

Gingival Crevicular Fluid [GCF]- Collecting Device for Analyzing Microvolume Sample Solutions

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The authors aim to develop noninvasive methods of measuring blood glucose levels by analyzing body fluids collected painlessly. In this report, we describe the manufacturing of a gingival crevicular fluid (GCF)-collecting device with a highly sensitive glucose testing tape that causes no pain for the subjects and has proven its usefulness and medical safety. The testing tape must be adequately colored for measurement. This study clarified the following: 1. The structure of a GCF-collecting device that can analyze very small volumes of GCF has been devised, and the quantity collected can be adjusted by varying the size of the testing tape. 2. Two types of GCF-collecting device are proposed which can be used even in cases of periodontitis. The GCF-collecting devices can be used as noninvasive devices in dolorimetry. 3. The manufacturing procedure for the glucose testing tape has been clarified with N, N-diethyl-p-phenylendiamine sulfate selected as a chromogen. The advantage of this procedure is that no materials with toxic or carcinogenic properties are used.

1. Introduction

It is essential for patients with diabetes mellitus to maintain their blood glucose level within a normal range. To achieve this goal, many diabetic patients carry a portable self-measuring glucose analyzer to withdraw their own blood to examine their glucose level. With this they can determine the appropriate level of exercise, insulin dose or restricted

food intake for maintaining their health. Thus, the development of a noninvasive method of measuring the blood glucose level is an urgent necessity, and putting such a method into practical use will not only enable us to reduce or eliminate the physical and mental stress and pain suffered by patients with diabetes mellitus, but will also allow us to utilize the method widely for the screening of patients who are at high risk of developing a diabetic condition.

We have continuously been focusing on the development of noninvasive methods of measuring the blood glucose level by analyzing body fluids such as painlessly collected saliva.^(1,2) We are the first investigators worldwide to recognize the importance of developing a noninvasive blood glucose analyzer that can measure blood glucose levels by determining the glucose concentrations in the fluid slowly secreted from gingival crevices.⁽³⁾ We are currently attempting to develop a noninvasive blood glucose analyzer and to put it into practical use.

There is a V-shaped crevice surrounding the teeth between the teeth and the gingiva (the gum). This crevice is called the gingival crevice or gingival pocket and is present around all teeth. The depth of the crevice within the clinically normal range is generally between 0.5 and 2.0 mm. When the gum becomes infected or injured because of an oral disease such as periodontitis or gingivitis, the crevice becomes deeper in proportion to the severity of the inflammation in the area. Gingival crevicular fluid (GCF) is an extracellular fluid secreted from the epithelia of the gingival crevice, whose presence was first reported by Brill *et al.* in 1958.⁽⁴⁾ The components of GCF are mainly derived from plasma, and its flow rate is 2 to 3 μl per hour per tooth. Accordingly, particularly in the United States and Europe, much attention is currently being paid to the attempt to establish methods of measuring the concentrations of specific substances contained in GCF and to apply this to noninvasive screening for disease and to biological determinations *in vivo*.⁽⁵⁾

To develop a working system for GCF analysis, however, a device which can not only collect a very small volume (only a few microliters) of the secreted GCF but which can also determine the concentrations of the secreted substance before evaporation and drying of the samples needs to be devised. Unfortunately, such a device, which can quantify specific substances contained in a very small volume of body fluid, has not yet been developed. Thus, most previous studies regarding GCF have mainly focused on the secreted volumes of GCF. In this report, we discuss the following topics as a first step in the development of noninvasive methods for the measurement of blood glucose level.

1. The structural features of the GCF-collecting device for the analysis of microvolume samples and a quantitative analyzing test.
2. Diagnostic test for pain (dolorimetry) in using the GCF-collecting device in 20 subjects.
3. Evaluation of the blood glucose testing tape, which is used for the determination of glucose concentrations and exhibits high biological safety.

