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Analysis of lipoic acid in biological samples by gas chromatography with flame photometric detection

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ABSTRACT

A selective and sensitive gas chromatographic method for the analysis of lipoic acid in biological samples has been developed. After base hydrolysis of the sample, the liberated lipoic acid was converted into its S,S-diethoxycarbonyl methyl ester derivative and measured by gas chromatography using a DB-210 capillary column and a flame photometric detector. The calibration curve was linear in the range 20–500 ng, and the detection limit was *ca.* 50 pg injected. The best hydrolysis conditions for the biological samples were obtained by using 2 *M* potassium hydroxide containing 4% bovine serum albumin at 110°C for 3 h. Using this method, lipoic acid in the hydrolysate could be selectively determined without any interference from matrix substances. Analytical results for the determination of lipoic acid in the mouse tissue and bacterial cell samples are presented.

INTRODUCTION

Lipoic acid is a naturally occurring cofactor that serves as an acyl carrier in the oxidative decarboxylation of the α-keto acids (pyruvate and α-ketoglutarate) [1–3] and an aminomethyl carrier in the glycine-cleavage enzyme system [4,5]. In the functional protein-bound form of the cofactor, the carboxyl group of lipoic acid is covalently bound in amide linkage to the ε-amino group of a lysyl residue [6–8]. Recently, lipoic acid has been shown to provide protection against attack by free radicals both *in vitro* and *in vivo* [9–15], and an interplay between lipoic acid and glutathione in the protection against lipid peroxidation has been demonstrated [9,11]. Moreover, patients diagnosed with liver cirrhosis, diabetes mellitus, atherosclerosis and polyneuritis have been found to contain a decreased level of endogenous lipoic acid [9]. Indeed, lipoic acid is presently used in the treatment of a various diseases, such as liver disease [16–18] and dysfunctions [11,19–22].

The determination of lipoic acid has been carried out by microbiological assay [23–25], colorimetric assay [26], high-performance liquid chromatography (HPLC) [27], gas chromatography (GC) [28,29] and gas chromatography–mass spectrometry (GC–MS) [30]. Biological assays are highly sensitive, but it is difficult to produce consistent results in different laboratories and these methods generally require laborious procedures to grow and maintain the bacterial strains. Colorimetric assay, HPLC and GC methods lack the sensitivity or specificity required for determining lipoic acid contents in biological samples. The GC–MS method is specific, but it requires expensive equipment. Enzyme immunoassay [31] as recently developed is specific and does not require release of protein-bound lipoic acid by hydrolysis of the sample, but it requires laborious

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preparation of anti-lipoic acid antibody and is not so sensitive.

This paper describes a selective and sensitive method for analysing lipoic acid in biological samples by GC with flame photometric detection (FPD), in which lipoic acid was converted into its S,S-diethoxycarbonyl (S,S-DEOC) methyl ester derivative after reduction to dihydrolipoic acid with sodium borohydride.

EXPERIMENTAL

Chemicals

D,L-α-Lipoic acid was purchased from Tokyo Kasei Kogyo (Tokyo, Japan), and was dissolved in methanol to obtain a stock solution at a concentration of 1 mg/ml and used after dilution with distilled water. Sodium borohydride (Nacalai Tesque, Kyoto, Japan) was used as a 100 mg/ml solution in 0.1 *M* sodium hydroxide. Ethyl chloroformate (ECF) was obtained from Tokyo Kasei Kogyo. Hydrogen chloride in methanol (HCl–MeOH) obtained from Toyko Kasei Kogyo was diluted with methanol at a concentration of 0.25 *M*. S,S-Dimethoxycarbonyllipoic acid, used as an internal standard (I.S.), was prepared as follows: 10 mg (48.5 μmol) of lipoic acid was dissolved in 1 ml of 50 mg/ml sodium borohydride in 0.05 *M* sodium hydroxide and then incubated at 60°C for 10 min. To the reaction mixture was added 0.3 ml (3.17 mmol) of methyl chloroformate, and the mixture was shaken for 20 min at room temperature. The reaction mixture was acidified to pH < 1 with 2 *M* hydrochloric acid and then extracted twice with 3 ml of pentane. The combined pentane extracts were washed with 1 ml of 0.2 *M* hydrochloric acid and evaporated to dryness at 60°C. The residue was reconstituted with methanol to prepare a 1 mg/ml solution. Bovine serum albumin (Fraction V, BSA) was obtained from Sigma (St. Louis, MO, USA) and used as a 50 mg/ml in 2.5 *M* potassium hydroxide. Commercial bacterial strains, *Aerobacter aerogenes* (*Enterobacter aerogenes* Type I), *Azotobacter vinelandii*, *Bacillus subtilis*, *Clostridium perfringens* (*Clostridium welchii*), *Escherichia coli* (strain B), *Micrococcus lysodeikticus* (*Micrococcus luteus*), *Pseudomonas fluorescens* (Type II) and *Saccharomyces cerevisiae* (baker's yeast Type I) were obtained from Sigma.

