Specific detection of acetyl-coenzyme A by reversed-phase ion-pair high-performance liquid chromatography with an immobilized enzyme reactor

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ABSTRACT

A selective chromatographic detection system for the determination of acetyl-coenzyme A (CoA) is reported. The short-chain acyl-CoA thioesters were separated by reversed-phase ion-pair high-performance liquid chromatography (HPLC), and then acetyl-CoA was selectively detected on-line with an immobilized enzyme reactor (IMER) as a post-column reactor. Thio-CoA liberated enzymatically from acetyl-CoA was determined spectrophotometrically after reaction with Ellman's reagent in the reagent stream. The IMER with phosphotransacetylase had a substrate specificity sufficient to determine acetyl-CoA and was active and stable in the mobile phase containing methanol and the ion-pair reagent. The calibration graph was linear between 0.2 and 10 nmol, with a detection limit of 0.05 nmol. This HPLC system with detection by IMER allows the selective identification and determination of acetyl-CoA in a mixture of acetoacetyl-CoA and 3-hydroxy-3-methylglutaryl-CoA, which are difficult to separate with ion-pair HPLC.

INTRODUCTION

Owing to the significance of coenzyme A (CoA) and its thioesters in metabolic regulation, several modifications of the reversed-phase high-performance liquid chromatographic (HPLC) method with ultraviolet (UV) detection for determining short-chain acyl-CoA esters have been published [1–6]. Acetyl-CoA plays an important role as the acetyl donor in the biosynthesis of acetylcholine, acetyl-L-carnitine and *N*-acetylserotonin. Working with an analytical approach for determining acetyl-CoA, a rapid and reliable flow injection technique has been reported with immobilized phosphotransacetylase (PTA) as a catalytic reactor [7].

Immobilized enzyme reactors (IMERs) are powerful analytical tools in flow injection analysis (FIA), with high selectivity and economy [8]. The application of IMERs for reaction detection systems in HPLC is another application in the analytical flow mode [9–11]. Although the on-line combination of HPLC separation and detection with an IMER provides high sensitivity and analytical selectivity, the effect on the activity and stability of the IMER towards organic modifiers in the mobile phase must be taken into consideration. Bowers and co-workers [9,12] examined the effect of the organic solvents methanol, ethanol, acetonitrile and ethylene glycol on immobilized β-glucuronidase, and observed that the organic modifiers in the mobile phase exert a reductive effect on the activity and stability of the immobilized enzyme.

In a previous paper, it was reported that immobilized PTA has a marked stability to changes in temperature and methanol concentration, and it has been shown that immobilized PTA retains its activity even in 60% methanol [7]. In this study, immobilized PTA was used in a post-column reaction detection system in HPLC, and this paper discusses the usefulness of this HPLC system with detection by IMER, that is, the selective determination of acetyl-CoA in a mixture of short-chain acyl-CoA esters.

EXPERIMENTAL

Materials

CoA, acetyl-CoA and other acyl-CoA esters were obtained from Sigma (St. Louis, MO, USA). PTA (acetyl-CoA:orthophosphate acetyltransferase, EC 2.3.1.8, 11 660 U/mg of protein) from *Bacillus stearothermophilus* was purchased from Seikagaku Kogyo (Tokyo, Japan), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB; Ellman's reagent) and glutathione (reduced) from Wako (Osaka, Japan) and AF-Tresyl Toyopearl 650 gel (–$CH_2OSO_2CH_2CF_3$ residue; 100 μmol/g of dry gel) from Tosoh (Tokyo, Japan). The ion-pairing reagent, tetra-*n*-butylammonium phosphate (TBAP), was from Nacalai Tesque (Kyoto, Japan). HPLC-grade methanol was purchased from Kanto Chemical (Tokyo, Japan).

