Glycated Hemoglobin Fractions in Normal and Diabetic Dogs Measured by High Performance Liquid Chromatography

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(Received 21 June 1990/Accepted 27 September 1990)

ABSTRACT. We established a new analytical condition to measure the canine glycated hemoglobin by high performance liquid chromatography (HPLC) using cation exchange column. The canine hemoglobin gave five peaks consisting of 2 major and 3 minor hemoglobin fractions such as HbA1a, HbA1b and HbA1c. Measurement was done in 38 clinically normal dogs and 10 diabetic dogs. Mean HbA1c values (% of total Hb) in normal and diabetic dogs were 2.60 and 6.41%, respectively. And mean HbA1 values were 3.58 and 7.41%, respectively. The mean values of the canine HbA1c and HbA1 in diabetic dogs was higher than those in normal dogs, significantly ($p < 0.01$). Advantages of the HPLC method and applicability for monitoring effectiveness of insulin therapy in the canine diabetes mellitus are discussed.—**KEY WORDS:** diabetes, dog, glycated hemoglobin, HPLC.

J. Vet. Med. Sci. 53(1): 65–68, 1991

The adult human hemoglobin (HbA) consists of a major (HbA0) and a minor (HbA1) component [1]. It was found that HbA1 was a glycosylated hemoglobin component, and increased about twofold in diabetics on agar-gel electrophoresis [15]. It was reported that a glucosylated hemoglobin component (HbA1c) could be separated from HbA1 by Bio Rex 70 column in normal and diabetic subjects [18]. Since then, clinical importance of HbA1 and HbA1c in diabetes mellitus has been mentioned.

HbA1c is formed nonenzymatically by the condensation reaction between glucose and α -valine residue, the N-terminus of the hemoglobin β -chain. The initial reversible condensation of glucose and hemoglobin forms an aldimine or Schiff base which is able to undergo a nearly irreversible intermolecular Amadori rearrangement. The Schiff base is known as labile HbA1c or pre-HbA1c and the ketoamine as stable HbA1c [4]. As each reaction proceeds nonenzymatically, the HbA1c is formed gradually but continuously in the red cell. Therefore HbA1c does not give a precise index for transitory changes of blood glucose concentration, but it provides an integrated measure of blood glucose levels over a preceding month. HbA1 and HbA1c have been measured extensively to monitor the human diabetes control.

Measurement of the canine glycosylated hemoglobin has been reported using various methods [5–8, 11, 12, 16, 17, 19–21], including ion-exchange mini-column or affinity-column chromatography

and colorimetry. The high performance liquid chromatography (HPLC) has not been applied to separate canine HbA1s yet, although the HPLC method for HbA1s has been used in the human clinics. Here after, the term glycated will be used to describe our results and the term glycosylated for citation. This is because of the recommendation for the terminology by National Diabetes Data Group [14]. As a pilot study, normal human and canine hemoglobin were separated by the HPLC (HLC-723GHBII; TOSOH) which was developed for specific measurement of the human glycated hemoglobin alone. Whole blood (3 μ l) with EDTA, was lysated in 1 ml of the hemolysating solutions (TOSOH), and then aliquot of the hemolysate (20 μ l) was applied to the column [13]. The human hemoglobin gave five peaks; HbA1a, HbA1b, HbF, HbA1c and HbA0 at 0.3, 0.5, 0.7, 1.1 and 2.6 min, respectively. The canine hemoglobin gave four peaks; HbA1a, HbF, HbA1c and HbA0 at 0.5, 1.2, 1.7 and 2.6 min, respectively. The third peak, expressed as HbA1c, represented 96.5%, not being the minor hemoglobin component. It was revealed that the analytical conditions of the human glycated hemoglobin were not applicable to the canine hemoglobin analysis. Therefore it was necessary to establish suitable analytical conditions for separation of the canine hemoglobin components by the HPLC. We report here that the established method of the canine glycated hemoglobin measurement by ion-exchange HPLC, evidences for a peak corresponding to HbA1c, and values of

HbA1s in clinically normal and diabetic dogs. These means will make it possible to monitor the insulin therapy in the canine diabetes mellitus.

