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

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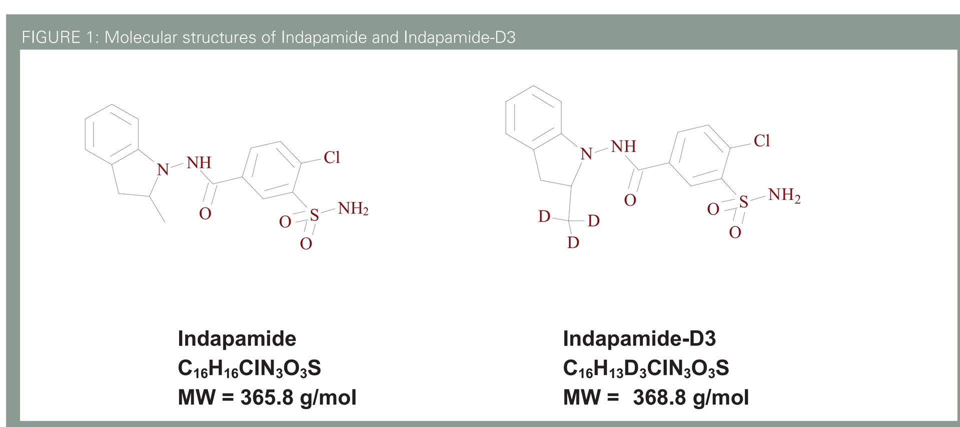
## INTRODUCTION

Indapamide[4-chloro-N-(2-methyl-2,3-dihydroindol-1-yl)-3-sulfamoyl-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. Quantitation used a D3-labeled internal standard targeting an LLOQ of 2 ng/mL.

Whilst the achieved LLOQ was sufficient for normal PK purposes the possibility to decrease the LLOQ was investigated. This would bring the method inline with existing whole blood determinations. With the LC-MS/MS conditions considered to be already optimised for sensitivity potential gains could only be expected to come from sample preparation. A systematic assessment of analyte losses during sample preparation was performed. The method could then potentially be optimised to minimise these losses with a positive impact on sensitivity and lowered LLOQ. This would also aid the understanding of the factors limiting sensitivity for DBS methods and help determine method development procedures for future methods for analytes requiring more challenging LLOQs.

FIGURE 1: Molecular structures of Indapamide and Indapamide-D3




## 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 Acquity UPLC system (Waters) connected to an API 4000 (Applied Biosystems/MDS Sciex). The chromatographic separation of a 30 µL sample injection was achieved on a BEH Phenyl (50x2.1 mm, 1.7 µm; Waters) column using a 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.

