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Liquid chromatographic determination of polythiols based on pre-column excimer fluorescence derivatization and its application to α-lipoic acid analysis

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ABSTRACT

We developed an LC method for the sensitive and selective fluorometric determination of polythiols. This method employs pre-column intramolecular excimer-forming fluorescence derivatization with *N*-(1-pyrene)iodoacetamide followed by LC separation. Polythiols were converted to the corresponding dipyrene-labeled derivatives, and the derivatives afforded intramolecular excimer fluorescence (440–540 nm). After the optimization using dithiothreitol and dimercaprol as model polythiols, α-lipoic acid (LA) and α-lipoamide were determined with high sensitivity and selectivity. The detection limits for polythiols were 0.6–3.5 fmol on column. Furthermore, this method could be successfully applied to the determination of LA in commercial dietary supplements and in human urine.

1. Introduction

α-Lipoic acid (LA; 1,2-dithiolane-3-pentanoic acid) has a polythiol structure and occurs naturally in the mitochondria as the coenzyme for pyruvate dehydrogenase, ketoglutarate dehydrogenase, and other enzymes [1–4]. In contrast to other endogenous thiols (e.g., glutathione or cysteine), LA is readily absorbed from the diet, transported, taken up by cells, and reduced to dihydrolipoic acid (DHLA) in various tissues. The cellular reduction of LA to DHLA has been reported in various mammalian cells and tissues [5–10]. LA and DHLA are well known for their biological antioxidant activities, not only directly through free radical quenching but also indirectly through the recycling of other cellular antioxidants such as vitamin E and glutathione [11–13]. Because of its antioxidant activity, LA has been shown to be beneficial in treating various forms of oxidative stress and is of interest as a therapeutic agent in numerous disorders ranging from diabetes to AIDS [14]. LA was also found to be a potent antioxidant in various drug-induced toxicities in experimental rat models [15–17]. Hence, the determination of LA-related compounds in biological specimens is important for the evaluation of its availability.

As described in the review by Kataoka [18], LA can be determined by gas chromatography (GC) after solvent extraction from biological specimens [19,20]. In addition, some determination methods employ liquid chromatography (LC) with several detection systems. In general, UV detection is neither highly sensitive nor highly selective. Electrochemical detection by using dual gold–mercury electrodes [21] is both selective and highly sensitive, but the linearity of the concentration–response curve is only over a narrow concentration range, and the method lacks reproducibility, mainly due to hysteretic degradation of the electrode. Therefore, a laborious reconstitution of the electrode is required to obtain repeatable results. Although LC coupled with mass spectrometry (LC–MS) [22] is sensitive and highly reliable, its apparatus and operating cost are too high for routine analysis. LA has been determined by LC with fluorescence derivatization methods, with carboxylic acid [23] or disulfide [24–26] as target functional groups. The former, however, is not suitable for simultaneous determination of LA-related compounds such as α-lipoamide (LAM), and the latter is not specific for LA among other thiol compounds. Therefore, we aim to develop an LC method for sensitive and selective fluorometric determination of polythiol compounds, including LA.

Recently, we have developed an intramolecular excimer-forming fluorescence derivatization method for sensitive and selective determination of poly-functional compounds such as polyamines [27–31], polyphenols [32,33], polycarboxylic acids [34,35], and amino acids [36–38]. These methods employ pre-column pyrene derivatization followed by reversed-phase LC

