The influence of the sample matrix on LC-MS/MS method development and analytical performance

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Chapter 3.1

What is the right blood hematocrit preparation procedure for standards and quality control samples for dried blood spot analysis?

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ABSTRACT

Since hematocrit effects on dried blood spot analysis is widely acknowledged, a correct preparation of the target hematocrit is vital. We compared two procedures for preparing specific hematocrit values using a hematology analyzer. In the first procedure, whole blood was centrifuged, red blood cells (RBC) and plasma were separated and the proper volumes of RBC and plasma were mixed to obtain the target hematocrit. This procedure resulted in a measured hematocrit which was 11% lower than the target value. In the second procedure, blood was centrifuged and a calculated volume of plasma was removed or added in order to adjust the hematocrit. No difference was observed between the measured hematocrit and the target value. The second procedure was therefore considered the preferred procedure. It is recommended that as part of quality assurance the target hematocrit should be measured after preparation using a hematology analyzer.

INTRODUCTION

Dried Blood Spot (DBS) sampling has many advantages and among others allows the patient to sample blood at home and send the DBS sample to the laboratory by mail. This sampling is simple; saves patients travel expenses and time, and is therefore considered to be patient friendly and cost effective. Other advantages of DBS sampling are lower risk of bio-hazard and a smaller amount of blood required for sampling, which is also an advantage for pre-clinical studies using rodents [1-4].

Several factors need to be taken into account in case therapeutic drug monitoring (TDM) using whole blood or plasma sampling is replaced by DBS sampling. The bias caused by the hematocrit (HT) variability of the sample can be considered a critical parameter related to DBS analysis [5]. The HT represents the relative volume of the red blood cells (RBC) in the blood and has a direct effect on the viscosity of the blood. The permeability of the DBS card is influenced by the HT of the blood. Blood with a high HT has a low penetration of the DBS card and thus forms a smaller spot. A fixed diameter punch would then contain a higher blood volume causing a higher bias. Earlier publications have investigated the effect on the measured concentration caused by the viscosity of blood over a range of HT values during validation. It is suggested to correct for this effect with the use of a linear relation between HT and measured concentration [6-8]. When a fixed volume of venous blood is drawn from the patient and applied on the DBS card, followed by full spot analysis, the DBS can be considered more as a sample storage method than as a sampling method. In this case the hematocrit effect caused by the viscosity of the blood would be of no influence on the analytical results. However, this would hamper self sampling by patients, because the applied volume cannot be checked for accuracy.

There are two procedures to prepare a target HT value of anticoagulated whole blood used for standard and quality control samples for DBS analysis. In the first procedure, anticoagulated blood is centrifuged, RBC and plasma are separated and the proper volumes of packed RBC and plasma are pipetted and pooled to obtain the target HT [9-15].

In the second procedure, a volume of anticoagulated blood with a known measured HT (by the microHT method or hematology analyzer) is centrifuged and the appropriate amount of plasma is removed or added to obtain the target HT [16-20]. The disadvantage of the second procedure is that the HT of each batch of blood needs to be measured by a hematology analyzer, but there's no tedious pipetting of the packed RBC. The first procedure seems to

