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화학발광법에 의한 전혈 중의 당 정량

李相學* · 崔詳燮†
경북대학교 자연과학대학 화학과
†안동과학대학 산업안전학과
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Determination of Glucose in Whole Blood by Chemiluminescence Method

Sang Hak Lee* and Sang Seob Choi†
Department of Chemistry, Kyungpook National University, Taegu 702-701, Korea
†*Department of Industrial Safety, Andong Science College, Andong 760-820, Korea*
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요 약. 흐름주입 장치를 이용하여 화학발광법에 의한 전혈 중의 당을 정량하는 방법에 대하여 연구하였다. 당의 효소반응에서 생성되는 과산화수소에 의하여 424 nm에서 발생하는 luminol의 화학발광 세기의 차이를 정량에 이용하였다. 효소반응기는 glucose oxidase를 aminopropyl glass bead에 혼입하여 만들었으며, 흐름셀에서 발생하는 화학발광의 세기는 광섬유다발을 이용하여 측정하였다. 최적 실험조건을 구하기 위하여 화학발광 시약 및 효소반응기의 pH, 흐름속도 및 온도가 화학발광 세기에 미치는 영향을 조사하였다. 최적 실험조건에서 구한 검정곡선은 1.0×10^{-1} - 7.0 mM에서 직선성이 성립하였으며, 검출한계는 6.0×10^{-2} mM이었다. 본 방법을 전혈 중의 당 정량에 적용하였으며, 그 결과를 기존의 분석법에서 구한 결과와 비교하였다. 또한, 회수율 측정을 통하여 본 방법의 신뢰성을 검증하였다.

ABSTRACT. A method for the determination of glucose in human whole blood by chemiluminescence method using a stopped flow injection system has been studied. The method is based on the differences in the chemiluminescence intensities of luminol due to the different amounts of hydrogen peroxide produced from the glucose oxidase catalyzed reaction. The enzyme reactor was prepared by immobilization of glucose oxidase on aminopropyl glass beads and the chemiluminescence from a flow cell was measured by means of an optical fiber bundle. In order to obtain the optimum experimental conditions, effects of pH for the chemiluminogenic solution and enzyme reactor, flow rate and temperature on the chemiluminescence intensity were investigated. The calibration curve obtained under optimum experimental conditions was linear over the range from 1.0×10^{-1} mM to 7.0 mM and the detection limit was 6.0×10^{-2} mM. The proposed method was applied to the determination of glucose in whole human blood sample and the results were compared with those obtained by an official method. The present method was also evaluated by the results of recovery experiments.

INTRODUCTION

Glucose sensors are of great interest since glucose content is one of the most important parameters checked in routine medical analysis. These sensors also have been used to measure glucose content in other biological fluids, molasses and fermentation processes. The biosensor to determine glucose in human whole blood is particularly important because it allows a shortening of the analytical time due to the elimination of the time required for the separation of serum and plasma. In addition, it could lead directly to the development of

implantable sensor to monitor glucose in blood vessel. The capacity of glucose oxidase to catalyze the oxidation of glucose to gluconic acid has been widely promoted as a model system for the design of glucose sensor. The enzyme is highly active, relatively stable and readily available, and thus has been extensively utilized to develop thermal,[1,2] transistor,[3,4] electrochemical[5-8] and optical biosensors.[9-11] An important consideration in the development of practical device is to improve the immobilization method. Various techniques such as physical adsorption,[12] covalent attachment,[13,14] cross linking[15,16] and electropolymerization[8,17,18] have been studied.

The principle of glucose determination by enzymatic method is to measure enzymatically produced hydrogen peroxide during the oxidation reaction of glucose in the presence of molecular oxygen [equation (1)].[7]

$$\beta\text{-D-glucos}-O_2+H_2O \xrightarrow{\text{glucose oxidase}} \text{gluconic acid}+H_2O_2 \quad (1)$$

Amperometric determination of hydrogen peroxide produced in the oxidation reaction has been extensively studied.[19-21] Another method developed for the determination of hydrogen peroxide produced in the enzymatic reaction is to measure the difference of chemiluminescence signal due to the change of hydrogen peroxide content.[9,22-25]