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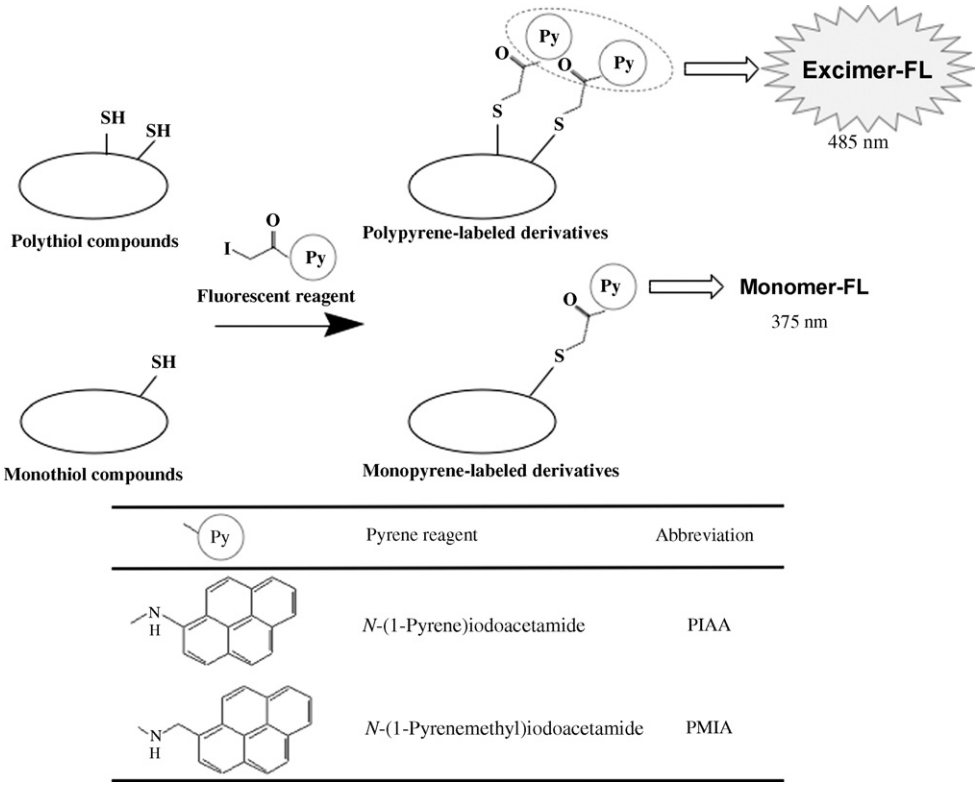

Fig. 1. Excimer-forming fluorescence derivatization reaction of polythiol compounds with PIAA and PMIA.

separation. The analytes containing poly-functional groups were converted to their corresponding polypyrene-labeled derivatives. The polypyrene-labeled derivatives afforded intramolecular excimer fluorescence (440–540 nm), which can be clearly discriminated from the monomer fluorescence (360–420 nm) emitted by monopyrene-labeled derivatives and reagent blanks. This chemistry allowed the selective analysis of poly-functional compounds, even in complex samples containing mono-functional compounds.

In the present paper, we describe an intramolecular excimer-forming derivatization method for the fluorometric determination of LA and LAM, which contain disulfide bonds, based on the above mentioned derivatization with the pyrene reagent (Fig. 1). In the preliminary experiment, dithiothreitol (DTT) and dimercaprol (BAL) were used as model polythiol compounds. For the derivatization reagent of thiol compounds, *N*-(1-pyrene)iodoacetamide (PIAA) and *N*-(1-pyrenemethyl)iodoacetamide (PMIA) were compared with each other for sensitivity, reproducibility, and efficiency of excimer formation. After optimization of analytical conditions, LA and LAM were determined with high sensitivity and selectivity by using this method. Furthermore, we applied this derivatization method to the determination of LA in commercial dietary supplements and in human urine without sample extraction or concentration. To the best of our knowledge, the method presented here is the first report of intramolecular excimer-forming fluorescence derivatization for polythiol compounds.

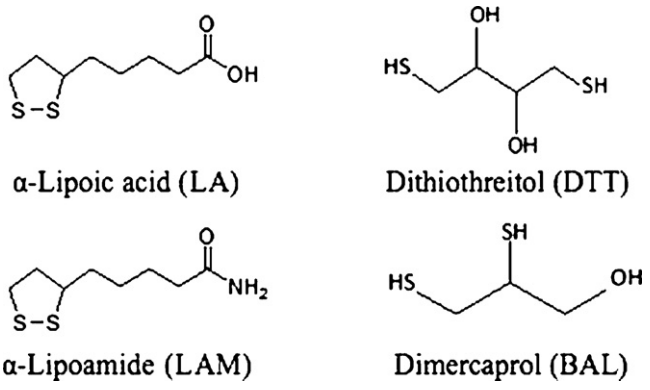

Fig. 2. Structures of the polythiol compounds examined.

