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# Simultaneous Assay of Glucose and Total Cholesterol in Blood Serum Using a Flow-Injection System with Immobilized Enzymes

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ABSTRACT

A flow-injection analytical method for simultaneous assay of glucose and total cholesterol in blood sera is reported. Glucose oxidase immobilized reactor and cholesterol esterase/cholesterol oxidase co-immobilized reactor were incorporated in parallel in a flow-injection system which was based on the splitting of the flow after the sample injection and subsequent confluence before reaching the peroxidase immobilized electrode. Because each channel has a different residence time, two peaks were obtained for total cholesterol and glucose. The peak-current of the first and second peaks was linearly related to total cholesterol in the range 2-220 mg/dl and to glucose in the range 4-400 mg/dl. Reliable results were obtained in the assays of total cholesterol and glucose in sera. The detection limits were 0.03 mg/dl for total cholesterol and 0.05 mg/dl for glucose when a 5-$\mu$l sample was injected. The assay speed was about 20 samples/h.

The assay of glucose and total cholesterol (free plus esters) in serum is an important test in clinical diagnoses. Enzymatic methods have been used for their assays in recent years [1-4], because their methods surpass chemical methods [5-7] with regard to specificity of assays.

The immobilization of the enzymes provides a preparation

that combines high selectivity with an increase in stability and that can be used repeatedly. Therefore, glucose oxidase [8-10] and cholesterol oxidase [11-13] immobilized on a variety of inert supports have been employed for the assay of glucose and cholesterol, respectively. In our earlier paper [13], a bioelectrochemical measurement system with immobilized cholesterol esterase and cholesterol oxidase was also proposed for the successive assay of free and total cholesterol.

In this work, we investigate the simultaneous assay of glucose and total cholesterol using a simple flow-injection analytical (FIA) system with immobilized enzymes.

## EXPERIMENTAL

### Reagents

Glucose oxidase (EC 1.1.3.4; 100 IU/mg, Type VII-S from Aspergillus niger) and bovine serum albumin were from Sigma. Cholesterol oxidase (EC 1.1.3.6; 3.4 IU/mg, from Nocardia erythropolis), cholesterol esterase (EC 3.1.1.13; 1 IU/mg, from microorganisms) and peroxidase (EC 1.11.1.7; 100 IU/mg, from horseradish) were from Boehringer-Mannheim. ORTHO control serum, cholesterol standard (200 mg/dl) and glutaraldehyde (20% solution) were from Wako Pure Chemicals. Cholesterol palmitate standard (300 mg/dl) was prepared as described previously [13]. All other chemicals were of analytical reagent grade and used without further purification.

### Preparation of immobilized enzyme reactors

The method was similar to that described previously [14]. The alkylamino-bonded silica (LiChrosorb $NH_2$ from Merck, particle size: 10 μm) was packed into two stainless steel columns (4mm i.d., 12mm long) and glutaraldehyde solution (5 v/v%) in 0.05M sodium hydrogen carbonate was circulated for activating the silica for 1.5h. After washing with 0.05M

phosphate buffer at pH 6.0, cholesterol oxidase and cholesterol esterase were co-loaded onto a column by circulating the mixed enzyme-solution (0.1M phosphate buffer at pH 6.0) composed of 5.0 U cholesterol oxidase and 4.5 U cholesterol esterase for 2h at room temperature. Glucose oxidase (200 U) was also loaded onto another column according to the similar procedure. The excess enzymes and aldehyde residue remaining onto the silica were removed by washing with the glycine buffer (0.1M, pH 8.0) for 3h. The reactors were stored at ca. 5 °C in the 0.1M phosphate buffer (pH 7.5) when not in use.

Preparation of peroxidase electrode

Peroxidase/bovine serum albumin membrane crosslinked by glutaraldehyde was bound to one side of a gold sheet (1x2cm) as reported earlier [13]. The amperometric flow-through peroxidase electrode was assembled with the enzyme modified gold sheet over the stainless steel-screw of a thin-layer electrochemical flow cell [15], which had a silver/silver chloride reference electrode and a stainless tube as an auxiliary electrode. The cell volume was 1.9 µl.

FIA system and procedures

The flow-injection system is similar to that reported previously [16] and is shown schematically in Fig. 1. The two immobilized enzyme reactors were positioned as shown. The configuration of the flow system was based on the splitting of the flow after the sample injection and subsequent confluence before reaching the peroxidase electrode. A constant potential (-50mV vs. Ag/AgCl) was applied to the peroxidase electrode with a Yanagimoto potentiostat (VMD-101), and the current was measured with a strip-chart recorder (Hitachi 056). A phosphate buffer (0.1M, pH 7.5), which was 2.0 %(v/v) in Triton X-100 and 1.0 mM in potassium hexacyanoferrate(II), served as the carrier solution and was

pumped at 3.0 ml/min with a Yanagimoto L-5000 high-pressure pump. Sample solutions (5μl) were injected with a micro-syringe. The calibration graphs for total cholesterol and glucose were prepared from the peak-currents of the separated two peaks obtained with standard solutions; the first peak corresponded to total cholesterol content and second to glucose in the sample.