Immobilized enzyme reactor

The detailed procedure for the preparation of immobilized PTA has been described previously [7]. The immobilized PTA was packed into a stainless-steel column (10 × 4 mm I.D.) and used as the IMER for FIA and HPLC. The active groups of the gel (AF-Tresyl Toyopearl 650) without enzyme to be bonded were entirely blocked with 0.1 *M* Tris-HCl buffer (pH 8.0) containing 0.5 *M* sodium chloride. The gel was then packed into another column of the same size and was used as a blank column.

Apparatus and procedures

The flow system for the FIA mode was almost the same as that reported previously [7], except that a blank column was constructed, as shown in the upper panel of Fig. 1. The carrier stream [30 m*M* Na_2HPO_4 as the substrate, 15 m*M* $(NH_4)_2SO_4$ as the enzyme activator and 0.1 m*M* DTNB as the colour-producing reagent in 0.1 *M* borate buffer (pH 7.5)] was pumped by a Shimadzu LC-6A pump (Kyoto, Japan) at a flow-rate of 1 ml/min. A 25-μl sample was introduced into the carrier stream through a Rheodyne Model 7125 injector (Cotati, CA, USA) with a 100-μl sample loop. CoA enzymatically liberated from acetyl-CoA by passage through the IMER was reacted with DTNB in the carrier stream. The columns with PTA [IMER, E(+)] and without PTA [the blank column, E(−)] were thermostated in an oven (CTO-6A, Shimadzu); the mixing coil tubing was 35 cm × 0.25 mm
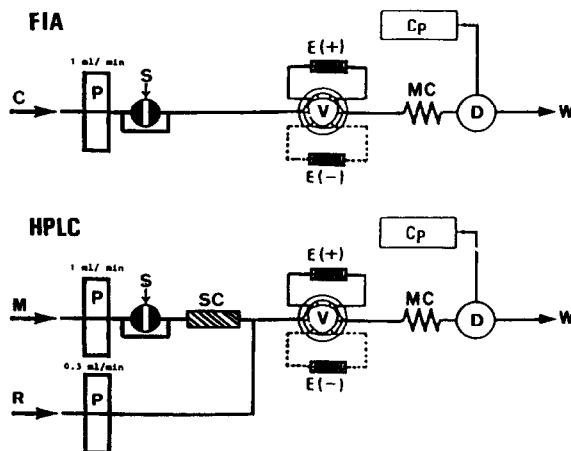

FIA

HPLC

Fig. 1. Schematic diagrams of the FIA and HPLC systems with IMER detection. P = pump; S = sample injector; SC = separation column; V = switching valve; MC = mixing coil; D = detector; Cp = computing integrator; M = mobile phase; R = reagent solution; E(+) = immobilized enzyme column; E(−) = blank column without immobilized enzyme.

I.D. The six-port switching valve (FCV-2AH, Shimadzu), controlled by a system controller (SCL-6A, Shimadzu), was used for column switching. The change in absorbance at 412 nm was monitored with a spectrophotometer (SPD-6AV, Shimadzu) equipped with an 8-μl flow cell. The chromatogram and integration were obtained with an integrator (C-R4A, Shimadzu). A trace amount of DTNB-reactive thiol compounds in the samples was corrected by subtracting the peak height or peak area obtained with the blank column, E(−), from those obtained with the IMER, E(+). The HPLC mode with the IMER detection system used is shown in the lower panel of Fig. 1.

An analytical column (15 cm × 6 mm I.D., Capcell Pak C_8, SG type, 5-μm particle, Shiseido, Tokyo, Japan) was used in the FIA flow mode described above. The mobile phase was pumped by a Shimadzu LC-6A pump at a flow-rate of 1 ml/min, and sample solutions of 25 μl were injected through a Rheodyne injector. After the separation of the compounds on the analytical column at ambient temperature, the reagent solution or distilled water was introduced through a T-piece with an another pump (LC-6A, Shimadzu) at a flow-rate of 0.3 ml/min. When the distilled water was pumped in, the column effluent was monitored by UV absorption

at 254 nm. When the reagent solution containing the enzyme substrate, the enzyme activator and the colour-producing reagent were introduced to the flow, the absorbance was monitored at 412 nm.

