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Determination of lipoic acid and dihydrolipoic acid in human plasma and urine by high-performance liquid chromatography with fluorimetric detection

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Abstract

A highly sensitive method for the determination of α-lipoic acid (LA) and dihydrolipoic acid (DHLA) in human plasma and urine has been developed. Samples were acidified and extracted with organic solvent, and the free sulfhydryls of DHLA protected as the dicarboxyethylate by treatment with ethylchloroformate. The free carboxylic function of LA and the SH-protected DHLA were converted into their amide derivatives with the strong fluorophore 2-(4-aminophenyl)-6-methylbenzothiazole in the presence of a coupling agent and a base catalyst. The resulting fluorescent amides of both LA and DHLA were separated on a reversed-phase column (Ultrasphere C_8) using simple isocratic elution with acetonitrile–water (80:20) and detected fluorimetrically (excitation 343, emission 423 nm). The method is highly sensitive, reproducible, and is easily applied for the simultaneous determination of LA and DHLA in biological samples.

Keywords: Derivatization, LC; Lipoic acid; Dihydrolipoic acid; Thiols; Aminophenylmethylbenzothiazole

1. Introduction

An intense interest in the biological activity of α-lipoic acid (LA) and dihydrolipoic acid (DHLA) has characterized the last decade since the observation that together (i.e., LA⇔DHLA) they constitute a redox couple with properties approaching the 'ideal' or 'universal' antioxidant. LA itself (known chemically either as 1,2-dithiolane-3-valeric acid or 6,8-dithiane octanoic acid, or more commonly as 6,8-thioctic acid) is an abundant naturally occurring compound with well established biological properties. These include: an accessory growth factor for a wide variety of microorganisms [1], a coenzyme in the glycine cleavage system [2,3], and a coenzyme in the dehydrogenase complex [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 DHLA in various tissues [5–7]. The reducing power for this reduction comes from both NADH and NADPH [8]. The DHLA thus formed is also exported from cells and can provide antioxidant protection to the extracellular compartment and to nearby cells. Thus, the remarkable ability of the LA⇔DHLA interconversion to affect cellular reducing capacity via modulation of NADH/

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NAD^+ and $NADPH/NADP^+$ ratios and other routes demonstrates how intimately LA is connected to cell metabolism and redox states. As such, LA has gained special interest as a therapeutic antioxidant. LA scavenges hydroxyl radicals, hypochlorous acid, peroxyl radicals, and singlet oxygen [9–11]. It also chelates iron, copper, and other transition metals [12–14]. In addition to those species acted upon by LA (including transition metals), DHLA also scavenges superoxide radicals and peroxyl radicals [15,16]. Because of these characteristics and its very low toxicity, LA has been successfully used as a therapeutic agent in human clinical trials for the treatment of conditions such as diabetes, ischemic-reperfusion injury, heavy metal poisoning, mitochondrial cytopathies, neurodegenerative disorders, radiation damage, and human immunodeficiency virus (HIV) infection [17,18].

At the analytical level, however, less attention has been devoted to the detection and quantitation of LA and DHLA, especially simultaneously (for a recent review and concise discussion, see Ref. [19]). So far, gas chromatography (GC) following extraction from biological material and conversion into a volatile derivative has been the method of choice in most studies [10–22]. However, only LA is determined by this method. Determination of LA and DHLA by HPLC with electrochemical detection has offered a major advantage over the GC method in that not only is the sensitivity increased, but also because simultaneous detection of LA and DHLA is feasible [23–25]. The use of a dual gold–mercury electrode offers an even more sensitive and selective method of detection [26]. However, with many of these methods the calibration curve is only linear in a narrow concentration range, and, especially in the dual electrode method, a laborious reconstitution of the electrodes is necessary.

Recently, progress was made where LA was determined by HPLC with fluorimetric detection after pre-column derivatization with monobromobimane [27]. This method is certainly superior to previous methods from the range of linearity point of view. However, it is neither less laborious nor offers the advantage of simultaneous determination of LA and DHLA in the same sample. In fact, the levels of DHLA can only be indirectly estimated after treatment of the sample with the S-alkylating agent *N*-ethylmaleimide to differentiate between the reduced and oxidized forms of LA. In addition to these limitations, the stability of the fluorescent adduct was not satisfactory and presented, at least in our hands, a serious limitation against automatization of this sensitive analytical procedure.

Here we report that conversion of LA and dicarboxyethyl-DHLA into their very stable fluorescent amide derivatives with a coupling agent and a base catalyst offers a novel, sensitive, and convenient HPLC method for the simultaneous determination of LA and DHLA in biological samples.

