Note

Improved high-performance liquid chromatographic method with fluorimetric detection for the determination of glycerol using an immobilized enzyme column reactor

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On-line post-column derivatization techniques have greatly extended the usefulness of high-performance liquid chromatography (HPLC). Post-column reactors are employed to improve the analytical sensitivity and/or detection selectivity. One of the disadvantages of the post-column techniques is band broadening due to post-column addition of reagents. To overcome this disadvantage, attempts have been made to develop pumpless reaction units[1].

We have previously reported an HPLC method for the determination of glycerol using a post-column reactor containing immobilized glycerol dehydrogenase (E.C. 1.1.1.6)[2]. Polyhydric alcohols were separated on a reversed-phase ODS column, followed by an enzymatic reaction of glycerol with nicotinamide adenine dinucleotide (NAD) and the resulting reduced form of NAD (NADH) was monitored with a fluorescence detector. Three pumps were used in the system, that is, a mobile phase pump, a buffer solution pump and an NAD solution pump. This paper describes a one-pump system for the determination of glycerol using immobilized glycerol dehydrogenase (GDH). Aminobutylpolystyrene beads were used as a support for covalent attachment of GDH.

GDH has been immobilized on the inner surface of nylon tubing and used as a reactor in a continuous-flow system[3,4]. However, an open-tubular reactor is unsuitable as a post-column reactor[5]. In this work the separation of polyhydric alcohols is effected by using a cation exchanger with carbonate buffer solution (pH 10.0) as the mobile phase. The analytical sensitivity and resolution are improved by changing the three- to the one-pump system. The method was applied to the determination of triglycerides in serum.

EXPERIMENTAL

Chemicals

Polystyrene beads (Bio-Beads SX-8, $55 \pm 20 \mu$m) were purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.), NAD (grade II) from Boehringer (Mannheim, F.R.G.), GDH (66.6 U/mg of solid, from *Cellulomonas* sp.) from Toyobo (Osaka, Japan) and lipoprotein lipase (1070 U/mg of solid, from *Pseudomonas* sp.) from Amano Pharmaceutrical (Nagoya, Japan). Anhydrous aluminium chloride, 1,4-

butanediamine and 1,1′, 2,2′-tetrachloroethane were used without further purification. Chloromethyl methyl ether, purchased from Wako (Osaka, Japan), was purified by distillation.

Preparation of the immobilized enzyme column reactor

Polystyrene beads (10 g) were allowed to swell in a mixture of 70 ml of chloromethyl methyl ether and 10 ml of 1,1′, 2,2′-tetrachloroethane for 6 h. Anhydrous aluminium chloride (15 g) was added and the mixture was stirred at 0°C for 1 h. After filtration, the beads were washed successively with tetrachloroethane, methanol, water and methanol and dried at 40°C under vacuum. The chloromethylated beads (10 g) were left to swell in 10 ml of benzene and then 20 ml of 1,4-butanediamine were added. The mixture was refluxed for 10 h.

The beads were removed by filtration and washed successively with benzene, methanol and water. The attached amine was measured by the Kjeldahl method[6] and amounted to 0.81 mequiv. per gram of dry beads.

The butylaminated beads (4 g) were slurried with 50 ml of methanol–water (1:1) and poured into a packing column, which was fitted with a stainless-steel column (150 mm × 4.0 mm I.D.). The methanol–water mixture was pumped through the column for 30 min, maintaining a pressure of 50 kg/cm^2 at room temperature. The stainless-steel column was disconnected from the packing column and connected to the HPLC pump. Glutaraldehyde (2.5%) in phosphate bufer (0.01 M, pH 7.0) was pumped through the column for 2 h at a flow-rate of 0.5 ml/min at room temperature, followed by deaerated water for 3 h at a flow-rate of 0.5 ml/min. Enzyme solution (10 ml) containing 10 mg of GDH in phosphate buffer (0.05 M, pH 7.0) was circulated through the column for 6 h at a flow-rate of 0.5 ml/min at room temperature. The enzyme solution was kept at 0–4°C in an ice-box throughout the immobilization procedure.

