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Changes in Glucose Measurement Kit Conditions in University Training

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ABSTRACT

We examined changes in the conditions of serum glucose measurement kit use in university biochemistry training. The kit is used in the field of clinical examination and can be applied to automatic analysis. The operation has been extremely simplified, and the reaction can proceed in one step, but the amount of sample used is very small, and it is difficult to measure the sample accurately with practice. We thought that it was necessary to consider increasing the sample volume to carry out measurements while maintaining accuracy and precision in practical training. Therefore, the amount of reagent, heating time, and a calibration curve was examined accordingly, and from the results of the recovery experiment, it was finally judged that changes were possible.

INTRODUCTION

Experiments and practical training in a university setting are considered important for actually applying the materials from classroom lectures and establishing knowledge[1),2)]. In addition, experience in practical training might affect subsequent graduation research and future courses. We are in charge of a practical training course called biochemical experiments at the Faculty of Nutrition, University of Kochi, and aim to solidify the contents of lectures through experiments on biochemical content. The experimental contents are diverse, but the quantification of serum glucose using a kit has been introduced. Similar experiments are not always conducted in biochemistry training at other universities. It is difficult to understand the basic biochemical events and principles because the kit structure and reagent composition are complicated or unclear, and the results are easy to obtain. Based on the intentions of the person in charge of the subject, it is important to think about future courses after graduating and to experience such practically used methods. Judging them comprehensively, the kit method and other classical experiments have been combined to form the entire practice.

The glucose kit currently used is based on the same method used in the field of clinical examination. The solution is adjusted to comprise a low volume such that testing with a device can be easily automated, and the result can be obtained in an extremely short time. However, this test is associated with an unsuitable aspect concerning its introduction into practice, which uses many methods[3)]. All reagents are contained in one solution, which is simply mixed with the sample and placed at a constant temperature to complete the reaction. Therefore, it is difficult to understand the principles underlying the reaction. In addition, the number of samples used is extremely small. As the details of the kit are published in the text[4)], it might be easy to consider introducing it into practical training. Probably because the same kit has already been introduced in other universities, the price has more than doubled over several years. Therefore, we decided to consider changing the conditions of use while maintaining its significance in practical training. When changing the conditions, we contacted the sales manufacturer before considering the conditions. In this training, we did not measure patient samples and determine treatment plans, and thus, we decided that it would be acceptable to change the conditions.

METHODS

The original use of the kit

The kit used in this training was the Glucose CII-Test Wako (Fuji Film Wako Pure Chemical Industries, Osaka)[5]. The kit consists of a color-developing solution, prepared by mixing a color-developing agent and a buffer solution at the time of use, and a standard solution of 200 and 500 mg/dl. The basic principle of the reaction is the *mutarotase/glucose oxidase* (GOD) method. The α-D-glucose in the blood is converted to β-D-glucose by *mutarotase* under neutral conditions, and hydrogen peroxide is generated in a dose-dependent manner by the action of GOD[6),7)]. The red quinone dye produced by the reaction of hydrogen peroxide with 4-amino antipyrine and phenol is detected at 505 nm. Since hydrogen peroxide is unstable and susceptible to the reducing action mediated by coexisting ascorbic acid, ascorbic acid oxidase is also present as a masking agent. Since it is easily oxidized by dissolved oxygen, it is a reagent that can react quickly in one step. All enzymes listed here (mutarotase, GOD, etc.) and substances other than glucose (4-amino antipyrine, pH 7.1 phosphate buffer, etc.) are contained in the color-developing solution. A constant pH was maintained using a buffer solution, and the reaction was carried out at 37°C in a constant-temperature bath. This method uses many enzymes and is considered less susceptible to interference owing to its substrate specificity compared to the unused method[8)].

The specific procedure was as follows: to 20 μl of glucose standard solution or serum sample, 3 ml of color-developing solution (a mixture of color-developing agent and buffer solution in advance) was added and heated at 37°C for 5 min; detect the resulting red pigment was detected at 505 nm. In an actual clinical examination site, it might be necessary to prepare a calibration curve using the data measured once with two standard solutions of 200 and 500 mg/dl. The kit can also be used to prepare standard solutions in 100 mg/dl increments from 0 to 500 mg/dl. According to the attached text of the kit, the reaction at 37°C is completed within 4 min and the coloration is constant, but heating for 20 min or more can cause fading and should be avoided[5)]. This method establishes a linear relationship with the absorbance at 505 nm for glucose concentrations of 0–500 mg/dl and can be used for quantification.

