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Simultaneous determination of α-lipoic acid and its reduced form by high-performance liquid chromatography with fluorescence detection

Soichiro Satoh, Toshimasa Toyo'oka*, Takeshi Fukushima, Shinsuke Inagaki

Division of Bio-Analytical Chemistry, School of Pharmaceutical Sciences, and COE Program in the 21st Century, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan

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Abstract

The simultaneous determination of α-lipoic acid (LA) and DHLA (reduced form of LA) was carried out by HPLC with fluorescence detection. DHLA in the sample was first labeled with ABD-F at room temperature for 10 min and then the LA was labeled with SBD-F at 50 °C for 1 h after conversion to DHLA using the reducing agent, TCEP. The resulting fluorophores, ABD-DHLA and SBD-DHLA, were separated by reversed-phase chromatography and detected at 510 nm (excitation at 380 nm). Both fluorophors were completely separated without any interference of endogenous thiols and disulfides in the sample and sensitively detected by fluorimetry. The proposed method was applied to the assay of the LA supplement and the determination in human plasma after the oral administration of LA tablets. The concentration (%) of LA in the tablet was reasonable to the stated amount. Furthermore, the result of a time course study in the plasma after the administration of LA did not differ from a previous report. Thus, the present method seems to be applicable to the simultaneous determination of LA and DHLA in various biological specimens.

Keywords: α-Lipoic acid (LA); Dihydrolipoic acid (DHLA); Thiols; Disulfides; 4-(Aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F); Ammonium 4-fluoro-2,1,3-benzoxadiazole-7-sulfate (SBD-F); Fluorescence; HPLC

1. Introduction

α-Lipoic acid (LA: 1,2-dithiolane-3-pentanoic acid) (Fig. 1) is a disulfide compound that is found naturally in mitochondria as the coenzyme for pyruvate dehydrogenase and ketoglutarate dehydrogenase [1–4]. LA is covalently bound to ε-NH_2 group in a lysine residue. It is found in most prokaryotic and eukaryotic microorganisms, as well as many plant and animal tissues [5]. The cellular reduction of LA to dihydrolipoic acid (DHLA) has been reported in various mammalian cells and tissues [6–11]. Due to its negative redox potential, DHLA possesses the capability of regenerating endogenous antioxidants such as vitamin E and glutathione [12–14]. LA and DHLA (reduced form of LA) 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 [12]. LA is an ideal antioxidant that acts in the membrane (hydrophobic phase) on the basis of the lipophilicity of LA and in the aqueous phase on the basis of the hydrophilicity of its reduced form, DHLA. Due to its antioxidant activity, LA has been shown to be beneficial in various forms of oxidative stress, and is also of interest as a therapeutic agent in numerous disorders ranging from diabetes to AIDS [15]. LA is also found to be a potent antioxidant in various drug-induced toxicities in an experimental rat model [16–18]. Hence, the determination of LA and DHLA in biological systems is important for the evaluation of its efficiency.

As described in the review by Kataoka [19], the detection of LA is possible by GC after solvent extraction from biological specimens [20,21]. However, the determination is mainly carried out by HPLC with electrochemical detection [22] and, more selectively, using dual gold–mercury electrodes [23]. The procedure using this electrode is highly sensitive, but the

* Corresponding author. Tel.: +81 54 264 5656; fax: +81 54 264 5593.
E-mail address: toyooka@ys2.u-shizuoka-ken.ac.jp (T. Toyo'oka).