2. Experimental

2.1. Chemicals and materials

2-(4-Aminophenyl)-6-methylbenzothiazole (the fluorescent reagent) was purchased (as brown plates) from Aldrich (Milwaukee, WI, USA) and was recrystallized from chloroform (white prisms) prior to use. LA was obtained from Merck (Darmstadt, Germany). For standardization, DHLA was freshly synthesized by reduction of LA by sodium borohydride. This DHLA was shown to possess all chemical and spectral properties of standard, commercially available DHLA (Sigma, Israel). Ethylchloroformate and other standard chemicals were of the highest analytical grade available and were purchased from Merck. The coupling (dehydrating) agent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) and all base catalysts used in this work were obtained from Aldrich. All solvents (and water) were of HPLC grade (Merck) and the mobile phase was passed through a 4 micron filter and degassed under vacuum prior to use.

Human plasma and urine were obtained either from healthy volunteers or diabetic patients dosed with commercially available LA capsules or tablets. Blank (drug-free) plasma and urine were obtained from the same individuals either before the start of LA dosing or 48 h after the last dose of LA. Blood was withdrawn into precooled EDTA tubes and separated within 5 min of withdrawal (4000 *g*, 4°C). Separated plasma (or collected urine) was either immediately processed as described below or frozen in liquid nitrogen and stored at −70°C until analysis.

A stock solution of LA was prepared by dissolving 10 mg of LA in 1 ml of pyridine and subsequent dilution to 10 ml with acetonitrile. The stock solution of DHLA was freshly prepared by reducing a known amount of LA with sodium borohydride in aqueous solution (0.05 *M* NaOH) maintained under a nitrogen atmosphere. This solution was found to be stable for several hours at room temperature and for longer periods at −20°C. The reagent solution (0.3%, w/v) was prepared by dissolving 3 mg of the reagent in 3 ml of pyridine, to which 700 mg of 4-dimethylaminopyridine (the base catalyst) was added, and subsequent dilution to 10 ml with acetonitrile. A solution of the coupling agent was prepared by dissolving 700 mg of EDAC in 10 ml acetonitrile. A solution of the internal standard (decanoic acid) was prepared by dissolving 10 mg of the acid in 10 ml of acetonitrile. A 100 μl aliquot of this solution was added to all samples before derivatization.

2.2. Reduction of LA to DHLA

Ten milligrams of LA is dissolved in 1 ml of a 0.05 *M* aqueous solution of NaOH containing 50 mg/ml of $NaBH_4$. The mixture is incubated at 60°C for 10 min, following which 0.4 ml of ethylchloroformate is added and the mixture shaken for an additional 15 min at room temperature. Under these conditions, quantitative conversion of DHLA into *S*,*S*-dicarboxyethyl-DHLA was observed by thin layer chromatography and NMR analysis. The reaction mixture is then acidified with 2 *M* HCl to pH<1 and extracted twice with 3 ml of *n*-hexane. The combined extracts were washed with 1 ml of 0.2 *M* HCl and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in acetonitrile to give 1 mg/ml. A 100 μl aliquot of this solution was used for the derivatization procedure.

2.3. Derivatization of LA and DHLA

The derivatization reaction proceeds as described in Fig. 1. To 100 μl each of LA and *S*,*S*-dicarboxyethyl-DHLA solutions in acetonitrile prepared as described in Sections 2.1 and 2.2, respectively, 100 μl of the coupling agent was added. The mixture was incubated at 60°C for 5 min, following which 100 μl of the reagent (fluorescent amine) solution containing the base catalyst was added and incubation continued at the same temperature for an additional 30 min. After cooling to room temperature, 10 μl of the resulting amide derivatives was directly injected into the chromatograph.

2.4. Extraction of LA and DHLA from plasma and urine

Blank plasma (or urine) was spiked with known amounts of LA and DHLA and mixed with 0.2 ml of 2 *M* HCl solution followed by the addition of 1.25 ml of dichloromethane. The resulting mixture was vortex mixed for a few seconds and immediately centrifuged at 4000 *g* (4°C) for 5 min to remove protein. The dichloromethane layer was then separated and evaporated to dryness under nitrogen. The residue was treated with ethylchloroformate and NaOH as described in Section 2.2. After completion of carboxyethylation, the reaction was acidified and extracted with *n*-hexane. After evaporation of the hexane extracts, the residue was reconstituted with acetonitrile containing the internal standard (decanoic acid) and derivatized as described above.