Apparatus

A schematic diagram of the system is shown in Fig. 1. The mobile phase was a mixture of carbonate buffer (sodium hydrogencarbonate–potassium carbonate, 0.4 M, pH 10.0) and NAD solution [10 mM in the phosphate buffer (0.05 M, pH 7.0)], both at flow-rates of 0.35 ml/min. The buffer and NAD solution were pumped by a double-plunger pump (Model KHU-W-52; Kyowa Seimitsu) through an injector (Model KHP-UI-130A; Kyowa Seimitsu) with a 100 µl loop, to a column (300 mm
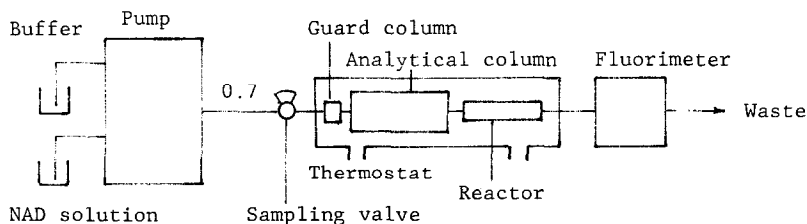

Fig. 1. Schematic diagram of the system. The number on the line is the flow-rate in ml/min. Buffer: sodium hydrogencarbonate–potassium carbonate (0.4 M, pH 10.0). NAD solution, 10 mM in phosphate buffer (0.05 M, pH 7.0). See text for details.

× 7.8 mm I.D.) of TSK gel SCX (5 μm) fitted with a guard column of Shodex Ionpak KS800P (50 mm × 6 mm I.D.). The solution then passed into the stainless-steel column reactor (150 mm × 4.0 mm I.D.). Both the analytical column and the reactor were thermostated at 40 ± 0.2°C. A Hitachi Model 650-10s fluorescence spectrophotometer fitted with a flow cell (18 μl) was operated at λ_{ex} = 340 nm and λ_{em} = 465 nm and the response was monitored on a Hitachi Model 056 recorder.

RESULTS AND DISCUSSION

Choice of support

For an immobilized enzyme column reactor, as the flow-rate is lowered the enzymatic reaction proceeds to completion. However, in general, in the column the peaks become broader at lower flow-rates because the elution times are longer and there is more time for diffusion to spread the peaks. Therefore, one should select a support the grain size of which is as small as possible in order to obtain a higher activity of immobilized enzyme and to minimize excessive peak broadening.

Another phenomenon that affects the shape of the peak in the column reactor is the adsorption of the reaction product on the surface of the support. As small beads have a large surface area, their adsorption capacity is high. TSK gel styrene-250 (grain size 10 μm) was examined as a support for covalent attachment of the enzyme. The column reactor (100 mm × 4.0 mm I.D.) which was packed with the beads gave pronounced peak tailing because of the adsorption of NADH, while the yield of the enzymatic reaction was about 100%.

The degree of cross-linking is also important. At low degrees of cross-linking the beads were crushed in the column reactor at high flow-rates and a gap was produced at the top of the reactor. On the other hand, the amounts of attached amino groups for beads with 2% cross-linking was 4.0 mequiv./g and with 12% cross-linking 0.3 mequiv./g. Bio-Beads (grain size 55 ± 20 μm, degree of cross-linking 8%) were chosen as a compromise. During the use of the column reactor which was packed with the beads, peak broadening was not observed even after 600 samples had been injected.

Evaluation and optimization of the enzyme column reactor

Effect of temperature. The reactor was placed in a water-bath and the temperature was varied between 30 and 50°C to determine the effect of temperature on GDH activity. A standard solution of glycerol (0.1 mM) was injected at each temperature. The reactor showed an increase in activity as the temperature was increased (Fig. 2). Although the reactor exhibited the highest activity at 50°C, this temperature reduced the lifetime of the reactor. For routine analysis, the reactor was operated at 40°C.

Effect of pH. The effect of pH on the activity of the immobilized enzyme was examined in the pH range 9.5–11.0. The results indicated a constant activity above pH 10.0 (Fig. 3). pH 10.0 was chosen for the mobile phase buffer. The concentration of carbonate buffer did not influence the peak height in the range 0.01–0.3 M.

Effect of concentration of NAD. Under the conditions used the Michaelis constant of the immobilized enzyme for NAD was 2 · 10^{-4} M. With an excess of NAD (above 2 · 10^{-3} M), the rate of the enzymatic reaction was independent of the concentration of NAD. We used an NAD concentration of 5 mM to shift the
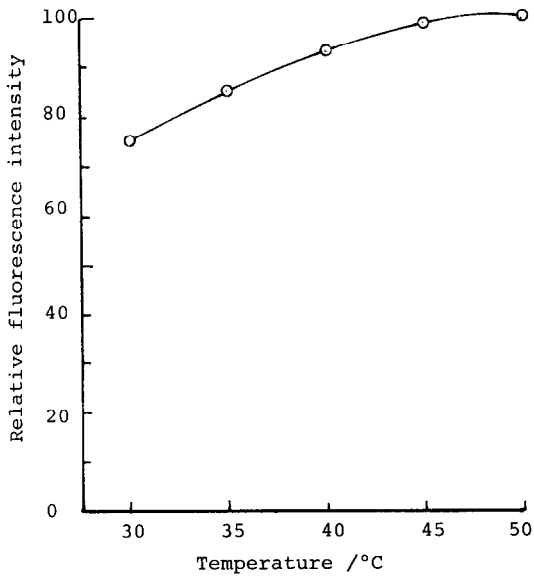
Fig. 2. Effect of temperature on the activity of immobilized glycerol dehydrogenase.

equilibrium of the reaction to the right. A higher concentration of NAD was undesirable for the chromatographic separation of polyhydric alcohols.