Points to be improved

The most problematic point is that the amount of sample used is 20 μl. The biochemical experiments that we are in charge of are for students in their second year of university. Since

it must be assumed that students who are not yet proficient in pipette operation will be conducting experiments, it is desirable to be able to perform measurements using a larger volume of samples. It is thought that this will improve the accuracy and provide a satisfactory result. Second, if possible, it would be better if the amount of reagent (color-developing agent) could be reduced to less than 3 ml. It is thought that human error is likely to occur during the experiment, but since it is for student training, it is necessary to consider the reason. A situation in which the result is not optimal. It is only measured once should be avoided because it is difficult to consider[3),5)]. We would like to carry out an experiment that considers reproducibility with a multi-point calibration curve, but if that happens, the amount of reagents used will inevitably increase (if six-point calibration curves are generated in triplicate, 18 measurements will be performed in total). Especially for kits, the cost must be taken into consideration. It is not a problem in terms of student training that the reaction time is longer than 5 min, but it is necessary to maintain accurate measurements.

Contents of examination and equipment/water used

We considered a sample volume of 50 µl. Precise measurement with a whole pipette might be difficult, but with a micropipette, sorting is relatively easy. Judging from the technical proficiency of the students, it was unacceptable to lower this below this level. In addition, the conditions for increasing the precision and accuracy were examined in terms of heating time and reagent amount. Although the calibration range was expected to be narrowed, we decided to consider linearity in the range of 0–300 mg/dl as a necessary condition. The wavelength was not changed in this study. This is because the manufacturer has already considered the construction of the kit. In addition, we did not test anything other than the usual 1 cm cuvette. A micro cuvette that can reduce the measured liquid volume to 50 µl or less can also be used, but it is meaningless to use it because the liquid volume is originally 3 ml or more, and it was considered that the liquid volume increases further with the examination. This is because even if the amount of the reagent is significantly reduced, it is easily affected by the coexisting substances in the sample, and the absorbance in the original measurement is sufficiently high. Since the reaction temperature is considered to have unintended effects, such as the deterioration of reagents, we decided not to change it from 37°C. It seems that outsourced inspection centers might dilute reagents with water or other liquids to reduce costs at their discretion, but we did not consider such changes in conditions.

A water bath TR-3A Thermal Robo (As One, Osaka) was used to maintain the temperature at 37°C. The water used to dilute the sample was produced by the distilled water production device WS200 Auto Still (Yamato Scientific, Tokyo). To make it easier to compare the conventional method and the modified method (hereinafter referred to as the improved method), it was assumed that a general-purpose cuvette would be used in the spectrophotometer. When the amount of reagent was reduced, we decided to add distilled water after the reaction and perform measurements with the same amount (for example, when reducing the amount of reagent to 1 ml, add 2 ml of distilled water to keep the amount constant, and then measure the absorbance). However, the final calibration curve of the improved method was generated by adding 3 ml of distilled water to make the entire volume approximately 5 ml. A U-1100 type (Hitachi, Ibaraki) was used as the spectrophotometer. Liquid control sera Wako C & C I and II (Wako Pure Chemical Industries, Osaka) were used for the recovery experiment. Distilled water was used to dilute the glucose solution and generate the blank for the absorbance measurement.

RESULTS AND DISCUSSION

Heating time and amount of color-developing solution

Figure 1 and Table 1 show the absorbance when 50 μl of the sample was used, the heating time at 37°C in a water bath was changed to 5,10, and 15 min, and the amount of color-developing solution was changed to 1,2, and 3 ml. The samples used were 50 mg/dl and 300 mg/dl glucose. When 50 mg/dl glucose was used as a sample, the absorbance was almost constant regardless of the amount of reagent or heating time. This was considered to be because the reaction proceeded rapidly and the amount of reagent was still sufficiently excessive, and thus, it was not affected by the differences in conditions. When 300 mg/dl glucose was used as a sample, a clear decrease in absorbance was observed when the amount of reagent was 1 ml, and a shortage of reagents was observed. Contrary to the content of the attached text for the reagent, it was observed that the absorbance tended to decrease even for 10 min or more when the heating time was extended. This tendency was remarkable at 300 mg/dl, but it was also observed at a minute level with 50 mg/dl glucose. Among the conditions examined, those having an absorbance ratio (ratio of absorbance obtained by treatment with 300 mg/dl glucose to that obtained with 50 mg/dl) close to 6 and a small variation in absorbance were considered good. Two conditions, 2 ml of color-developing solution with a reaction time of 5 min and 3 ml of color-developing solution with a reaction

time of 10 min, seemed optimal. Above all, the condition comprising 2 ml of color-developing solution with a reaction time of 5 min led to a reduction in the number of reagents, and thus, the following experiments were continued under this condition.

