Simultaneous Determination of Three Species by Flow Injection Analytical Method Using Enzyme-Immobilized Open-Tubular Reactors

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A simultaneous determination of three species by a flow injection analytical (FIA) method using enzyme-immobilized open-tubular reactors in parallel is described. The design of the optimal geometry of the FIA system is especially important to avoid overlap of any pairs of peaks. Ours was carried out based on the Taylor-Aris equation. Despite a somewhat complex splitting-confluence flow system, reproducible results were obtained in the simultaneous determination of L-lactate, β -D-glucose and glycerol by the detection of the resulting common product, NADH. Assignment of the enzyme reactors to the channels should be made by taking into account the concentration ranges of the analytes to be measured and the differences in the rates of individual enzyme reactions.

Keywords Flow injection analysis, enzyme-immobilized open-tubular reactor, simultaneous determination, L-lactate, β -D-glucose

One of favorable features of the flow injection analytical (FIA) method is that the flow configuration can be constructed freely by analysts to suit their purposes. The authors have reported a splitting-confluence system in urate-determination by FIA method¹ using a uricase-immobilized open-tubular reactor. We have obtained satisfactorily reproducible results despite a somewhat complex flow system. Simultaneous determination should be feasible with an FIA system equipped with multiple enzyme-immobilized reactors in parallel and a single detector by only a single injection. Some methods for the simultaneous determination have been proposed including serial or parallel configuration with several detectors², multi-injection³ and parallel configuration based on differential kinetics⁴; these have been reviewed by Luque de Castro *et al.*⁵

It is a remarkable feature of enzymatic reactions that analytically useful reactions can be classified into a limited number of types, that is, the same common products are formed in many enzymatic reactions. This suggests that enzymatic reactions are favorable for the simultaneous determinations. For example, the reduction of nicotinamide adenine dinucleotide (NAD) takes place together with oxidation of substrates in dehydrogenase-catalyzed reactions. Many substrates can be determined by monitoring the resulting hydrogenated NAD (NADH) by use of a single UV or fluorimetric detector. Recently, Masoom *et al.*⁶ described the simultaneous determination of glucose

and sucrose by an FIA using immobilized invertase/mutarotase and glucose oxidase. But no papers have described the simultaneous determination of more than two species in such a system.

In this paper, the simultaneous determination of three species by an FIA method with enzyme-immobilized open-tubular reactors will be described and, in particular, the design of the flow system will be discussed.

Experimental

The following enzymes were chosen by attaching importance to a preliminary investigation of the FIA

Fig. 1 Schematic diagram of flow injection analytical system. a, carrier solution; b, pump; c, pulse damper; d, injector; e1-e3: enzyme-immobilized reactors; f, fluorimetric detector; g, waste; h, recorder.

system for simultaneous determination rather than practical applications: lactate dehydrogenase (LDH, E.C.1.1.1.28, Toyobo), glycerol dehydrogenase (GDH, E.C.1.1.1.6, Toyobo), glucose dehydrogenase (GluDH, E.C.1.1.1.47, Sigma). L-lactate, glycerol and β -D-glucose can be determined by using these enzymes. Triglycerides may be determined by coupling a lipase-immobilized reactor to a GDH reactor. A schematic diagram of the FIA system is shown in Fig. 1. The carrier solution was fed by a single-head reciprocating pump (Milton-Roy, Type NSI-33R). The carrier solution consisted of 0.033 M phosphate buffer (pH 8.0) containing 2 mM NAD, 1 mM EDTA and 0.1 mM dithiothreitol (DTT). NAD, EDTA and DTT were purchased from Oriental Yeast Co., Dohjin Kagaku Kenkyujo and Wako Pure Chemical Co., respectively. Sample solutions were injected through a loop injector (Japan Spectroscopic Co., Model VL611, volume of the sample loop: 22 to 50 μ l). The enzyme-immobilized reactors were prepared according to the procedure described in the previous papers^{7,8}; in brief, after the amino group was introduced by the reaction of 3-aminopropyltriethoxysilane and silanol group within the whisker-walled capillary tube, an enzyme was immobilized by cross-linking with glutaraldehyde. The inside diameter and the coil diameter of the reactors were 0.022 to 0.037 cm and 11 cm, respectively. The reactor length was determined from calculations shown in the next section. T-shape mixing joints (internal vol.: 1 μ l) and Teflon tubes (0.025 cm i.d., less than 10 cm/a tube) were used to connect the reactors with the injector and the detector. The reactors were thermostated at 34°C during the measurement. The resulting NADH was monitored by a fluorimetric detector (Schoeffel, Model FS-970, ex. 340 nm, em. 470 nm, cell volume 10 μ l).

