Evaluation of a colorimetric method for canine glycosylated hemoglobin

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SUMMARY

Glycosylated hemoglobins can be used to monitor glucose status and the effectiveness of insulin therapy in canine diabetes mellitus. We have compared a colorimetric method with a time-consuming chromatographic method for measuring glycosylated hemoglobins in canine blood. Although the colorimetric method is sensitive to hemoglobin concentration, its convenience and sample size make it a possible alternative to other methods.

The treatment of canine diabetes mellitus requires monitoring of blood and urine glucose concentrations, insulin dosage, and energy expenditure. Traditionally, blood urine and glucose concentrations are measured periodically to monitor the clinical response to insulin. Unfortunately, an individual glucose measurement only represents the glucose concentration at the time the sample was taken. Blood glucose can be increased or decreased within a short time by a number of factors, thus possibly giving an erroneous indication of therapy.

Recently, hemoglobin A1c, a glycosylated hemoglobin, has been used to evaluate blood glucose control in persons and dogs. During the life span of the erythrocyte, glucose is nonenzymatically added to hemoglobin. Because the reaction seems to follow the law of mass action, hemoglobin A1c concentrations are determined by the intraerythrocytic glucose concentration and are not affected by short-term fluctuations in blood glucose. In dogs, 2 weeks are required for hemoglobin A1c to increase significantly after acute onset of continual hyperglycemia.

Although canine hemoglobin A1c can be used to monitor insulin therapy, the present method is cumbersome. In an attempt to find an easier technique, we evaluated a commercial colorimetric procedure (Glycospec). It is based on the spectra changes in hemoglobin when it interacts with certain organic phosphates, such as phytic acid and 2,3-diphosphoglycerate, both of which bind to the \( \beta \) chains of hemoglobin. That binding is accompanied by a decreased absorbance of hemoglobin. When hemoglobin is glycosylated, organic phosphate binding is prevented, and the change (decrease) in absorbance is less. The procedure measures total glycosylated hemoglobin (hemoglobin A1a, hemoglobin A1b, and hemoglobin A1c). If there is a change in total glycosylated hemoglobin concentration, the change is due to hemoglobin A1c changes. Hemoglobin A1a and hemoglobin A1b do not significantly change in diabetes mellitus.

Materials and Methods

Blood samples were obtained in EDTA from 25 adult, clinically healthy dogs, 4 diabetic dogs, and 4 dogs with chronic hemolytic anemia. Of the 4 diabetic dogs, 3 had diabetes mellitus induced chemically with the combination of streptozotocin (30 mg/kg) and alloxan (50 mg/kg); the 4th diabetic dog had naturally occurring diabetes mellitus. The dogs with hemolytic anemia were Alaskan Malamutes, homozygous for hereditary stomatocytosis.

Glycosylated hemoglobins were determined chromatographically by a modification of the method of Trivelli et al. The colorimetric determination was performed in a Quantum bichromatic analyzer; reagents were supplied by the manufacturer. Imidazole-lysing-potassium ferricyanide reagent (1 ml) was pipetted into a disposable round cuvette. Thoroughly mixed whole blood (20 μl) was added to the cuvette, mixed, and incubated at 30 C. After 5 minutes, the absorbance was measured in the analyzer (Mode 5). Phytic acid reagent (0.1 ml) was added to the cuvette and mixed, and absorbance was redetermined. The percentage of glycosylated hemoglobin was determined automatically by the programmed calculator in the bichromatic analyzer. The assay was calibrated against 3 glycohemoglobin standards provided by the manufacturer. (All specimens and controls were run in duplicate.) The data were analyzed statistically by standard methods.