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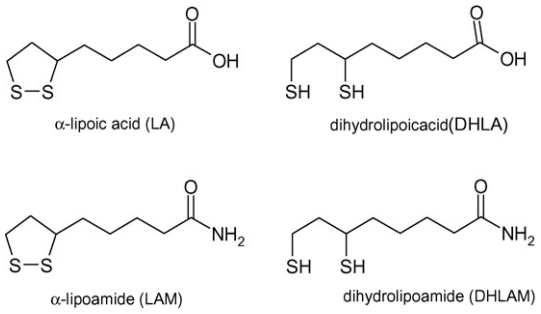

Fig. 1. Structures of LA, DHLA, LAM and DHLAM.

linearity of the concentration–response curve is only in a narrow concentration range. Furthermore, laborious reconstitution of the electrode is required to obtain repeatable results. Fluorometry is an alternative means for the detection of LA and DHLA [24]. Pre-column derivatization with *o*-phthalaldehyde and phenylalanine has been reported, but the differentiation between LA and DHLA was not described in the papers [25,26]. Another approach is the use of a thiol-reactive monobromobimane (mBBr) for pre-column derivatization. The reagent readily and quantitatively forms relatively stable fluorescence derivatives with various thiols under mild reaction conditions [27–29]. However, the direct determination of both the oxidized form (LA) and the reduced form (DHLA) could not be performed by this method utilizing mBBr. In this method, DHLA was first determined by HPLC with fluorescence detection after labeling with mBBr, while the concentration of LA was calculated by the subtraction of free DHLA from the total DHLA, which includes both intrinsic DHLA and DHLA obtained from the reduction of LA. As a simultaneous determination of LA and DHLA, Haj-Yehia et al. [30] report a fluorescence labeling method using 2-(4-aminophenyl)-6-methylbenzothiazole by HPLC. Although the method is highly sensitive and reproducible, the specificity to LA and DHLA seems to be less because the fluorescence reagent reacts with a carboxylic acid (−COOH) to form amide derivatives. Various compounds possessing −COOH group(s) could thus possibly react with the reagent. The aim of the present research is to develop a selective, sensitive and simultaneous determination method for LA and DHLA by HPLC with fluorescence detection. The application to food supplement and human plasma is also described in this paper.

2. Experimental

2.1. Materials and reagents

4-(Aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F) [31,32], ammonium 4-fluoro-2,1,3-benzoxadiazole-7-sulfate (SBD-F) [33,34], glutathione (reduced form: GSH) and 2-mercaptoethyl octanonate (MEO) were purchased from Wako Pure Chemicals (Osaka, Japan). (±)-α-Lipoic acid (LA), tris(2-carboxyethyl) phosphine hydrochloride (TCEP) and ethylenediaminetetraacetic acid disodium salts (EDTA·2Na) were obtained from Tokyo Kasei (Tokyo, Japan). DHLA, (±)-α-lipoamide (LAM), glutathione (oxidized form: GSSG), L-cystine (CySSCy), L-cysteine (CySH), *N*-acetyl-L-cysteine (NAC) and DL-homocysteine (HCySH) were from Sigma-Aldrich (St. Louis, MO). Trifluoroacetic acid (TFA), acetonitrile (CH_3CN), acetone and ethylacetate (AcOEt) were of special reagent grade (Kanto Chemicals, Tokyo, Japan). All other chemicals were of analytical-reagent grade and were used without further purification. De-ionized and distilled water was used throughout.

2.2. HPLC

A Shimadzu (Kyoto, Japan) HPLC system consisting of two LC-10AD pumps, an SCL-10A system controller, an auto-injector (SIL-10A_{XL}) and a degasser (DGU-12A) was used. The analytical column for reversed-phase chromatography was a 5-μm particle size ULTRON VX-ODS (150 mm × 4.6 mm i.d.; Shinwa Chemicals, Kyoto, Japan) with a guard column GL CARTO (Inertsil ODS-3, 5 mm × 4.6 mm i.d., 5 μm; GL Sciences, Tokyo, Japan). The columns were maintained at 40 °C by a CTO-10A column oven (Shimadzu). The effluent was monitored by SPD-10AV_{VP} UV–vis and RF-10A_{XL} fluorescence detectors equipped with an 8-μL flow cell (Shimadzu). The fluorescence detector was set at 380 nm (excitation) and 510 nm (emission). A linear gradient elution from H_2O–CH_3CN (95:5) containing 0.1% TFA to H_2O–CH_3CN (5:95) containing 0.1% TFA over 40 min was adopted. The flow rate of the mobile phase was 1.0 mL/min. The peak areas obtained from both detectors were calculated using Vstation chromatography software (GL Science, Tokyo, Japan).