2. Experimental

2.1. Reagents and solutions

All chemicals otherwise stated below were of the highest purity available and were used as received. BAL, LA, and LAM were obtained from Tokyo Chemical Industry (Tokyo, Japan), and DTT was from Nacalai Tesque (Kyoto, Japan). The structures of the analytes are presented in Fig. 2. All organic solvents were of LC grade. Both PIAA and PMIA (Fig. 1) were purchased from Invitrogen (Carlsbad, CA, USA) and were used without further purification. These reagents and solvents are toxic to the eyes, lungs, and skin and should be carefully handled according to the guidelines provided in the latest material safety data sheets. Ultra-pure water, further purified using a Milli-Q Gradient system (Millipore, Billerica, MA, USA), was used for all aqueous solutions.

Stock solutions (10 mM) of thiol compounds were prepared in methanol and stored at 4 °C. These solutions were stable for at least 1 week and were diluted further with methanol to the required concentrations before use. Borate buffer (100 mM, pH 9) and diluted acetic acid [1% (v/v)] were prepared in water and stored at room temperature. The solutions of PIAA (2 mM in DMF), PMIA (2 mM in DMF), and tributylphosphine (TBP) [0.1% (v/v) in DMF] were prepared before use and used within one day.

2.2. Derivatization of thiols

The sample solution (40 μl) was placed in a 1.5-ml Reacti-vial (Pierce, Rockford, IL, USA), and 10 μl each of 100 mM borate buffer

(pH 9) and 0.1% TBP (v/v), and 40 μl of 2 mM PIAA (or PMIA) were then successively added. The vial was tightly sealed and was left at room temperature for 15 min. After derivatization, 10 μl of 1% acetic acid (v/v) and 500 μl of 50% methanol (v/v) were added to the vial. Twenty microliter of the reaction mixture was directly injected into the chromatograph. To prepare the reagent blank, a 40-μl aliquot of methanol was subjected to the same procedure.

2.3. Determination of LA

2.3.1. Pretreatment of LA supplement

LA tablets (racemate, 33 mg per tablet) were purchased from a city market in Fukuoka (Japan) and then were weighed and vigorously pulverized. The average amount (*ca.* 0.25 g) corresponding to one tablet of the resulting powder was then accurately weighed and dissolved in 3 ml of methanol. The supplement solution was diluted further with methanol to a concentration corresponding to 100 μM LA. The mixture was vortex mixed for a few seconds and immediately centrifuged at 1,000 × *g* for 5 min at 4 °C to remove protein. The supernatant was passed though a disposable filter (0.45 μm, 13 mm i.d., cellulose acetate; Millipore) and was then subjected to derivatization.

2.3.2. Pretreatment of urine sample

Urine samples were obtained from a healthy male volunteer (23 years old, 57 kg) before and at 1 h after the oral administration of two LA tablets (containing 33 mg LA per tablet). He understood the purpose and the importance of the experiments, and took the tablets of his own free will. The obtained urine samples were immediately diluted 5-fold with methanol, and the diluted urine samples were centrifuged and filtered in the same manner with the pretreatment procedure of LA supplement. The filtrate was immediately subjected to the derivatization.

2.4. LC with fluorescence detection

We used an isocratic LC system consisting of a Jasco (Tokyo, Japan) PU-2085 semi-micro LC pump, a Jasco AS-2057 intelligent autosampler, a Jasco LG-2080-02 low-pressure gradient unit, a Jasco DG-2080-53 3-line degasser, a reversed-phase Zorbax Eclipse Plus C_{18} column (150 × 4.6 mm i.d., 3.5 μm; Agilent, Palo Alto, CA, USA), and a Hitachi (Tokyo, Japan) L-7485 spectrofluorometer fitted with a 12-μl flow cell. The mixtures of acetonitrile: methanol: water: acetic acid (35:35:30:1, v/v) and acetonitrile: water: methanol: acetic acid (50:40:10:1, v/v) were used as the mobile phase for the preliminary examination and lipoic acid analysis, respectively. The flow rate was set at 1.0 ml/min, and the column temperature was ambient (23 ± 3 °C). The fluorescence detector was operated at the excitation and emission wavelengths of 345 and 485 nm, respectively, and the slit-widths of both the monochromators were set at 5 nm.