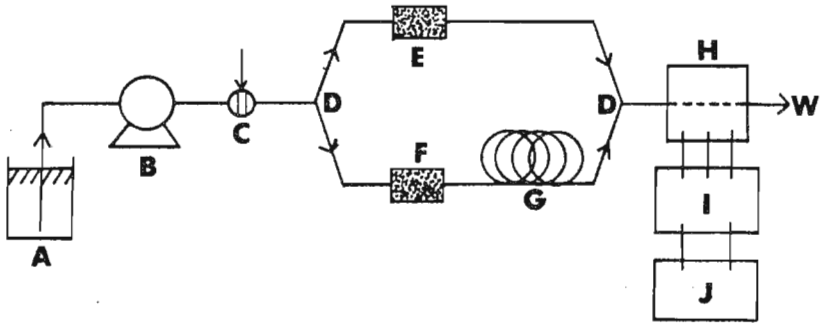


Fig. 1. FIA manifold for the simultaneous assay of total cholesterol and glucose. (A) Carrier reservoir [carrier solution: 0.1M phosphate buffer, pH 7.5, 2.0%(v/v) in Triton X-100 and 1.0mM in potassium hexacyanoferrate(II)]; (B) pump; (C) sample injector; (D) T-connector; (E) cholesterol oxidase /cholesterol esterase co-immobilized reactor (4mm i.d., 12mm long); (F) glucose oxidase immobilized reactor (4mm i.d., 12mm long); (G) delay coil (0.5mm i.d., 6m long); (H) peroxidase electrode; (I) potentiostat; (J) recorder; (W) waste. Arrows show the direction of flow.

## RESULTS AND DISCUSSION

An injected sample-zone is splitted to two sub-zones at the T-connector after the sample injection point, passing the two portions through two channels with different dimensions, and subsequent confluence of the streams before reaching the peroxidase electrode. Splitted ratio of sample-zone at the T-connector was 1:1.42. The cholesterol oxidase/

cholesterol esterase co-immobilized reactor catalyses specifically the successive enzymatic reactions shown in Eqns. (1) and (2). The glucose oxidase immobilized reactor catalyses the reaction shown in Eqn. (3). The hydrogen peroxide generated in the two enzyme reactors was amperometrically detected with the peroxidase electrode in the presence of hexacyanoferrate(II) as the mediator [Eqns. (4) and (5)].

$$\text{cholesterol esters} + H_2O \xrightarrow{\text{cholesterol esterase}} \text{cholesterol} + \text{acids} \quad (1)$$

$$\text{cholesterol} + O_2 + H_2O \xrightarrow{\text{cholesterol oxidase}} \text{cholest-4-en-3-one} + H_2O_2 \quad (2)$$

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{D-gluconic acid} + H_2O_2 \quad (3)$$

$$H_2O_2 + 2\ Fe(CN)_6^{4-} + 2\ H^+ \xrightarrow{\text{peroxidase}} 2\ Fe(CN)_6^{3-} + 2\ H_2O \quad (4)$$

$$Fe(CN)_6^{3-} + e \longrightarrow Fe(CN)_6^{4-} \quad (5)$$

Because each channel has a different residence time, two peaks, for total cholesterol and glucose, are obtained (Fig. 2). The separation of these peaks depends on the relative lengths of the channels; a delay coil of 0.5mm i.d. and 6.0m in length was needed to separate the two peaks completely.

Some experiments were conducted to establish the optimum pH and optimum concentration of Triton X-100 and potassium hexacyanoferrate(II) for the simultaneous assay of total cholesterol and glucose under flow conditions. The phosphate buffer (0.1M, pH 7.5), 2.0 %(v/v) in Triton X-100 and 1mM in potassium hexacyanoferrate(II), was consequently selected as the carrier solution.

Figure 3 shows the effect of the carrier flow rate on the peak-current of the first and second peaks obtained by the use of the FIA system shown in Fig. 1. The peak-current of the first peak obtained by the injections of the mixed solu-

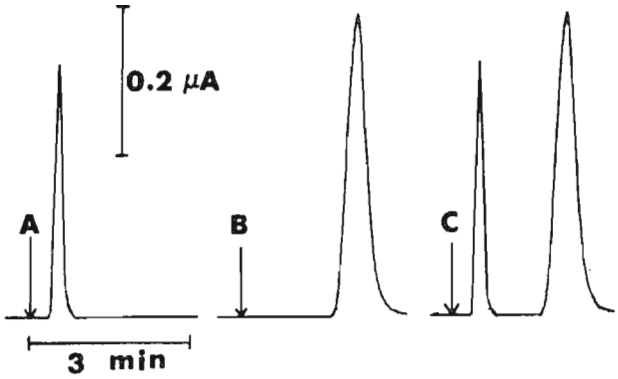


Fig. 2. FIA signals from solutions containing: (A) 100mg/dl cholesterol palmitate; (B) 100mg/dl glucose; (C) 100mg/dl cholesterol palmitate and 100mg/dl glucose.