2. Gingival Crevicular Fluid Collecting Device

2.1 Collection and analysis

GCF is secreted at less than $1 \mu\text{l}$ per hour, and the liquid usually dries up completely in about one min at room temperature. With a GCF-collecting device it must be possible to collect the GCF in about 30 s and to analyze the concentration before evaporation. The GCF-collecting device and built-in glucose testing tape must satisfy at least four conditions as follows:

1. The device must have a structure such that the collected sample solution is automatically absorbed in a highly sensitive glucose testing tape. Before the concentration changes due to evaporation of the particle sample, the testing tape must be adequately colored for measurement.
2. The collecting device must be able to be inserted into the gingival crevice with a width of 0.1 mm without pain.
3. The quantity of the sample solution is to be determined by the structure of the collecting device.
4. The safety of the materials used for the device must be confirmed in order for it to be inserted orally.

2.2 GCF-collecting device

We manufactured a GCF-collecting device that has a capillary drain and built-in high sensitivity glucose testing tape as shown in Fig. 1. Capillary action is used to pump GCF automatically. Three sheets of film are laminated to form the capillary drain. The middle film has a drain and the plane films on each side function as covers. The drain leads

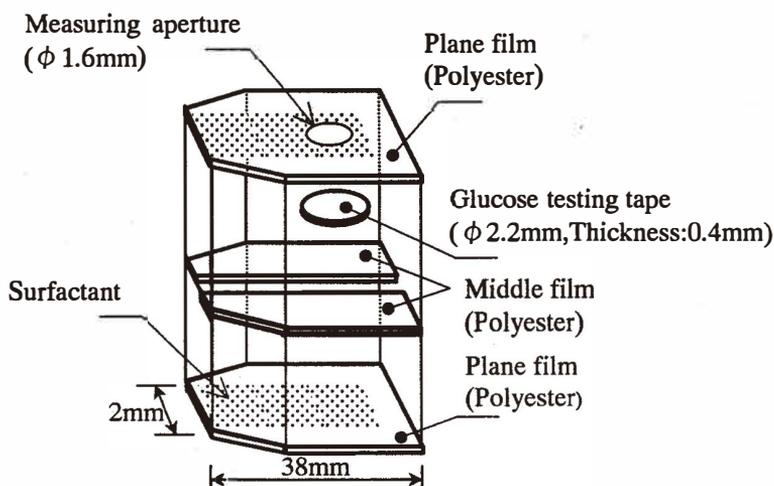


Fig. 1. Structure of gingival crevicular fluid (GCF) collecting device.

samples to a built-in testing tape between the plane films. Since the plastic film is water repellent, a surfactant (sucrose fatty acid ester, DK ester, Daiichi Kogyo Seiyaku Co., Ltd) diluted to 5% in ethanol is applied to the inside face of the two plane films. This sucrose fatty acid ester is in a food additive, so organic and environmental safety are fully confirmed by the manufacturer. Since the collecting device is to be placed in the mouth of the subjects, it is desirable that it be designed to be disposable. Therefore, only low-cost polyester film (Teijin. Co. Ltd.) is used for these plastic films. To enable insertion into a gingival crevice with a width of about 0.1 mm, the thickness of the film is selected to be 30 μm , and the three layers are contacted by an ultrasonic welding method.

Figure 2 shows a standard device (a) and a device for the elderly (b). These two devices cover a range of depths associated with periodontitis and also corresponding to the increase of gingival crevicular depth due to aging. Figure 3 shows the relation between the water-holding volume and the diameter of the GCF-collecting device with built-in testing tape. The experimental results shown are the average of five measurements with glucose standard liquid used as a sample solution. The difference in the water-holding volume between the standard device and the device for the elderly corresponds to the length of these capillary tubes. The water-holding volume is proportional to the diameter of the testing tape, and for the purposes of calibration, the variation (CV) in the capillary is a maximum of 7%.

3. Dolorimetry

To test the pain during insertion, dolorimetry is performed on twenty normal subjects using these GCF-collecting devices. (Fig. 4) Each subject inserts the device twice in the upper and lower front teeth and in the bicuspids at random. None of the subjects complained of excruciating pain. More than 85% using the standard device and 70% using the device for the elderly collected GCF without pain. (Table 1) This result corresponds to the physiological viewpoint that, although tactile points exist around the gingival crevice, there are no pain points. In a previous experiment where the device was inserted by a dentist, GCF could be collected without pain in more than 90% of subjects.⁽³⁾ The results suggest that these devices are usable for collecting GCF, and that the pain can be decreased by increasing the degree of skill of the user.

