Capillary Blood on Filter Paper for Determination of HbA_{1c} by Ion Exchange Chromatography

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OBJECTIVE — To facilitate HbA_{1c} determination, we evaluated an HbA_{1c} filter paper system enabling capillary blood sampling at home by the patients.

RESEARCH DESIGN AND METHODS — Capillary blood (two drops) was applied to a filter paper (HbA_{1c} Via Post) and sent to the laboratory where a small disc was punched out on the filter paper. Hemoglobin was eluted from the disc in a buffer containing cysteine to eliminate the interfering glutathione adduct (HbA_3) formed during storage. Analysis was performed by ion-exchange chromatography (Mono S, high-performance liquid chromatography), and the eluate was compared with hemolysate of venous blood from 41 patients. The stability of blood impregnated on filter paper was checked at different temperatures over different periods of time.

RESULTS — There was an excellent agreement ($r = 0.99$) between HbA_{1c} values from capillary blood on filter paper and HbA_{1c} values from venous blood. HbA_{1c} values were constant when stored on filter paper for 5–7 days at 20–21°C (room temperature) or at 4–6°C (refrigerator) for 10 days as well as at −70°C for several months after blood sampling. A new chromatographic-interfering hemoglobin fraction both from venous and capillary samples was identified as free α-chain of hemoglobin.

CONCLUSIONS — The HbA_{1c} filter paper system enables capillary blood sampling at home, eliminates the need of vein puncture in children and adults, and provides the diabetologist with an HbA_{1c} value when the patient visits the clinic without a need for a previsit phlebotomy.

HbA_{1c} has been used for almost two decades as a marker of long-term glycemic control in diabetic patients (1). The importance of HbA_{1c} in this context has increased during the years, and HbA_{1c} is now considered as the gold standard for metabolic control in diabetic patients; the improved glycemic control, as reflected by a near normalization of HbA_{1c}, prevents the development of microvascular complications in type I diabetic patients (2,3). Indeed, without HbA_{1c} determinations, it simply would not have been possible to demonstrate that improvement in metabolic control is associated with prevention of diabetic complications (4).

HbA_{1c} is usually assayed from venous whole-blood samples. To enable analysis of capillary blood sampled on filter paper, a thiobarbituric acid colorimetric method and affinity chromatography have been introduced (5,6). When capillary blood was preserved and transported in liquid solutions, the correlation between venous and capillary HbA_{1c} improved (7,8). Liquid specimens are difficult to handle for the patient; capillary blood samples on a filter paper are less complicated.

Assaying HbA_{1c} from venous blood and mailing the HbA_{1c} results directly to the diabetic patient improves glycemic control (9). We have now evaluated whether capillary blood samples applied on a novel filter paper could be used in this context in combination with ion-exchange chromatography. In our HbA_{1c} program, the patient takes a capillary blood sample at home and applies the blood to the filter paper. The filter paper is mailed to the laboratory, where the sample is eluted from the paper and HbA_{1c} assayed. The aims of the current study were to optimize our conventional HbA_{1c} method for the filter paper system, clarify essential technical steps in the procedure, relate capillary filter paper HbA_{1c} values to HbA_{1c} values obtained from venous whole blood, and to clarify whether HbA_{1c} values are altered when dried spot blood samples on filter paper have been stored.

RESEARCH DESIGN AND METHODS

Blood samples

Venous blood was collected in EDTA-containing vacuum tubes (Becton Dickinson, Rutherford, NJ). Capillary blood (two drops) obtained from the fingertip was applied to a filter paper (Schleicher & Schuell, Keene, NH) attached to a request card: HbA_{1c} Via Post (Boehringer Mannheim Scandinavia AB, Bromma, Sweden). The patient sampling was supervised by a nurse at the outpatient diabetes unit. The adult patients were selected at random at the same time they were venipunctured for ordinary HbA_{1c} analysis. The dried capillary blood on the filter paper was stored in a small plastic bag and sent by regular mail (1–2 days in Sweden, ambient temperature 15–25°C) to the laboratory. If analysis was not performed directly on arrival to the laboratory, filter papers were maintained at 4°C (refrigerator) until analysis. To evaluate the effect of prolonged mailing, samples from 10 patients were sent around Sweden for 5 days at 15–25°C. The filter papers were eluted on the 6th day, and results were compared with venous blood from the 1st day when the capillary blood was applied on the filter paper.

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HPLC, high-performance liquid chromatography.

