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Rapid assay of A1c-type glycosylated hemoglobin in blood of diabetic rats using fast protein liquid chromatography[1]

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KEY WORDS glycosylated hemoglobin A; liquid chromatography; experimental diabetes mellitus

ABSTRACT

AIM: To develop a fast protein liquid chromatography (FPLC) method for the assay of A1c-type glycosylated hemoglobin in diabetic rats. **METHODS**: Venous blood was collected from rats. After the erythrocytes were washed and sedimented, the hemolysate was prepared and diluted with isotonic saline as a sample for the assay. Samples were then separated on Mono_S_HR_5/5 cation exchange column by a lithium chloride gradient elution system. The A1c-type glycosylated hemoglobin was monitored by measuring the absorbance at 415 nm. **RESULTS**: The A1c-type glycosylated hemoglobin was well separated from total hemoglobin. The average HbA1c amount in diabetic rats was determined to be (3.6 % ± 0.6 %, $n = 7$), while the corresponding figure in normal rats was (1.4 % ± 0.4 %, $n = 7$, $P < 0.01$). **CONCLUSION**: This FPLC method is easy, rapid and reproducible, and can be used for an assay of diabetic rat HbA1c to evaluate and screen new drugs for diabetes mellitus therapy.

INTRODUCTION

A1c-type glycosylated hemoglobin (HbA1c) is a minor component of hemoglobin (Hb) formed through an irreversible non-enzymatic catalytic reaction combining glucose with the N-terminus of the β chain of hemoglobin[1]. The percentage of HbA1c in total hemoglobin has been accepted as an reliable marker in monitoring long-term diabetic patients[2,3] and has been considered to be a better marker than fasting blood glucose assay since HbA1c reflects the average level of blood glucose concentration 1 – 2 months before assaying and does not fluctuate during short time. Several methods for human HbA1c assay have been developed[4].

Although rats are commonly used as laboratory animal model for experimental diabetes studies, HbA1c in rats has rarely been reported due to its hemoglobin heterogeneity. Recently, fast protein liquid chromatography (FPLC) has been shown to be more efficient new technology than HPLC. We have found it to be an easy, rapid, accurate, and reproducible method for HbA1c assay in normal and diabetic rats using fast protein liquid chromatography. Hence rat HbA1c can be used as an important marker in evaluating and screening new drugs for diabetes mellitus therapy.

MATERIALS AND METHODS

Reagents Alloxan was purchased from Sigma chemical Co (St Louis, MO63178, USA); The blood glucose assay kit was purchase from Beijing ZhongSheng High-Tech Bioengineering Co (Beijing 100083, China). Other reagents were of AR grade unless specified otherwise. Redistilled water was used for all experiments.

Animals Adult ♂ Sprague Dawley rats weighing 200 g ± 20 g ($\bar{x} \pm s$) were provided by the Experimental Animal Center, Institute of Zoology, Chinese Academy of Science, Beijing. The rats were housed in a temperature – controlled (20 – 26 ℃) facility with 12-h light/dark cycle and were given free access to food and water. Diabetic rats were induced by intravenous injection of Alloxan which was dissolved in the 0.9 % NaCl saline (injectio natril chloridi). After being injected alloxan, rats raised 5 wk were selected with blood glucose level more than 3000 mg/L and used as diabetic rats for study. The blood glucose was assayed by following the kit protocol that based on glucose oxidase method.

Sample preparation One hundred μL blood

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samples collected from the tail vein of diabetic and normal control rats were put into sample tube containing 10 μL of 1 % edetic acid and 3.5 % sodium fluoride. Blood samples 25 μL were taken and mixed with isotonic saline 500 μL. Erythrocytes were sedimented by centrifugation at 2000 × *g* for 1 min. The supernatant was discarded. Redistilled water 500 μL and toluene 500 μL were added to the pellet and mixed for 1 min and centrifuged again at 2000 × *g* for 3 min. The toluene phase with a thin lipid layer were aspirated and discarded. The hemolysate 50 μL was taken from the aqueous phase and diluted to 500 μL with redistilled water. The diluted hemolysate was centrifuged before assay.

Fast protein liquid chromatography The FPLC system of AKTA Explorer and the cation exchange column of Mono_S_HR_5/5 are the products of Amersham Pharmacia Biotech, Uppsala, Sweden. Buffer A contained 10 mmol/L of sodium malonate and 0.2 g/L of sodium azide (pH 5.7). Buffer B was the buffer A plusing lithium chloride 0.3 mol/L. Prior to the assay, all buffer solutions were filtered through 0.45 μm filter membrane. The separating parameters of FPLC were set as: the flow rate was 2 mL/min; the detecting wavelength of absorbance was 415 nm; the column temperature of separation was 20 ℃. Before sample injection, the system was equilibrated with 5 mL of buffer A. The samples (500 μL) were injected through an automatic sample injector with a 500 μL loop. After injection, the sample loop and the column were washed separately by 2 mL buffer A. The gradient elution profile was used as follows:

Elution solution volume/mL	Buffer B/%
0	0
11	40
15	50
25	100
28	100

Finally, after elution the column was re-equilibrated with 2 mL buffer A. The total assay time was about 27 min.

The HbA1c peak area and total hemoglobin area were integrated separately, the HbA1c percentage in total hemoglobin was calculated automatically by FPLC operation software.

Ion-exchange micro-column method An ion-exchange micro-column kit from Bio-Rad Diagnostics Group, 4000 Alfred Nobel Drive Hercules, California, USA, was used as standard for rat HbA1c assay. The HbA1c value in diabetic rats and the normal control were determined by following the kit protocol.

Affinity micro-column method An affinity micro-column kit from Biotech Development Department, Fu Rei Da Commercial Center, Beijing, China, was used for GHb assay as another standard reference. The GHb values in diabetic rat blood and normal control rat were determined by following the kit protocol.

