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Development of filter paper Hemoglobin A1c assay applicable to newborn screening

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Abstract

Purpose—Gestational diabetes influences risk for future metabolic disease including type 2 diabetes. Hemoglobin A1c (HbA1c) measurement assesses hemoglobin A glycosylation, and could theoretically be used as a test to estimate gestational glucose exposure. HbA1c assay on dried blood spots (DBS) is needed before potential application to statewide newborn screening (NBS) population studies. The study aimed to establish a reliable method to measure HbA1c on NBS DBS specimens. De-identified blood was used to generate trials to evaluate stability of HbA1c in DBS, optimal elution time, and stability of eluted blood.

Results—Analysis of DBS stability HbA1c measurements from 3–6 days after collection overestimated HbA1c values by a bias factor between 0.83 and 0.87. Sixty minutes of elution time produced maximal reproducibility and minimal bias of results. Within assay standard deviation: 0.058; average bias: -0.02%. Stability of eluted blood did not vary significantly between days 0–2 after DBS elution.

Conclusions—Measurement of HbA1c levels on DBS from human blood is feasible. Results suggest new method using DBS to measure HbA1c level with the following characteristics: optimal time for sample analysis 3–6 days after collection, elution time of 60 minutes and eluted blood analysis within 2 days of elution. Measurement of neonatal HbA1c could provide insight regarding the infant's *in utero* exposure to glucose.

Keywords

Hemoglobin A1c; filter paper; diabetes; obesity; newborn screening

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Introduction

Low[1,2] and high[3] birth weight infants and infants of diabetic mothers[4] are at increased risk for future development of obesity and type 2 diabetes (T2D), yet the pathogenesis of this connection is not fully understood. Variation in birth weight reflects diverse bio-social factors including *in utero* metabolic imprinting of the fetus by nutritional, endocrine, and genetic variables. These factors also appear to contribute to a child's postnatal risk of obesity, T2D, and cardiovascular disease [3,5-8]. Mechanistic theories of how these risks develop include Barker's Thrifty Hypothesis, which posits that poor *in utero* nutrition and other perinatal factors result in fetal prioritization of nutrients to vital organ development, at the expense of muscle and pancreas development; ultimately resulting in insulin resistance geared toward survival in poor-nutrient environments [9]. Alternatively, the Insulin Hypothesis posits that an insulin-resistant genotype is linked to the dual phenotype of low birth weight and T2D via insulin resistance and impaired insulin-mediated growth [5, 9].

Although it is known that extremes of birth weight predict higher risk for adult metabolic and cardiovascular disease, specific laboratory indicators of this risk related to *in utero* programming are lacking. Hemoglobin A1c (HbA1c) is an indicator of irreversible glycosylation of the NH₂-terminus of the beta chain of adult hemoglobin (HbA), and is widely used to assess glycemic control in diabetic children and adults [10]. HbA1c is the major fraction of total glycosylated hemoglobin (GlyHb) and is a measure of red cell glucose exposure over the previous two to three months. *In utero*, the shift from fetal hemoglobin (HbF) to HbA synthesis begins as early as 24 weeks gestation [11], and HbA is exposed to maternal glucose for the remainder of gestation, positioning newborn HbA1c as a potential marker of fetal glucose exposure and glycosylation of HbA during the last trimester of pregnancy [9]. Variation in newborn HbA1c values, and by inference, *in utero* glycemic exposure, may also indicate a predisposition to subsequent metabolic disease and aid in targeting interventions to those identified early as higher risk. Normal ranges for values of HbA1c have not been established in neonates, and establishment of normal reference range of HbA1c in neonates is required to assess HbA1c value as a predictive marker of prenatal pathophysiology that predisposes to insulin resistance, obesity and T2D.

Newborn screening (NBS) programs provide a large population of newborn blood samples, accompanied by information about each infant including birth weight, sex, race, and gestational age. Blood samples are collected from infants at 24-48 h of life and dried on filter paper, and sent for analysis at a NBS laboratory. In Wisconsin (WI), statewide newborn screening began in 1965 with screening for phenylketonuria (PKU); the NBS Laboratory is within the WI State Laboratory of Hygiene (WSLH) [12].

1.2 Material and Methods

Hemoglobin was eluted from blood spots prepared using de-identified residual blood from the University of WI Hospital and Clinic Laboratory of Chemistry. Whatman 903 filter paper was used for blood spotting, as is routine for newborn screening. HbA1c measurement, was performed using the ion-exchange HPLC Tosoh G7 HbA1c assay, which is certified by the National Glycohemoglobin Standardization Program (NGSP) and is designed to minimize interference by HbF. This assay is routinely calibrated using

manufacturers's control solution and quality assurance maintained per Chemistry laboratory standards as it is used daily for clinical sample analysis [13]. Multiple assay trials were conducted to compare HbA1c results between DBS and liquid blood samples specifically to (1) Evaluate stability of HbA1c in blood spots, (2) Evaluate optimal elution time and (3) evaluate stability of eluted blood. Whole blood EDTA specimens, <12 h after collection were selected from the UWHC Chemistry Laboratory conventional workload to represent HbA1c levels observed in patient population of adults and children. All samples were sent to the State Laboratory of Hygiene where blood spots were prepared and then stored at 4 °C. This study was determined to be exempt from review per the University of Wisconsin Institutional Review Board.