2.3. Stock solutions

As the stock solutions, 1-mM concentrations of CySSCy, CySH and HCySH were prepared with 0.1 M hydrochloric acid (HCl). The other thiols and disulfides including LA, DHLA and LAM were prepared at 10 mM concentrations with distilled water containing 1 mM EDTA·2Na. The stock solutions were stored in a refrigerator at 5 °C just prior to use. The reducing reagent (TCEP) [35] and fluorescent labeling reagents (ABD-F and SBD-F) were dissolved just before the reaction.

2.4. Simultaneous determination of LA and DHLA

The stock solutions of thiols and disulfides were diluted to 120-μM concentrations with 0.1 M sodium tetraborate (pH 9.3) containing 2 mM EDTA·2Na. To 400 μL of the diluted solution, 200 μL of CH_3CN containing ABD-F (25 mM) was added and the mixture was allowed to stand for 10 min at room temperature. After the labeling reaction of the thiols, 1 mL of AcOEt was poured into the solution and the two-phase solutions were vigorously mixed and then centrifuged at 3000 rpm for 5 min. The aqueous phase was separated and the organic solvent was removed. The extraction procedure was repeated two additional times. To 600-μL portions of the aqueous solution, a 100 μL H_2O solution of SBD-F (40 mM) and an equal volume of TCEP

(160 mM), dissolved with 0.1 M sodium tetraborate (pH 10.0) containing 2 mM EDTA·2Na, were added and the mixture was heated at 50 °C for 1 h. After the reductive labeling of the disulfides with SBD-F, 10 μL of the reaction mixture was diluted with 350 μL of the dilution solution (CH_3CN:H_2O = 1:1). Ten microliter of the solution was then injected into HPLC.

For the determination of thiol compounds, a 10-μL portion of the solution that had reacted with ABD-F was diluted with 470 μL of the dilution solution (CH_3CN:H_2O = 1:1) and a 10-μL portion of this solution was injected into HPLC.

2.5. Determination of LA in supplement

Ten tablets containing LA (racemate, 20 mg per tablet), which were purchased from a city market, 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 transferred to a vial. Five milliliter of acetone including LA (0, 8.0, 20 and 32 mM) was added to the vial, ultrasonicated for 20 min and subsequently centrifuged at 3000 rpm for 5 min. The clean supernatant was transferred to a round-bottom flask. The same extraction procedure was repeated two additional times. The combined extracts were evaporated to dryness under stream of nitrogen gas. The resulting residues were re-constituted with 10 mL of CH_3CN and filtered through a 0.45-μm membrane. The solution was diluted to 100 times with 0.1 M sodium tetraborate (pH 9.3) containing 2 mM EDTA·2Na.

The diluted solution (300 μL) was reacted with 50 μL of ABD-F (40 mM) and 50 μL of TCEP (160 mM in 0.1 M sodium tetraborate (pH 10.0) containing 2 mM EDTA·2Na) at room temperature for 10 min. A 20-μL portion of the reaction mixture was diluted with 280 μL of dilution solution (CH_3CN:H_2O = 1:1) and then an aliquot (10 μL) was injected into HPLC. The final concentrations of LA spiked were 0–80 pmol/10 μL injection.