Comparison of the calibration curve and recovery rate

Figure 2 shows the calibration curve when measured using conventional and improved methods. In the conventional method, the calibration curve, regression equation, and correlation coefficients in the glucose concentration ranges of 0–500 mg/dl and 0–300 mg/dl are shown. In the improved method, the calibration curve, regression equation, and correlation coefficient before and after dilution with water are displayed in the range of 0–300 mg/dl. Unfortunately, in the improved method, linearity was slightly inferior to that of the conventional method based on the value of the correlation coefficient. In the improved method, the addition of water after the reaction might have increased the variation in the amount of solution; therefore, results without the addition of water are also shown. When water was not added, the absorbance tended to be very high, and linearity tended to deteriorate. In the improved method, it seems that this method was superior because the linearity was improved and the correlation coefficient approached 1 when the absorbance was suppressed to 1 or less by adding water.

It was expected that the linearity would be maintained up to a glucose concentration of 200 mg/dl under the reaction condition of 5 min based on the ratio of the sample and the color-developing solution with the conventional method and that it could be used for quantification. From the actual reactivity data, the linearity was considered to be maintained even up to 300 mg/dl because a larger amount of reagent was present. This was supported by the results shown in Figure 3, which were obtained separately.

Table 2 shows the results of recovery determination when commercially available control serum and standard solution were measured as samples by the conventional method, the improved method, and the improved method without water dilution. There was almost no difference in the results in either case. The accuracy or precision of the improved method was judged to be equivalent to that of the conventional method, and it was concluded that 0–300 mg/dl glucose could be sufficient for practical use.

It is possible to consider changing the sample volume to 100 µl in the future. From the calibration range of the conventional method and the result of Figure 3, the range was

considered to be narrowed to 0–200 mg/dl or less even if 3 ml of the color-developing solution was used. As a result, it was suggested that this would not fit the purpose of the training. If the raw data for preparing the calibration curve included 100 mg/dl intervals, the number of experimental points (different glucose concentrations) would be four instead of six, which are used in the conventional training. The amount of reagent used per measurement would thus be two-thirds. Because the amount of reagent for one measurement would also be reduced, the amount of reagent can be reduced by approximately 56% overall. If seven-points calibration curves are used with 50 mg/dl intervals, the number of reagents could be reduced by approximately 22%. Therefore, the results of this study are considered highly applicable for practical training.

CONCLUSION

From the results of this study, we considered that if the glucose concentration is in the range of 0–300 mg/dl, the measurement can be performed by increasing the sample amount and reducing the reagent amount while maintaining good accuracy and precision. The final solution volume was approximately 5 ml, and there was a sufficient reaction solution volume to allow for co-washing and measurements with a normal cuvette. The sample volume was 50 µl, subject to the constraint that the concentration of the sample used must be less than 300 mg/dl. It is thought that this would easily correspond to the level of student proficiencies with the required procedures and instruments, such as micropipettes, which are usually used. For next year's student training, we would like to change the training content based on the examination results.

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Table no 1: Absorbance ratio and precision of reaction products

Reaction time and amount of color-developing solution	Absorbance and CV (%) during reaction[*1]		Absorbance ratio[*2]
	300mg/dlglucose	50mg/dlglucose	
5 min - 1 ml	1.276 / 0.56	0.313 / 0.92	4.07
10 min - 1 ml	1.119 / 0.45	0.308 / 0.19	3.63
15 min - 1 ml	0.897 / 1.27	0.308 / 0.86	2.91
5 min - 2 ml	1.563 / 0.65	0.320 / 0.65	4.89
10 min - 2 ml	1.510 / 0.99	0.316 / 0.80	4.77
15 min - 2 ml	1.495 / 1.17	0.311 / 0.93	4.80
5 min – 3 ml	1.592 / 0.31	0.327 / 1.27	4.86
10 min - 3 ml	1.579 / 0.78	0.321 / 0.82	4.92
15 min - 3 ml	1.550 / 0.52	0.316 / 1.14	4.91

[*1]: Absorbance was measured after setting the reaction volume to 3.05 ml.

[*2]: Ideally, the absorbance ratio should be 6.00, but this is not possible owing to reagent blanks. However, a higher value is better.

Shading represents good reproducibility (less than 1%) or a high absorbance ratio (4.8 or more).

n = 3.