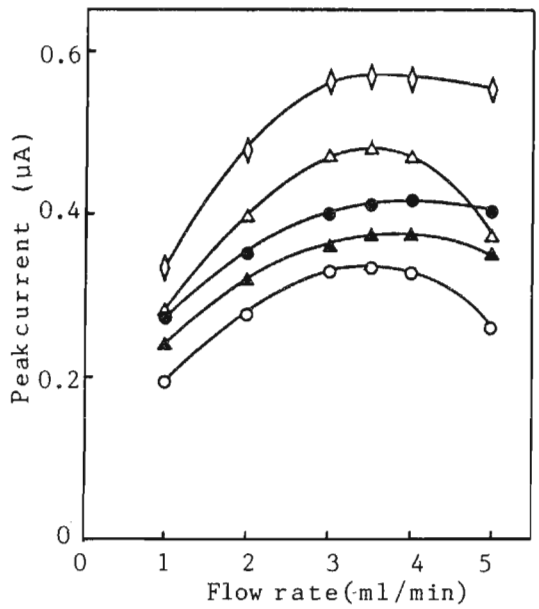


Fig. 3. Effect of flow rate on the peak current. The first peak (○) and second peak (●) were obtained for the injection of a 5-μl aliquot of solution containing 100mg/dl cholesterol palmitate and 100mg/dl glucose. Similarly the first peak (△) and second peak (▲) were obtained for control serum (5μl). Only the first peak (◇) was obtained for 100mg/dl cholesterol (5μl). Conditions are described in the text.

tion of cholesterol palmitate and glucose decreased at higher flow rates because of incomplete hydrolysis of cholesterol ester to cholesterol. However, the ratio of the peak-current for cholesterol and cholesterol palmitate remained constant at flow rates below 3.5 ml/min. This means that the enzymatic conversion of cholesterol ester to cholesterol is complete in such flow rates. Consequently, the carrier flow rate was adjusted to 3.0 ml/min in all further experiments. Assay speed was 20 samples/h at this flow rate.

Table 1 Coefficient of variations of peak height for the first and second peaks

| Sample type | | First peak[a] | Second peak[b] |
|---|---|---|---|
| cholesterol palmitate (mg/dl) | glucose (mg/dl) | | |
| 100 | 50 | 1.2% | 1.5% |
| 100 | 100 | 1.3% | 1.3% |
| 50 | 100 | 1.8% | 1.2% |
| 100 | 300 | 1.2% | 1.2% |
| Control serum[c] | | 1.3% | 1.6% |

Note. Ten measurements on each solution

[a] This peak corresponds to cholesterol palmitate or total cholesterol.

[b] This peak corresponds to glucose.

[c] Manufacture's data: 149±9 mg/dl for total cholesterol and 89±5 mg/dl for glucose.

The calibration graphs for total cholesterol and glucose can be prepared from the first and second peaks obtained by

injecting the mixed solutions of cholesterol palmitate and glucose. A linear relationship was obtained over the range 2-220 mg/dl for total cholesterol and 4-400 mg/dl for glucose, for 5 μl injections; the slope, y-intercept, and linear correlation coefficients, were 3.2 $nA(mg/dl)^{-1}$, 8.2nA, and 0.9999 for total cholesterol; and 4.1 $nA(mg/dl)^{-1}$, 5.8nA, and 0.9997 for glucose. The lowest concentration which could be detected by the present simultaneous assay-system was 0.03 mg/dl for total cholesterol and 0.05 mg/dl for glucose (signal-to-noise ratio = 3).

The reproducibility of the assays was also tested by measurements of the assays on repeated 5-μl injections (n=10) of the mixed solutions of cholesterol palmitate and glucose and a control serum. The coefficient of variation was less than 1.8 % for all samples (Table 1). Total cholesterol and glucose in human sera were assayed by both the chemical methods [6,7] and the present simultaneous assay-system. The correlation coefficient between the methods were found to be 0.968 for total cholesterol and 0.972 for glucose, for 20 pairs of results.

The two immobilized enzyme reactors and the peroxide electrode were reasonably good in their stabilities; even after repetitive use for two months, they retained over 85% of their original activities.

In conclusion, the combination of simultaneous FIA system and immobilized enzymes in the forms of reactor and electrode afforded the rapid, sensitive, selective and simultaneous assay of total cholesterol and glucose in sera.

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