Sample preparation

Venous blood. Of the mixed whole blood, 17 μl was diluted with 900 μl sodium phosphate-citrate buffer also containing a detergent (0.02 and 0.05 mol/l, respectively, and 0.1% Triton X-100, pH 5.4). After incubation at 37°C for 30 min to enhance the elimination of pre-HbA_{1c} (the labile fraction), the tubes were centrifuged, and 5 μl was injected into the chromatographic system.

Capillary blood on filter paper. A small filter paper disc (3-mm diameter) was punched out from each paper on a spot with maximum amount of dried blood. Hemoglobin was eluted from the filter paper by adding 75 μl of the sodium phosphate-citrate Triton X-100 buffer described above. After elution at 37°C during 30 min (to eliminate pre-HbA_{1c}), an equal volume (75 μl) of a cysteine-containing buffer, pH 8.0 (Boehringer Mannheim Scandinavia AB), was added according to the manufacturer's instruction. After 45 min at room temperature, 5 μl of the hemolysate was injected into the chromatographic system, and HbA_{1c} analysis was performed as for venous blood.

Equipment

The high-performance liquid chromatography (HPLC) system consisted of a pump (no. 2941, Pharmacia Biotechnology, Uppsala, Sweden), a Jasco 870 detector (Tokyo, Japan) equipped with a 415-nm filter, and a 10-mm flow cell together with a hydrogen lamp. The system contained an autoinjector for microtiter plates (BioRad, Richmond, CA) with a cooling system for 96 samples. A Shimadzu CR5A integrator (Tokyo, Japan) was used for calculating the peak areas using the valley-valley mode.

Procedure

HbA_{1c} was separated from other hemoglobin fractions by ion-exchange chromatography on a Mono S HR 5/5 column (Pharmacia, product no. 17–1040-01) by salt gradient elution at pH 5.7 (10). New Guard AX-300 (Applied Biosystems, Foster City, CA) was used as a precolumn. The flow rate was 2.0 ml/min, and the complete cycle time was 9 min. To prevent bacterial growth, sodium azide (0.02%, wt/vol) was added to all buffers, which were degassed under vacuum before use. If venous hemolysates and filter eluates were run on the same column, 100 μl of the cysteine buffer solution was injected followed by a blank gradient before the patient samples.

Stability

EDTA blood samples with different HbA_{1c} concentrations from three patients were applied on multiple filter papers and stored at 20–21°C, 4–6°C, and −70°C for different time periods.

Assay of imprecision

The between-day imprecision was determined by analyzing filter paper samples stored at −70°C during 6 months and by analyzing lyophilized blood samples (Bio-Rad, Richmond, CA).

Isolation of an interfering peak

One minor fraction between HbA_{1c} and the major HbA_0 was pooled, dialyzed against water, and lyophilized. The α- and β-chains were prepared, and their amino acid content and sequence were determined as previously reported (11).

RESULTS

Development of the method

Among the different fractions of hemoglobin appearing when dried blood is stored on filter paper, methemoglobin and a glutathione adduct of hemoglobin interfere with the chromatography pattern. Red cells contain a high amount of the tripeptide glutathione (glycine-cysteine-glutamic acid). The free thiol group of this peptide forms a disulfide bond with the β-chain of hemoglobin, and this compound increases during storage. The extracarboxyl group in glutamic acid contributes to an extra charged group in the hemoglobin molecule and forms a new hemoglobin fraction (HbA_3) in the chromatographic profile (Fig. 1). The addition of a cysteine-containing buffer thereafter reduces the methemoglobin and eliminates the glutathione adduct. Hence, the chromatography profile after the addition of the cysteine-containing buffer was quite similar to that of native venous hemoglobin.

Interpretation of chromatographic profile

The difference in net charge between the HbA_{1c} fraction and other minor hemoglobin fractions may be small. Using the described salt gradient, a small fraction located to the right of HbA_{1c} in the chromatography profile was noticed (*X* in Fig. 1).