Preparation of mobile phase and reagent solution

The mobile phase solvents consisted of the ion-pairing reagent (TBAP) and methanol. Two mixtures, namely, 2 mM TBAP (pH 6.4) in methanol–water (42:58) and 2 mM TBAP (pH 6.5) in methanol–water (45:55), were used throughout this study. The pH was adjusted with 1% H_3PO_4. The solvents were filtered through 0.4-μm pore size filters (Fuji Photo Film, Tokyo, Japan) and degassed. The concentration of the reagent solution used for the HPLC system with IMER detection was twice that of the carrier stream, i.e. the reagent solution contained 60 mM Na_2HPO_4, 30 mM $(NH_4)_2SO_4$ and 0.2 mM DTNB in 0.2 M borate buffer, pH 7.5.

RESULTS AND DISCUSSION

Effects of ion-paired reagent and methanol on IMER activity

The reversed-phase ion-pair chromatographic (RP-IPC) technique using the ion-pairing reagent TBAP and methanol reported by Baker and Schooley [2,6] provided a rapid isocratic separation of CoA, acetyl-CoA and other acyl-CoA thioesters. If our immobilized PTA is used as the IMER in a RP-IPC system, it is necessary to evaluate the level of the enzymatic activity of the IMER in the presence of TBAP and methanol. The effects of the concentrations of TBAP and/or methanol on the IMER activity were examined using the FIA flow system and the carrier stream, as described under *Apparatus and procedures*. The TBAP had no inhibiting or acitvating effect on the IMER at concentrations less than 4 mM in the carrier tested. The effects of methanol on the IMER activity were studied in the presence of 2 mM TBAP. A decrease in the relative response with an increasing concentration of methanol was observed. When the carrier stream contained 2 mM TBAP and 25% methanol, the enzymatic activity of the IMER was 65% of its original level. This decreased effect was assumed (as reported earlier) not to be enzyme inactivation but to result from a decrease in the rate of the enzymatic reaction [7].

Substrate specificity of the IMER

The enzyme specificity of the IMER was also examined using the FIA flow system, in which the formation of CoA from a series of related acyl-CoAs at a concentration of 2.5 nmol per 25-μl injection was measured. The activities obtained from peak-area and peak-height measurements were corrected by subtracting the values with the blank column from those with the IMER; the relative responses are given in Table I.

The IMER, as far as tested, can catalyse the transfer reaction of acetyl-CoA, propionyl-CoA and isobutyryl-CoA. Other acyl-CoAs were not catalysed. When the acetyl-CoA of the substrate was injected together with acetoacetyl-CoA and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), with 2.5 nmol of each component per 25-μl injection, no inhibitory activity was observed. These results show that neither acetoacetyl-CoA nor HMG-CoA had any competitive effect against the catalytic reaction of the IMER for acetyl-CoA.

Chromatographic separation and specific detection of acetyl-CoA

The separation of CoA and other acyl-CoA esters was performed with a reversed-phase ion-paired

TABLE I

ENZYMATIC ACTIVITY OF IMMOBILIZED ENZYME REACTOR (IMMOBILIZED PHOSPHOTRANSACETY-LASE)

Enzymatic activity was measured using the FIA flow system. Samples contained 2.5 nmol of each ingredient per 25-μl injection. Each value is expressed as the mean of five experiments. A trace amount of DTNB-reactive thiol compounds in the samples was corrected. See text for details.

