HbA1c levels Measured by Enzymatic Assay and Immunoassay during Off-Site Health Checkups Are Both Lower than Those Measured by On-Site HPLC Assay

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It has already been reported that HbA1c levels measured by immunoassay (IA) (IA-HbA1c) during off-site health checkups present falsely lower results. We also reported that HbA1c levels measured by enzymatic assay (EA) (EA-HbA1c) during off-site health checkups are lower. In the present study, we compared IA-HbA1c levels or EA-HbA1c levels during off-site health checkups with on-site high-performance liquid chromatography (HPLC)-HbA1c levels using the same samples. Subjects were 88 non-diabetic individuals who had health checkups in Nishinomiya Municipal Central Hospital. Subjects with a history of diabetes mellitus and those with HPLC-HbA1c ≥ 6.5% were excluded. IA-HbA1c levels (Study 1) or EA-HbA1c levels (Study 2) in the health checkups were compared with on-site HPLC-HbA1c levels using the same samples. Both IA-HbA1c levels and EA-HbA1c levels had positive correlations with HPLC-HbA1c levels (p <0.0001 for both), although both were significantly lower than HPLC-HbA1c levels (p <0.0001 for both). The degrees of reduction in the IA-HbA1c levels and EA-HbA1c levels compared with HPLC-HbA1c levels were almost same to each other. Similarly to IA-HbA1c levels, EA-HbA1c levels during the health checkups were lower than HPLC-HbA1c levels. It was demonstrated that HbA1c levels decrease similarly if measured by either EA or IA during off-site health checkups.

INTRODUCTION

HbA1c is widely used as a therapeutic target marker and as a diagnostic marker for diabetes mellitus (1). This has led to an increasing frequency of HbA1c measurements in current health checkups throughout Japan. Previously, the major HbA1c assay has been the high-performance liquid chromatography (HPLC) method that allows accurate measurement with high resolution. In recent years, assays, such as the affinity assay, immunoassay (IA), and enzymatic assay (EA), have been developed, and HbA1c is measured by various methods. In the early phase of the popularization of these assays, HbA1c results differed by analytic device and reagent. In Japan, the Japan Diabetes Society and the Japan Society of Clinical Chemistry made an effort to standardize HbA1c tests, resulting in a reduction in the differences between assays or kits (2). However, Oikawa et al. reported that HbA1c levels measured by IA (IA-HbA1c) during health checkups were lower than HbA1c levels measured by HPLC (HPLC-HbA1c) (3). In addition, we reported that HbA1c levels measured by EA (EA-HbA1c) during off-site health checkups were lower than on-site HPLC-HbA1c levels (4).

The HbA1c measurement method of the laboratory facility that undertakes test measurements at Nishinomiya Municipal Central Hospital has been changed from IA to EA as of October 2019. The present study compared HbA1c levels measured during off-site health checkups by IA and EA with on-site HPLC-HbA1c levels in the hospital and compared the degree of reduction relative to HPLC-HbA1c between IA-HbA1c and EA-HbA1c.
**SUBJECTS AND METHODS**

**Study subjects**

Eighty-eight subjects who had health checkups in Nishinomiya Municipal Central Hospital were used in the present study. Subjects with a history of diabetes mellitus and those with HPLC-HbA1c ≥ 6.5% were excluded.

In Study 1, off-site IA-HbA1c levels were compared with on-site HPLC-HbA1c levels using the same samples in 36 non-diabetic subjects (16 males and 20 females; age: 70.6 ± 14.1 years) who had health checkups in the hospital from July to September 2019 (Table I). In Study 2, off-site EA-HbA1c levels were compared with on-site HPLC-HbA1c levels using the same samples in 52 non-diabetic subjects (17 males and 35 females; age: 69.8 ± 10.6 years) who had health checkups in the hospital from October to November 2019.

The reported investigations have been carried out in accordance with the principles of the Declaration of Helsinki as revised in 2000. The study was approved by the institutional research board of Nishinomiya Municipal Central Hospital (date of approval; June 25, 2019, approval no.: 506).