2.6. Calibration curve for the determination of LA in supplement

A series of working solutions of LA (2, 20, 60 and 200 μM) were prepared from the stock solution with the addition of 0.1 M sodium tetraborate (pH 9.3) containing 2 mM EDTA·2Na. Each solution (300 μL) was reacted with 50 μL of ABD-F (40 mM) in the presence of 50 μL of TCEP (160 mM) at room temperature for 10 min, as described in Section 2.5. A 20-μL aliquot of the reaction mixture was then diluted with 280 μL of dilution solution (CH_3CN:H_2O = 1:1). Each 10-μL portion of the solutions was subjected to HPLC and detected by fluorimetry. The peak areas of the fluorescent derivative were plotted against the concentrations of LA. The injected concentration range was 1–100 pmol (n = 3).

2.7. Determination of LA in plasma after supplement intake

Healthy male volunteers (n = 5, age 22–26 years, body weight 63.4 ± 8.1 kg) were fasted for 12 h (overnight) before LA administration. The fasting state was maintained till the end of blood collections. All subjects gave written informed consent on entry into this study and the study protocol was approved by the Ethics Committee of the University of Shizuoka.

Six tablets containing LA (20 mg per tablet) were administered with tap water (100 mL) at 9 a.m. At a fixed time interval, 2.5 mL of blood was drawn from an arm vein before (0 min) and after administration of the LA supplement (10, 30, 60, 120 and 180 min), and the blood sample was then collected into the tubes containing EDTA·2Na as an anticoagulant. The samples were immediately centrifuged at 3000 rpm for 10 min. The supernatant plasma was transferred to clean tubes and kept at −80 °C until analyzed.

Each 500-μL portion of the plasma was added to a 100 μL CH_3CN solution of MEO (10 μM) used as the internal standard (IS). The solution was then acidified with 80 μL of 10% TFA. A 700-μL aliquot of AcOEt was added to the solution for the extraction of LA and MEO (IS). The solution was vigorously mixed and centrifuged at 3000 rpm for 5 min. The extraction treatment was repeated two additional times and the combined organic solvent was evaporated to dryness under reduced pressure. To the residues, 200 μL of ABD-F (15 mM), 100 μL of TCEP (120 mM) and 300 μL of 0.1 M sodium tetraborate (pH 9.3, 2 mM EDTA·2Na) were added, and the mixture was allowed to stand for 10 min at room temperature. After reductive fluorescence labeling, the reaction mixture was filtered through a 0.45-μm membrane. Ten microliter of each filtrate was then subjected to HPLC.

The plasma LA concentration–time data were subjected to a non-compartmental analysis using the nonlinear least-squares regression program WinNonlin (Pharsight, Cary, USA). The maximum plasma concentration (C_{max}) and times to peak (t_{max}) were taken directly from the plasma concentration–time profiles. The terminal half-lives ($t_{1/2}$) were obtained by linear regression analysis of the last data points after log transformation of the data by means of the WinNonlin software. The area under the curve values (AUC_{last}) were determined using trapezoidals from time 0 to the last quantifiable concentration (C_{last}). The area under the curve values through infinite time (AUC_∞) were calculated by adding $C_{last}/\lambda z$ to AUC_{last}.

2.8. Calibration curve for the determination of LA in plasma

A series of LA solutions (1.2, 24, 60 and 120 μM) were prepared from the stock solution by adding 0.1 M sodium tetraborate (pH 9.3) containing 2 mM EDTA·2Na. Each 450-μL portion of the blank human plasma (LA-free plasma) before administration of the LA tablets was added to 50 μL of the LA solutions (1.2–120 μM) and 100 μL of MEO (10 μM). The solutions were treated, reacted and analyzed by HPLC with FL detection, as described in Section 2.7. The peak area ratios of LA to MEO were plotted against the concentrations of LA. The injected concentration range (n = 3) was 1–100 pmol (IS concentration, 16.7 pmol).

The absolute calibration curve was also drawn from the peak areas against the concentrations of LA (injection concentration 1–100 pmol).