4. Glucose Testing Tape

4.1 Manufacturing method

A glucose testing tape used for measuring the glucose concentration in GCF requires the following characteristics:

1. The sensitivity of detection of a testing tape compared with ones currently available on the market (for example, ureal testing tape) must be improved by two digits. The required accuracy is 0.5 mg/dl.

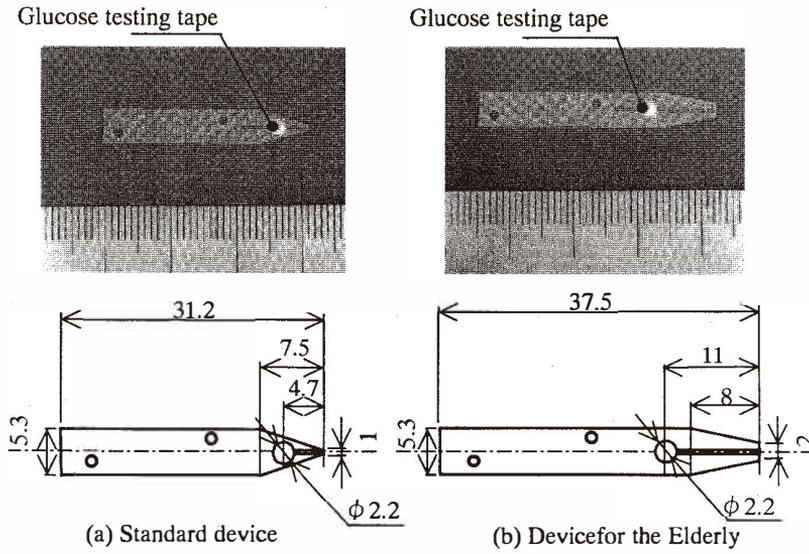


Fig. 2. Comparison of the two kinds of GCF-collecting devices (values in mm).

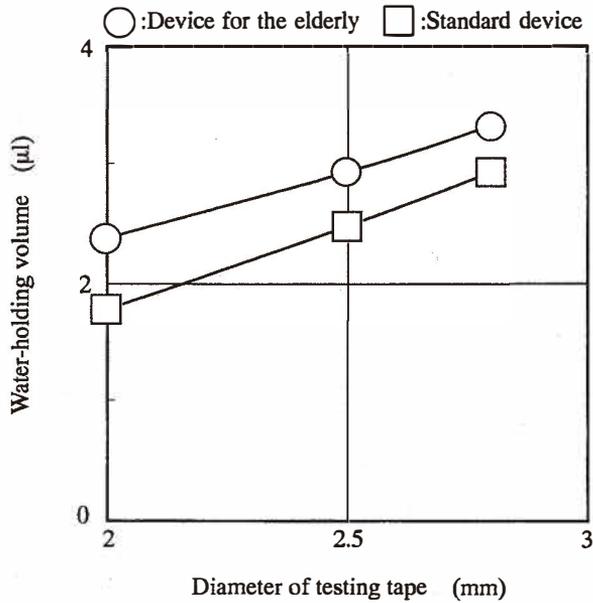


Fig. 3. Comparison of water-holding volume with diameter of testing tape of the GCF-collecting device.

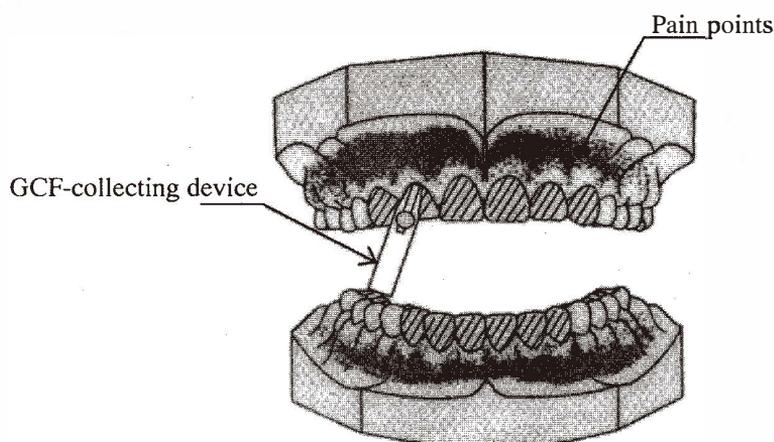


Fig. 4. Method of dolorimetry.