Table no 2: Recovery of glucose using control serum and standard solution

Calibration curve type and range	Recovery (mg/dl; upper) / Recovery rate (%, average; lower)			
	Control I[*1]	Control II[*1]	100mg/dlstandard[*2]	200mg/dlstandard[*2]
Conventional method 0–500 mg/dl	91.0 ± 0.7 100.0	197.3 ± 14.7 98.2	100.8 ± 0.9 100.8	208.3 ± 2.7 104.2
Conventional method 0–300 mg/dl	91.1 ± 0.7 100.1	193.2 ± 14.1 96.1	100.5 ± 0.8 100.5	203.7 ± 2.6 101.9
Improved method 0–300mg/dl	90.4 ± 1.3 99.3	201.2 ± 6.5 100.1	101.7 ± 0.9 101.7	208.3 ± 2.1 104.2
Improved method (undiluted) 0–300 mg/dl	90.6 ± 0.3 99.6	203.9 ± 1.5 101.4	103.7 ± 0.3 103.7	216.7 ± 3.8 108.4

Numerical values are shown as the mean ± standard deviation.

[*1]: The glucose concentration shown in the control is I: 91.0 ± 3.1, II: 201.0 ± 6.3 mg/dl.

[*2]: The concentration of the standard solution data was calculated by recalculating the original absorbance to prepare the calibration curve.

Shading represents the numerical value (100% ± 5%), which seemed to be acceptable.

n = 3.

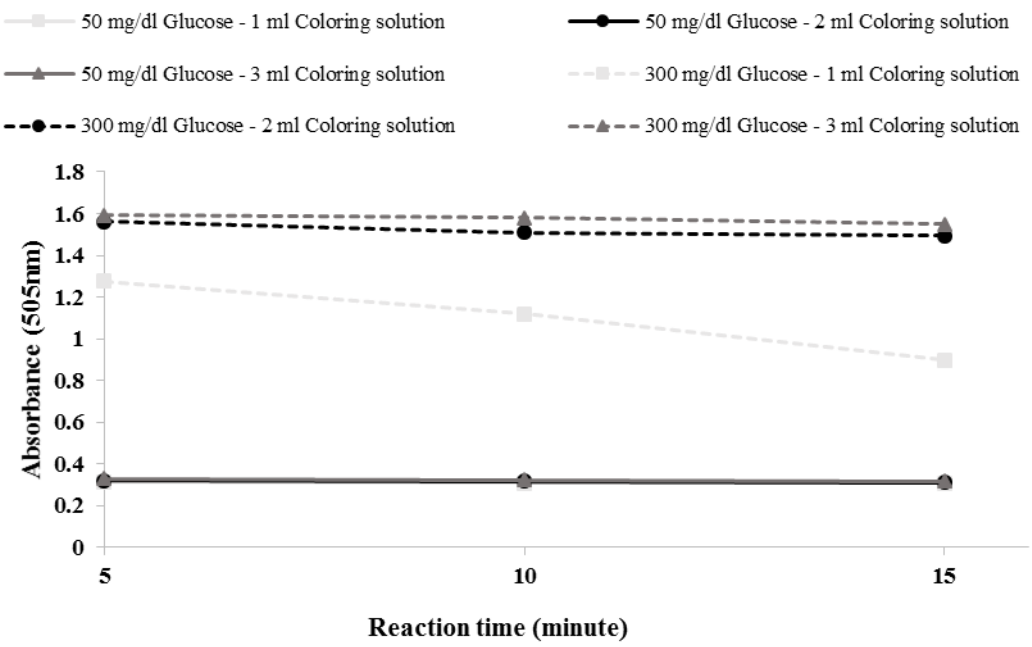

Figure no. 1 Relationship between reaction time and absorbance when 50 mg/dl and 300 mg/dl glucose are used for the reaction in a color-developing solution.