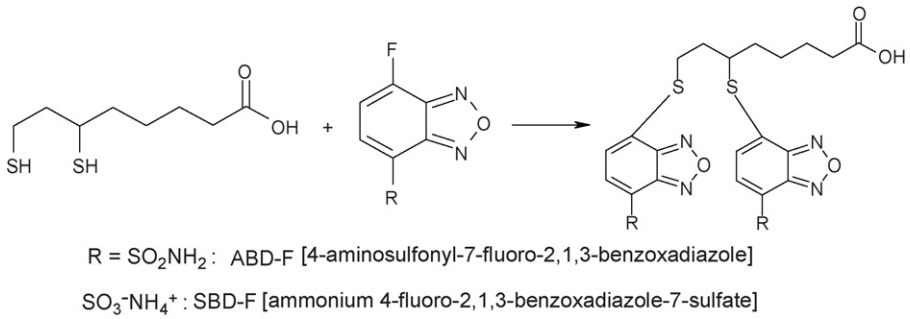

Fig. 2. Fluorescence labeling reactions of DHLA with ABD-F and SBD-F.

3. Results and discussion

In our laboratory, fluorigenic reagents, i.e. SBD-F [33,34] and ABD-F [31,32], have been developed for the sensitive and selective determination of thiols. These reagents are now commercially available and purchased from reagent company in this research. The derivatization reactions with these reagents are relatively mild and the resulting derivatives fluoresce in a long-wavelength region (emission at 510 nm, excitation at 380 nm). The methods using these reagents have been applied to the determination of biological thiols and thiol-containing drugs. The simultaneous determination of thiols and disulfides in biological specimens has also been reported in previous papers [36,37]. Briefly, thiols are first labeled with ABD-F and then the unre-