Preparation of samples

Six male ddY mice (six weeks old) and several commercial bacterial strains were used in the experiments. Immediately after dissection, each mouse organ was removed and frozen, and stored at −20°C until used for each assay. Each pooled tissue was chopped up, and an aliquot (*ca.* 1 g) was homogenized in four volumes of distilled water with a Model LK-21 ultra-disperser (Yamato Kagaku, Tokyo, Japan), and then made up to 5 ml with water. An aliquot (0.2 ml) of tissue homogenate or 5–50 mg of bacterial cell were placed in a polypropylene tube (75 × 9 mm I.D.), and 0.8 ml of 50 mg/ml BSA in 2.5 *M* potassium hydroxide was added. The volume was made up to 1 ml with water. The mixture was hydrolysed for 3 h at 110°C under vacuum in the Pico-Tag workstation (Waters Assoc., Milford, MA, USA). To the resulting hydrolysate were added 0.05 ml of 1 mg/ml 2-mercaptoethanol and 0.4 ml of 6 *M* hydrochloric acid, and the solution was transferred to the reaction tube (10-ml Pyrex glass tube with a PTFE-lined screw-cap). The mixture was extracted twice with 2 ml of methylene chloride, and the pooled methylene chloride extracts were evaporated to dryness. The residue was dissolved in 1 ml of 0.01 *M* sodium hydroxide and used for the analysis as a hydrolysate sample.

Derivatization procedure

To the hydrolysate sample was added 0.05 ml of 100 mg/ml sodium borohydride solution, and the mixture was incubated at 60°C for 5 min. Then 0.05 ml of 2 *M* sodium hydroxide and 0.05 ml of ECF were added, and the mixture was shaken on a shaker set at 300 rpm (up and down) for 3 min at room temperature. The reaction mixture was acidified to pH < 1 by addition of 0.2 ml of 2 *M* hydrochloric acid, and 0.1 ml of 1 μg/ml I.S. solution was added. The mixture was extracted with 3 ml of pentane, and the extract was evaporated to dryness at 60°C. To the resid-

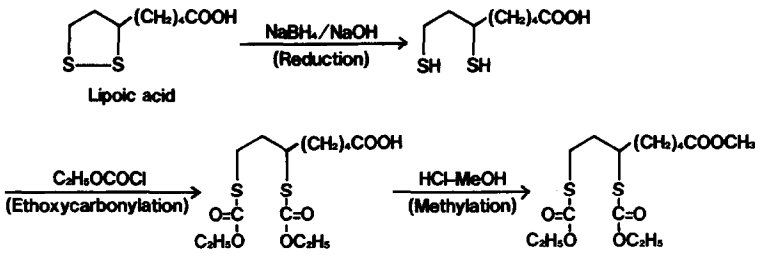

Fig. 1. Derivatization process of lipoic acid.

ue was added 0.3 ml of 0.25 *M* HCl–MeOH, and the mixture was incubated at 80°C for 10 min. After the solvent had been evaporated to dryness at 80°C, the residue was dissolved in 0.1 ml of ethyl acetate, and 1 μl of this solution was injected into the gas chromatograph. The derivatization process is summarized in Fig. 1.

