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## Determination of hemoglobin content in whole blood and in dried blood spots in lambs

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### Abstract

The use of filter paper for the collection and analysis of human blood has begun in the 1960s. In those days Dr. Robert Guthrie used dried blood spot (DBS) specimens to measure phenylalanine in newborns. DBS are whole blood samples collected from blood obtained by finger-sticks or heel pricks and placed onto filter paper, dried and eluted later for laboratory analyses.

The purpose of this study was to develop and evaluate a capillary filter paper method that could be employed as a reliable technique for hemoglobin (Hb) determination in animals.

Heparinised blood (10 ml) was taken from healthy lambs (n=20). DBS were prepared as venous whole blood samples were aliquoted (20 µL) onto Whatman 903 (Schleicher & Schuell GmbH Inc., Germany) filter paper. Either the entire DBS were cut from the paper card with scissors for extraction, or DBS punches were removed from each DBS by using a standard paper hole punch. The discs were placed into test tubes for elution in Drabkin reagent and the eluates were measured spectrophotometrically. Hb values obtained on filter paper were compared with values from fresh whole blood samples.

As our results indicate the standard punches gave identical results as the whole blood determinations under similar conditions (2.5 ml reagent and 20 min elution time). After 10 day storage period the elution required more time (40 or 60 min) for the same results.

The use of DBS offers certain advantage over using traditional methods and conventional samples. Not only in case of infants but also in animal investigations the DBS are a promising alternative to venipuncture.

**Key words:** dried blood spot, DBS, hemoglobin, lamb

### Introduction

The use of filter paper for the collection and analysis of human blood has begun in the 1960s. In those days Dr. Robert Guthrie used dried blood spot (DBS) specimens to measure phenylalanine in newborns for the identification of inborn errors for the detection of phenylketonuria (Mei et al., 2001). Since that time additional possibilities of using DBS cards for inborn errors of metabolism in neonates are proceed. These include investigations of disorders of amino acid metabolism, fatty acid oxidation, organic acid metabolism, congenital hypothyroidism, and more recently cystic fibrosis (Lacher et al., 2013). Nowadays, thanks to the development of new techniques, the possible applications of DBS have greatly been widened (Snijdewind et al., 2012).

DBS are whole blood samples collected from blood obtained by finger-sticks or heel pricks and placed onto filter paper, dried and eluted later for laboratory analyses. The general procedure is that the well formed blood drop should be applied on the filter paper card to allow an appropriate quantity of blood to steep and entirely fill a pre-printed circle. Blood should consistently penetrate and soak through the filter paper to ensure reliable result. Due to the minimal invasiveness of sampling and small sample requirement collecting blood on DBS cards also offer the chance to obtain specimens from infants and young children in whom venipuncture is problematic to perform.

The purpose of this study was to develop and evaluate a capillary filter paper method that could be employed as a reliable technique for hemoglobin (Hb) determination. In this study the DBS samples were tested as an alternative method of Hb measurement in whole blood samples. Objective of this investigation also was to optimize Hb measurement from DBS by measuring the stability of Hb in filter paper dried blood spots collected from lambs. The optimal extraction time required for the measurement of Hb from DBS was also defined.

## **Materials and Methods**

### *Specimen collection*

Heparinised blood (10 ml) was taken from healthy lambs (n=20). In the laboratory DBS were prepared from anticoagulated venous blood samples taken for routine measurement of Hb.

The Hb values were studied in filter paper blood spots that were stored at room temperature (25 °C) for different time (1 day and 11 days).

### *DBS analyses*

The venous whole blood samples were aliquoted (20 µL) onto Whatman 903 (purchased from Schleicher & Schuell GmbH Inc., Germany) filter paper. This paper is made from high purity cotton linters that absorb a fixed volume of blood in a given diameter of paper.

Ten DBS were made from each sample using standard filter paper. Blood spots were created by dispensing blood on the filter paper using a pipette with constant pipetting speed and pressure.

After preparation all DBS cards were placed in a place away from direct sunlight in horizontal position on a non-absorbent surface and allowed to dry overnight at ambient temperature before measurement of the Hb concentration by the cyanmethemoglobin procedure. After thorough air-drying DBS cards were put in plastic bags with desiccant packs, sealed and stored at room temperature.

### *Hemoglobin assays*

Control whole blood samples used to monitor DBS extraction efficiencies were prepared by conventional volumetric dilution of whole blood (20 µl blood + 2.5 ml Drabkin reagent). After 20 minutes incubation period the samples were measured spectrophotometrically (Sós, 1974).