1.2.1 Stability of HbA1c in Blood Spots—Bias and reproducibility of HbA1c measurements were assessed from eluted residual blood from DBS that had been spotted 3, 4, 5, and 6 days prior, and compared to HbA1c measurements from residual liquid blood. This timeframe was chosen to mimic potential application in NBS program, where NBS specimens are analyzed on the day they arrive. Specimens are typically collected within 24 – 48 h after birth, and arrive at the lab with 3–4 days after collection. For elutions specimens were routinely shaken at room temperature for 1 hour.

1.2.2 Optimal Elution Time—This study evaluated bias and reproducibility of HbA1c measurements from eluted blood from DBS that had elution times of 10, 15, 20, 30, 60, 90, 120 min, compared to HbA1c measurements from residual liquid blood. The optimal elution time point was determined by maximizing the reproducibility while minimizing the bias. Specifically, the following utility function was used to determine the optimal elution time point: $F(t) = 0.5 \times \% \text{ from total variability} + 0.5 \times \% \text{ from total bias}$. [14] The time point t which maximizes $F(t)$ was defined as the optimal elution time.

1.2.3 Stability of Eluted Blood—Bias and reproducibility of HbA1c measurements from eluted blood from DBS 0, 1 and 2 days after elution were compared to HbA1c measurements from residual liquid blood.

1.2.4 Statistical Analysis—Bias was reported in terms of means along with the corresponding 95% CI. Reproducibility was reported as the percent of variability between repeated HbA1c assessments from the total variability. Data analysis was conducted using SAS software, ver 9.3.

Results

Stability of HbA1c in Blood Spots: HbA1c measurements from 3, 4, 5 & 6 days after DBS exceeded control samples by a consistent factor (bias). Bias and 95% CI for each respectively 0.83 (0.78-0.89), 0.87 (0.81-0.92), 0.83 (0.66-0.99), 0.87 (0.81-0.92). Table 1 shows one example blood sample, where liquid blood HbA1c value was 5.7% and DBS HbA1c results of same sample are shown in triplicates 3 to 6 days after initial preparation of DBS.

Optimal Elution Time: Sixty minutes of elution time (Step 3) produced highest reproducibility and lowest bias when compared to 10, 15, 20, 30, 90 and 120 min. Within assay SD was 0.058 and average bias was -0.02%.

Stability of Eluted Blood: The bias and reproducibility of the HbA1c assay results did not vary significantly between days 0, 1 and 2 after DBS elution (time between Step 3 and 4). Bias -0.06, 0.07, 0.03, % total variability 34.29, 35.34, 30.36 respectively using 1-day-old DBS with 60 minutes elution. Table 2 shows one example, where liquid blood HbA1c value was 4.9% and DBS HbA1c results of same sample are shown in triplicates 0 to 2 days after blood elution from DBS.

Discussion

Newborn screening programs were originally designed to detect treatable life-threatening disorders in asymptomatic neonates (e.g. PKU), but over time the NBS has also proven that early recognition and treatment of some chronic diseases can improve long term outcomes (e.g. cystic fibrosis). It is becoming apparent that prevention of obesity and its long-term morbidity optimally starts in infancy [15,16]. Several studies have shown that rapid weight gain in the first year of life increases obesity-risk in adulthood and children who are obese by age 5 y have a much increased chance of continuing to be obese as adults [17]. Thus, NBS programs could have potential to detect biomarkers for obesity risk, and by doing so, allow early beneficial intervention in high risk children. Neonatal HbA1c, as a candidate indicator of future obesity, may fit into this contemporary NBS model.

Previous studies of HbA1c measurement in neonates have limited applicability to newborn screening in large populations due to utilization of cord blood *liquid* specimens, reliance on total glycosylated hemoglobin measurement, or small sample size [18-20]. One small study[18] (n=5; HbA1c assay using diethylaminoethyl cellulose chromatography) reported HbA1c levels in *liquid* cord blood and found infants of diabetic mothers had HbA1c values (mean $3.3\% \pm 1.5\%$) that were higher than infants of non-diabetic mothers ($2.1\% \pm 0.5\%$; $p > 0.05$). This is not surprising given the higher expected fetal exposure to glucose in a diabetic pregnancy. Of note, infants of diabetic mothers are known to be at increased risk for future obesity and T2D. A second small study[19] (n=5; HbA1c assay by Tosoh G8 High Performance Liquid Chromatography (HPLC), similar to assay used in this study), reported mean *liquid* cord blood HbA1c measurements of $1.5\% \pm 0.2\%$. In comparison, HbA1c levels in non-diabetic older children and adults range from 4.3–6.0%. Lower newborn HbA1c measurements in neonates are likely due to several factors including timing of HbF to HbA switch which could reduce duration of glucose exposure of HbA, rate of red cell turnover *in utero*, and variable Hb concentrations. One large study[20] (n=1295; Total glycosylated Hb assay HPLC Variant Total Glycated Hemoglobin Assay) reported mean total glycosylated Hb from *liquid* cord blood of $4.5 \pm 0.5\%$. Total glycosylated Hb has the advantage of including both HbF and HbA, but this assay is no longer routinely clinically available, so may not be as practical as HbA1c for large, non-research populations such as in NBS programs.

