRIED BLOOD SPOT ASSAY FOR THE DETERMINATION OF INDAPAMIDE.

M. Zaugg, S. Wood, M. Bachmann, P. Struwe
Celerion Switzerland AG, 8320 Fehraltorf, Switzerland

INTRODUCTION

Indapamide (4-[[1-((2-methyl-1H-1,2,4-triazol-3-yl)methyl)phenyl]sulfonyl]benzamide) is a sulphonamide diuretic agent for the treatment of hypertension. A robust LC/MS/MS method for the determination of indapamide in human dried blood spots (DBS) was established in our laboratory. Indapamide was extracted from human dried blood spots and measured using UPLC with MS/MS detection. Quantiﬁcation was achieved using a 0.3 µL internal standard targeting an LOD of 2 ng/mL.

METHOD OVERVIEW

A 3 mm diameter disk was punched from a 20 µL blood spot dried on a Whatman FTA DMPK-B card into a 96-well plate. The disk was then extracted by the addition of 100 µL methanol containing Indapamide-D3, followed by shaking for 15 minutes at room temperature. An aliquot of 75 µL of the supernatant was then transferred to a fresh 96-well plate and diluted with 75 µL water.

The LC/MS/MS system consisted of an Agilent UPLC system (Waters) connected to an API 4000 (Applied Biosystems/MDT Xic). The chromatographic separation of a 30 µL sample injection was achieved on a Phenomenex (50x2.1 mm, 1.7 µm, Waters) column using methanol/ammonium formate gradient at a flow rate of 0.8 mL/min. The Indapamide-characteristic precursor to product ion transition m/z 366.2 to 132.1, were analysed using an electrospray in the MRM positive mode. Conditions as described above were found to give best results for sensitivity, having optimised the method for solvent composition, chromatography (peak shape) and injection volume.

METHOD PERFORMANCE

Method validation was performed for all appropriate parameters with a range of 2 to 200 ng/mL, including:

- Standard sample preparation for DBS used a single 3 mm card punch with subsequent extraction in methanol, dilution for chromatographic reasons then injection to LC/MS/MS. This method may not necessarily provide optimal conditions for sensitivity for this or any analyte.
- A systematic sampling of the number of punches, matrix suppression losses and card suppression losses. Additionally evaporative losses and reconstitution losses were investigated to cover the way these experiments were conducted.

EXTRACTION RECOVERY

Recovery experiments involved the assessment of analytical recovery from the DBS sample punch and comparison on different card types, into different solvents and with and without the addition of pH modifier. The effect of solvent volume was also investigated. The recovery was determined by comparing the peak area response of extracted tandem mass spectrometry (MS/MS) isolated ion chromatogram (IC) responses assuming 100% concentration level. Recovery was calculated using a 3 mm punch and compared this to the mean weight of a complete 20 µL blood spot. Approach was used the diameter of the punch and compared this to the diameter of a 20 µL blood spot. In both cases an assumed sample volume per punch of 2.86 µL was determined. The results showed difference in extraction recovery for polar and apolar extraction solvents (Figure 3).

For all extraction recoveries, a 2 mm punch of a 20 µL spot was extracted with 100 µL solvent and evaporated after removal of the punch. The residue was reconstituted and analysed. For post-extracted spiked blank samples, the analyte was diluted directly in the reconstitution solution and this added to dried extraction blank samples. In order to calculate the 100% concentration level the amount of sample within a punch had been estimated. Two different approaches were used. Approach one used the mean weight of a 3 mm punch and compared this to the mean weight of a complete 20 µL blood spot. Approach two used the diameter of the punch and compared this to the diameter of a 20 µL blood spot. In both cases an assumed sample volume per punch of 2.86 µL was determined. The results showed difference in extraction recovery for polar and apolar extraction solvents (Figure 3). No pH adjustment was used for the apolar solvents but an increase of the extraction recovery might be anticipated with correct pH adjustment.

A different in the overall suppression patterns was observed between untreated (Whatman FTA DMPK-A, Whatman 903 Protein saver) and treated (Whatman FTA DMPK-B, Whatman FTA DMPK-C) card samples. The ionisation suppression was much reduced as chromatographic retention increased. At the retention time of indapamide the quantitative experiments were confirmed with only small differences observed. At the dead volume of the system the treated cards showed a large suppression area (Figure 4) whereas virtually no suppression could be observed with untreated cards (Figure 5). Suppression coming from the treated card material masked any effect from the matrix components. On the untreated cards an additional suppression coming from matrix components was seen. Due to the overall retention of Indapamide under the chromatographic conditions used the dead volume results of course have less relevance but are a good indicator of the need for good chromatographic retention.

EVAPORATION AND RECONSTITUTION LOSSES

As the loss evaluations was used a dry down and reconstitution approach (for consistency between solvents) the analyte loss during evaporation and reconstitution was evaluated at different concentration levels.

Total recovery was evaporated and reconstituted. This solution was compared against a non-treated solution. No losses could be found during the evaporation and reconstitution steps.