In case of DBS samples two pretreatments were used. Either the entire DBS were cut from the paper card with scissors for extraction, or DBS punches were removed from each DBS by using an ordinary standard paper hole punch. For "small" DBS samples 1/8 inch diameter discs were punched from the center of the blood spot. For Hb determination the punches were placed into test tubes for elution and then analyzed by the specific assay (cyanmethemoglobin method). As in case of conventional Hb determination equivalent amount of Drabkin reagent (2.5 ml) was added to DBS discs. After mixing each disc was eluted for different times (20, 40 or 60 minutes) at room temperature in Drabkin reagent. Finally the hemoglobin concentrations of DBS eluates were measured spectrophotometrically. After establishing the stability over a ten days period, Hb values obtained on filter paper were compared with values from fresh whole blood samples.

## **Results**

The Hb concentrations of the lamb's blood samples are shown in Table 1. The expected Hb content of the standard (1/8 inch) punches was established by measuring the width of a single 20 µL blood spots.

Table 1

**Comparison of the Hb concentrations of the fresh whole blood samples and the DBS spots in lambs**

2.5 ml Drabkin reagent			Hb cc.	Diameter	Estimated Hb cc.
Elution time: 20 min			(g/L)	(mm)	based on area (%)
Whole blood	(20 µl blood)		118.37±10.19		
DBS	whole spot (20 µl blood)	1 <sup>st</sup> day	95.26±9.90	7.33±0.37	100
DBS	punched spot (1/8 inch)	1 <sup>st</sup> day	21.69±2.62	3.175	121.90±16.35

The results of the established necessary elution times after 10 days storage of DBS samples are found in Table 2.

As our results indicate on the 1<sup>st</sup> day the standard punches gave identical results as the whole blood determinations under similar conditions (2.5 ml reagent and 20 min elution time). At later investigations (after 10 day storage periods) the elution required more time (40 or 60 min) for the same results.

Table 2

**Hb concentrations of the DBS samples after storage**

2.5 ml Drabkin reagent			Hb concentration		
			Elution time		
			20 min	40 min	60 min
Whole blood	(20 µl blood)		(g/L) 118.37±10.19		
			(%) 100		
DBS	whole spot (20 µl blood)	10 day storage	(g/L) 104.40±11.85	117.38±11.37	
			(%) 88.52±9.52	99.59±9.94	
DBS	punched spot (1/8 inch)	10 day storage	(g/L) 20.25±1.87	24.84±2.21	
			(%) 91.84±13.08	112.57±15.54	

**Discussion**

Filter paper sampling has several important advantages in contrast to similar technologies. Compared to conventional methods (venipuncture), DBS can be collected by non-phlebotomists in non-clinical settings (Lacher et al., 2013). The DBS sampling offer opportunities to measure and analyze samples in resource-limited settings, in areas lacking specialized laboratories. To prevent degradation DBS do not need to be handled in the laboratory on the same day and centrifuged or immediately refrigerated or frozen following collection in contrast to serum or plasma samples (Snijdewind et al., 2012).

DBS card samples are easy to collect and require a relatively small amount of blood; it can be prepared from a few drops of blood, obtained from a capillary blood stab, i.e. a finger prick.

A filter paper disk punched from a blood-filled circle provides a volumetric measurement that is similar to liquid measuring devices. It was agreed that 3.2 mm punch (universal punch size) normally contain the equivalent of 3.2 µl of whole blood. Increasing the number of disks may be used to enlarge the sample volume.

The effect of haematocrit (Ht) must be evaluated as part of any method development because it has a considerable effect on blood viscosity and may thereby affect flux and diffusion properties of the blood through the paper and hence the size of the blood spots. In our investigation the Ht values were identical in lambs (0.32±0.03 L/L). In addition, due to chromatographic effects there may be a substantial difference of metabolite concentrations between central and peripheral areas within the DBS (Holub et al., 2006).

Storage and transport of DBS cards provides some important advantages over the traditionally used liquid forms of whole blood, serum and plasma. DBS samples are very easy to store, they can be stored at ambient temperature for a prolonged period of time when stored with desiccant in closed bags. A maximum storage of 2 weeks at room temperature is suggested. Freezing at -20 °C is recommended when DBS cards are stored for more than 2 weeks for maintaining enzyme activities for longer durations (Mei et al., 2001). Therefore, the cards are stable and can be sent by regular mail to a laboratory in a tightly sealed thick envelope at room temperature safely. DBS specimens can be kept at -20°C for many weeks or years.

For the appropriate analyses and results in case of DBS sampling reliable and reproducible spots need to obtain. The limitations of the small sample sizes in DBS cards require sensitive methods. DBS methods need to be carefully developed and validated against venous methods.

### Conclusions

Based on our results the advantages of DBS Hb method compared to whole blood assay could be summarized as follows. Using venous whole blood method as a reference, the Hb concentration correlated well between DBS and venous method in case of adequate elution time. Taken together, these results indicate that this blood-spot procedure is a useful means of monitoring blood Hb level in animals.

The use of DBS offers certain advantage over using traditional methods and conventional samples. Not only in case of infants but also in animal investigations the DBS are a promising alternative to venipuncture. Development of a quantitative method for determining blood parameters in small amounts of blood will be very important also in animal investigations.

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