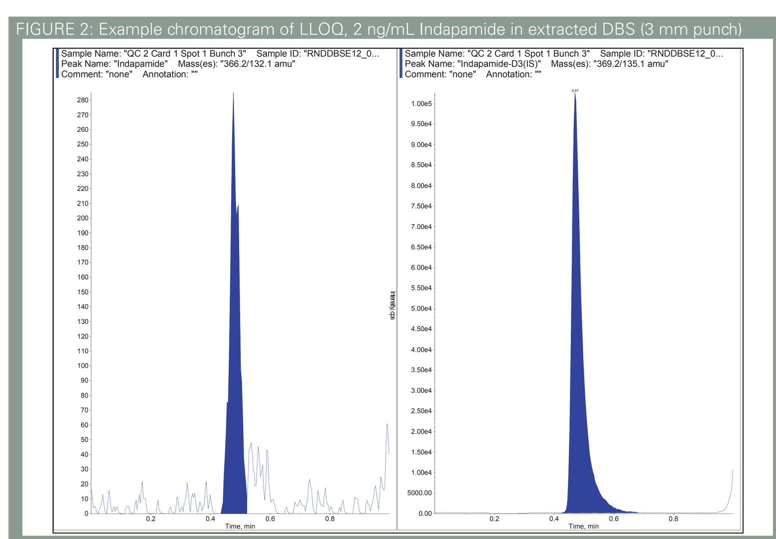
## METHOD PERFORMANCE

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

TABLE 1: Validation parameters

| Quality Control Samples | | Precision (%) | Mean Accuracy (%) |
|---|---|---|---|
| Intra-spot | LLOQ | 8.4 | 96.1 |
| | Low | 4.9 | 90.4 |
| | Medium | 8.5 | 89.5 |
| | High | 5.5 | 89.7 |
| Intra-card | LLOQ | 8.1 | 95.2 |
| | Low | 4.4 | 91.3 |
| | Medium | 5.9 | 88.2 |
| | High | 4.4 | 90.8 |
| Intra-run, Run 1 | LLOQ | 6.7 | 95.1 |
| | Low | 3.9 | 91.3 |
| | Medium | 5.0 | 88.2 |
| | High | 4.2 | 90.8 |
| Intra-run, Run 2 | LLOQ | 7.7 | 100 |
| | Low | 4.5 | 92.6 |
| | Medium | 4.7 | 89.1 |
| | High | 4.8 | 89.0 |
| Recovery | | Recovery (%) | |
| | Low | 80.4 | |
| | Medium | 74.3 | |
| | High | 80.5 | |
| Short-term stability (whole blood) | | 20 hours at room temperature | |
| Long-term stability (FTA card) | | 21 days at room temperature | |
| Blood spot homogeneity | | Demonstrated (samples taken at periphery quantified against samples taken from centre of blood spot) | |
| Dilution integrity | | up to 1000 ng/mL | |
| Processed sample integrity | | 72 hours at approximately 5°C in autosampler | |
| Batch size | | Up to 192 injections | |

FIGURE 2: Example chromatogram of LLOQ, 2 ng/mL Indapamide in extracted DBS (3 mm punch)




## 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 approach may not necessarily provide optimal conditions for sensitivity for this (or any) analyte.

A systematic analyte loss evaluation investigated recovery losses, 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 losses 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 samples with post-extracted spiked blank samples assuming 100% concentration level. Different concentration levels were investigated.

For all extraction recovery experiments, a 3 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 extracted blank samples. In order to calculate the 100% concentration level the amount of sample within a punch had to be 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 a 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.

FIGURE 3: Comparison of the extraction recovery on different extraction solvents on the Whatman DMPK-B card. The error bars indicate the variation with concentration.

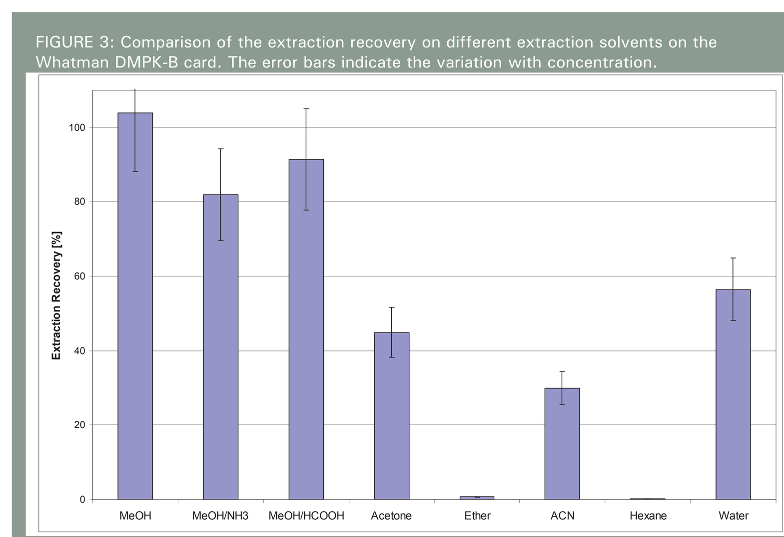
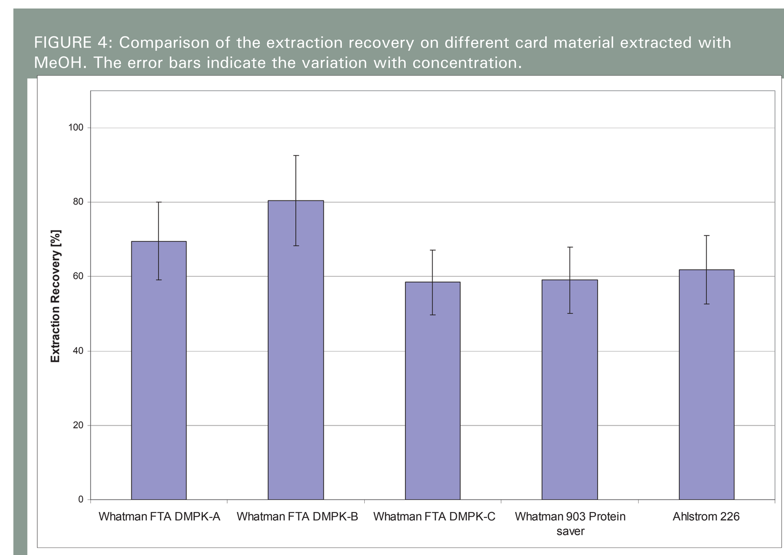



FIGURE 4: Comparison of the extraction recovery on different card material extracted with MeOH. The error bars indicate the variation with concentration.




An increase of 10 to 20% in extraction recovery on treated card material (FTA DMPK-A and DMPK-B) could be observed in comparison to the untreated card material (Figure 4). Indapamide is known to be highly bound to red blood cells. The influence of increased cell lyses on the treated card material could account for the better extraction recovery.