2.5. LC–MS system

A Thermo Fisher Scientific (San Jose, CA, USA) LCQ, ion-trap mass spectrometer equipped with an electrospray ionization (ESI) interface, was used in the place of a fluorescence detector. Other separation conditions were the same as those described in the previous section. The effluent from the LC column was directly introduced into the LC–MS interface without splitting. The ion source voltage and temperature of the heated capillary were set at 4.2 kV and 270 °C, respectively. The scan range was set at *m/z* 50–2000.

2.6. Fluorescence spectral characterization

Fluorescence spectral measurements were performed using a Hitachi (Tokyo, Japan) F-2500 spectrofluorometer in 10 × 10 mm quartz cells; a spectral bandwidth of 5 nm was used for both the excitation and emission monochromators.

2.7. Method validation

In order to obtain the validation parameters, peak areas integrated automatically were used for the quantification of all thiols. For the quantitative analysis of polythiol compounds, seven calibration solutions ($n = 3$ each) with a concentration range from 0.75 to 120 μM (0.75, 1.5, 3.8, 7.5, 15, 38, and 120 μM) were prepared by diluting the stock solutions. The equation of the calibration lines was calculated by least-squares linear. Precision (intra-day and inter-day) of the present method was determined throughout the full analytical procedures (sample dilution, derivatization, and LC separation) using the standard solutions (15 μM). The intra-day and inter-day precisions by using PIAA and PMIA were assessed by analysis five times on the same day and on five different days within one month, respectively. The detection limits were determined as the lowest concentration yielding a signal-to-noise ratio of 3.

3. Results and discussion

3.1. LC separation

In the preliminary experiment, we used DTT and BAL as model polythiol compounds. The derivatives of DTT and BAL were moderately retained on a reversed-phase column by using the acidic mobile phase. Generally, chromatograms obtained from the reaction mixture by using intramolecular excimer-forming fluorescence derivatization are so simple that acceptable separation is easily achieved. In this examination, a good separation of the PIAA or PMIA derivatives of thiols and reagent blank components (intermolecular excimer fluorescence peaks of the pyrene reagent [27,32,34]) were achieved within 30 min on an ODS column by using acetonitrile: methanol: water: acetic acid (35:35:30:1) as the mobile phase. Typical chromatograms obtained with a standard mixture of DTT and BAL derivatized with PIAA and PMIA are illustrated in Fig. 3. Regardless of the reagents used, DTT and BAL yielded respective

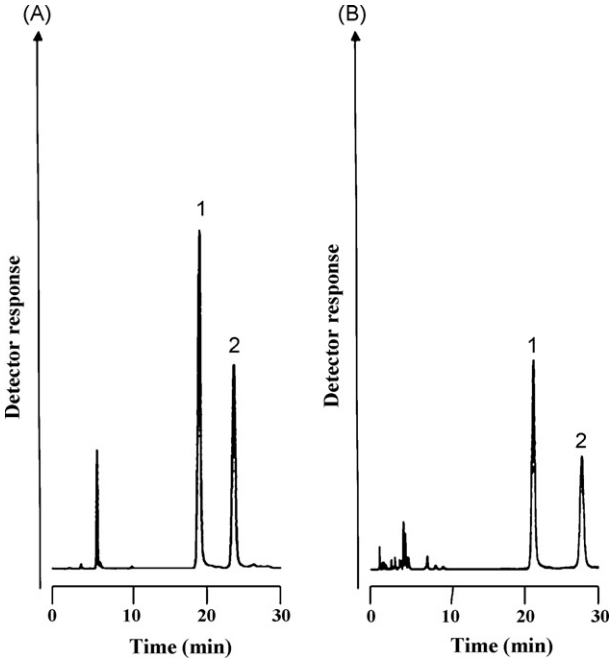

Fig. 3. Chromatograms obtained with the (A) PIAA- and (B) PMIA-labeled DTT and BAL (20 pmol each on column). Peaks: 1, DTT; 2, BAL; others, reagent blanks.