<table>
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<th>Table I. Clinical characteristics in individuals in study 1 and study 2</th>
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**Laboratory analysis**

Blood samples were collected into two sodium fluoride-containing blood collection tubes for glucose measurement. For the HbA1c test in the health checkups, one sample was placed in cool boxes and transported to the laboratory facilities, where HbA1c was measured on the blood-sampling days by immunoassay (Determiner, Kyowa Medex Co., Ltd. Tokyo, Japan) in Study 1 and by enzymatic assay (MetaboLead, Kyowa Medex Co., Ltd.) in Study 2. The samples were centrifuged at 1500 g for 5 minutes and 800 g for 5 minutes, respectively, before measuring HbA1c in the blood cells. The same sample as those for health checkups was used to measure whole blood HbA1c in the hospital using the standard mode HPLC (Adams A1c HA-8190V, Arkay, Inc., Kyoto Japan). HbA1c results were indicated according to the National Glycohemoglobin Standardization Program (NGSP).

**Statistical analysis**

Results are expressed as means ± SD. For statistical analyses, the paired Student’s t test, the unpaired Student’s t test and χ² test were used to compare two parameters, as appropriate. To analyze correlation between two parameters, Pearson’s correlation coefficient was performed with the StatView computer program (Version 5.0 for Windows, Abacus Concepts, Berkeley, CA). P values of <0.05 were considered to be statistically significant.

**RESULTS**

No significant differences were noted in age, sex, fasting plasm glucose, or HPLC-HbA1c levels between Study 1 and Study 2 (Table I).

In Study 1, IA-HbA1c levels had a strong significant correlation to HPLC-HbA1c levels (R = 0.954, p < 0.0001). The regression equation between the two values was IA-HbA1c = 0.915 x HPLC-HbA1c + 0.340 with the regression line shifted downward compared to the line of y = x (Fig. 1). IA-HbA1c levels were significantly lower than HPLC-HbA1c levels (5.52 ± 0.25% vs. 5.66 ± 0.29%, P < 0.0001). In Study 2, EA-HbA1c levels had a strong significant correlation to HPLC-HbA1c levels (R = 0.893, P < 0.0001). The regression equation between the two values was EA-HbA1c = 0.858 x HPLC-HbA1c + 0.665 with the regression line shifted downward compared to the line of y = x (Fig. 2). EA-HbA1c levels were significantly lower than HPLC-HbA1c levels (5.52 ± 0.25% vs. 5.66 ± 0.29%, P < 0.0001).
**DISCUSSION**

In the present study, the HbA1c levels measured by immunoassay (IA) and enzymatic assay (EA) during off-site health checkups were similarly lowered compared with the HPLC-HbA1c levels measured in the hospital. Previously, Oikawa et al. reported that IA-HbA1c levels in off-site health checkups were lower than HPLC-HbA1c levels (3), and we also reported that the EA-HbA1c levels in health checkups were lower than the on-site HPLC-HbA1c levels estimated based on HPLC-HbA1c levels measured before and after the health checkups (4). Furthermore, we hypothesized and demonstrated that these phenomena were attributed to hemolysis associated with transportation of the samples (5). The present study demonstrated that both IA-HbA1c and EA-HbA1c levels during off-site health checkups were similarly lowered compared with the on-site HPLC-HbA1c levels.

There are two types of medical checkups: on-site health checkups where HbA1c is measured at the medical institutions and off-site health checkups where HbA1c is measured in laboratory facilities outside the medical institutions. The on-site health checkups measure HbA1c immediately after blood sampling without transporting the samples, while the off-site health checkups transport the samples to a laboratory facility and then measure HbA1c within a day after sampling. As the subjects in the present study had HbA1c measured in off-site checkups, the samples were transported from the hospital to the laboratory facility where HbA1c was measured within the day of blood sampling. Including the present study, a number of institutions sample blood using blood glucose test tubes containing sodium fluoride, which have high salt concentrations to elevate osmotic pressure, and it is therefore known that the samples are prone to hemolysis (6). Usually, samples undergo no hemolysis when HbA1c is measured immediately within the institute allowing accurate measurement of HbA1c, while hemolysis tends to occur in off-site health checkups because of the effect of vibrations during transportation. Aged erythrocytes are more vulnerable to hemolysis (6). After sample centrifugation, aged erythrocytes containing more HbA1c are hemolyzed and distributed in the upper layer, and young erythrocytes containing less HbA1c are distributed in the blood cells, reducing the blood cells HbA1c in the samples with hemolysis. This seemed to be the result in the EA-HbA1c lower than HPLC-HbA1c. Since IA-HbA1c is measured as HbA1c in the blood cell fraction after centrifuging the blood samples collected in tubes for glucose measurement similarly to measurement of EA-HbA1c, and therefore, IA-HbA1c results seemed to be lowered in a mechanism similar to that in EA-HbA1c.
Fig. 2. Comparison of HbA1c measured by enzymatic assay (EA-HbA1c) with HbA1c measured by HPLC (HPLC-HbA1c) in Study 2.
A. Correlation of EA-HbA1c levels with HPLC-HbA1c levels was shown. The line y = x is shown with a dotted line.
B. Comparison of EA-HbA1c levels (open column) with HPLC-HbA1c levels (closed column) was shown.