The generic starting conditions (Whatman DMPK-B card extracted with methanol) were found to be optimal for extraction recovery with mean losses minimised to less than 20%. Solvent volume increases could not improve on this.

## IONISATION RECOVERY

Matrix and card suppression experiments were performed quantitatively and qualitatively to ascertain the effect of co-extracted matrix and card components.

For quantitative experiments the ionisation recovery was determined by comparing the peak area response of post extracted spiked blank samples with that of pure solution samples. Different concentration levels were investigated. For all ionisation recovery experiments, a 3 mm punch of a 20 µL blank blood spot was extracted with 100 µL solvent and evaporated after removal of the punch. The residue was reconstituted in appropriate solvent containing analyte and analysed. This same fortified reconstitution solution was used for pure solution samples.

FIGURE 5: Comparison of the ionisation recovery on different extraction solvents on the Whatman DMPK-B card. The error bars indicate the variation with concentration.

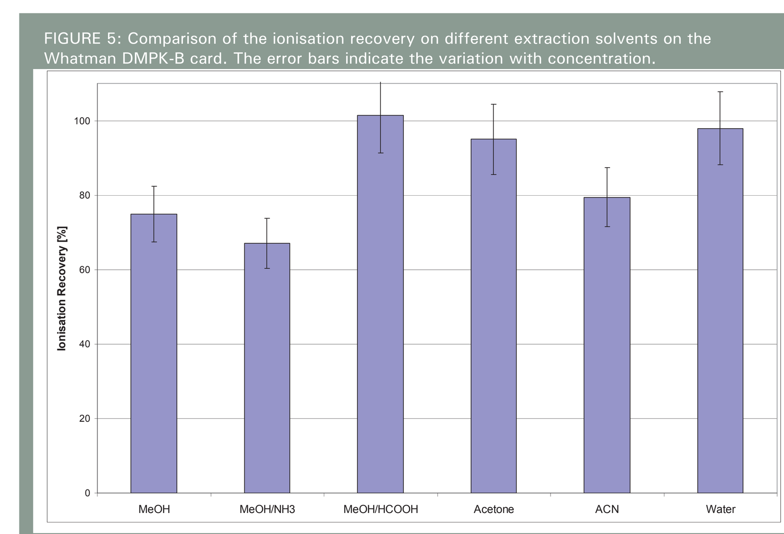
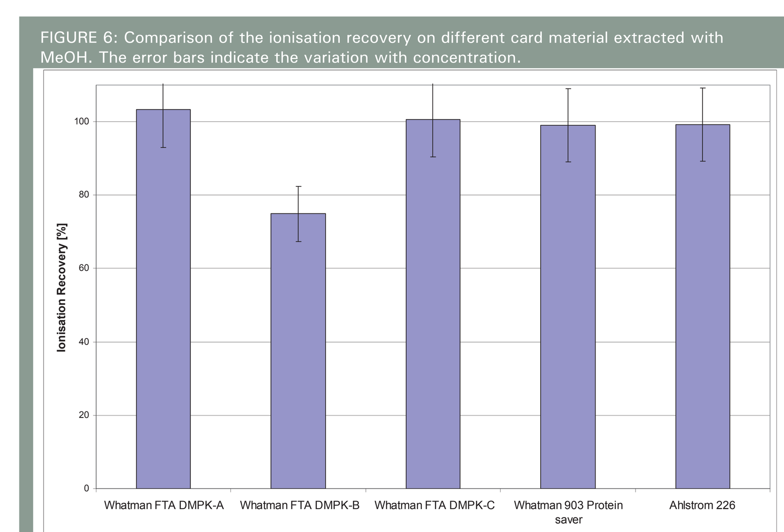



FIGURE 6: Comparison of the ionisation recovery on different card material extracted with MeOH. The error bars indicate the variation with concentration.




For solvent assessment methanol, 0.25% ammonia in methanol and acetonitrile gave an ionisation loss of 20% or more, whereas 1% formic acid in methanol showed minimal ionisation loss (Figure 5).

For card material assessment, the only material which showed a significant ionisation loss was the Whatman FTA DMPK-B card (Figure 6).

Quantitative ionisation recovery experiments were confirmed by post column infusion experiments.