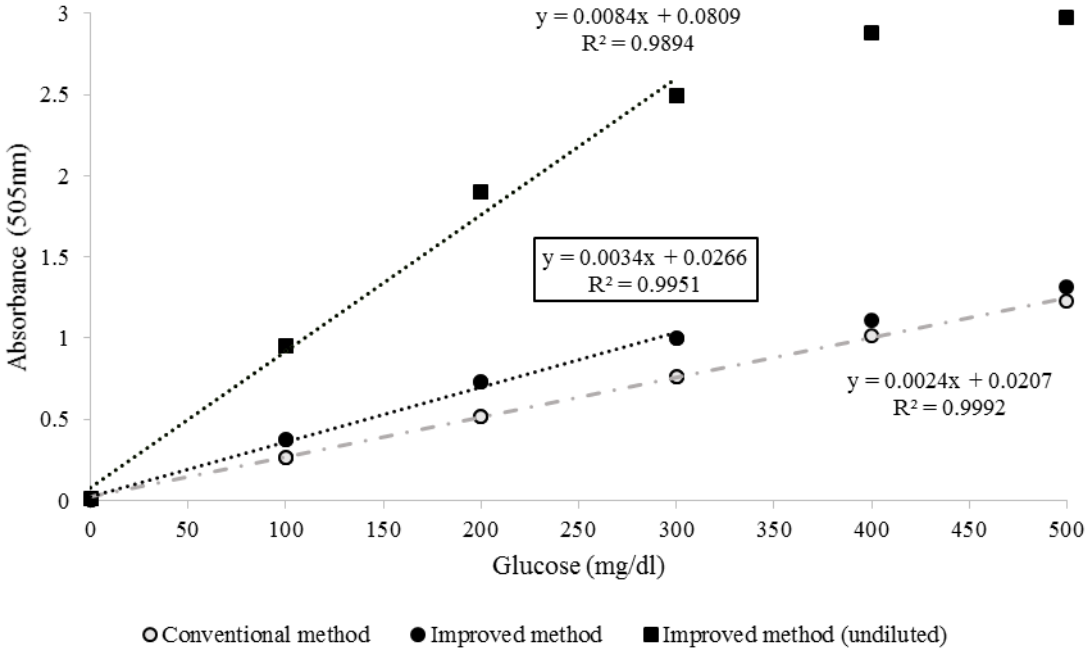

Figure no. 2 Calibration curves.

In the conventional method, 3 ml of the color-developing solution was added to 20 µl of the standard solution to carry out the reaction, and the absorbance was measured.

In the improved method, 2 ml of the color-developing solution was added to 50 µl of the standard solution to carry out the reaction; then, 3 ml of distilled water was added, and the absorbance was measured.

In the undiluted method, the absorbance was measured without adding distilled water.

The calibration curve and correlation coefficient of the conventional method are in the range of 0–500 mg/dl, and the improved method is shown in the figure at 0–300 mg/dl.

The regression equation and correlation coefficient are the calculation results within the respective straight-line ranges.

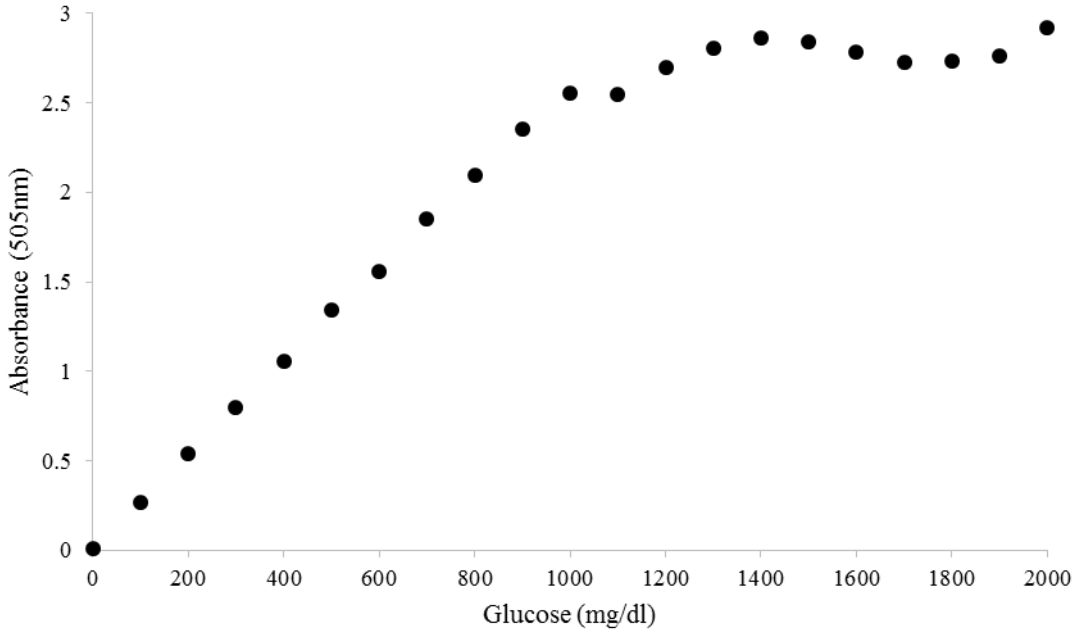

Figure no. 3 Relationship between absorbance and glucose concentration in the conventional method.

The absorbance measurement results are shown when 3 ml of the color-developing solution was added to a sample volume of 20 µl and heated at 37°C for 5 min (blank is distilled water).

n = 1.