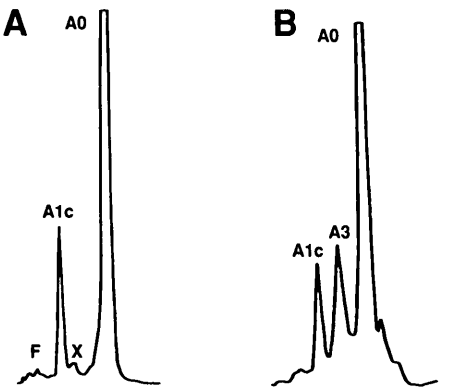

Figure 1—*Ion-exchange chromatography of hemoglobin fractions from eluate of dried blood stored for 5 days on filter paper at room temperature: A) elution in the phosphate-citrate Triton X-100 buffer, pH 5.4, followed by addition of a cysteine-containing buffer, pH 8.0; B) results after elution in the phosphate-citrate Triton X-100 buffer. The fraction marked F represents the normal occurring fetal hemoglobin. X indicates free α-chain that is separated from and not included in the HbA_{1c} fraction. HbA_3 is the glutathione adduct formed during storage both in filter paper and aged venous EDTA blood.*

Amino acid analysis and sequence determination of this fraction (in nondiabetic and diabetic subjects) demonstrated an identity with purified α-chain, indicating that this interfering fraction contained dimers or tetramers of the α-chain of hemoglobin. The fraction was visible in chromatography patterns both from fresh venous blood and filter paper eluate.

Comparison between venous blood and capillary blood

HbA_{1c} from venous blood and capillary blood sampled on the same occasion from 41 patients were compared using a similar chromatography system for analysis of HbA_{1c} in venous and capillary blood. Figure 2 shows the close correlation between HbA_{1c} values obtained from venous blood analyzed on day 1 (when the blood sample was taken) and HbA_{1c} values obtained from dried capillary blood samples (taken simultaneously with the venous blood sample) but stored on filter paper at room temperature in darkness and kept in plastic bags for 2 days before the assay. The correlation was as close for low values of HbA_{1c} as for high values. The mean value and range for filter paper and venous blood was 7.49% (4.37–12.28) and 7.33% (4.13–12.21), respectively. Filter paper gave an average of 0.16% higher values than did venous blood in this study.

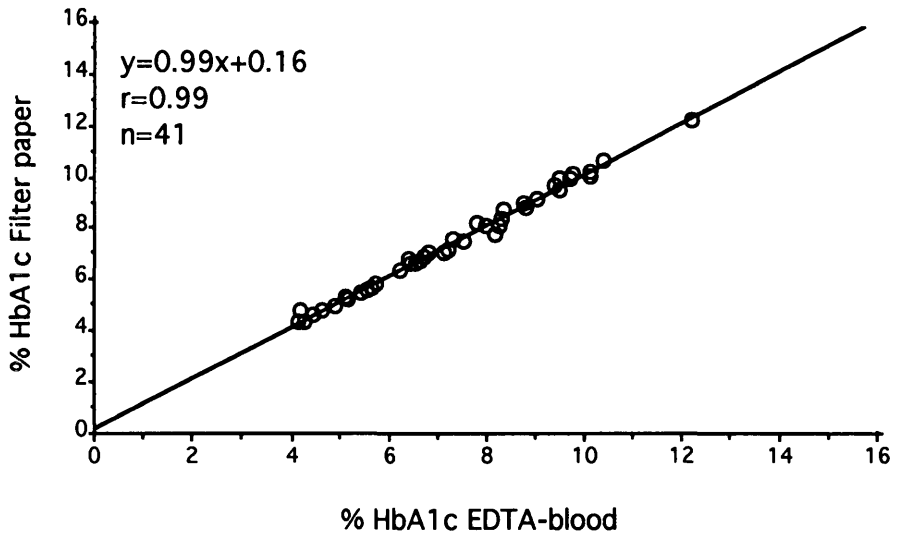

Figure 2—*Comparison of HbA_{1c} obtained from filter paper (capillary samples) and EDTA blood (venous whole blood) on a Mono S column for HbA_{1c} on a standard HPLC system.*

Storage and stability of capillary samples

Samples from individual patients showed no significant differences in HbA_{1c} results when stored for 5 days at room temperature (in darkness) or 4–6°C for 10 days and for several months at −70°C after blood sampling (Fig. 3).

Mailing for 5 days during a weather period with a daily outside temperature of 15–25°C did not produce any change in the chromatographic profile. The between-day imprecision (coefficient of variation) of venous and capillary blood samples within the reference range was 1.35 and 1.54%, respectively.