control both the volume of RBC and the volume of plasma. However, the centrifuged red blood cell fraction still contains plasma between the cells. The volume of plasma which is still present in the red blood cell fraction may depend on the centrifugal procedure and may result in a lower HT value than the target HT value. Various centrifugal procedures may produce different HT values. In current literature, the HT preparation procedure is often not accurately described and may have had a negative influence on the target HT [7, 8, 15, 21-27]. Articles that describe the preparation of a target HT by pipetting RBC and plasma use varying centrifugal procedures like 2 minutes at 1000g [9], 10 minutes at 1543g [10, 12], 4 minutes at 7000g [28] and 10 minutes at 3506g [14]. It seems uncommon to refer to the DIN 58933-1 (German National Standard) reference method, the CLSI H7-A3 (Clinical Laboratory Standards Institute) standard or another reference method in the preparation of HT values for DBS analysis [29, 30]. The DIN 58933-1 document mentions that the product of the minimum relative centrifugal force (RCF ≥ 5000g) acting on the erythrocytes and the centrifuging time in minutes is at least 100,000, implying a centrifugal time for example of 20 minutes at 5000g (20 x 5000 = 100,000). The product of the centrifugal time and the RCF of 100,000 was not met by the earlier described centrifugal procedures for the preparation of the target HT by pipetting RBC and plasma [9-14] , which ranged from 2000 (2 min x 1000g) [9] to 35060 (10 min x 3506g) [14]. The CLSI H7-A3 standard describes the use of a microhematocrit centrifuge spinning at 10,000g to 15,000g. The centrifuge method should be calibrated by testing the minimum packing time which is required to obtain a stable minimum packed cell volume for two consecutive time series [30]. For this study, the optimal centrifuge method was derived from both the DIN 58933-1 reference method and the CLSI H7-A3 standard. Since HT effects are common in DBS analysis, the preparation procedure of the target HT was considered a critical step in DBS analysis.

The varying HT preparation procedures described in literature may cause a variation in the prepared HT values and may influence the analytical results in DBS analysis. This effect may be underestimated at this time and was thus subject of this investigation.

The aim of the study was to establish the best procedure for the preparation of target HT values based on two common procedures. The first procedure involved pipetting RBC and plasma while the second procedure involved removing or adding plasma. These procedures were compared with some different HT preparation procedures described in literature and were all evaluated with the HT assay results of a hematology analyzer.

MATERIALS AND METHODS

Chemicals and materials

Citrate whole blood was purchased from Sanquin (Amsterdam, The Netherlands) and was used within two weeks after blood donation and was always checked on hemolysis before use. The whole blood was stored at 4°C. A Hettich centrifuge model 460R (Tuttlingen, Germany) was used to centrifuge the whole blood for HT preparation.

A XN-9000 hematology analyzer from Sysmex (Hyogo, Japan), using flow cytometry, was applied for all HT analyses and was an integral part of the routine patient HT analyses in the authors' hospital.

The preparation of the target HT

In order to test the HT preparation procedure, HT calibration curves from three different lots were each prepared in triplicate by the two described different preparation procedures in order to achieve target HT values of 0.10, 0.20, 0.30, 0.40, 0.50, and 0.60 L/L. The whole blood was first allowed to reach ambient temperature under very gentle tumble mixing before any centrifugation tests were performed.

The first preparation procedure was performed by centrifuging citrate whole blood and separating the plasma supernatant and the RBC. Then, the RBC and plasma were mixed together in order to obtain the target HT. The RBC were pipetted using an Eppendorf air displacement pipette with two times pre-wetting of the pipette tip. Pre-wetting is a standard procedure for viscous samples recommended by Eppendorf AG (Hamburg, Germany), the producer of the Eppendorf pipette.

The second preparation consisted of centrifuging citrate whole blood with a known HT (measured by the Sysmex XN-9000 analyzer), followed by removing or adding the necessary volumes of plasma to achieve the target HT values. The two optimized HT preparation procedures are described in table 1.

Centrifugation procedures found in DBS publications, which make use of the pipetting of RBC and plasma, were used to prepare target HT values of 0.50 L/L in triplicate, as described above. The following centrifugation procedures were tested: 2 minutes at 1000g [9], 10 minutes at 1543g [10, 12], 4 minutes at 7000g [28] and 10 minutes at 3506g [14].

Table 1 The two optimized hematocrit (HT) preparation procedures

Step	First procedure	Second procedure
1	Centrifuge for 30 minutes at 4637g	Measure the HT with a hematology analyzer
2	Transfer plasma supernatant to fresh tube	Centrifuge for 5 minutes at 2000g
3	Discard approximately 300 µL of the top layer to minimize the amount of plasma in the RBC section	Remove or add the necessary volume of plasma
4	Gently homogenize the RBC	Gently homogenize the blood tube
5	Pipette the necessary volumes of RBC and plasma together	Measure the HT with a hematology analyzer
6	Measure the HT with a hematology analyzer	

The pipetting of the red blood cells (RBC) was performed by reverse pipetting with two times pre-wetting, using an Eppendorf air displacement pipette.