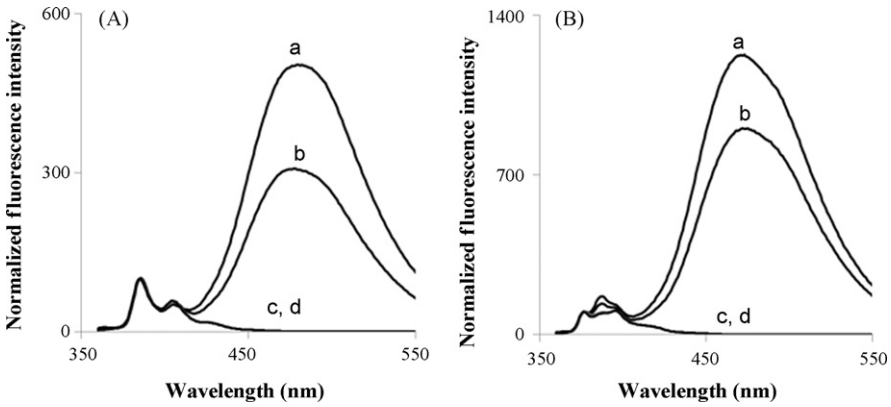

Fig. 4. Fluorescence emission spectra (excitation 345 nm) of the eluates for (A) PIAA- and (B) PMIA-labeled thiols. Spectra in A and B were normalized at 385 and 377 nm, respectively. Spectra: a, BAL; b, DTT; c, 6-mercaptohexanol; d, 1-octanethiol.

single peaks, and these peaks were well separated from each other and from the intermolecular excimer fluorescence peaks of the pyrene reagents. On the other hand, monothiols such as cysteine, glutathione, 2-mercaptoethanol, and 1-octanethiol did not show any peaks in excimer fluorescence detection (data not shown). Thus, this derivatization method permits easy optimization of separation conditions for analytes and highly selective determination of polythiol compounds in samples containing monothiols.

3.2. Excimer fluorescence from pyrene-labeled derivatives

Fig. 4 shows normalized fluorescent emission spectra of the LC peak components from pyrene-labeled polythiols and monothiols. As in the case with polypyrene-labeled derivatives obtained from other pyrene reagents [27–38], the PIAA (Fig. 4A) and the PMIA (Fig. 4B) derivatives of polythiols afforded excimer fluorescence, which was characterized by a structureless and broad peak around 480 nm. On the other hand, labeled monothiols (6-mercaptohexanol and 1-octanethiol) exhibited only the expected monomer fluorescence (<420 nm). The effect of solvent on the emission of excimer fluorescence was quite similar to that in the polypyrene-labeled derivatives of polythiols. All the derivatives showed intense excimer fluorescence in aqueous 50–90% (v/v) solutions of water-soluble organic solvents (THF, acetonitrile, methanol, and ethanol).