The most widely used chemiluminogenic reagent is 3-aminophthalhydrazide(luminol). As seen in equation (2), luminol reacts with hydrogen peroxide produced by the enzymatic reaction of glucose, and produce chemiluminescence. The chemiluminescence spectrum shows a broad band whose maximum intensity appears at 425 nm.[26,27]

$$2H_2O_2 + \text{luminol} \xrightarrow[OH^-]{\text{Catalyst}} \text{3-aminophthalte}^- + N_2 + \text{light} \quad (2)$$

Within certain limits, the chemiluminescence intensity of luminol is directly proportional to the concentration of either the peroxide, the catalyst, or the luminol. Transition metal ions such as Co^{2+}, Cu^{2+} and Fe^{3+} or various metal complexes have been used for the catalysts.[25,28] The chemiluminescence method is particularly useful for the determination of peroxide because of the low detection limit, the high sensitivity, the high selectivity, the ease of manipulation and the inexpensive instrumentation. Recently, fiber optic cables have been widely used to measure light intensity. For example, they were used to transmit emission signal to determine oxygen based on its quenching effect on fluorescence dyes,[29,30] to determine Eu(III) by fluorescence spectrometry,[31] and to analyze antioxidants by chemiluminescence method.[32]

This paper describes a method for the determination of glucose in human whole blood by chemiluminescence method using a stopped flow injection system. Luminol has been used for the chemiluminogenic reagent in the present work. The optimum analytical conditions for calibration were obtained on the basis of the results of the effects of pH for the chemiluminogenic solution and enzyme reactor, flow rate and temperature on the chemiluminescence intensity. The method proposed was applied to the determination of glucose in human whole blood sample and the results were compared with those obtained by the official method.

EXPERIMENTAL

Reagents. Analytical grade anhydrous D(+)-glucose was purchased from Fluka(Buch, Switzerland), glucose oxidase([E.C.1.1.3.4.], Type II, from *Aspergillus niger*, activity of 25,000 units/g solid) from Sigma (St. Louis, MO, USA) and luminol (3-aminophthalhydrazide, 97%) from Aldrich(Milwaukee, WI, USA). Activated aminopropyl glass bead(125~177 μm) and aqueous glutaraldehyde used for the preparation of enzyme reactor were purchased from Sigma. All other chemicals were of analytical reagent grade and were used as received. Deionized water was obtained by means of a Millipore (Bedford, MA, USA) Milli-Q water system and used throughout the whole experiment. The human blood samples were kindly provided by the Lee Sang Mu Clinic.

Apparatus. A schematic diagram of the automated stopped flow injection analyser used in the chemiluminescence measurements is shown in *Fig.* 1.

The flow system employed in this work consisted of two peristaltic pumps(Ismatec Model MS-4 Reglo/6-100, Glattbrugg-Zürich, Switzerland). One(P1) delivered a chemiluminogenic reagent solution(R1) and a 1.0×10^{-1} M borate buffer solution(pH 12.0, R2). The other(P2) delivered a sample solution(R3) and a 1.0×10^{-1} M phos-

phate buffer solution(pH 7.0, R4). The two streams were mixed in a Y-shaped flow line and the mixture was delivered to a column of immobilized glucose oxidase enzyme reactor(E). The solution passed through the enzyme reactor was mixed with the mixture of P1 stream in a Y-shaped element positioned at 15 mm before the flow cell inlet. PTFE tubing (0.8 mm i.d.) was used to connect all components in this system.

A bifurcated optical fiber bundle(Model 77533, Oriel, Stratford, CT, USA) was screwed to the flow cell for the position of the sensing tip of the optical fiber to be the same for each measurement. The flow cell was housed in a laboratory made light tight chamber to remove all the unnecessary stray light.

One end of the fiber bundle was fixed at 10 mm before the emission port and the other end was fixed at 10 mm before the excitation port of the cell component of a spectrofluorometer(Model FL111, Spex, Edison, NJ, USA). To record emission and excitation spectra, a 450 W Xe lamp was used. To measure chemiluminescence intensity, the Xe lamp was shut off and the luminescence emitted from the cell was fed to a photomultiplier tube(Model R928, Hamamatsu, USA). The voltage used for the photomultiplier tube was 850 V. The aquisition mode used for the excitation and emission spectra, and for the chemiluminescence measurements was signal/reference and signal, respectively. The chemiluminescence intensity at 424 nm was monitored for the determination of glucose. The integration time was 1 s and slit width used in this study was 1 mm and 5 mm for the emission and excitation spectra and the chemiluminescence measurements, respectively.