Results

Effect of hemoglobin concentration—The hemoglobin concentration was altered by removing or adding plasma to blood obtained from a healthy person, a healthy dog, and a diabetic dog. The glycosylated hemoglobin percentage of human blood was independent of the hemoglobin concentration when the hemoglobin was greater than 10 g/dl (Fig 1). In contrast, the percentage of glycosylated hemoglobin of canine blood increased as the hemoglobin concentration increased. Samples with hemoglobin greater than 16 g/dl could not be measured. In all subsequent experiments, the hemoglobin concentration

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* Glycospec, Abbott Laboratories, North Chicago, Ill.
* Quantum Bichromatic Analyzer, Abbott Laboratories, North Chicago, Ill.
* Walter Sanstedt Inc. Princeton, NJ.
of canine blood was adjusted to between 12 and 13 g/dl, so that the influence of hemoglobin concentration would be minimized.

Correlation with chromatographic method—Samples of blood from 8 healthy dogs and 4 diabetic dogs were examined by the chromatographic and the colorimetric methods. The values obtained from the colorimetric method correlated significantly \( (r = 0.96; P < 0.01) \) with those obtained from the chromatographic method (Fig 2).

Precision—Within-run precision was studied by assaying blood from healthy and diabetic dogs, in 10 replicates each. The coefficients of variation were 6.96% and 12.9% for glycosylated hemoglobin concentrations of 10.9% and 4.4%, respectively.

Effect of glucose—When glucose (equivalent to 500 mg/dl) was added to blood samples, glycosylated hemoglobin concentrations remained unaltered after 10 minutes at 37 C or overnight at 4 C.

Effect of storage—Samples stored for longer than 3 days had significant increases in the glycosylated hemoglobin measured colorimetrically. That phenomenon was observed with samples from healthy and diabetic dogs.

Reference values—In the analyses of blood samples from 25 clinically healthy dogs, the mean glycosylated hemoglobin was 5.1% (with SD ± 0.86).

Effect of shortened erythrocyte life—In the 4 dogs with chronic hemolytic anemia associated with hereditary stomatocytosis, determination of their glycosylated hemoglobin values (6.1% ± 1.2) by the colorimetric method was not significantly different from those values obtained from healthy dogs. In contrast, the chromatographic values for both hemoglobin \( A_{1c} \) (1.02% ± 0.37) and total glycosylated hemoglobin (2.37% ± 0.438) were significantly lower \( (P < 0.01) \) than those observed for 13 healthy dogs (2.81 ± 0.35; 4.80% ± 0.191; respectively).

Discussion
This colorimetric assay provides an alternative to the chromatographic method previously used to measure glycosylated hemoglobins. A single sample can be measured within 30 minutes; additional samples require 2 to 3 minutes each. In contrast, the chromatographic method requires approximately 8 hours per sample and the time does not decrease with additional samples. Adjustment of hemoglobin for the colorimetric method requires some additional time, but ensures the necessary accuracy.

Hemoglobin \( A_{1c} \) concentrations depend not only on the intraerythrocytic glucose concentration, but also on the length of exposure of glucose to hemoglobin. In those species with short erythrocytic life spans (eg, mouse), the hemoglobin \( A_{1c} \) is less than in those species with a longer erythrocytic life span (eg, dog). In nondiabetic animals, the hemoglobin \( A_{1c} \) concentration reflects the glycosylation rate of half of the erythrocytic life span. Similarly, those animals with hemolytic disorders should have a lower concentration of hemoglobin \( A_{1c} \) than do healthy individuals in the same species. The normal glycosylated hemoglobin concentration observed from the dogs with hemolytic anemia, by using the colorimetric method, was unexpected. Other investigations will be necessary to determine whether that is a peculiarity of hereditary stomatocytosis or if other species having a shorter erythrocytic life span would have normal glycosylated hemoglobin concentrations by this method.

Some chromatographic procedures (particularly those using small columns) erroneously measure a labile form of hemoglobin \( A_{1c} \). The labile form is produced by acute changes in blood glucose or by incubating blood spiked with additional glucose. Because the colorimetric assay is unaffected by incubation of samples with high glucose concentration, it should not be influenced by short-term hyperglycemia.

Although we have evaluated the colorimetric method by using a discrete analyzer, this test can be automated readily.

References