Application of the method Using the FPLC method, HbA1c of diabetic rats ($n = 7$) and normal rats ($n = 7$) were determined. The results were compared with that of the ion-exchange micro-column method (diabetic rat $n = 33$, normal control $n = 10$) and the affinity micro-column method (diabetic rat $n = 5$, normal control $n = 5$) respectively.

Statistical analysis Data were expressed as $x \pm s$ and compared with t-test.

RESULTS AND DISCUSSION

A typical FPLC elution pattern of HbA1c from blood samples of diabetic and normal control rats were given in Fig 1. It can be seen that when same amount (500 μL) of sample from diabetic or normal rat was injected into a separate column, the peak of HbA1c was observed in around the 18th mL eluant. A marked increase in elution peak area in diabetic rats was seen as compared with normal controls. After integration and calculation, the differences in HbA1c value between diabetic and normal rat were significant as seen in Tab 1. In our experiment, when any sample (diabetic or normal control) was injected again, the almost same elution pattern and its integrated area value could be obtained. This means that this method is reproducible.

Further comparison between FPLC, ion-exchange micro-column, and affinity micro-column methods was made (Tab 2). First, the three methods showed the same trend, ie value of HbA1c of diabetic rats was higher than that of normal one. Secondly, with ion exchange micro-column method as a standard reference, the HbA1c value both in diabetic and normal rat were slightly lower in FPLC method, and higher in affinity method. This is because that the FPLC is more efficient to separate HbA1c from Hb than ion-exchange micro-column, and that the affinity micro-column can only separate HbA1 (HbA1a + HbA1b + HbA1c) from Hb. Finally, when compared with ion-exchange micro-column and affinity micro-

Tab 1. HbA1c values of diabetic and normal rat blood determined by FPLC method.

Sample number	HbA1c/%
Normal rats	
1	0.97
2	1.88
3	1.06
4	1.21
5	2.03
6	1.35
7	1.32
$\bar{x} \pm s$	1.4±0.4
Diabetic rats	
1	4.23
2	4.03
3	3.62
4	4.21
5	2.80
6	3.72
7	2.67
$\bar{x} \pm s$	3.6±0.6

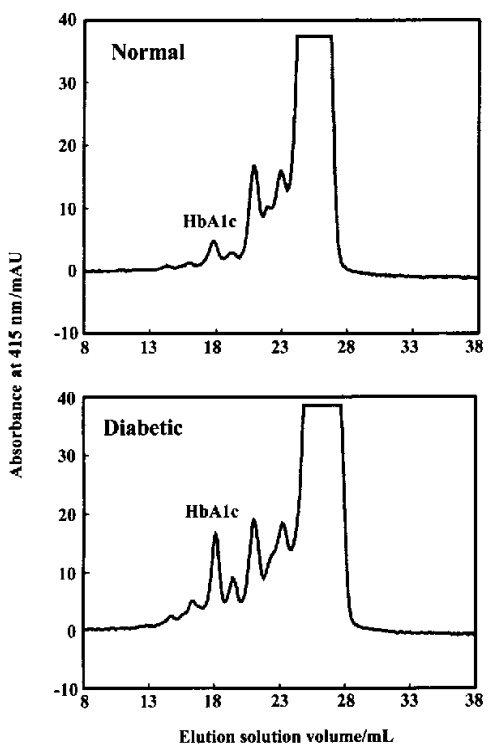

Fig 1. A typical elution pattern of HbA1c in blood samples from diabetic rat and normal control.

Tab 2. The comparison of FPLC, ion-exchange micro-column, and affinity micro-column method for HbA1c assay in diabetes and normal control rats blood. $\bar{x} \pm s$.

Method	Normal rats/%	Diabetic rats/%	Result *t*-test
FPLC	1.4±0.4 (n=7)	3.6±0.6 (n=7)	$P<0.01$
Ion-exchange micro-column	1.8±0.5 (n=10)	3.8±0.8 (n=33)	$P<0.01$
Affinity micro-column	2.4±0.6 (n=5)	3.6±0.6 (n=5)	$P<0.05$

column method, FPLC method can be run automatically by setting fixed the separating parameters, such as column temperature and separating time, that makes the assay procedure more easy, rapid, reproducible, and accurate than the other two method. In conclusion, FPLC method is easy, raqid, reproducible and accurate for determination of rat HbA1c for screening experimental drugs for use in diabetes mellitus.

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快速蛋白质液相色谱法测定糖尿病大鼠 A1c 型糖基化血红蛋白含量[1]

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关键词 糖基化血红蛋白 A; 液相色谱法; 实验性糖尿病

目的: 建立快速测定糖尿病大鼠 A1c 型糖基化血红蛋白的 FPLC 方法. **方法**: 大鼠血样经洗涤, 离心, 甲苯破膜, 稀释后; 用丙二酸钠溶液(A 液)和丙二酸钠-氯化锂溶液(B 液)为基本流动相, 采用梯度洗脱程序在 Mono_S_HR_5/5 阳离子交换柱上进行分离测定, 检测波长为 415 nm. **结果**: 在所给色谱条件下, 大鼠 A1c 型糖基化血红蛋白能很好地从总血红蛋白中分离出来. 糖尿病大鼠中 HbA1c 含量为 3.6 % ± 0.6 % (n = 7) 显著高于正常大鼠的 1.4 % ± 0.4 % (n = 7), $P < 0.01$. 测定一个样品所需的总时间约为 27 min. **结论**: 本方法简便, 快速, 准确, 重现性好, 可用于大鼠糖尿病模型中糖基化血红蛋白含量的研究和糖尿病治疗药物的筛选.

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