MATERIALS AND METHODS

Animals: Thirty-eight clinically normal and ten diabetic dogs were used in the present study. In the former case, the normal beagle dogs bred in the Central Research Laboratory of Nihon Zenyaku Kogyo Co., LTD. The age of the dogs ranged between 1.9 and 8.6 (mean, 3.2) years old. Twenty-nine females and 9 males were included. Normality was confirmed in each dog to be free from any signs of diabetes, such as turbid lens, hyperglycemia (fasting blood glucose: FBG > 100 mg/dl), and glycosuria. In the latter case, the diabetic dogs diagnosed at our college hospital and other veterinary hospitals were used. Included were three Maltses, and each one Toy Poodle, Japanese Spitz, and Pomeranian breeds and 4 mongrels. The age of the dogs ranged 2.0 and 14.0 (mean, 7.9) years old. Eight females and 2 males were included. The blood samples were collected from diseased animals before starting the insulin therapy.

Preparation of canine hemoglobin solution: Each blood sample was drawn into a heparinized syringe, and was transferred into a tube containing EDTA at 4°C. The blood was centrifuged for 10 min at 3,000 r.p.m. at 4°C to remove plasma and buffy coat. Three washings with 5 times volume of 0.9% NaCl at 4°C was followed. The packed red cells (20 μ l) were hemolysed with 4 ml of a triton X-100 solution (triton X-100: 1 ml/l, EDTA-2Na: 1 g/l, pH 6.5). The hemolysate was passed with the membrane filter (Cellulose Nitrate 0.45 μ m, ADOVANEC, Tokyo Roshi Kaisha, LTD.) to remove the cell membrane. Aliquots (20 μ l) of the hemolysate was sampled and applied to the HPLC.

Flow diagram of HPLC and analytical conditions in dogs: The canine hemoglobin was separated by cation-exchange chromatography using the CM-3SW column (TOSOH). The HPLC system, CCPM as pump and UV-8010 as U.V. detector (TOSOH), were all controlled by a system controller (TOSOH). Stepwise gradient of the phosphate buffers, 20 and 60 mM/l K_2HPO_4 (pH 6.5), were used for elution. The U.V. absorbance was measured at 415 nm. All the chromatographic procedures were carried out at a constant temperature ($25 \pm 1^\circ\text{C}$).

Identification of canine HbA1c peak in HPLC chromatogram: Blood samples were obtained from 3 clinically normal dogs and processed separately. An increase in labile HbA1c by incubation of the RBC with 5% glucose solution was examined. The washed packed red cells were incubated in vitro for 6 hours at 37°C with equal volume of modified PBS (NaCl 8.5 g, KCl 0.2 g, Na_2HPO_4 1.15 g, KH_2PO_4 0.2 g/l; pH 7.30) with (5% Glu-PBS) and without (only-PBS) glucose. After incubation, the packed red cells were washed three times with 0.9% NaCl, and were hemolysated to apply to the HPLC.

Statistical analysis: Data were expressed as mean $\pm$ SD. Comparison was made by Student's t-test.

RESULTS

A chromatogram of the canine hemoglobin under the new analytical condition is shown in Fig. 1. Five peaks (designated as P1-P5) were detected at 2.0, 2.6, 5.0, 9.0 and 10.5 min. The latter 2 peaks (P4 and P5) are not shown separately in Fig. 1, since they are too high. These 2 peaks accounted for 97% of the total peak area. Three minor peaks were recognized in front of them. The separation of the canine hemoglobin was finished in 18 min.

The chromatograms of the hemoglobin after incubation of normal canine RBC with 5% Glu-PBS or only-PBS are shown in Fig. 2. The peak level at five minute (P3) of 5% Glu-PBS (3.40%) was significantly higher than that of only-PBS (1.66%).

Peak levels in normal and diabetic dogs are shown in Table 1. The mean values of P1 ($p < 0.05$) and P3 ($p < 0.01$) in diabetic dogs were significantly higher than those in normal dogs. The mean value of P4-P5 was significantly lower in diabetic dogs than in normal ($p < 0.01$).

Fig. 1. Typical chromatogram of the hemoglobin in normal dog by the new analytical conditions. Solid line is for elution profile and broken line for concentration of elution buffer.

Fig. 2. Chromatograms of the hemoglobin after incubation of normal canine RBC with equal volume of PBS with (5% Glu-PBS) and without (only-PBS) glucose for 6 hours at 37°C.