Preparation of enzyme reactor. The glucose enzyme oxidase reactor was prepared according to a modified method of the published procedure.[33] After 2.0 g of aminopropyl glass beads(125-177 μm) were added to 30 mL of pH 7.0 phosphate buffer solution containing 2.5 % glutaraldehyde, the mixture was stirred for 5 hours at room temperature. The beads were then filtered, washed with phosphate buffer solution. The resulting beads were added to 10 mL of pH 5.6 acetate buffer solution containing 5 g of glucose oxidase and the mixture was incubated for 5 hours at 4 °C under constant agitation. The immobilized enzyme beads were filtered and packed into a 70 mm glass column (2.5 mm i.d.). Both ends of the column were furnished with glass wool.

Measurement procedure. The blood sample collected from human blood vessel was diluted 10-fold with $1.0 \cdot 10^{-1}$ M sodium phosphate buffer (pH 7.0) solution containing $5.0\ 10^{-2}$ M NaF, $2.0\ 10^{-1}$ M NaCl and $3.5\ 10^{-3}$ M EDTA. A 500 mM glucose stock solution was prepared by dissolving D(+)-glucose monohydrate in a buffer solution containing the same ingredients as those used for the blood samples. The stock solution was stored in a dark glass bottle at 4 °C. The standard solutions were freshly prepared by appropriate dilution of the stock solution with a buffer solution containing the same ingredients as that used for the stock solution. A chemiluminogenic reagent solution containing $1.0\ 10^{-3}$ M luminol and $1.0\ 10^{-2}$ M ferricyanide was used in this work. The chemiluninogenic reagent solution was not stable under ambient conditions The chemiluminogenic reagent solution was not stable under ambient conditions, and a fresh solution was made daily.

The sample and phosphate buffer streams were pumped at the flow rate of 3.8 mL/min. The borate buffer and chemiluminogenic reagent streams were pumped at the flow rate of 2.5 mL/min. The time intervals selected for sampling and washing with blank solution were 100 and 150 s, respectively. Calibration plots of chemiluminescence intensity measured at 424 nm *vs.* concentration of glucose in standard solution were carried out, and the content of glucose in sample solution was determined from the calibration curve. For each standard and sample solution, three successive measurements were conducted.

RESULTS AND DISCUSSION

Excitation, emission and chemiluminescence spectra of luminol. The excitation spectrum of chemiluminogenic reagent solution containing $1.0\ 10^{-3}$ M luminol and $1.0 \cdot 10^{-2}$ M ferricyanide was recorded by pumping only a chemiluminogenic reagent stream of the stopped flow injection analyzer (R1 in *Fig.* 1). The excitation spectrum obtained at 412 nm for emission wavelength *via* a bifurcated optical fiber is shown in *Fig.* 2. Two rather broad bands are observed and their wavelength maxima appear at 298 and 358 nm.

The corrected emission spectrum obtained at 298 nm excitation for the same solution is shown in *Fig.* 3(a).

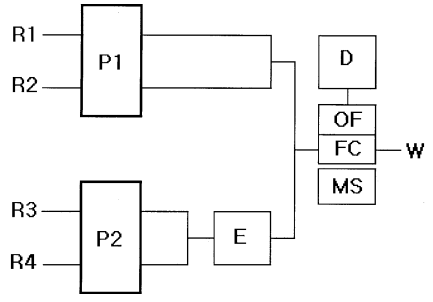

Fig. 1. Schematic diagram of the stopped flow analyser for chemiluminescent determination of glucose: R1, chemiluminogenic reagent solution; R2, borate buffer solution; R3, sample stream; R4, phosphate buffer solution; P1 and P2, peristaltic pump; E, glucose oxdase enzyme reactor; FC, flow cell; MS, magnetic stirrer; OF, bifurcated optical fiber bundle; D, detector; W, waste.