TOTAL RECOVERY

The combined effects of extraction and ionisation recovery are best expressed as total recovery (Figure 8).

For total recovery, the combination of Whatman DMPK-B card extracted with 1% formic acid in methanol was found to give best conditions (total recovery of 90% in total loss of 10%). This represents an increase of only 10% in total recovery compared to the generic starting method (Whatman DMPK-B card extracted with methanol).

ADDITIONAL SENSITIVITY EXPERIMENTS

Given this increase does not allow for any factor improvement in sensitivity, another approach to increase sensitivity was required. Sample volume increase could be achieved by using a larger punch diameter 6 mm representing 11.4 µL blood sample or by extracting multiple 3 mm punches.

Using a 6 mm punch at an LOD of 0.5 ng/mL, could be achieved with the same method performance as 2 ng/mL at a 3 mm punch. No adverse effects were observed on extraction recovery or ionisation recovery.

CONCLUSION

For DBS methods requiring low LODs it is to be expected that only small sensitivity gains may be possible by minimising recovery and ionisation losses (but this should be investigated on an analyte specific basis). Instead sensitivity optimisation should perhaps centre on sample volume and absolute LC/MS/MS sensitivity improvement.

ACKNOWLEDGEMENTS

We gratefully acknowledge the contribution of our method validation and sample analysis colleagues at Celerion Switzerland during this work.

EVALUATION OF ANALYTE LOSSES

This standard sample preparation approach for DBS used a single 3 mm card punch with subsequent extraction in methanol, dilution for chromatographic reasons then injection to LC/MS/MS. This method may not necessarily provide optimal conditions for sensitivity for this or any analyte.

A systematic sampling of the number of punches, matrix suppression losses and card suppression losses. Additionally evaporative losses and reconstitution losses were investigated to cover the way these experiments were conducted.

For all extraction recoveries, a 2 mm punch of a 20 µL spot was extracted with 100 µL solvent and evaporated after removal of the punch. The residue was reconstituted and analysed. For post-extracted spiked blank samples, the analyte was diluted directly in the reconstitution solution and this added to dried extraction blank samples. In order to calculate the 100% concentration level the amount of sample within a punch had been estimated. Two different approaches were used. Approach one used the mean weight of a 3 mm punch and compared this to the mean weight of a complete 20 µL blood spot. Approach two used the diameter of the punch and compared this to the diameter of a 20 µL blood spot. In both cases an assumed sample volume per punch of 2.86 µL was determined. The results showed difference in extraction recovery for polar and apolar extraction solvents (Figure 3). No pH adjustment was used for the apolar solvents but an increase of the extraction recovery might be anticipated with correct pH adjustment.

A different in the overall suppression patterns was observed between untreated (Whatman FTA DMPK-A, Whatman 903 Protein saver) and treated (Whatman FTA DMPK-B, Whatman FTA DMPK-C) card samples. The ionisation suppression was much reduced as chromatographic retention increased. At the retention time of indapamide the quantitative experiments were confirmed with only small differences observed. At the dead volume of the system the treated cards showed a large suppression area (Figure 4) whereas virtually no suppression could be observed with untreated cards (Figure 5). Suppression coming from the treated card material masked any effect from the matrix components. On the untreated cards an additional suppression coming from matrix components was seen. Due to the overall retention of Indapamide under the chromatographic conditions used the dead volume results of course have less relevance but are a good indicator of the need for good chromatographic retention.

EVAPORATION AND RECONSTITUTION LOSSES

As the loss evaluations was used a dry down and reconstitution approach (for consistency between solvents) the analyte loss during evaporation and reconstitution was evaluated at different concentration levels.

Total recovery was evaporated and reconstituted. This solution was compared against a non-treated solution. No losses could be found during the evaporation and reconstitution steps.

TOTAL RECOVERY

The combined effects of extraction and ionisation recovery are best expressed as total recovery (Figure 8).

For total recovery, the combination of Whatman DMPK-B card extracted with 1% formic acid in methanol was found to give best conditions (total recovery of 90% in total loss of 10%). This represents an increase of only 10% in total recovery compared to the generic starting method (Whatman DMPK-B card extracted with methanol).

ADDITIONAL SENSITIVITY EXPERIMENTS

Given this increase does not allow for any factor improvement in sensitivity, another approach to increase sensitivity was required. Sample volume increase could be achieved by using a larger punch diameter 6 mm representing 11.4 µL blood sample or by extracting multiple 3 mm punches.

Using a 6 mm punch at an LOD of 0.5 ng/mL, could be achieved with the same method performance as 2 ng/mL at a 3 mm punch. No adverse effects were observed on extraction recovery or ionisation recovery.

CONCLUSION

For DBS methods requiring low LODs it is to be expected that only small sensitivity gains may be possible by minimising recovery and ionisation losses (but this should be investigated on an analyte specific basis). Instead sensitivity optimisation should perhaps centre on sample volume and absolute LC/MS/MS sensitivity improvement.

ACKNOWLEDGEMENTS

We gratefully acknowledge the contribution of our method validation and sample analysis colleagues at Celerion Switzerland during this work.