Gas chromatography

The GC analysis was carried out with a Shimadzu 12A gas chromatograph equipped with a flame ionization detector and a flame photometric detector (S-filter). A fused-silica capillary column (15 m × 0.53 mm I.D., 1.0 μm film thickness) of cross-linked DB-210 (J & W, Folsom, CA, USA) was used. The operating conditions were as follows: column temperature, programmed at 5°C/min from 200 to 250°C; injection and detector temperature, 260°C; nitrogen flow-rate, 10 ml/min.

Gas chromatography–mass spectrometry

A Hewlett-Packard 5890A gas chromatograph was used in conjunction with a VG Analytical 70-SE mass spectrometer and a VG 11-250J mass data system. The GC column was of the same type as used for GC analysis, with an ionizing voltage of 40 eV, an ion-source temperature of 240°C, and helium flow-rate of 8 ml/min.

RESULTS AND DISCUSSION

Although standard lipoic acid can be readily gas chromatographed as its methyl ester derivative, it is difficult to detect the lipoic acid isolated from biological samples as the same derivative because it forms mixed disulphides [32] and disulphide polymers [33]. Therefore, these disulphides must be reduced to the thiols and then derivatized with appropriate reagent for GC analysis. White [28,30] reported that the lipoic acid isolated from tissue could be successfully analysed as its S,S-dibenzylmethyl ester derivative after reduction with sodium borohydride. However, this method requires time-consuming derivatization, and samples that contain less than a total of 300 ng of lipoic acid must be purified by thin-layer chromatography after derivatization in order to reduce solvent tailing during GC analyses.

We investigated a simple and rapid derivatization method (outlined in Fig. 1) using ECF as a thiol-derivatizing reagent. The reduction of lipoic acid to dihydrolipoic acid was accomplished within 2 min at 60°C by using 5 mg of sodium borohydride in aqueous alkaline media. Subsequently, the ethoxycarbonylation of reduced lipoic acid was accomplished within 2 min in aqueous alkaline media by shaking at room temperature, and lipoic acid was converted into its S,S-DEOC derivative. The S,S-DEOC lipoic acid was quantitatively extracted into pentane in acidic media, and methylated with HCl–MeOH. This methylation was accomplished within 5 min at 80°C. The derivative preparation could be performed within 30 min.

The structure of the derivative was confirmed by GC–MS analysis. As shown in Fig. 2A, a molecular ion peak (M^+) with postulated *m/z* 366 and the prominent fragment ion peaks, *m/z* 293 [$M^+ - 73$ ($COOC_2H_5$)], *m/z* 249 [$^+COSCH_2CH_2CH(SH)(CH_2)_4COOCH_3$], *m/z* 189 [$M^+ - 177$ ($2COOC_2H_5$ and OCH_3)], *m/z*

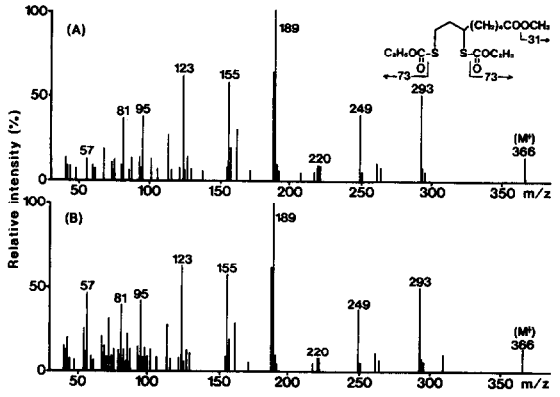

Fig. 2. GC–MS profiles of S,S-diethoxycarbonyl methyl ester derivatives obtained from (A) authentic lipoic acid and (B) mouse liver.