Results and Discussion

When a sample solution is injected in the FIA system depicted in Fig. 1, a train composed of three peaks corresponding to individual substrates appears in a FIA-gram. The design of the optimal geometry of the FIA system might be especially important to avoid overlap of any pairs of the three peaks. The control of time interval between peaks by insertion of delay tubes should be avoided because residence in these tubes does not contribute to the progress of reaction but only to band broadening. Broadening of the band due to the substrate and the product can be represented by the Taylor-Aris equation because the time ascribed to the capture of substrates or products by enzymes is very short: the ratios of the times when they are retained by enzymes to the times when they are in the carrier solution were observed to be 0.07, 0.04 and 0.03 for LDH, GDH and GluDH, respectively. These values were estimated from the observed residence times within the reactor and the ratios of the reactor length

to the velocity of the carrier solution. Additional consideration of secondary flow within a coiled tube leads to the following equations^{1,8,9}:

$$\sigma_{T,i}^2 = \sigma_{r,i}^2 + \sigma_{ex}^2 \quad (i=1 \text{ to } 3) \quad (1)$$

$$\sigma_{ex}^2 = \frac{V_s^2}{12F^2} + \frac{V_d^2}{F^2} \quad (2)$$

$$\sigma_{r,i}^2 = \frac{\kappa_i d_i^2 t_i}{96D} \quad (i=1 \text{ to } 3) \quad (3)$$

where $\sigma_{T,i}^2$ is the total variance of the band passing through the i -th channel, $\sigma_{r,i}^2$ and σ_{ex}^2 are the variance in the i -th reactor and the extra-reactor variance, respectively, V_s and V_d are volumes of injected sample and the detection cell, respectively (V_d also includes the volume of the connecting tubes and the mixing joints), F is the flow rate of the carrier solution, d_i is the inside tube diameter of the i -th reactor, t_i is the residence time of the sample sub-bolus within the i -th reactor and D is the diffusion coefficient of the substrate and the product in the carrier solution. κ is a correction factor due to the secondary flow in a coiled tube and is dependent on the Schmidt and the Dean numbers. Since resolution between each pair of peaks must be 1.5 to avoid peak-overlapping, the following equations are obtained:

$$\frac{t_{i+1} - t_i}{2(\sigma_{T,i} + \sigma_{T,i+1})} = 1.5 \quad (i=1 \text{ or } 2) \quad (4)$$

$$\frac{\Delta t + t_1 - t_3}{2(\sigma_{T,1} + \sigma_{T,3})} = 1.5 \quad (5)$$

$$t_1 < t_2 < t_3 \quad (6)$$

$$S = \frac{3600}{\Delta t} \quad (7)$$

where S is the number of sample injections per hour and Δt is the time interval between two successive injections. Equation 5 represents the resolution between the third peak of the first injection and the first peak of the second injection. The pressure drop for laminar flow through a straight tube is represented by the Poiseuille equation. The secondary flow in a coiled tube causes an increase in the pressure drop but this increase can be neglected for the Dean number < 25 ;

$$\Delta P = \frac{32\eta L_i u_i}{d_i^2} \quad (i=1 \text{ to } 3) \quad (8)$$

where ΔP is the pressure drop across the reactor, u_i is the linear velocity of the carrier passing through the i -th reactor of the length L_i and η is the viscosity of the carrier. Furthermore, the following equations hold:

$$F = \sum_{i=1}^3 F_i \quad (9)$$

$$F_i = \frac{\pi}{4} d_i^2 u_i \quad (i=1 \text{ to } 3) \quad (10)$$

$$t_i = L_i / u_i \quad (i=1 \text{ to } 3) \quad (11)$$

Fig. 2 Reactor lengths (L_1 – L_3) and total flow rate (F) required to achieve a given pair of S and t_1 values and resulting peak-height ratios ($H_{1/2}$ and $H_{1/3}$). $d_1=d_2=d_3=0.03$ cm, $V_s=V_d=0.05$ ml, $\eta=0.01$ P, $D=1\times 10^{-5}$ cm² s⁻¹.

Fig. 3 Repeatability of measurement by a two-channel FIA system. Sample: 1, 0.51 mM L-lactate; 2, 2.01 mM β-D-glucose. Flow rate, 0.73 ml min⁻¹.

where F_i is the flow rate of the carrier passing through the i -th reactor. For a given combination of S , t_1 , d_1 , d_2 and d_3 , the optimum set of L_1 , L_2 and L_3 values is obtained from these equations. Figure 2 shows plots of L_i , the total flow rate (F) and the peak-height ratios ($H_{1/2}$, $H_{1/3}$) against t_1 , which are calculated based on supposition that $D=1\times 10^{-5}$ cm² s⁻¹, $\eta=0.01$ P, $V_s=0.05$ ml and $V_d=0.05$ ml. The peak-height ratios are calculated by assuming the same yield for the three reactions. These plots could be obtained easily in a short time by using a microcomputer. For example, when S and t_1 are required to be 20 h⁻¹ and 40 s, respectively, for the reactors with the same inside diameter ($d_1=d_2=d_3=0.03$ cm), L_1 , L_2 and L_3 are required to be 247, 374 and 496 cm, respectively. Taking into consideration the asymmetry in the observed peak shapes and the uncertainty in supposed

Fig. 4 Simultaneous measurement of three species. Sample: 1, L-lactate; 2, β-D-glucose; 3, glycerol. Flow rate, 0.54 ml min⁻¹.

Fig. 5 Plots of relative peak height against the reaction time (t). The reactor lengths are kept constant. A: GluDH-immobilized reactor (0.031 cm i.d.×227 cm), 1.3 mM β-D-glucose (22 μl) injected. B: GDH-immobilized reactor (0.031 cm i.d.×224 cm), 3.5 mM glycerol (22 μl) injected. C: LDH-immobilized reactor (0.031 cm i.d.×217 cm), 0.45 mM L-lactate (22 μl) injected.

values, somewhat shorter L_1 and somewhat longer L_3 than the corresponding calculated values should be used in a practical FIA system.

First, the repeatability in the two-channel FIA system was evaluated. LDH and GluDH immobilized reactors (290 and 520 cm, respectively) were inserted in both channels. Figure 3 shows a FIA-gram obtained by 20 successive injections of the sample (50 μl) containing 0.51 mM L-lactate and 2.01 mM β-D-glucose. The relative standard deviations of the peak heights are 0.52 and 1.21% for L-lactate and β-D-glucose, respectively. These results suggest that the variation ascribable to the splitting of flow is extremely small.

A three-channel FIA system was constructed by

Fig. 6 Plot of peak height for L-lactate against reaction time (t). The flow rate of carrier solution is kept constant.

Fig. 7 Effect of varying split ratios among the three channels. (a): 1, LDH (0.022 cm i.d.×190 cm); 2, GluDH (0.029 cm i.d.×367 cm); 3, GDH (0.037 cm i.d.×740 cm). Flow rate, 0.54 ml min⁻¹. (b): 1, GDH (0.037 cm i.d.×200 cm); 2, GluDH (0.029 cm i.d.×275 cm), 3, LDH (0.022 cm i.d.×336 cm). Flow rate, 0.48 ml min⁻¹.

inserting a GDH immobilized reactor in the third channel. The reactor lengths were 220, 360 and 540 cm for LDH, GluDH and GDH reactors, respectively. Figure 4 shows a FIA-gram measured in this system. Sample solution (50 μ l) were injected at about three-minute intervals. The mean values of relative standard deviations were 0.60, 1.17 and 2.45% for L-lactate, β -D-glucose and glycerol, respectively, in the concentration ranges seen in Fig. 4. These satisfactory results suggest that a splitting and confluence FIA system using enzyme-immobilized reactors in parallel can be useful for the simultaneous determination of more than two species.