3.3. Optimum derivatization conditions

Optimization studies for PIAA and PMIA were carried out simultaneously to maximize the excimer fluorescence peak area by using DTT and BAL as model polythiol compounds. The optimum concentrations of the pyrene reagents were 0.5–5 mM in the respective reagent solutions; 2 mM was selected as the optimum. The derivatization reaction of the thiol group with an iodoacetamide-type reagent efficiently proceeded in the presence of a base and a water-miscible organic solvent. Of the tested bases [pyridine, triethylamine, potassium carbonate, and borate buffer (pH 7–13)], 100 mM borate buffer (pH 9.0) afforded maximum peak areas. As water-miscible organic solvents, methanol, ethanol, acetonitrile, acetone, THF, DMF, and DMSO were investigated. The maximum peak areas for all the thiols were obtained when DMF was used for the preparation of the pyrene reagent. The reaction proceeded more rapidly with increasing reaction temperature in the range 2–70 °C, and the peak areas for the thiols reached maxima after reactions of 30 min at 2 °C, 15 min at room temperature, 5 min at 40 °C, and 2 min at 70 °C. The derivatization reaction at room temperature for 15 min was selected for obtaining high sensitivity and reproducible results. Whereas, the addition of 0.1% TBP (v/v) to the reaction solution for the reduction of disulfide bond did not interfere with the derivatization reaction nor the stabilities of the other reagents.

The pyrene-labeled derivatives of the thiols in the final reaction mixture were stable and still showed constant fluorescence intensities after standing for at least 24 h in the dark at 4–25 °C.

3.4. Structural analysis by LC–MS

The structures of pyrene-labeled DTT and BAL were confirmed by LC–MS with the ESI interface in the negative-ion mode. As listed in Table 1, the selected ion chromatograms suggested that diPIAA- and diPMIA-labeled derivatives were formed from both DTT and BAL. Mass spectra for the peak components also provided the corresponding quasi-molecular ion ($[M-H]^-$) as their respective base peaks. When detected at the *m/z* ($[M-H]^-$) corresponding to the monopyrene-labeled derivatives of DTT and BAL and unlabeled DTT and BAL themselves, no significant peaks were observed in the

Table 1
Structural analysis of pyrene derivatives and validation of the presented method.

Pyrene reagent	Analyte	Structural analysis by LC–MS		Fluorescence detection			
		Molecular weight of dipyrene derivative	Detected base peak, *m/z* (corresponded ion)	Detection limit (fmol)[a]	r^2[b]	RSD (%)[c] (*n* = 5)	
						Intra-day	Inter-day
PIAA	DTT	668.8	667.4 ($[M-H]^-$)	2.1	0.996	3.3	4.8
	BAL	638.8	637.2 ($[M-H]^-$)	3.1	0.993	3.4	8.3
PMIA	DTT	696.9	695.2 ($[M-H]^-$)	3.3	0.994	2.1	6.7
	BAL	666.9	665.2 ($[M-H]^-$)	6.4	0.992	2.1	11.7

[a] Defined as the amount per 20-μL injection volume yielding a signal-to-noise ratio of 3.
[b] Correlation curve in the range of 1–160 pmol per 20-μL injection volume.
[c] Relative standard deviation of peak height in 20 pmol per 20-μL injection volume.

respective ion chromatograms. Thus, we concluded that all thiol moieties in DTT and BAL were derivatized quantitatively with the pyrene reagent under the present derivatization conditions, and that the dipyrene-labeled derivatives yielded the respective fluorescent peaks.

From these observations, we concluded that the present method is highly selective for polythiol compounds by measuring the intramolecular excimer fluorescence from their dipyrene-labeled derivatives.

3.5. Method validation (calibration graph, precision, and detection limits)

The validation data by using PIAA and PMIA are listed in Table 1. With both pyrene reagents, the relationships between the amounts of polythiols (DTT and BAL) and the peak heights were linear over the concentration range of 0.75–120 μM in the sample solution, which corresponded to 1–160 pmol per 20-μl injection volume. The linear correlation coefficients were more than 0.992. The intra- and inter-day precision values throughout the entire process were established by repeated determinations ($n = 5$ each) by using the mixtures of the thiols (15 μM each in a sample solution, 20 pmol each per 20-μl injection volume); the relative standard deviations were within 3.4 and 11.7%, respectively.

The detection limits (signal-to-noise ratio = 3) for polythiols by using PIAA and PMIA were less than 3.1 and 6.4 fmol per 20-μl injection volume, respectively. This intramolecular excimer-forming fluorescence derivatization method is more sensitive than the methods in which other fluorescence derivatization reagents for LA are used [23–26]. In the following examinations about LA analysis, PIAA was used for highly sensitive and reproducible analysis of polythiols.