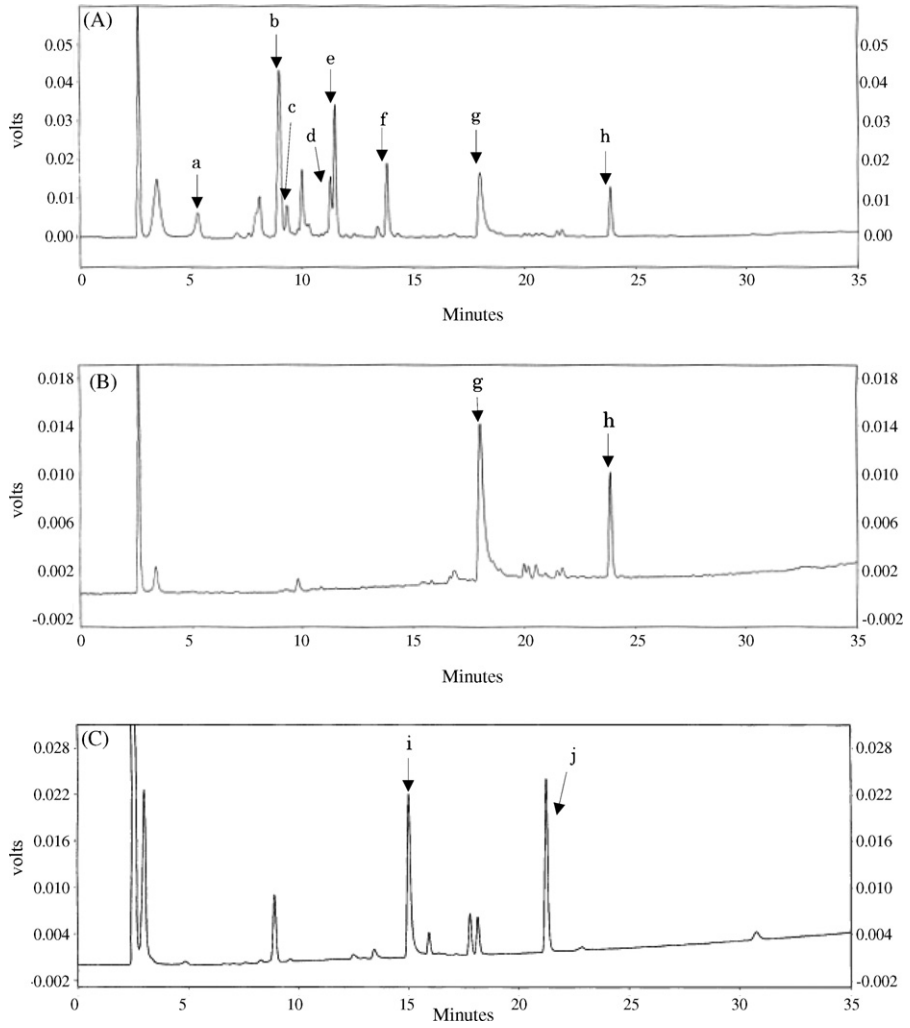

Fig. 3. Chromatograms of thiols and disulfides obtained after labeling with the fluorescent reagents, ABD-F and SBD-F. Chromatograms: (A) derivatives of thiol and disulfide compounds; (B) derivatives of DHLA and LA; (C) derivatives of DHLAM and LAM. Peaks: (a) SBD-CySH; (b) SBD-GSH; (c) ABD-CySH; (d) ABD-GSH; (e) ABD-HCySH; (f) ABD-NAC; (g) SBD-DHLA; (h) ABD-DHLA; (i) SBD-DHLAM; (j) ABD-LAM. HPLC conditions are shown in Section 2.

acted disulfides are labeled with SBD-F in the presence of a reducing agent. Thus, the simultaneous determination of LA and DHLA seems to be carried out by this strategy.

The reactivities of ABD-F and SBD-F to DHLA were tested in advance (Fig. 2). The recommended labeling conditions using ABD-F and SBD-F were different in each reagent: room temperature for 10 min for ABD-F and 50 °C for 1 h for SBD-F in an aqueous alkaline solution (pH 9.3). Although the suitable reaction conditions were different in both reagents, DHLA was completely labeled with these reagents and the resulting derivatives showed high fluorescence intensity.

The simultaneous determinations of LA and DHLA in the presence of endogenous thiols and disulfides were investigated next. The labeling procedures of LA and DHLA are as follows. DHLA in the sample was initially labeled with ABD-F. After extracting out the excess amount of ABD-F, LA was reduced to DHLA with TCEP (a reducing agent) and then labeled as a fluorescence derivative with SBD-F. Fig. 3 shows the typical chromatograms obtained from the thiols/disulfides mixture (chromatogram A), LA/DHLA (chromatogram B) and LAM/DHLAM (reduced form of LAM) (chromatogram C). The peaks corresponding to DHLA, LA, DHLAM and LAM were completely separated from the endogenous thiols and disulfides. The limits of detection (LOD) of LA, calculated from signal-to-noise ratio (S/N) on the chromatogram, are approximately 0.3 pmol (S/N = 3). On the contrary, the limits of quantification (LOQ) are less than 1.0 pmol (S/N = 10). Thus, the simultaneous determination of DHLA and LA seems to be possible by the present procedure.

As one of the applications, the assay of LA in a supplement was carried out by the present method. In a preliminary experiment, DHLA was not included in the tablet (data not shown). Therefore, the determination of LA in the tablet was performed by labeling using ABD-F in the presence of TCEP because the labeling reaction with ABD-F was faster than that with SBD-F. Fig. 4 shows the calibration curves of LA. Curve A is obtained from LA dissolved in CH_3CN, while curve B is of the samples spiked in the powder of the LA tablet. The linearity of the calibration curves was validated with three different calibration curves ($n = 3$). Since almost the same linearity is obtained from both curves, LA in the tablet seems to be quantitatively recovered by the proposed procedure. The recovery calculated from the calibration curves was approximately 97.0 ± 2.7% ($n = 3$, mean ± RSD). When the content in the LA tablet was determined on the basis of the calibration curve by the standard addition method, the value was 95.7 ± 11.7% ($n = 3$, mean ± RSD) of the stated amount (20 mg per tablet).