Peak heights can be varied by changing the enzyme reactors assigned to the channels. So assignment of the reactors to the channels should be carried out by taking into account the concentration ranges of the substrates to be measured and differences in the rates of individual enzyme reactions. An extremely great difference in the peak height of the adjacent peaks could result in an extensive error in the measurement of the smaller peak. Figure 5 shows the plots of the relative peak height against the reaction time. They were obtained with a single-channel FIA system. The reaction time was varied by changing the flow rate while keeping the reactor length constant. The extent of dispersion of the band within the reactor varies with the flow rate; the relative peak height indicated as ordinate is normalized by dividing the measured peak height by the standard deviation of the band in volume units. While the heights of the peaks corresponding to β -D-glucose and glycerol increase almost linearly with the reaction time, that of L-lactate rises sharply and reaches a plateau after a short reaction time. Figure 6 shows the plots of the peak height for L-lactate against the reaction time, which was obtained by changing the

Table 1 Reaction times (t_2 , t_3), reactor lengths (L_1 – L_3), pressure drop across the reactor (ΔP) and peak-height ratios ($H_{1/2}$, $H_{1/3}$) to achieve given values of sampling rate (S) and reaction time (t_1)

	Case(i)	Case(ii)	Case(iii)
d_1 /cm	0.030	0.022	0.037
d_2 /cm	0.030	0.029	0.029
d_3 /cm	0.030	0.037	0.022
t_2 /s	91	91	97
t_3 /s	160	159	163
L_1 /cm	247	195	234
L_2 /cm	374	389	281
L_3 /cm	496	668	278
ΔP /atm	0.54	0.63	0.30
$H_{1/2}$	2.01	0.86	4.38
$H_{1/3}$	3.46	0.75	13.1

$S=20$ h⁻¹, $t_1=40$ s.

$V_s=0.05$ ml, $V_d=0.05$ ml, $\eta=0.01$ P, $D=1\times 10^{-5}$ cm² s⁻¹.

reactor length while keeping the flow rate constant. The peak height approaches the maximum rapidly, and decreases at an excessively long reaction time because the increase in dispersion of band within the reactor becomes greater than the increase in the product yield. The results seen in Figs. 5 and 6 lead to a recommendation that LDH should be assigned to the first channel, while GDH or GluDH to the second or the third.

Peak heights can be varied by changing the inside tube diameters of three reactors due to changes in the splitting ratios of the carrier solution. Now let us consider an attempt to increase the peak height corresponding to glycerol, which is rather small compared to the other peaks in Fig. 4. Table 1 shows the peak-height ratio together with the reaction times and the reactor length, which are calculated for different combinations of inside tube diameters of reactors, assuming that the other conditions are the same in every case. Case (i) approximates the conditions under which the FIA-gram in Fig. 4 was measured. In case (ii), the enzymes are assigned to the same channels as in case (i), but the inside diameter of the LDH reactor becomes narrower and that of the GDH reactor wider. Thus the peak-height ratio $H_{1/3}$ becomes lower. On the other hand, in case (iii), the channels for the LDH and GDH reactors are exchanged and the peak-height ratio $H_{1/3}$ rises greatly. In both cases it is expected that the peak height of glycerol relative to that of L-lactate should become higher than in case (i). Figures 7(a) and 7(b) show the FIA-grams obtained under the conditions corresponding to cases (ii) and (iii), respectively. The split ratios

among the channels were evaluated by injecting a NADH-containing solution. Figure 7(a) shows the satisfactory results, as expected, but Fig. 7(b) shows poor results because the reaction time in the GDH reactor is too short. The results seen in Figs. 5 to 7 demonstrate the importance of the assignment of the reactors to the channels.

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