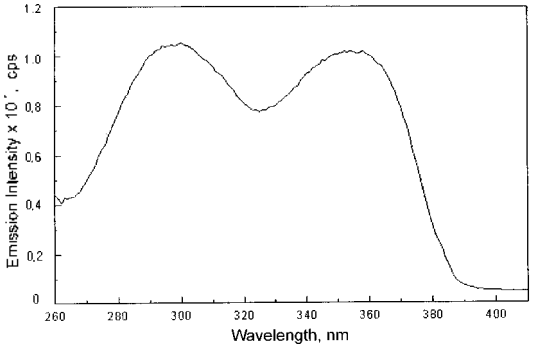

Fig. 2. Excitation spectrum of the aqueous chemiluminogenic reagent solution obtained by using the automated stopped flow analyser: [luminol], 1.0×10^{-3} M; [$Fe(CN)_6^{-3}$], 1.0×10^{-2} M; λ_{em}, 412 nm.

The chemiluminescence spectrum recorded for a solution containing chemiluminogenic reagent, borate buffer and hydrogen peroxide is shown in *Fig.* 3(b). For the delivery of 1.0×10^{-1} M hydrogen peroxide solution, the R3 line in *Fig.* 1 was used after disconnecting the enzyme reactor. The chemiluminescence spectrum shows a similar shape but a slight blue shift in comparison to the emission spectrum. The peak maximum of chemiluminescence spectrum observed at 424 nm is in good agreement with those reported by Kulmala *et al.*[26] and Collaudin *et al.*[27] The results indicate that glucose can be determined by measuring the change of chemiluminescence intensity due to the different amounts of hydrogen peroxide produced from the enzymatic reaction of glucose.

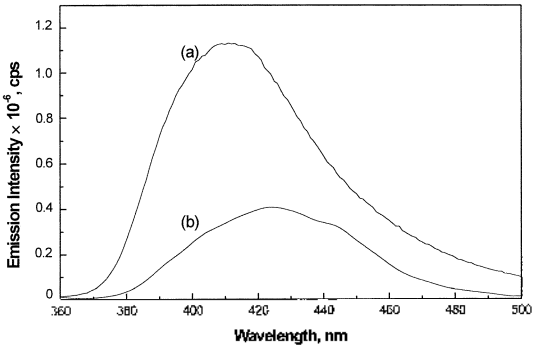

Fig. 3. Emission spectrum of the chemiluminogenic reagent solution(a) and chemiluminescence spectrum of the luminol-Fe(III)-H_2O_2 system obtained by using the automated stopped flow analyser(b): [luminol], 1.0×10^{-3} M; [$Fe(CN)_6^{-3}$], 1.0×10^{-2} M in R1 stream; 1.0×10^{-1} M borate buffer solution in R2 stream; [H_2O_2], 1.0×10^{-1} M in R3 stream. R4 stream and enzyme reactor shown in *Fig.* 1 were not used.

Effect of pH for the enzymatic activity on chemiluminescence intensity. The effect of pH in the range 5~8 for the glucose oxadiase enzyme reactor on the chemiluminescence intensity was investigated by measuring chemiluminescence intensity emitted from the flow cell at 424 nm. A flow rate of 2.5 mL/min was selected for the chemiluminogenic reagent and borate buffer solutions and 3.8 mL/min for the standard and phosphate buffer solutions. An injection volume of 2.0 mL and 2.5 mL for the chemiluminogenic reagent and borate buffer solutions, and the standard and phosphate buffer solutions, respectively. The concentration of glucose in the standard solution used was 5.0×10^{-1} mM. The results are shown in *Fig.* 4.

The chemiluminescence intensity increased with pH up to about 7 and then decreased. This result is in good agreement with the results for enzyme activity reported by Sittampalam *et al.*(pH 7.3).[34] Yao(7.5).[35] and Guilbault *et al.*(6.9).[36] In the present work, pH 7.0 was used for the 1.0×10^{-1} M phosphate buffer solution in all subsequent studies.

Effect of pH for chemiluminogenic reagent on chemiluminescence intensity. The effect of pH in the range 9.6~12.7 for the 1.0×10^{-1} M borate buffer solution on the chemiluminescence intensity from 1.0×10^{-3} M luminol and 5.0×10^{-1} mM glucose was studied with the stopped flow injection system (see *Fig.* 1). The flow rate