Table 1
Dolorimetry of GCF-collecting devices.

Sensation	Standard device		Device for the elderly	
No pain	140	(87.5%)	118	(73.8%)
Some pain	20	(12.5%)	42	(26.2%)
Excruciating pain	0	(0%)	0	(0%)

2. The testing tape must be made using only materials of which the biocompatible safety has been confirmed by experiment. The materials should be neither toxic nor carcinogenic.

The procedure for manufacturing the glucose testing tape, which used N, N-diethyl-p-phenylenediamine sulfate (DPD sulfate) as a chromogen, is shown in Fig. 5.

1. A surfactant, 0.05 g of sodium dodecyl sulfate ($\text{CH}_2(\text{CH}_2)_{11}\text{OSO}_3\text{Na}$, Wako Pure Chemical Industries, Ltd.), and a stabilizer for various chemicals, 0.5 g of sodium alginate, are dissolved in 30 ml of distilled water. This is solution A.

2. Glucose oxidase (GOD, EC1.1.3.4, 220 units/mg, Wako Pure Chemical Industries, Ltd.) and peroxidase (POD, EC1.11.1.7, 100 units/mg, Wako Pure Chemical Industries, Ltd.) are dissolved in 10 ml of distilled water. This is solution B.

3. Solution C-1 is made from 1.44 g of gelatin dissolved in 30 ml of distilled water at 40°C , after which 30 ml of neutral phosphoric acid buffer solution (pH6.86) is added to keep it warm. Solution C-2 is made from 1.44 g gelatin dissolved in 60 ml of boric acid buffer solution (pH9.18) at 40°C . A chromogen, DPD sulfate, is added to C-1 and C-2. Table 2 shows six types of buffer solutions made using various solvents and solute concentrations.

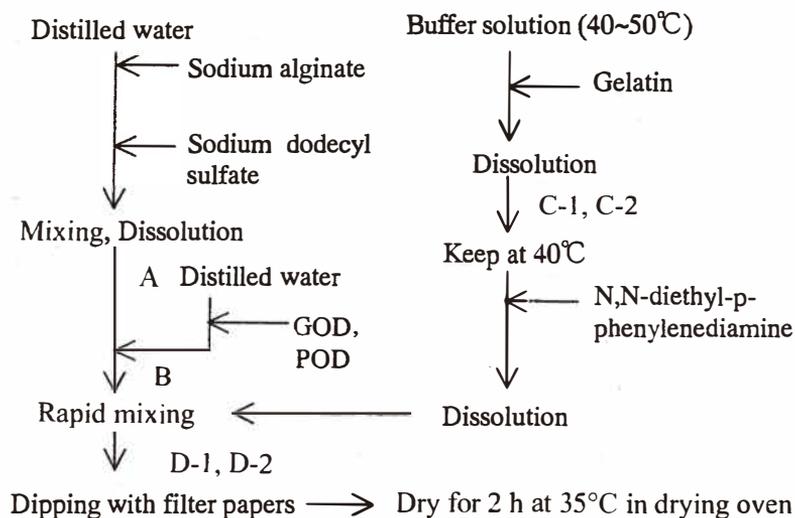


Fig. 5. Manufacturing procedure for glucose testing tape.

Table 2

Combinations of various concentrations of chromogen and solvent.