3.6. α-Lipoic acid analysis

To investigate the practicality of this derivatization method in real sample analysis, two applications, the determinations of LA in commercial dietary supplements and in human urine, were conducted. LA and its amide form, LAM, were used for this experiment (Fig. 2). LA and LAM could not react with PIAA because their sulfur atoms exist in the oxidized disulfide form (-S-S-). Therefore, a reduction step was necessary prior to excimer fluorescence derivatization to thiol moiety. The addition of TBP solution did not interfere with the derivatization reaction or the stabilities of the other reagents and pyrene derivatives.

The best separation of the PIAA derivatives of LA, LAM, other urinary substances, and reagent blank components was achieved within 60 min on an ODS column by using acetonitrile: water: methanol: acetic acid (50:40:10:1) as the mobile phase. A typical chromatogram obtained with a standard mixture of LA and LAM is illustrated in Fig. 5A. On the other hand, as shown in Fig. 5B, no peaks from LA and LAM could be detected without performing the reduction procedure. The detection limits (signal-to-noise ratio = 3) for LA and LAM were 3.5 and 0.6 fmol per 20-μl injection volume, respectively; these values corresponded to analyte concentrations of 2.9 and 0.5 nM, respectively. In addition, this analysis method has good calibration curves and reproducibility.

Fig. 6 shows the calibration curves of LA from samples of the standard and samples of the supplement spiked with standard. The linearity of the calibration curves was validated with three different calibration curves ($n = 3$). Since almost the same linearity and slope were obtained from both curves, LA in the tablet seems to be quantitatively recovered and determined by the proposed procedure. The recovery calculated from the calibration curves was approximately 98.0 ± 3.3% ($n = 3$, mean ± SD). When the content in the LA tablet was determined on the basis of the calibration curve by the standard addition method, the value was 98.0 ± 5.6% ($n = 3$, mean ± SD) of the stated amount (33 mg per tablet).

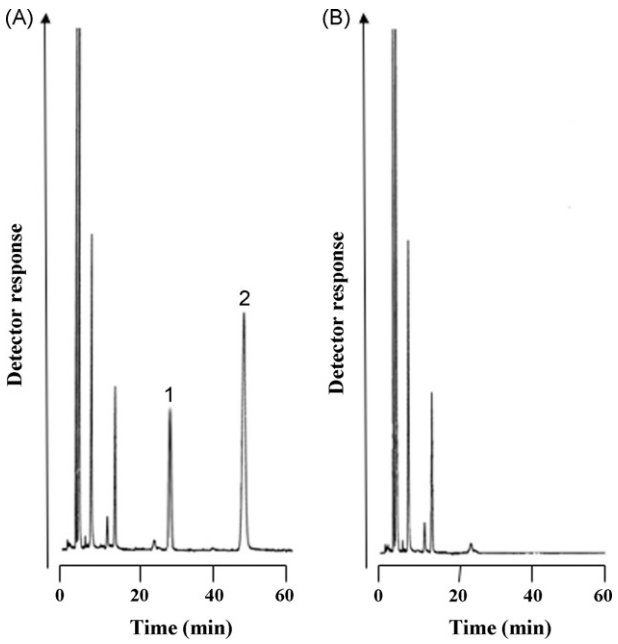

Fig. 5. Chromatograms obtained with the PIAA-labeled LA and LAM (A) with and (B) without reduction. Peaks and amounts (pmol on column): 1, LAM (13); 2, LA (130); others, reagent blanks.