While previous small studies have suggested feasibility of HbA1c measurement using *liquid* neonatal blood, the results of this study support feasibility of HbA1c measurement using NBS program *filter paper*. A limitation of this study is that it did not directly test *neonatal* blood samples on filter paper, but instead de-identified residual blood from a general chemistry lab, which receives proportionally few neonatal samples. Given the distinct HbF and HbA characteristics of neonatal blood, more information is needed to validate *filter paper* HbA1c measurement of *neonatal* blood samples.

Development of assays used in NBS programs require consideration of key sample conditions which were evaluated in this study. Results of this study show that dried filter paper blood samples provide HbA1c measurements with minimal bias and maximal reproducibility when processed within 6 days of blood collection (stability of HbA1c in DBS prior to elution), eluted for 60 min (elution time), and analyzed with Tosoh G7 within 2 days of elution (stability of eluted blood). Stability of HbA1c in blood spots must be assessed in NBS programs because the time it takes between sample collection at distant site and sample processing at the analyzing laboratory has potential to introduce variability in assay results. In the WI NBS program, most samples are received within 2-3 days after collection and are processed on the day they arrive. HbA1c levels from DBS in this study were higher than the levels from liquid blood by a consistent factor. While the need to apply a correction factor is not ideal, filter paper analysis of this is amendable since this was by a consistent factor and blood spots were stable well within the usual WSLH processing timeframe. After a period of time post-elution from filter paper, the sample is analyzed via the HbA1c assay. Results in this study did not vary significantly if samples were analyzed 0, 1, 2 days after elution, providing some flexibility in scheduling laboratory procedures if applied on a larger scale.

In NBS programs, samples must be processed in an efficient standardized way to yield high quantity of accurate results in a short period of time. This study of an HbA1c assay on dried blood spots using the NBS workflow indicated that it may be feasible to measure HbA1c on DBS: (1) spots could be tested within a practical window to time after collection, (2) standardized elution time yielded consistent results and (2) HbA1c assay results were reliable up to 2 days after blood elution. Next steps to expand this to the large population of neonates screened involve validating HbA1c measurement from filter paper samples from neonatal blood. This would set the stage for longitudinal trials correlating neonatal HbA1c with subsequent weight gain trajectory and other risk factors for obesity and T2D.

1.5 Conclusions

In conclusion, measurement of neonatal HbA1c could provide insight regarding the infant's *in utero* exposure to glucose. This study establishes parameters required to use HPLC HbA1c assay by Tosoh to analyze blood samples from neonatal dried blood spots. Results support taking the next step of measuring HbA1c in a study group of neonates within a NBS program to assess any potential differences with neonatal samples, which include a proportion of HbF. With results from this neonatal sample, normal range of HbA1c could be established, providing support for further evaluation of HbA1c as a possible indicator of late gestation glucose exposure in neonates, and perhaps of their future risk for T2D and obesity.

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Abbreviations

GlyHb	total glycosylated hemoglobin
NBS	newborn screening
PKU	phenylketonuria
WSLH	Wisconsin State Laboratory of Hygiene
DBS	dried blood spots
NGSP	National Glycohemoglobin Standardization Program

Highlights

- Measurement of HbA1c levels on DBS from human blood is feasible.
- Optimal time for sample analysis is 3-6 days after collection.
- Optimal elution time is 60 minutes.
- Optimal timing of eluted blood analysis is within 2 days of elution.
- Measurement of neonatal HbA1c could provide insight into in utero glucose exposure.

Table 1
Stability of HbA1c in dried blood spots

Days since blood collection	Reproducibility: Triplicate HbA1c results from same sample (5.7% liquid blood)		
3	6.6%	6.5%	6.5%
4	6.5%	6.6%	6.6%
5	6.3%	6.5%	6.7%
6	6.5%	6.6%	6.6%

Table 2
Stability of HbA1c in eluted blood

Days since blood collection	Reproducibility: Triplicate HbA1c results from same sample (4.9% liquid blood)		
0	5.0%	4.9%	5.0%
1	5.1%	5.2%	5.2%
2	5.2%	5.1%	5.1%