We reported that EA-HbA1c levels during health checkups were lower than HPLC-HbA1c levels (4). However, because no HPLC-HbA1c was measured on the day of the checkup, estimated checkup-day HPLC-HbA1c was calculated from HPLC-HbA1c before and after the day of the checkup to compare with EA-HbA1c during the health checkups. In the present study, IA-HbA1c was measured on the same day of the HPLC-HbA1c measurement using the same samples to compare the two levels, and the same procedure was performed for EA-HbA1c. As a result, the IA-HbA1c levels and EA-HbA1c levels were both lower than the HPLC-HbA1c levels.

In the present study, the laboratory facility switched the method of HbA1c measurement from IA to EA while the present study was being carried out, allowing a comparison of the decreases in HbA1c between the two methods. There were no significant differences in subject backgrounds, such as sex, age, and fasting plasma glucose, before and after the switching, and both populations were therefore considered to be comparable. In addition, HPLC-HbA1c levels were almost identical between both groups. Although IA-HbA1c and EA-HbA1c differ in the measurement principle, both methods measure the blood cells HbA1c using blood collection tubes for glucose measurement. As the degree of decrease was identical between both methods, it was revealed that the decrease in HbA1c levels was not caused by a difference in the measurement principles but was due to hemolysis caused by transportation of the samples in test tubes for plasma glucose before measurement of HbA1c. In the HPLC assay, on the other hand, because HbA1c was measured in homogeneous samples mixed beforehand, there was no decrease in HbA1c due to transportation (5).

In recent years, the number of HbA1c measurements has markedly increased because of the increasing morbidity of diabetes mellitus and the popularization of HbA1c measurements as a test of diabetes mellitus. For health checkups, the number of institutions measuring HbA1c by IA or EA is increasing because they have high processing power and are cheap. Although the present study was performed in samples for health checkups, HbA1c measurement following transportation was performed in various situations, such as multicenter clinical studies/trials with central measurement and clinics without HPLC measurements available. Therefore, this exerted a significant impact on society.

From now on, actions should be taken immediately for accurate measurement of IA-HbA1c or EA-HbA1c in off-site health checkups. First, HPLC is capable of accurate measurement, although it requires facility investments and has problems of high cost and low processing power. Second, IA-HbA1c or EA-HbA1c measured in whole blood could be a solution. However, a majority of kits used are blood cell test kits, and kits capable of whole blood tests are limited in both methods. Third, EDTA tubes not prone to hemolysis could be used instead of blood glucose test tubes prone to hemolysis. Fourth, glycated albumin (GA) could be used as the glycemic control index in health checkups instead of HbA1c. HbA1c samples in blood glucose test tubes are
tested in order to reduce the cost by using the same tubes as those for blood glucose tests. Because GA can be measured in serum and plasma, it can be measured by automatic analyzers along with other blood chemistry test items and can be measured in frozen samples as well. In addition, recent epidemiological studies reported that GA was as useful as similar results (7,8) or more useful than HbA1c (9). Whatever the case may be, the test method should be switched to any of the above proposals immediately.

IA-HbA1c levels and EA-HbA1c levels in off-site health checkups were similarly lower than those measured by on-site HPLC assay. In the present observation, both IA-HbA1c and EA-HbA1c levels in off-site health checkups were measured as blood cells HbA1c. The result supported our hypothesis (5) that the cause was hemolysis that occurred during transportation of blood samples in sodium fluoride-containing blood collection tubes for glucose measurement.

CONFLICTS OF INTEREST AND FUNDING

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REFERENCES