In addition, the present method was applied to the analysis of LA in human urine as another application of biological analysis. In recent studies about LA determination in biological samples, pretreatment such as liquid–liquid extraction [22–26] and solid-phase extraction [39] have been utilized for the purpose of deproteinization and concentration of analytes. However, deproteinization by methanol was used in this study as the simplest pretreatment for biological samples. Fig. 7 presents the typical chromatograms obtained with the 5-fold diluted urine samples collected before and after oral administration of the LA tablets. Both the peak components of peaks 2A and 2B in Fig. 7A and B were identified as the single PIAA derivative of LA, on the basis of their retention times by a comparison with those in Fig. 5A, and also by co-chromatography with the use of various mobile phases. This intramolecular excimer-forming derivatization method was so selective that quite simple chromatogram was obtained even in the biological analysis. The concentrations of LA in urine samples collected before and after

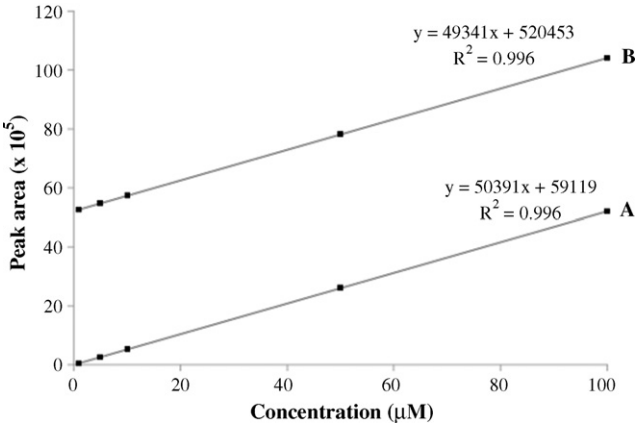

Fig. 6. Calibration curves for the determination of LA in a dietary supplement. Curves: (A) spiked to methanol, (B) spiked to the extract of an LA tablet.

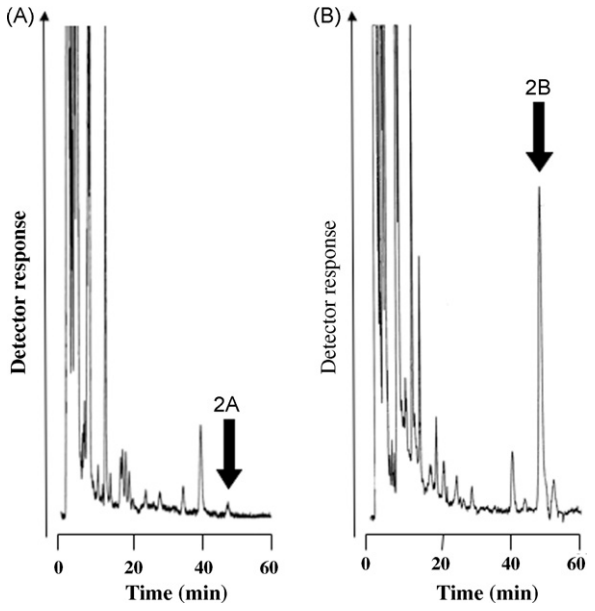

Fig. 7. Chromatograms obtained with urine samples collected (A) before and (B) at 1 h after oral administration of LA tablets. Peaks and LA concentrations (nmol/ml urine): 2A, (1.8); 2B, (43).

the supplement intake were determined to 1.8 and 43 nmol/mL urine, respectively, by using the absolute calibration method. This derivatization method was so sensitive that LA in human urine collected before LA administration could be detected without special pretreatments such as solid-phase extraction.

4. Conclusions

By using the present intramolecular excimer-forming fluorescence derivatization method, polythiol compounds were found to be converted to the respective polypyrene-labeled derivatives and could be detected with high sensitivity and selectivity after LC separation. The derivatization conditions of the polythiol group with *N*-(1-pyrene)iodoacetamide were optimized in order to achieve maximal fluorescence yields of the resulting intramolecular excimers. This optimized method was successfully applied to the determination of not only standards of dithiothreitol, dimercaprol, α-lipoic acid, and lipoamide but also α-lipoic acid in a commercial dietary supplement and in human urine without extraction or concentration.

This method might be useful for biomedical and clinical investigations of the above-examined compounds as well as other polythiol and disulfide compounds. In addition, the introduction of ultra-fast LC to this method might be effectable for rapid analysis of them. These studies are currently in progress.

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