Size of reactor. In post-column HPLC systems using immobilized enzymes in a post-column reactor, it is difficult to optimize the size of the reactor. Generally, the selection of the reactor involves a compromise between obtaining an adequate
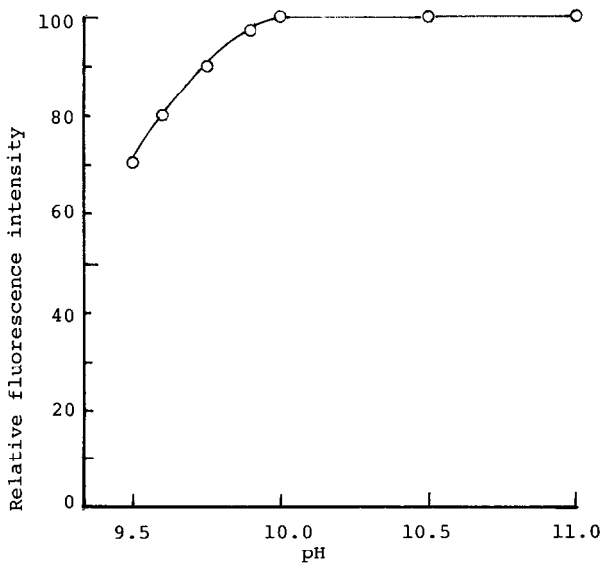

Fig. 3. Effect of pH on the activity of immobilized glycerol dehydrogenase.

sensitivity and avoiding peak broadening. As pointed out by Shih and Carr[7], a fast reaction in the reactor gives the negligible peak broadening. As the rate of enzymatic reactions is directly proportional to the activity of the enzyme, a decrease in activity causes an increase in peak width. To obtain immobilized GDH with higher activity levels and to use it for long periods in practical analyses, we selected a column of length 15 cm and I.D. 4 mm. By using this column reactor, peak broadening was controlled by the chromatographic column, and the contribution of the reactor to the peak broadening was about 10% of the peak width. The reactor could be used for 2 months without pronounced peak broadening. A column of length 5 cm could be used for about only 2 weeks in a similar manner to the above; this reactor was not suitable for practical use because of its short lifetime.

The specificity and storage stability of immobilized GDH were similar to those reported previously[2].

Separation

GDH oxidizes not only glycerol but also ethylene glycol, propylene glycol and 1,2-butanediol[2]. In certain instances, samples contain compounds that fluoresce at the monitored wavelength. Alcohols and other such compounds must be separated from glycerol. Ion exchangers were screened for their ability to separate polyhydric alcohols at higher pH (about 10). A good separation was achieved with the anion exchanger TSK gel SAX ($5 \mu m$) (150 mm × 6 mm I.D. column) with borate buffer (0.1 M, pH 9.5) as the mobile phase. The retention times of ethylene glycol, glycerol, propylene glycol and 1,2-butanediol were 7.1, 8.5, 12.0 and 14.3 min, respctively. However, the borate complexes of the alcohols were too stable to act as substrates of the enzyme.
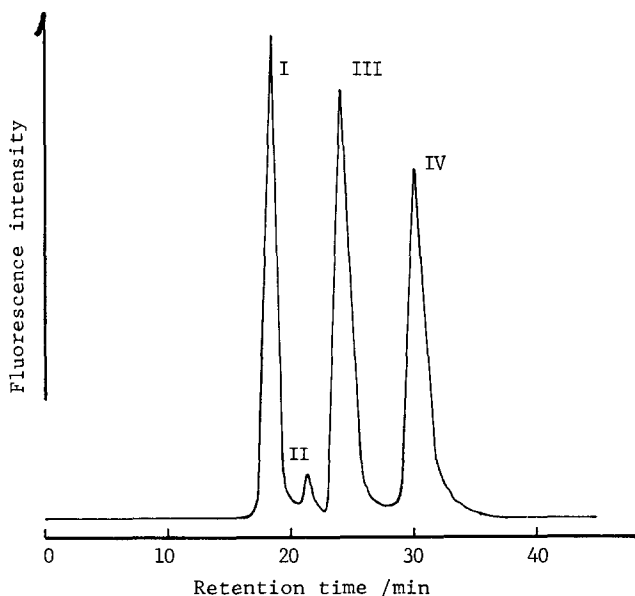

Fig. 4. Chromatogram of (I) glycerol, (II) ethylene glycol, (III) propylene glycol and (IV) 1,2-butanediol. Sample, 50 nmol of each in 100 μl. Mobile phase, 0.2 M carbonate–5 mM NAD (pH 10). Flow-rate, 0.7 ml/min. Column, TSK gel SCX ($5 \mu m$) (300 × 7.8 mm I.D.) at 40°C. Fluorescence monitored at 340/465 nm.