155 ($^{+}CH_2CH_2CH=CHCH_2CH_2-CH_2COOCH_3$ and *m/z* 123 ($CH_2=CHCH=CHCH_2CH_2CH_2C^{+}O$) were observed, and these peaks were useful for structure elucidation. The derivative was found to be very stable under normal laboratory conditions, and no decomposition was observed during GC analysis.

As shown in Fig. 3A, the derivative was eluted as a single and symmetrical peak and provided an excellent FPD response. The minimum detectable amount of lipoic acid, at a signal-to-noise ratio of 3, was *ca.* 50 pg injected. In order tot test the linearity of the calibration graph, various amounts of lipoic acid ranging from 20 to 500 ng were derivatized, and aliquots representing 0.2–5 ng of lipoic acid were injected. A linear relationship was obtained from both logarithmic plots, and the regression line was $\log y = 1.464 \log x - 3.104$ ($r = 0.9950$, $n = 15$), where y is the peak-height ratio and x is the amount of lipoic acid.

Because the majority of lipoic acid in biological samples is present as a protein-bound form with an amide linkage, the release of lipoic acid by hydrolysis of the sample is necessary. Although the lipoic acid tends to be oxidized to its thiosulphinate or thiosulphonate during hydrolysis, this oxidation is far more resistant to base than to acid hydrolysis, and the presence of albumin prevents the degradation of lipoic acid [34]. Therefore, we studied the hydrolysis under basic conditions with the addition of BSA. In the hydrolysis of mouse liver, 2–3 *M* potassium hydroxide proved to be adequate for maximum recovery of lipoic acid. As shown in Fig. 4, free lipoic acid was completely degraded by hydrolysis in 2 *M* potassium hydroxide at 110°C for 3 h, but this degradation was prevented by addition of BSA. Similarly, the addition of BSA to mouse liver homogenate increased the recovery, and maximum recovery was obtained by hydrolysis at 110°C for 2–4 h. The optimum concentration of BSA was 2–6%. The released lipoic acid was easily extracted into methylene chloride. In this extraction, the

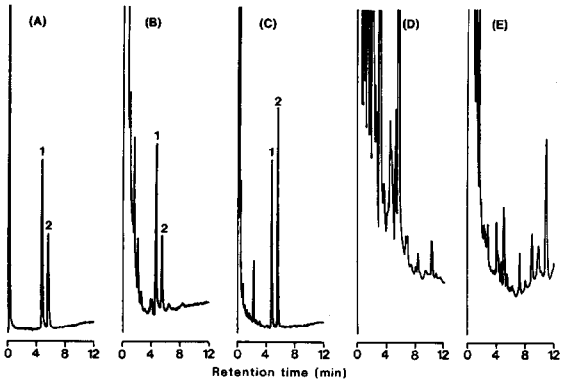

Fig. 3. Gas chromatograms obtained from the standard solution and biological samples. (A) Standard (containing 100 ng of lipoic acid); (B) mouse liver (50 mg); (C) *Escherichia coli* (5 mg); (D) mouse liver (50 mg); (E) *E. coli* (50 mg). The derivatized samples were analysed by either GC–FPD (A–C) and GC–FID (D and E). GC conditions were as in Experimental. Attenuations: GC–FPD, 10 × 8; GC–FID, 10^2 × 4. Peaks: 1 = S,S-dimethoxycarbonyllipoic acid (I.S.); 2 = lipoic acid.

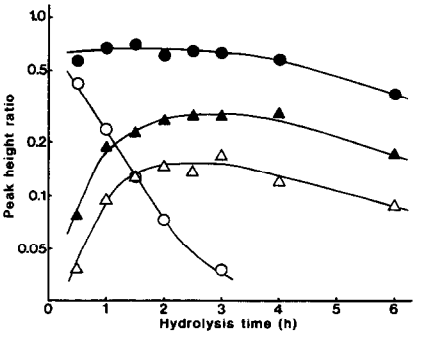

Fig. 4. Effect of the addition of BSA on the base hydrolysis of standard lipoic acid and mouse liver. (○) Lipoic acid without BSA; (●) lipoic acid with BSA; (△) mouse liver without BSA; (▲) mouse liver with BSA.