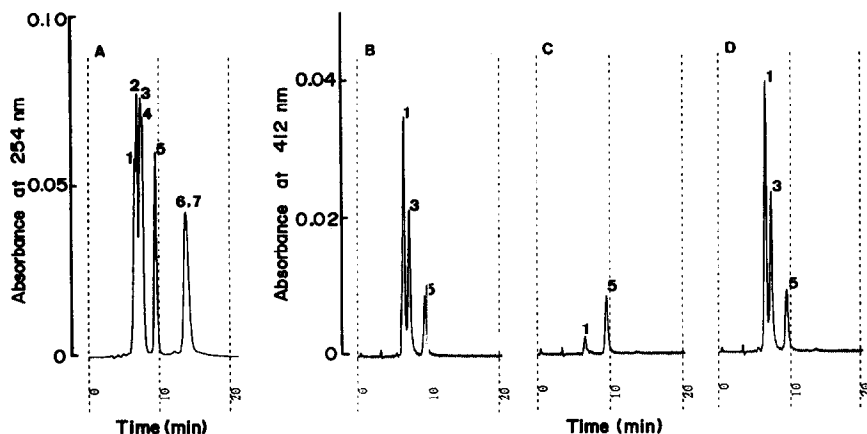| Sample	Relative activity (%)	
	Peak area	Peak height
Acetyl-CoA	100	100
Acetoacetyl-CoA	0.7	0.9
3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA)	0	0
Propionyl-CoA	96.4	96.5
n-Butyryl-CoA	0.3	0.6
Isobutyryl-CoA	16.8	17.1
Acetyl-CoA + acetoacetyl-CoA	101.5	99.2
Acetyl-CoA + HMG-CoA	99.3	100.9
Acetyl-CoA + acetoacetyl-CoA + HMG-CoA	100.5	98.7

Fig. 2. Chromatograms of reversed-phase ion-pair HPLC with (A) ultraviolet detection at 254 nm and (B)–(D) visible detection at 412 nm. Samples comprised mixtures of acyl-CoAs at a concentration of 2.5 nmol per 25-μl injection. Samples in (A) and (D) contained CoA, acetyl-CoA, acetoacetyl-CoA, HMG-CoA, propionyl-CoA, n-butyryl-CoA and isobutyryl-CoA; in (B), CoA, acetyl-CoA, propionyl-CoA and n-butyryl-CoA; and in (C), acetoacetyl-CoA, HMG-CoA, propionyl-CoA and isobutyryl-CoA. Peaks: 1 = CoA; 2 = acetoacetyl-CoA; 3 = acetyl-CoA; 4 = HMG-CoA; 5 = propionyl-CoA; 6 = isobutyryl-CoA; 7 = n-butyryl-CoA. Mobile phase, 0.002 M TBAP, pH 6.4, in methanol–water (42:58).

system. The mobile phase was pumped at a flow-rate of 1.0 ml/min and distilled water was introduced through a T-piece with another pump at a flow-rate of 0.3 ml/min. The column effluent was monitored at 254 nm. The separation conditions onto a Capcell Pak C_8 column were selected on the basis of the excellent results reported previously, for which 50 mM TBAP was used [6]. TBAP is an expensive and corrosive reagent, so its concentration was reduced to 2 mM. At this low TBAP concentration, acetyl-CoA was weakly adsorbed to the column, and was therefore readily eluted from the column with the lower methanol concentration. The lower methanol concentration was also favourable for the functioning of the IMER. At the lower pH, acetyl-CoA had a long retention time, but, at pH values below 5.5, the activity and the stability of the IMER were decreased. Consequently, a mobile phase was selected consisting of 2 mM TBAP (pH 6.4) in methanol–water (42:58).

A typical chromatogram of a mixture of acyl-CoA esters monitored at 254 nm is shown in Fig. 2A. Although CoA and the six acyl-CoA esters in the samples were eluted within 15 min, the resolution of acetyl-CoA (peak 3) from acetoacetyl-CoA (peak 2) and HMG-CoA (peak 4) presented considerable difficulties. Isobutyryl-CoA and n-butyryl-CoA (peaks 6 and 7) were co-eluted. The pumping

of distilled water was replaced with reagent solution pumping, and the column effluent passing through the IMER was monitored at 412 nm. As Fig.2 B–D shows, the IMER could selectively detect acetyl-CoA (peak 3) and propionyl-CoA (peak 5), but not the other acyl-CoA esters. This selective detection is in accord with the data shown in Table I. Peak 1 in Fig. 2C, which has a retention time corresponding to CoA, is assumed to be the contamination or degradation product originating from acetoacetyl-CoA.