FIGURE 7: Post column infusion of extracted blank card vs. extracted blank whole blood on a DMPK-B card (treated card material)

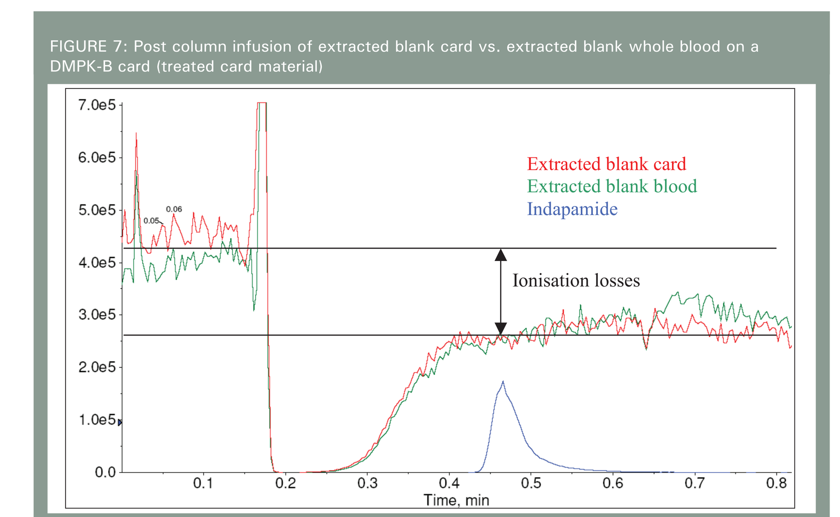
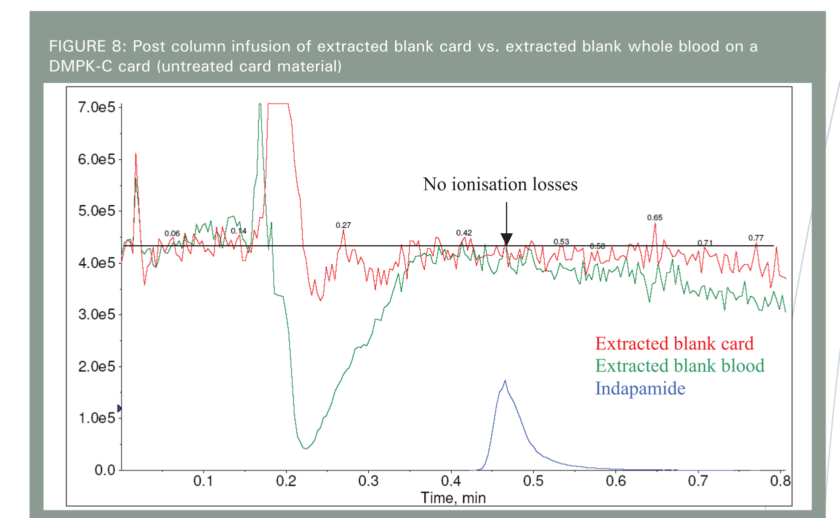



FIGURE 8: Post column infusion of extracted blank card vs. extracted blank whole blood on a DMPK-C card (untreated card material)




A difference in the overall suppression patterns was observed between untreated (Whatman FTA DMPK-C, Whatman 903 Protein saver, Ahlstrom 226) and treated (Whatman FTA DMPK-A, Whatman FTA DMPK-B) cards. 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 extensive suppression area (Figure 7) whereas virtually no suppression could be observed with untreated cards (Figure 8). 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 good 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

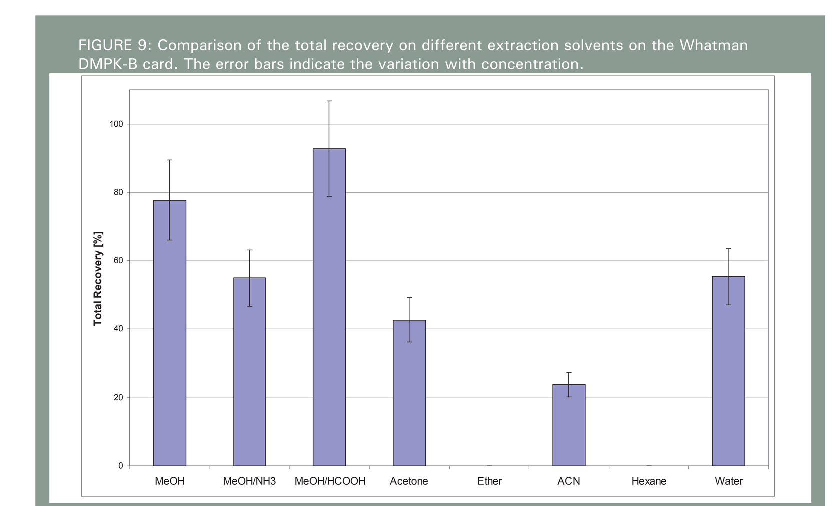
As the loss evaluations used a dry down and reconstitution approach (for consistency between solvents) the analyte loss during evaporation and reconstitution was evaluated at different concentration levels.

Pure solution 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 9).

FIGURE 9: Comparison of the total recovery on different extraction solvents on the Whatman DMPK-B card. The error bars indicate the variation with concentration.




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% ie 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 (eg 6 mm representing 11.4 µL blood sample) or by extracting multiple 3 mm punches.

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

## CONCLUSION

For DBS methods requiring low LLOQs 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

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