	Concentration of N,N-diethyl-p- phenylenediamine sulfate (g/dl)	Solvent for N,N-diethyl-p- phenylenediamine sulfate	pH
Type 1	0.125	Diluted neutral	6.00
2	0.250	phosphoric acid	4.25
3	0.375	buffer solution (pH6.86)	3.50
Type 4	0.250	Borate standard	8.25
5	0.375	solution (pH9.18)	7.50
6	0.500		5.50

4. Solution D-1 is made by mixing solutions A, B and C-1. Solution D-2 is made by mixing solutions A, B and C-2.

5. Filter papers are dipped in the solution D (D-1, D-2) for 60 s, then dried for about 2 h at 35°C in a drying oven.

Generally, as the coloring characteristics of DPD sulfate change depending on the buffer solution, the gradient and linearity (correlation coefficient) of the coloring characteristics are calculated to determine the difference due to using different solvents. Six types

of buffer solutions are selected as shown in Table 2. The final pH of the buffer solution decreases in proportion to the concentration of chromogen.

The reflectance of these six glucose testing tapes is measured with a spectrophotometer (U-3010, Hitachi, Ltd.) using a glucose standard solution. The reflectance changes depending on whether the paper is dry or wet. Using the wet condition as standard, the relative reflectance can be calculated using the following equation:

$$R = \frac{R_{\text{mes}}}{R_0}, \quad (1)$$

where R_0 is the reflectance measured with zero mg/dl glucose standard solution, and R_{mes} is the reflectance at each glucose concentration.

The optical density, OD , is obtained from this relative reflectance by the equation:

$$OD = -\log R. \quad (2)$$

The calibration curves between the optical density, OD , and the glucose level of these six testing tapes have been measured. Figure 6 shows the correlation between the gradient

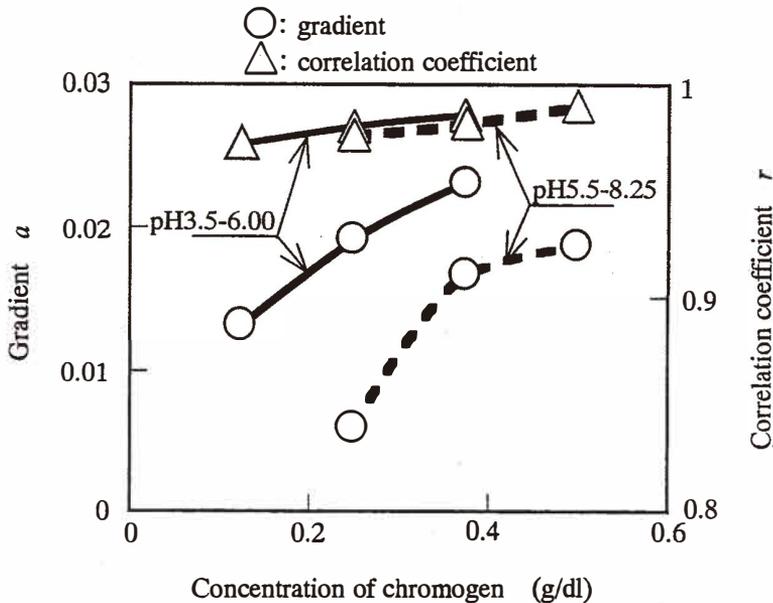


Fig. 6. Measured results of coloring characteristics of testing tape as a function of chromogen concentration. (wavelength: 530 nm)

and the correlation coefficient of these calibration curves. From the results, the following become clear:

1. The sensitivity of the reciprocal of absorbance (reflectance) of the glucose testing tape shows a maximum at a wavelength of about 530 nm. The gradients of these buffer solutions are measured at this wavelength, and it is found that the gradient increases in proportion to the final pH of the solution.

2. The correlation coefficient increases in proportion to the decrease in the final pH. In this evaluation, the best sensitivity is obtained when the solute concentration is 0.375 g/dl of DPD sulfate (chromogen) and when a mixed solution of diluted neutral phosphoric acid buffer solution is used as the solvent.