CONCLUSIONS — In this study, we demonstrate that capillary-sampled blood collected on filter paper shows HbA_{1c} values corresponding to those obtained by conventional venous whole-blood samples. Moreover, storage of capillary samples on filter paper at 20–21°C for 5 days did not change the HbA_{1c} results. The tested HbA_{1c} system therefore seems appropriate for use in diabetic patients. This system will allow the diabetic patient to take a capillary blood sample at home and then send the sample by mail to the laboratory for a reliable HbA_{1c} determination. Two years of experience with frequent comparisons between venous and capillary blood samples show that the introduced technique works in a home setting if the patient is orally informed by a nurse at the first sampling. Every request form also contains a clear explanation and instruction to the patient. HbA_{1c} from both venous blood and filter paper eluates was quantitated after a minor modification (addition of sodium azide)

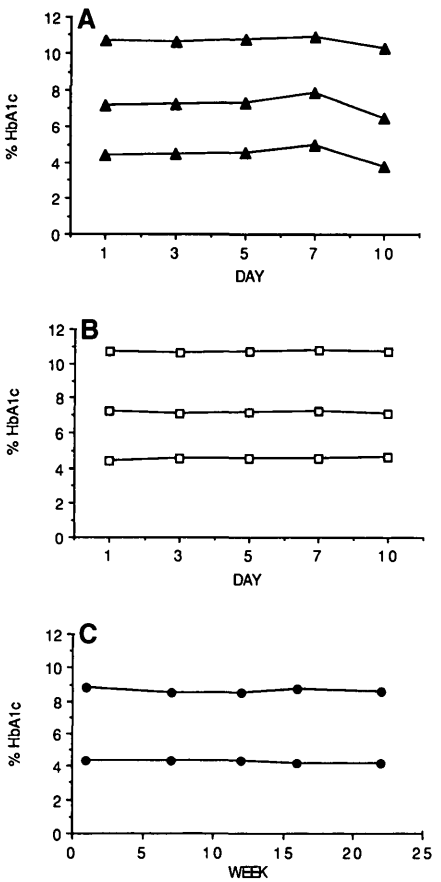

Figure 3—*Storage of filter paper samples in A) room temperature (20–21°C), B) refrigerator (4–6°C), and C) freezer (−70°C).*

of our original ion-exchange chromatography method (12). The modified system had sufficient resolution to give accurate results for the filter paper eluate.

The 97.5 percentile value of HbA_{1c} from healthy individuals in this study was 4.9% and in good agreement with Bisse and Wieland (13) but considerably lower than that reported by Little et al. (14). It is well known, however, that the same blood specimen tested in different HbA_{1c} systems, such as ion-exchange chromatography, affinity chromatography, electrophoresis, or mono- and polyclonal antibody techniques, produces different results. To avoid confusion, an international calibrator for HbA_{1c} has to be introduced (15). Nevertheless, capillary blood samples on filter paper have the potential to be used in most HbA_{1c} assays, including immunoassay (16), and not only HPLC, as in this study.

A filter paper–based HbA_{1c} system allows HbA_{1c} results to be available when patients visit their doctor. At our hospital, >2,000 capillary samples have now been handled in this way, eliminating previsit venipunctures. Besides reducing the need for the patient to come to the clinic for phlebotomy, capillary HbA_{1c} also diminishes the burden for the diabetic team; the need for venipunctures by diabetic nurses or laboratory technicians are substantially reduced. The gain in the diabetic child is obvious; fear of phlebotomy that might interfere with the goal in diabetic care is avoided. Intensive attention improves glycemic control (17), and this is promoted by mail delivery of HbA_{1c} results directly to the diabetic patient at home (9). This is easily accomplished by the capillary blood HbA_{1c} filter paper system. To further facilitate for the diabetic patient, we provide our HbA_{1c} results directly in a graphic form from the laboratory computer presenting the current and previous HbA_{1c} test results (absolute values together with an HbA_{1c} graph). The form is mailed to the patient simultaneously as the result is sent to the patient's diabetologist. Another advantage with this HbA_{1c} system is the quality. Submitting samples by mail will allow laboratories to conduct adequate HbA_{1c} assays with the same accuracy and precision from both diabetic wards and outpatient units.

Certainly, after the Diabetes Control and Complications Trial (2), HbA_{1c} will remain a focus for many years. A

normal or slightly deviated HbA_{1c} value without the occurrence of major adverse hypoglycemic occasions has to be the characteristic of diabetes care in the future. To promote this, an HbA_{1c} system where the patient takes a capillary blood sample and mails it to a laboratory that uses a valid HbA_{1c} assay must take place. The result may not only be provided to the diabetologist but also to the patient on an appropriate and didactic form.

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