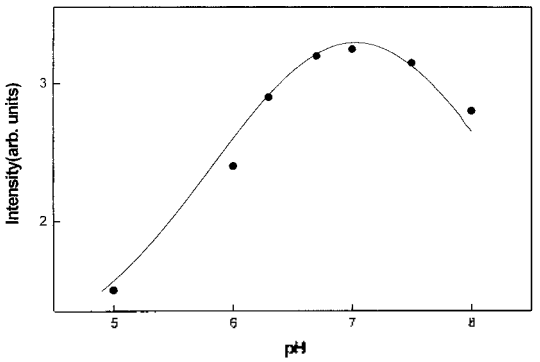

Fig. 4. Effect of pH for the glucose oxidase enzyme reactor on the chemiluminescence intensity: [luminol], $1.0 \cdot 10^{-3}$ M; [glucose], $5.0 \cdot 10^{-1}$ mM; λ_{em}, 424 nm; flow rate, 2.5 mL/min for P1 streams and 3.8 mL/min for P2 streams; volume injected, 2.0 mL for P1 streams and 2.5 mL for P2 streams.

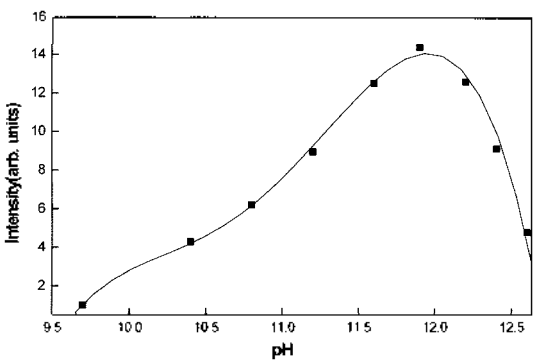

Fig. 5. Effect of pH for $1.0 \cdot 10^{-1}$ M borate buffer on the chemiluminescence intensity: [luminol], $1.0 \cdot 10^{-3}$ M; [glucose], $5.0 \cdot 10^{-1}$ mM; λ_{em}, 424 nm; flow rate, 2.5 mL/min for P1 streams and 3.8 mL/min for P2 streams; volume injected, 2.0 mL for P1 streams and 2.5 mL for P2 streams.

and injection volume selected for the P1 streams were 2.5 mL/min and 2.0 mL, respectively, and for the P2 streams 3.8 mL/min and 2.5 mL, respectively. The results obtained are shown in *Fig.* 5.

The optimum pH was about 12 above which the chemiluminescence signal decreased. Therefore, pH 12.0 was selected for the borate buffer solution.

Effect of flow injection variables. Flow rates of the reagents delivered to a flow cell is an essential factor for chemiluminescence measurements using a flow injection system because flow rates determine the contact time between reactants and sensing tip. Flow rate also control, to some extent, the diffusion of reactants from the flow

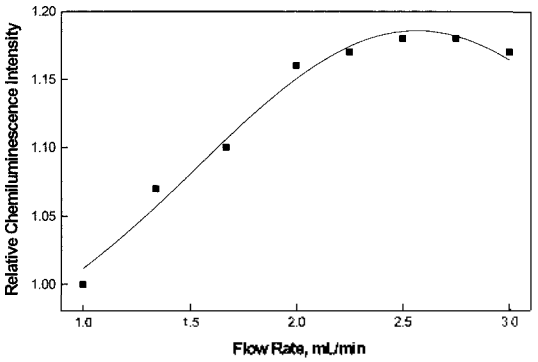

Fig. 6. Effect of flow rate for chemiluminogenic reagent solution on the chemiluminescence intensity to determine glucose using the proposed stopped flow injection system: [luminol], $1.0 \cdot 10^{-3}$ M; [glucose], $5.0 \cdot 10^{-1}$ mM; λ_{em}, 424 nm; flow rate, 3.8 mL/min for P2 streams; volume injected, 2.0 mL for P1 streams and 2.5 mL for P2 streams.

solution to the sensing tip. Therefore, the influence of the flow rate of the chemiluminogenic reagent solution on the chemiluminescence response was investigated in the 1.0–3.0 mL/min range. The results are shown in *Fig.* 6.

The lower flow rates resulted in higher contact time for the sensing tip of optical fiber but they were found to be unfavorable for the sensitivity because the chemiluminescence reaction was a very fast process. A flow rate of 2.5 mL/min was chosen in this work for optimum value to have a fast response as well as a high chemiluminescence intensity. The effect of the flow rate of the sample stream was also investigated by using similar method as that used for the chemiluminogenic reagent solution. In this case, a flow rate of 3.8 mL/min was selected for the optimum flow rate.