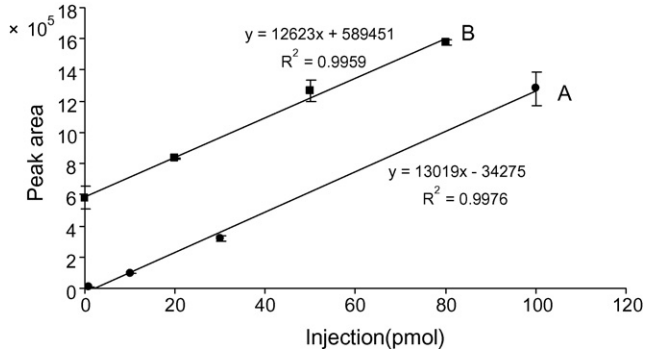

Fig. 4. Calibration curves for the determination of LA in tablet. (A) Spiked to acetonitrile. (B) Spiked to extract of LA tablet.

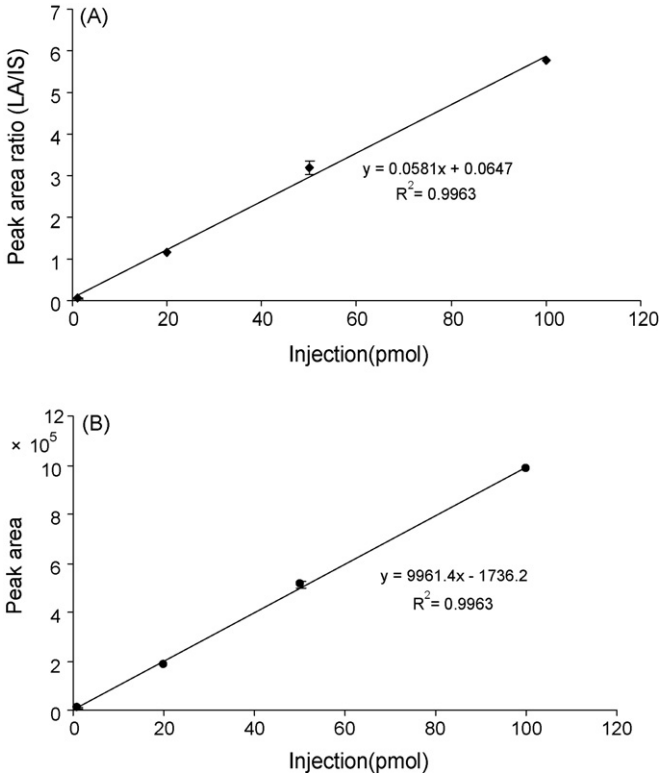

Fig. 5. Calibration curves for the determination of LA in plasma. (A) Internal standard method. (B) Absolute method.

The concentration in the plasma after the oral administration of the LA tablet was determined as another application of the present method. The calibration curves by internal standard and absolute methods were determined for the plasma sample analysis. The linearity of the calibration curve was validated with three different calibration curves ($n = 3$). As shown in Fig. 5A, a good linearity was achieved from the calibration curve, obtained by plotting the peak area ratios of LA relative to the MEO (IS) against the injected amounts of LA spiked in the LA-free plasma. The recovery calculated from the IS method at 2.4 μM and 12 μM concentrations, spiked to human plasma, was 95.4 ± 3.4% and 98.3 ± 1.2% ($n = 3$, mean ± RSD), respectively. A good linearity and similar recovery (%) were also obtained from the absolute calibration method.