The prepared samples were analyzed for their HT using the Sysmex XN-9000. The prepared target HT and the measured HT of the HT calibration curves were compared using Passing and Bablok regression analysis with Analyse-it® (version 2.30) software (Leeds, United Kingdom).

RESULTS AND DISCUSSION

The pipetting of the RBC appeared to be a critical step in the DBS procedure because of the high viscosity of the RBC. The use of a Gilson positive displacement pipette, which is very suitable for pipetting viscous samples, caused hemolysis by the friction of the plunger. An air displacement pipette (Eppendorf Hamburg, Germany) with careful reversed pipetting and two times pre-wetting proved to be the best approach. Centrifuging at 4637g for 30 minutes resulted in no further decrease in RBC levels compared with the preceding centrifugation time, which complied with the CLSI H7-A3 standard. The centrifugal procedure also complied with the DIN 58933-1 guideline, where the RCF multiplied with the time in minutes, was in this case 138,000 and was higher than the recommended value of >100,000 (where the RCF should be >5000).

For the procedure of pipetting the RBC and plasma, the Passing and Bablok results of the three prepared HT curves in triplicate showed a difference of -11% between measured HT relative to target HT according to the following equation: $y = 0.89x – 0.01$, with 95% confidence intervals (CI's) of 0.86 to 0.95 for the slope and -0.02 to 0.01 for the intercept (figure 1).