Quantitative chromatograms were obtained by injecting samples containing various amounts of acetyl-CoA (peak 3) in a definite amount of glutathione (peak 1) and CoA (peak 2). As shown in Figs. 3A–E, the peak of acetyl-CoA increased linearly with increasing amounts of acetyl-CoA injected into the HPLC system with IMER detection. In contrast, when the sample solution identical with that used in Fig. 3D was injected into the system and the blank column was used in place of the IMER, no acetyl-CoA peak was detected (Fig. 3F).

Calibration graph, precision and stability

A calibration graph for acetyl-CoA, obtained by measurement of the peak area, was linear over the range 0.2–10 nmol per 25-μl injection. The detection limit was 0.05 nmol at a signal-to-noise ratio of
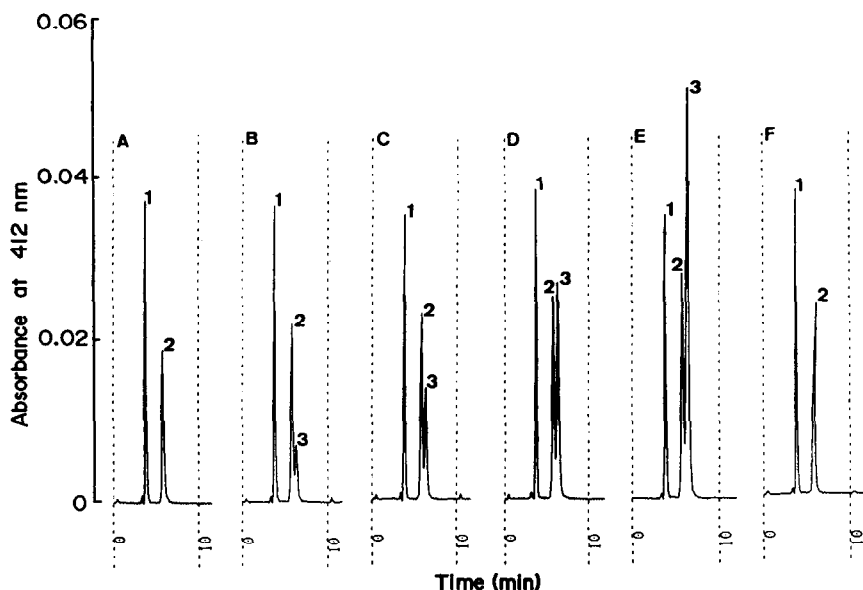
Fig. 3. Typical chromatograms for the selective determination of acetyl-CoA using the HPLC system with IMER detection. Samples comprised various amounts of acetyl-CoA: (A) none; (B) 0.625 nmol per 25-μl injection; (C) 1.25 nmol per 25-μl injection; (D) 2.5 nmol per 25-μl injection; (E) 5 nmol per 25-μl injection; (F) 2.5 nmol per 25-μl injection, each in a definite amount of glutathione (1 nmol per 25-μl injection) and CoA (1.25 nmol per 25-μl injection). Peaks: 1 = glutathione; 2 = CoA; 3 = acetyl-CoA. Mobile phase, 0.002 M TBAP, pH 6.5, in methanol–water (45:55).

3. The regression equations and the correlation coefficients (r) were $y = 233.38x - 8.81, r = 0.9994$ ($n = 30$) for peak area (mV · S) and $y = 9.63x + 0.21, r = 0.9987$ ($n = 30$) for peak height (mV). The intra-assay relative standard deviation (R.S.D.) was 2.8% (1 nmol per 25-μl injection, $n=9$). The IMER retained 80–85% of its original activity after 1 month with repeated use for 5 h per day, even using in the organic modifier (methanol) at 40°C.

The HPLC system with IMER detection described here provides a simple, rapid and selective method for determining acetyl-CoA. The sensitivity should be further increased if a thiol-specific electrochemical detection system [13,14] is used.

ACKNOWLEDGEMENT

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