A typical chromatogram obtained from the plasma after the single oral administration of the LA tablets is also shown in Fig. 6. The peaks corresponding to LA and MEO were completely separated without any interference of the endogenous compounds. Judging from the chromatogram, the concentration of LAM in plasma after LA administration seems to be negligible and thus the determination of LAM was not performed in the present research. Fig. 7 shows the LA concentration–time curve in human plasma after single oral administration ($n = 5$). The error bar at each sampling time shows the SD value. The

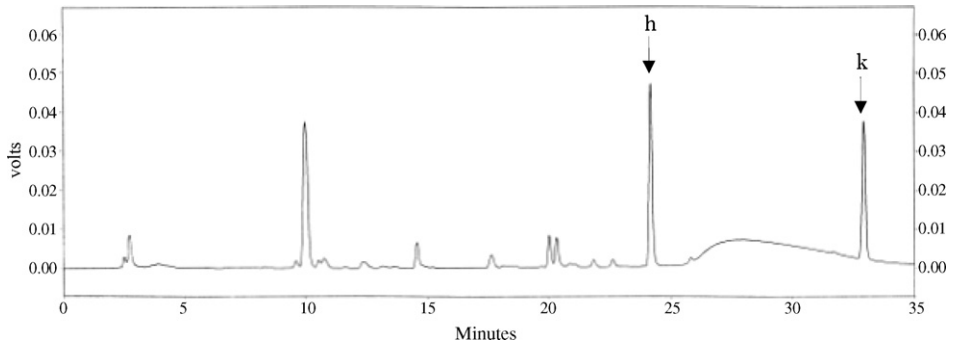

Fig. 6. Typical chromatogram obtained from human plasma 30 min after the single oral administration of the LA tablets. Peaks: (h) ABD-DHLA; (k) ABD-MEO. HPLC conditions are shown in Section 2.

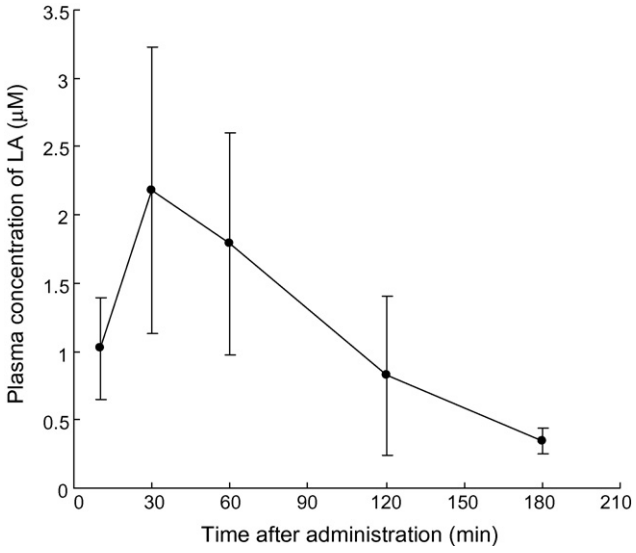

Fig. 7. Mean plasma concentration–time curve after single oral administration of 120 mg LA (six tablets). The concentrations of LA were obtained from healthy male volunteers ($n = 5$). The error bar at each sampling time means the SD value ($n = 5$).

shape of the curve was almost comparable to that of a previous report [26]. The mean pharmacokinetic parameters of LA are summarized in Table 1. The maximum plasma concentration, C_{max} (2.23 ± 0.90 nmol/mL), and the time of occurrence, t_{max} (42.0 min), found in this study appear comparable to that previously reported in fasted volunteers [38]. The proposed method seems to be applicable not only for human plasma but also for various biological specimens such as urine and tissues. Further studies are currently in progress in our laboratory.

Table 1
Pharmacokinetic parameters of LA after single oral administration of LA (120 mg) to healthy male volunteers ($n = 5$)

Parameters	Mean ± SD
$t_{1/2}$ (min)	55.04 ± 15.27
C_{max} (nmol/mL)	2.228 ± 0.9014
t_{max} (min)	42.00 ± 16.43
AUC_{last} (min nmol/mL)	210.1 ± 89.71
AUC_{∞} (min nmol/min)	238.3 ± 91.12

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