Table 1. Chromatographed peaks in diabetic and normal dogs

	n	Peak 1	Peak 2 (% of total Hb)	Peak 3	Peak 4-5
Diabetes	10	0.56±0.119 ^{a)} (0.38–0.71)	0.44±0.156 ^{b)} (0.25–0.79)	6.41±3.241** (3.74–14.29)	92.57±3.431** (84.22–95.60)
Normal	38	0.44±0.061 (0.32–0.56)	0.54±0.130 (0.32–0.81)	2.60±0.357 (1.61–3.13)	96.43±0.534 (95.54–97.66)

a) Significantly different between diabetes and normal dogs: *, $p < 0.05$, **, $p < 0.01$.

b) Mean±SD of n observations.

DISCUSSION

In the present study, we examined the analytical conditions of the canine glycated hemoglobin using the HPLC, and succeeded in separating HbA1s. The canine hemoglobin gave the five peaks (P1-P5). The P4 and P5 are the canine HbA0, since they were accounted for 97% of the total peak area. It is obvious that the P3 at five minute is the canine HbA1c, as it's level was significantly increased, when the canine RBC was incubated with glucose solution. This is further supported by the fact that the mean value of the P3 in the diabetic dogs was significantly higher than that in the normal dogs. It seems likely that the first two peaks (P1 and P2) are the canine HbA1a and HbA1b, respectively, and the total area of the three peaks (P1-P3) in front of the P4 is the canine HbA1. In adult human, the fetal hemoglobin (HbF) is recognized as a peak before the HbA0 peak, while in the dog HbF nearly disappears soon after birth [9]. The mean value of this HbA1 is also higher in diabetic dogs ($7.41 \pm 3.438\%$) than in normal dogs ($3.58 \pm 0.534\%$),

significantly ($p < 0.01$).

In the pilot study using HLC-723GHBII, the peak of the human HbA0 was chromatographed consistently at 2.6 min and was account for 89.9%. In the canine hemoglobin, the peak at 1.7 min was recognized as "HbA1c" and was account for 96.5%. Base on the present analysis of eluting conditions, it was concluded that most of the peak at 1.7 min by HLC-723GHBII may be consisted of HbA0. Association with HbA0 and HbA1s and faster elution time obtained by the human conditions suggest that the negative charge of the canine hemoglobin is much more than those in the human hemoglobin in the eluting solution of HLC-723GHBII. This might result from the structural difference of the hemoglobin between dogs and human [2–3, 10]. Thus, the canine HbA1c or HbA1 cannot be measured with the analytical condition for the human hemoglobin.

Several investigators reported various methods for measuring the canine glycosylated (glycated) hemoglobin [5–8, 11, 12, 16, 17, 19–21]. The HPLC method has never been included. The HbA1 level in our HPLC ($3.58 \pm 0.534\%$) is lower than that in mini-column chromatography ($5.63 \pm 0.68\%$ [12],

$5.25 \pm 0.647\%$ [19]) and in colorimetric method ($5.1 \pm 0.86\%$) [17], significantly ($p < 0.01$ both). The HbA1c level is significantly higher in the present study ($2.60 \pm 0.357\%$) than in affinity-column chromatography method ($1.68 \pm 0.285\%$) [16] ($p < 0.01$). In the measurement of the canine hemoglobin by other methods else ours, every component of the hemoglobin was not recognized, and it took a long time. In our HPLC method, the canine hemoglobin could be separated to make 5 definitely recognizable peaks, and the reproducibility was good ($CV = 1.0\%$, $n = 10$). Since the measurement of the canine glycated hemoglobin was possible to be finished in 18 minutes, many samples could be applied.

The mean values of HbA1c and HbA1 were significantly higher in the diabetic dogs before insulin therapy was started than in the normal dogs ($p < 0.01$). It may suggest that the canine HbA1c can provides an integrated measure of blood glucose levels over the preceding month(s) and is valuable for monitoring effectiveness of insulin therapy in the canine diabetes mellitus, as shown in human.

ACKNOWLEDGEMENTS. The authors thank Mr. T. Tohji at TOSOH for technical advice and Dr. K. Suzuki at Dept. Vet. Physiol., NVZC for his critical readings of this manuscript.

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