3. In all cases, the correlation coefficient increases in proportion to the density of DPD sulfate.

4.2 Calibration curve and time-course changes of testing tape

The glucose testing tape is made using 0.375 g/dl of chromogen and a diluted neutral phosphoric acid buffer solution. Figure 7 shows the calibration curve of the absorbance converted from the reflectance. When the glucose concentration is more than 5 mg/dl, the reflectance saturates. The correlation coefficients in the ranges from 0 to 5 mg/dl and from 0 to 10 mg/dl are 1.00 (calibration curve A) and 0.99 (calibration curve B), respectively. The coefficient of variation (CV) obtained from each concentration for both calibration curves shows a maximum of 12%. Figure 8 shows the changes over a period of time in a

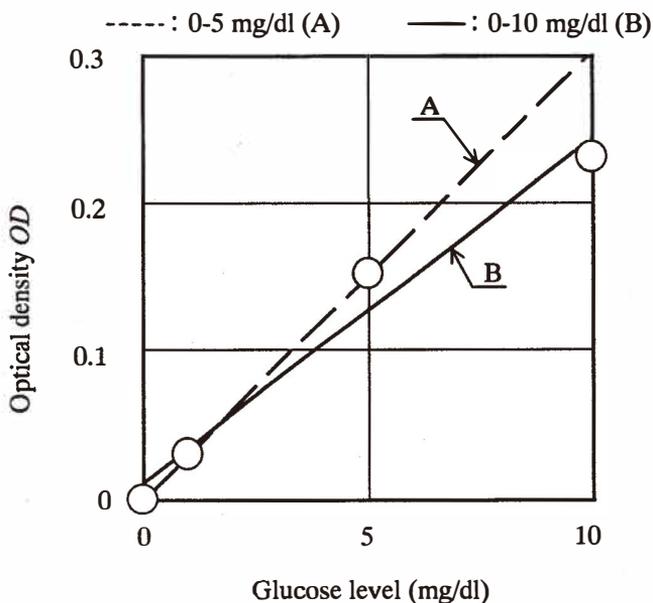


Fig. 7. Calibration curve of testing tape measured using the spectrometer.

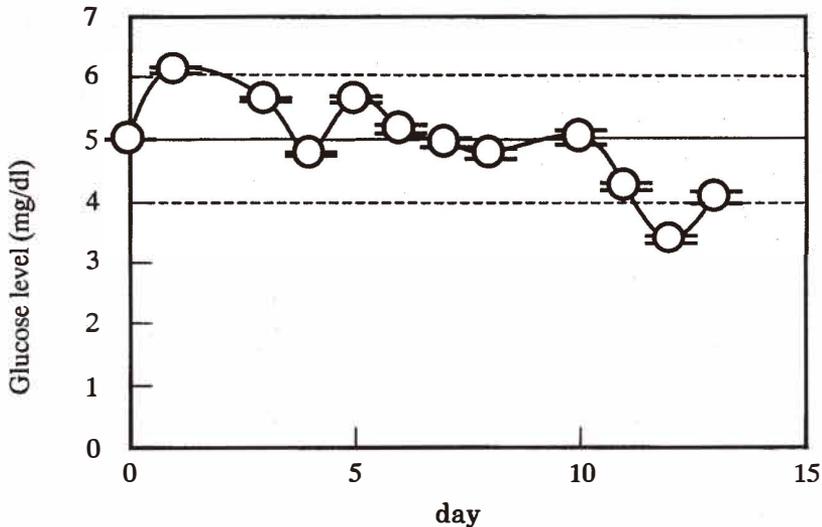


Fig. 8. Time-course changes of testing tape with 5 mg/dl of glucose standard solution.

standard solution of 5 mg/dl glucose calculated using calibration curve B. In the first four days the glucose levels changed by 22%. Thereafter, they varied by $\pm 10\%$ for 5 mg/dl for about seven days. Based on these results, it is desirable to use this glucose testing tape after four days aging.

4.3 Calibration curve of GCF-collecting device

Figure 9 shows the results of the calibration curve obtained using a GCF-collecting device. This curve is measured using a manufactured optical measuring system in the range from 0 to 10 mg/dl. In this measurement, a green LED (E1L51-3G, Toyoda Gosei Co., Ltd.) irradiates the glucose testing tape, and the reflectance is measured by a photodiode (S2506-02, Hamamatsu Photonics., K.K.). For comparison, a calibration curve measured by the spectrophotometer is also shown. The gradient of the spectrophotometer is 2.32×10^{-2} and the coefficient of variation (CV) is 12% maximum. On the other hand, the gradient of the optical measuring system is 2.07×10^{-2} and the CV is 9% maximum. Both correlation coefficients are more than 0.98. If the condensing characteristics of the green LED can be improved further, it may be possible to improve the accuracy of the measurement. In conclusion, with the GCF-collecting device the necessary precision for measuring glucose concentration can be achieved.