Effect of temperature. The performances of enzyme reactor are directly affected by temperature for most enzymatic reactions. The effect of temperature of enzyme reactor on the chemiluminescence intensity was studied in the 15–45 °C range. The results showed that the chemiluminescence response increased with temperature up to about 35 °C. The response decreased at temperature above 35 °C, probably due to the denaturation of glucose oxidase. Considering both the lifetime of the reactor and the practical convenience, 25 °C was selected as the operational temperature for the present method to determine glucose.

Calibration curve of glucose. The average of peak

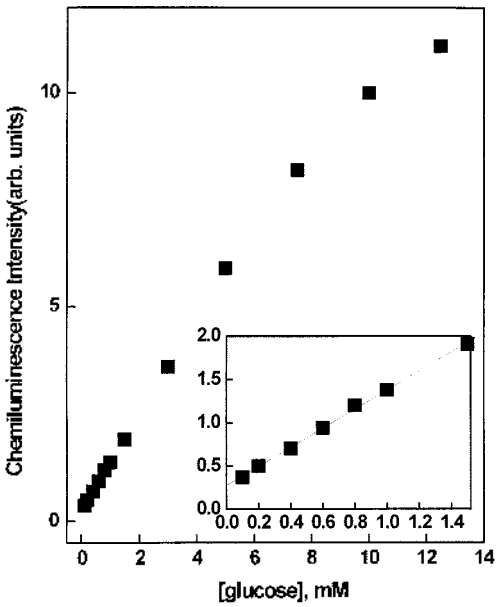

Fig. 7. Calibration curve of glucose obtained by the chemiluminescence method using the proposed flow injection system.

heights of three successive chemiluminescence signals obtained under the optimum experimental conditions for each glucose standard solution was used for calibration. One complete measurement took less than 5 minutes. *Fig.* 7 shows a typical calibration curve for different glucose concentrations up to 12 mM. A linear response to glucose concentration was established over the range of $1.0 \cdot 10^{-1}$ mM to 7.0 mM. The correlation coefficient in this range was 0.9987. The detection limit (3σ) was found to be $6.0 \cdot 10^{-2}$ mM.

The stability of the enzyme reactor used for the determination of glucose was investigated by performing 20 successive injections of $5.0 \cdot 10^{-1}$ mM glucose standard solution once in every three days for 2 months. The enzyme reactor was stored at 4 °C when it is not used. For the first 15 measurements, the responsibility of chemiluminescence intensity was acceptable with a variation coefficient of 5.8%. For the last 5 measurements, the chemiluminescence signal considerably decreased giving a variation coefficient of 11%.

Analysis of human whole blood. The present method was applied to the determination of glucose in human whole blood. Ten blood samples were analysed using the same experimental conditions as those used for the calibration curve, and the results were compared with the data obtained by the clinic using an official method. The results are presented in *Table* 1.

Table 1. Analytical results of glucose in human whole blood samples obtained by chemiluminescence method using the proposed stopped flow injection system[a]

Sample	Sex[b]	Proposed method[c]	Official method[d]
1	M	5.12±0.15	5.0
2	M	5.54±0.20	5.7
3	M	4.89±0.18	5.2
4	M	9.72±0.22	11.3
5	M	4.63±0.17	5.8
6	F	5.28±0.02	5.3
7	F	10.4±0.52	10.0
8	F	4.95±0.05	5.1
9	F	5.03±0.32	5.5
10	F	5.24±0.22	5.5

[a]Concentration unit is mM. Experimental conditions are the same as those in *Fig.* 1. [b]M, male; F, female. [c]Errors indicated are standard deviations of three measurements. [d]Spectrophotometric method (CIBA-CORNING Model 550 EXPRESS was used.). The data in mg·dl, received from the clinic was converted to mM.

Table 2. Recovery of glucose added to whole blood sample

Orignal, mM	Added, mM	Recovered, mM	Recovery, %
0.512	0.200	0.210	105
0.512	0.300	0.288	96
0.972	0.200	0.190	95
0.972	0.300	0.282	94

A satisfactory agreement between the two results was obtained with the exceptions of sample 4 and 5. The accuracy of the proposed stopped flow injection method for the determination of glucose was further examined by performing recovery experiments. To each of the two blood samples two different amounts of glucose were added, and the spiked samples were analysed. The results are shown in *Table* 2. The recovery range was found to be 94–105%. The results indicate that the present method can successfully determine the glucose content in human whole blood sample.

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