ASSESSMENT OF FACTORS INFLUENCING METHOD PRECISION IN A HUMAN DRIED BLOOD SPOT ASSAY FOR THE DETERMINATION OF A NOVEL ANALYTE

S. Wood, L. Neudert, L. Meunier, P. Struwe
Celerion Switzerland AG, 8320 Fehraltorf, Switzerland

INTRODUCTION

A robust bioanalytical LC-MS/MS method for the determination of a novel analyte in human dried blood spots (DBS) was established in our laboratory. Quantification used a di- or tri-labelled internal standard targeting an LLOQ of 0.100 ng/mL. In order to fully evaluate the variables for the assay and further optimise processes for dried blood spot analysis, this assay was used as a model for testing the factors that may affect reproducibility. Assay precision particularly at challenging LLOQs can have a significant impact on data quality for bioanalytical assays. This also has implications for the quality of repeat analysis and insured sample reproducibility. Several factors are unique to DBS bioanalysis in influencing this precision and are focused around on card homogeneity and blood spot thickness. Spotting technique, spotting volume, punching method, card type, anticoagulant choice and internal standard addition may all contribute to the reproducibility of the assay. This presentation explores which factors may influence precision and outlines the potential methodology optimisations for overall method reproducibility.

METHOD OVERVIEW

Fresh human whole blood (stored at 5°C for a maximum of two weeks after bleed date) was fortified with analyte (2% by volume) and immediately spotted onto an on-card microfluidic extraction card (ID Biological Systems and Whatman DMPK-B, GE Healthcare). Cards were dried at ambient temperature for 15 min. Spots of defined size were punched into DBS cards using a semi-automated punching instrument (Biodesign Australia) and matrix extract injections at corresponding concentration levels). This presentation explores which factors may in influence this precision and are focused around on card homogeneity and blood spot thickness. Spotting technique, spotting volume, punching method, card type, anticoagulant choice and internal standard addition may all contribute to the reproducibility of the assay. The presentation explores which factors may influence precision and outlines the potential methodology optimisations for overall method reproducibility.

RESULTS AND DISCUSSION

Factors potentially affecting the precision of DBS assays were evaluated systematically. Defined sets of samples were prepared according to the standardization procedure as described in the method overview section. This standard procedure was only modified at one variable per sample set (as described in the corresponding section) allowing appropriate impact evaluation of the particular factor tested. Sample sets consisted of Human whole blood (Sodium Heparin) fortified with analyte at analyte concentration levels (eight replicates assayed per concentration level). A sample volume of 20 μL per spot was applied to the DBS card by Multipipette using a 1-mL tip allowing the automated punching of one 3mm punch per spot. Samples from each assessment were then extracted and analysed in the same analytical batch.

Results are presented as analyte/standard peak area ratio reflecting the response algorithm as used for data reduction.

IMPACT OF SPOT PUNCHING TECHNIQUE

In order to address sufficient sample throughout automatic spotting and its impact on data precision were evaluated. For this purpose semi-automated punching using a BSD 600 dust puncting instrument (BDS Robotics, figure 4) was compared to manual punching using a Harris Uni-Core/GE Healthcare figure 4(μ)mm diameter punch size in order generate inter spot precision data.

Manual and automated punching methods gave similar absolute response at all concentration levels (table 1). Both methods showed overall improved precision compared to the manual approach (table 5).

IMPACT OF SPOT PUNCH SIZE

Another aspect to be considered within bioanalytical DBS studies is punch size as this can impact how repeat analysis either for analytical realising or assessment of incurred sample reproducibility will be performed. Validation of appropriate intra and inter spot precision is therefore important. Since the spot punch diameter directly corresponds to sample volume this will consequently directly impact assay sensitivity. Therefore the following evaluations were performed at one concentration level only using automated punching at a spot size of 6mm (representing 11.2 μL blood and allowing 1 punch per spot) and 3.2mm (representing 3.2 μL blood and allowing a maximum of 3 punches per spot).

Results are presented in table 6. Although inter and intra spot precision were found to be in line with measured baseline precision, intra spot precision (total 24 replicates) was found to be marginally worse than inter spot precision (from the corresponding punch size). Inter spot precision on 6mm punch size was found to be the best since this punch size almost complete the on-card area of a single spot.

IMPACT OF INTERNAL STANDARD ADDITION PROCEDURE

The addition of internal standard prior to sample extraction is a common bioanalytical practice to overcome potential precision issues derived from the sample extraction variability. In this standardized procedure previously described the addition of internal standard directly in extraction solvent will not correct for any extraction recovery differences. Therefore alternative methods of internal standard addition were investigated and compared against the standardized procedure.

Two alternative approaches were used. Firstly a defined volume of internal standard (2 μL) was added in methanol was spotted onto the DBS cards (with addition of a visual marker dye) prior to the spotting of whole blood sample; Secondly whole blood was fortified directly with internal standard as well as analyte and this was then spotted in the normal manner. This represents an ideal control for comparison only and was not intended for practical application. These samples were then extracted using methanol only and compared to the samples prepared according to the standardised protocol.

The results presented in table 7 show that the overall precision is not significantly worse when internal standard is added in extraction solvent (in this case). As expected the different procedures for internal standard addition did not provide comparable absolute values since any recovery and on-card distribution issues are not covered by the standard extraction procedure. Addition of internal standard directly to the card resulted in wider distribution of results compared to the blood spot. This lead to a reduced inter-spot precision for a defined punch area. Additionally this pre-addition of internal standard prior to spotting will reflect the final on-card distribution even though the cards were allowed to dry in between spotting steps.

CONCLUSION

These evaluations indicate the general robustness of the dried blood spot technique in the respect of sample extraction. Whilst many of the factors evaluated here did not cause additional impact on method precision it is clear that a defined and consistent procedure should be used both for blood spotting and card punching to maximise method performance. This needs to be balanced with practicalities of applying the DBS technique to facilitate throughout for larger sample numbers associated with clinical studies. Choice of punch size was driven by required LLOQ also has implications for method reproducibility. In our laboratory we have chosen to standardise on 3.2mm and 6mm punch diameters. Precision assessment therefore needs to reflect how repeat analysis will be performed in each case.

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