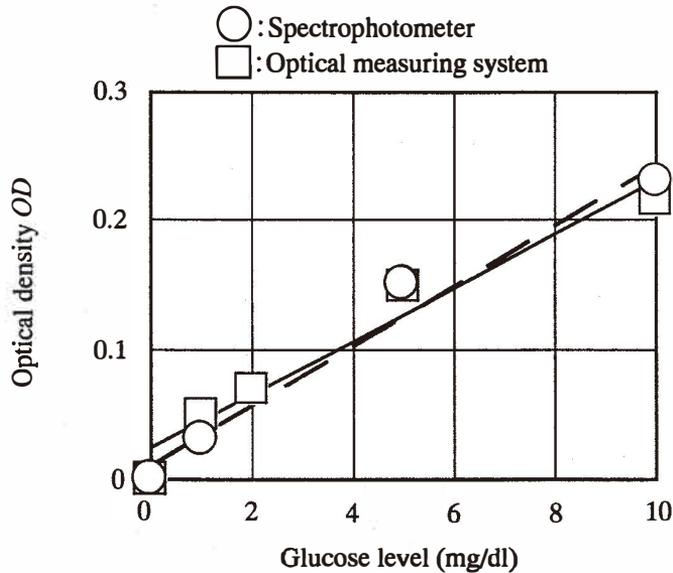


Fig. 9. Calibration curve of GCF-collecting device measured by an optical measuring system.

5. Conclusions

We manufactured a GCF-collecting device with a highly sensitive glucose testing tape that causes no pain to the subjects and proved its usefulness and medical safety. The following results became clear:

1. The structure of a GCF-collecting device that can analyze very small volumes of GCF is devised, and the quantity collected can be adjusted by the shape of its capillary drain and the size of the testing tape.

2. Two types of GCF-collecting device are proposed which can be used even in cases of periodontitis. Based on results from dolorimetry, the GCF-collecting devices can be used as noninvasive devices.

3. The manufacturing procedure for the glucose testing tape is clarified with N, N-diethyl-p-phenyldiamine sulfate selected as a chromogen. The advantage of this procedure is that no materials with toxic or carcinogenic properties are used. From the measurements, the coefficient of variation of this GCF-collecting device in the range 0–10 mg/dl is a maximum of 9%.

In the future, we aim to establish methods for noninvasive measurement for blood glucose levels by performing bench tests using these GCF-collecting devices.

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Masaki Yamaguchi was born in Nagoya, Japan, in 1963. He received the B.S. and M.S. degrees in engineering from Shinshu University, Japan, in 1985 and 1987. He joined the Research Laboratory of Brother Industries, LTD., Japan, in 1987. He received the doctoral degree in engineering from Shinshu University in 1994. He started an assistant professor at Tokyo University of Agriculture and Technology since 1995. He is currently working as an associate professor in faculty of engineering at Toyama University since 1999. His primary research interests focus on the development of noninvasive medical sensors and welfare apparatuses. His noninvasive measurement approaches include

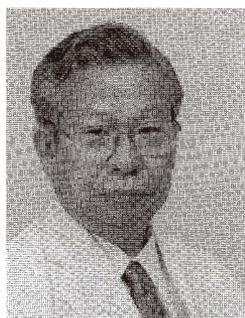
blood glucose, human stress, and other clinical analytical items. In 2001 he received Nikkei BP Technology Awards in Medicine/Biotechnology Section from Nikkei Business Publications, Inc. for his achievements in the study of noninvasively measuring blood glucose via gingival crevicular fluid. He is a member of the IEEE EMBS, the Institute of Electrical Engineers of Japan (IEEJ), the Japan Society of Medical Electronics and Biological Engineering (JME), and the Japan Diabetes Society.



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