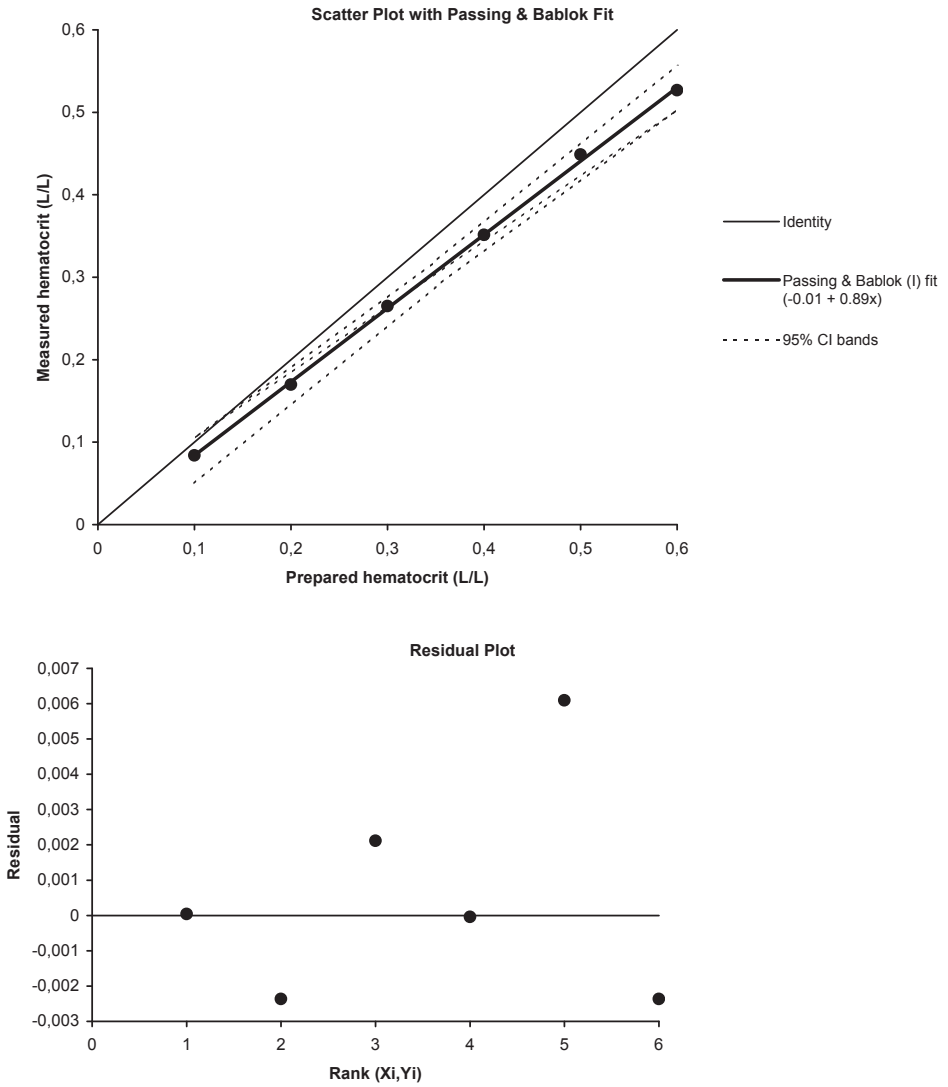

Figure 1 Results of the Passing and Bablok and residual plots where the prepared target hematocrit was compared with the measured hematocrit using a Sysmex XN-9000 analyzer.
The target hematocrit was prepared by pipetting red blood cells and plasma in triplicate for each of the 3 batches of blood at each hematocrit level. N = 9 at each of the 6 hematocrit levels. The following equation was found: y = 0.89x – 0.01, with 95% confidence intervals (CI's) of 0.86 to 0.95 for the slope and -0.02 to 0.01 for the intercept.

The coefficients of variation (CV's) for the HT measurements ranged between 3.95% and 7.04% per HT value (n=9). The procedure of adding or omitting plasma from blood with a measured HT showed a deviation in HT of 0% according to the following equation: y = 1.00x – 0.00, with 95% CI's of 0.95 to 1.03 for the slope and -0.01 to 0.01 for the intercept (figure 2).