TABLE I

RECOVERIES OF LIPOIC ACID ADDED TO MOUSE TISSUE AND BACTERIAL CELL SAMPLES

Sample	Amount added (μg/g)	Amount found[a] (μg/g)	Recovery (%)
Mouse brain	0	0.83 ± 0.03	
	2.00[b]	1.83 ± 0.01	50.0
	2.00[c]	2.70 ± 0.06	93.5
Mouse liver	0	1.23 ± 0.03	
	2.00[b]	2.42 ± 0.04	59.5
	2.00[c]	3.02 ± 0.01	89.5
Mouse kidney	0	1.54 ± 0.06	
	2.00[b]	2.69 ± 0.05	57.5
	2.00[c]	3.36 ± 0.16	91.0
Mouse muscle	0	0.78 ± 0.02	
	2.00[b]	1.90 ± 0.04	56.0
	2.00[c]	2.49 ± 0.02	85.5
Escherichia coli	0	23.45 ± 0.44	
	20.0[b]	34.80 ± 0.28	56.8
	20.0[c]	40.20 ± 1.19	83.8
Saccharomyces cerevisiae	0	1.54 ± 0.01	
	2.00[b]	2.71 ± 0.14	58.5
	2.00[c]	3.23 ± 0.08	84.5

[a] Mean ± S.D. (n = 4).
[b] Added to sample.
[c] Added to hydrolysate.

TABLE II

LIPOIC ACID CONTENT IN MOUSE TISSUES AND BACTERIAL CELLS

Sample	Content[a] (μg/g)
Mouse tissue	
Brain	0.83 ± 0.03
Heart	2.03 ± 0.07
Lung	0.80 ± 0.03
Liver	1.23 ± 0.03
Spleen	0.52 ± 0.03
Pancreas	0.84 ± 0.05
Kidney	1.54 ± 0.06
Testis	1.06 ± 0.05
Muscle	0.78 ± 0.02
Bacterial cell	
Aerobaster aerogenes	10.62 ± 0.62
Azotobacter vinelandii	34.96 ± 2.39
Bacillus subtilis	17.65 ± 1.38
Clostridium perfringens	ND[b]
Escherichia coli	23.45 ± 0.44
Micrococcus lysodeikticus	4.34 ± 0.18
Pseudomonas fluorescens	16.25 ± 0.25
Sacchromyces cerevisiae	1.54 ± 0.01

[a] Mean ± S.D. (n = 4).
[b] Not detectable.

addition of 2-mercaptoethanol to the hydrolysate increased the recovery. As shown in Table I, the overall recoveries of lipoic acid added to mouse tissue and bacterial cell samples were 50–60%, and the relative standard deviations calculated from four independent determinations were 0.5–5.3%. On the other hand, the recoveries of lipoic acid added to hydrolysates were above 83%. Therefore, 20–30% of free lipoic acid was lost by oxidation during hydrolysis.

Fig. 3B–E show typical chromatograms obtained from mouse tissue and bacterial cell samples by FPD and flame ionization detection (FID). It was difficult to determine the lipoic acid in these samples by GC–FID because of the interfering peaks and low sensitivity. However, the lipoic acid in these samples could be analysed by GC–FPD without any such interference. The lipoic acid peak obtained from each biological sample was confirmed by GC–MS analysis. As shown in Fig. 2B, the GC–MS spectrum obtained

from mouse liver agreed with that of the authentic lipoic acid. Table II shows the analytical results for lipoic acid in mouse tissue and in several commercial bacterial cell samples.

CONCLUSIONS

A convenient and reliable method for the analysis of lipoic acid in biological samples has been established. The method consists of base hydrolysis of the sample to release the bound lipoic acid, methylene chloride extraction of the lipoic acid, and subsequent conversion into S,S-DEOC methyl ester derivative prior to GC–FPD analysis. This method is selective and sensitive, and biological samples can be analysed without any interference from other coexistent substances. We believe that this method provides a useful tool in biochemical and biomedical research.

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