TABLE I

SEPARATION PARAMETERS FOR POLYHYDRIC ALCOHOLS

Alcohol	Retention time (min)	Capacity factor	Resolution	Separation factor
Glycerol	18.4	0.28		
			1.27	1.70
Ethylene glycol	21.2	0.37		
			1.08	1.41
Propylene glycol	24.0	0.67		
			1.89	1.63
1,2-Butanediol	30.0	1.08		

The separation of a mixture of the polyhydric alcohols into its components was effected by the cation exchanger TSK gel SCX (5 μm) (300 mm × 7.8 mm I.D. column) with carbonate buffer (0.2 M, pH 10.0) as the mobile phase, as shown in Fig. 4. The separation parameters of the alcohols are given in Table I.

The resolution was not influenced by the presence of NAD at concentrations below 5 mM in the carbonate buffer. The carbonate buffer (0.4 M, pH 10.0) and NAD solution [10 mM in 0.05 M phosphate buffer (pH 7.0)] were applied at equal flow-rates and were combined before the injector because NAD is unstable in a basic solution. The optimal flow-rate for good sensitivity and reasonable resolution was 0.70 ml/min.

The chromatographic column was thermostated at 40°C. At a column temperature of 60°C the chromatographic peak was sharper than at 40°C. However, NAD was decomposed to some extent, leading to a smaller analytical peak. On the other hand, at 20°C, the resolution was inferior to that at 40°C.

There is a linear relationship between the peak height and the concentration of glycerol and calibration graphs were prepared for the range 0.005–0.5 mM. The detection limit was 0.001 mM of glycerol.

Application

Table II gives results for the determination of triglycerides in serum (Precilip; Boehringer). The serum (100 μl) was added to 1.0 ml of phosphate buffer (0.1 M, pH 7.0) containing lipoprotein lipase (1000 U) and incubated for 15 min at 35°C. The supernatant liquid was withdrawn from the suspension with a syringe through a guard column (pore size 0.45 μm). An aliquot (100 μl) of the solution was injected into the

TABLE II

RESULTS FOR TRIGLYCERIDES IN CONTROL SERA

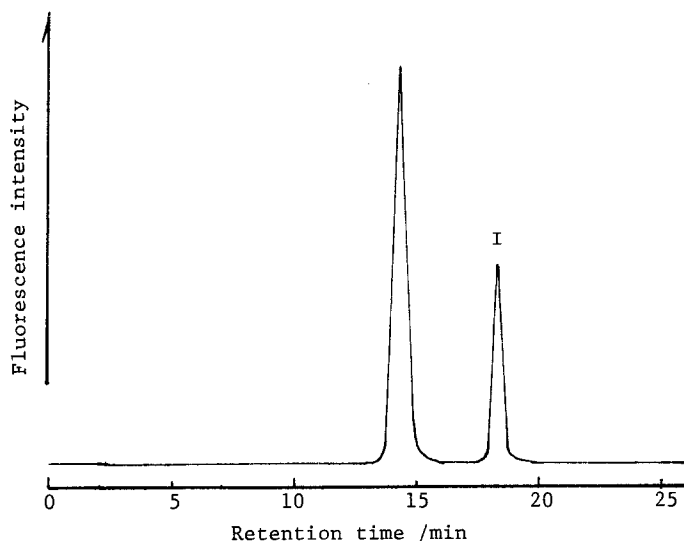| Serum sample	Triglycerides found (mM)	Relative standard deviation (n = 10) (%)		Certified value (mM)
		Within day	Between-day	
Lot 1-375	1.52	3.2	3.6	1.54
Lot 151 662	2.11	3.0	3.6	2.10

Fig. 5. Chromatogram of serum. Peak I corresponds to glycerol. Analytical conditions as in Fig. 4.

column. The concentration of triglycerides was calculated from the calibration graphs for glycerol. A typical chromatogram for the serum is shown in Fig. 5.

CONCLUSION

The HPLC system with an immobilized GDH column reactor was highly selective for the determination of glycerol. Glycerol has previously been determined fluorimetrically by reversed-phase chromatography using a post-column immobilized reactor[2] and the detection limit was 0.01 mM. The use of the pumpless reagent unit (one-pump system) provided a 10-fold increase in sensitivity.

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