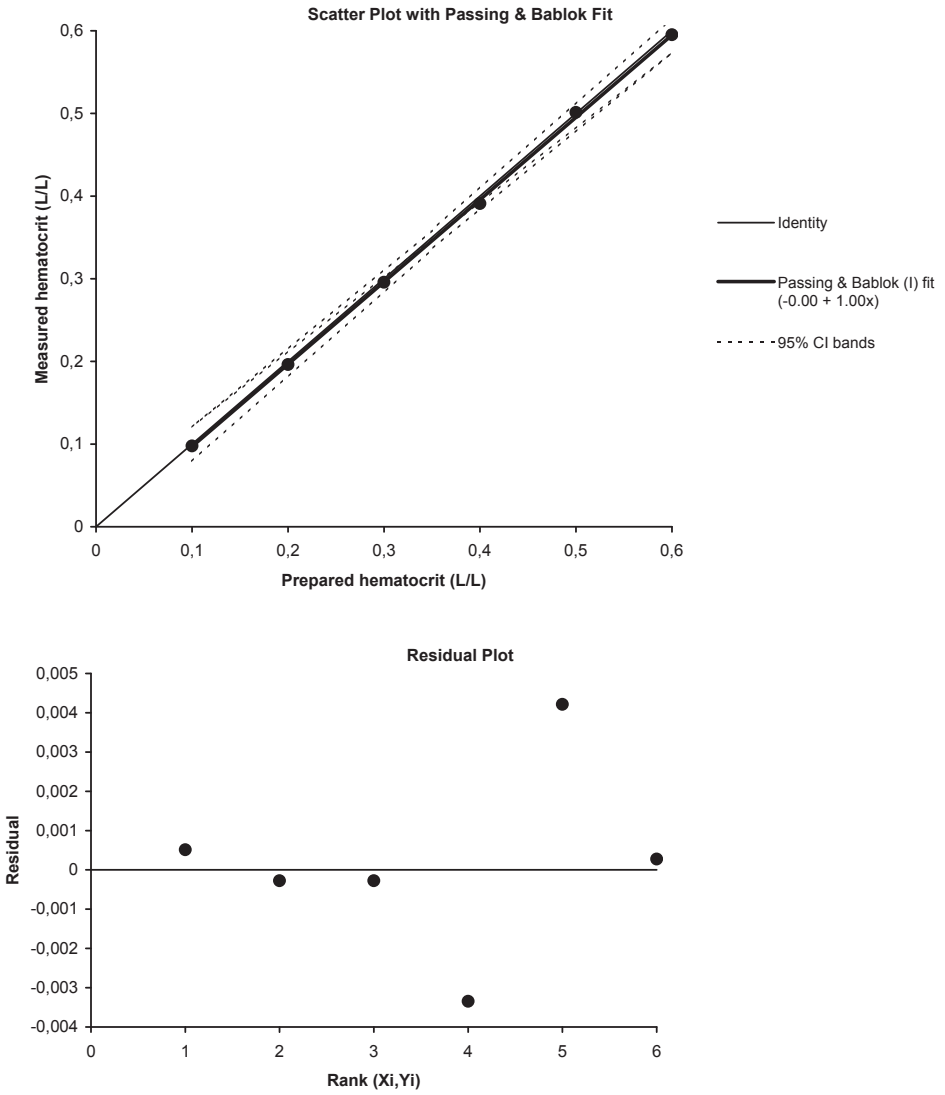

Figure 2 Results of the Passing and Bablok and residual plots where the prepared target hematocrit was compared with the measured hematocrit using a Sysmex XN-9000 analyzer.
The target hematocrit was prepared by removing or adding plasma in triplicate for each of the 3 batches of blood at each hematocrit level. N = 9 at each of the 6 hematocrit levels. The following equation was found: y = 1.00x – 0.00, with 95% CI's of 0.95 to 1.03 for the slope and -0.01 to 0.01 for the intercept.

The CV's for the HT measurements ranged between 2.13% and 3.65% per HT value (n=9). The procedure of adding or omitting plasma yielded lower CV's compared to the pipetting of RBC and plasma, indicating that this is a more robust procedure.

The varying centrifugal times and forces combined with pipetting of the RBC and plasma as mentioned in previous publications provided similar deviations of the HT, as can be seen in table 2.

The procedure of centrifuging for 2 minutes at 1000g showed by far the highest deviation, and can be considered as inadequate. The differences between the other centrifugal procedures were of much less influence on the HT preparation procedure. However, all the centrifugal procedures that used the pipetting of RBC and plasma yielded biases between -7.9% and -16.1%. It can be concluded that the procedure of pipetting RBC and plasma will unavoidably cause an error in the prepared HT. And this procedure should only be used after correction by HT measurements for every HT preparation using a hematology analyzer. Maybe this procedure could be validated over time with various batches of blood, where a constant bias and a low batch to batch variability may provide a correction factor.

As a consequence of these discrepancies, the HT effects mentioned in the literature should be interpreted with caution, since real HT values may deviate from the mentioned HT values. A HT preparation procedure that causes deviating HT values is especially advised against when a HT effect has been observed and standards and quality control samples are prepared with a HT value that was meant to represent the patient population. This prepared HT value may already cause analytical biases due to the different HT values of the standards and the patient population.

Table 2 The varying centrifugal times and forces combined with pipetting of the RBC and plasma derived from previous publications

Centrifugal parameters	Bias (%)	CV (%)
2 minutes at 1000g	-16.1	0.14
10 minutes at 1543g	-12.1	0.57
10 minutes at 3506g	-9.7	0.78
5 minutes at 10,000g	-9.2	1.14
30 minutes at 4637g	-9.1	1.25
4 minutes at 7000g	-7.9	0.13

The prepared target hematocrit values were compared to the hematology analyzer resulting in the stated biases (n=3).

CONCLUSIONS

The preparation of the target HT with the pipetting of RBC and plasma (first procedure) caused a significant bias in the HT and can only be used when the HT is corrected with the measured HT using a hematology analyzer. The procedure of adding or removing plasma from blood with a known HT (second procedure) caused no error in the HT and was considered the preferred HT preparation procedure. It is recommended that as part of quality assurance the target HT should be measured after preparation using a hematology analyzer.

FUTURE PERSPECTIVE

The use of dried blood spot (DBS) analysis for therapeutic drug monitoring will play an increasing role in the future. The investigation of critical parameters such as the preparation of the target hematocrit value for calibration curves and quality control samples will lead to improved procedures for DBS validation and analysis. This will ensure that future DBS analysis results will be generated with improved quality standards.

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