2.5. Extraction recovery and assay reproducibility

The recoveries of LA and DHLA from plasma and urine (drug-free samples) were determined at four different concentrations of both acids, separately and simultaneously, with recovery calculated as the mean of six replicates at each concentration. Known amounts of LA and DHLA were added to blank plasma (or urine), extracted and derivatized as described above. The detector response of both acids in extracted and derivatized spiked samples was compared with the detector response obtained following direct derivatization of standard solutions containing equivalent amounts of LA and DHLA. Assay reproducibility was assessed at three concentrations of LA and DHLA (1, 50 and 200 ng/ml each). The within-day reproducibility was calculated from quintuplicate analyses which were performed on three (consecutive) days to determine the day-to-day variability and reproducibility.

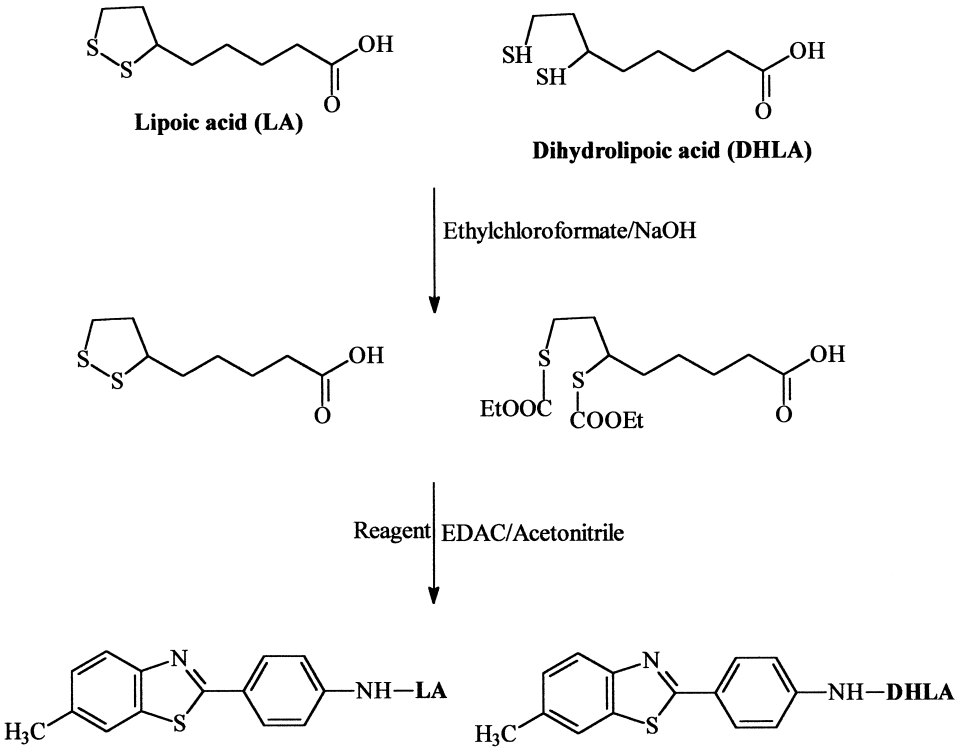

Fig. 1. Scheme for the derivatization reaction of LA and DHLA.

2.6. Apparatus and chromatographic conditions

The HPLC system consisted of a Spectra Series P200 pump and a Shimadzu RF 551 fluorescence detector operating at excitation and emission wavelengths of 343 and 423 nm, respectively. Samples were either injected via an autosampler (Spectra) or manually using a Rheodyne 20 μl sample loop (Rainin, USA). The HPLC apparatus was connected to an Ultrasphere C_8 column (25 cm×4.6 mm, 5 μm, Beckman, USA). The samples were eluted isocratically with acetonitrile–water (80:20) at a flow-rate of 1 ml/min.

3. Results and discussion

Because of their multidisciplinary therapeutic application, a reliable method of analysis for LA and DHLA that can be easily applied by most standard laboratories is required. At this level, various chromatographic methods have been developed for the determination of LA and DHLA in biological and food samples. In fact, such a determination is crucial in studies involving biochemical, nutritional and pharmacokinetic–dynamic investigations related to the role of LA in homeostasis of the redox status, as well as for understanding the beneficial role of treatment with antioxidants in diseases for which an indication exists for supplementation with LA. The GC–MS method is a powerful technique for such an objective, although so far only detection of LA, but not DHLA, has been reported [28–30]. However, the basic equipment for this sophisticated technique is highly expensive and is beyond the reach of many standard laboratories. The measurement of endogenous levels of LA and DHLA requires hydrolysis of the peptide linkage between LA and a lysine side chain. Mattulat et al. measured endogenous levels of LA in different animal tissues following sulfuric acid hydrolysis of the covalent bond, followed by GC–MS analysis [31]. Sulfuric acid treatment, however,

oxidizes DHLA and also destroys 30–40% of LA [31] (more in our hands). Although our method can be easily applied to the measurement of endogenous levels of LA and DHLA in biological tissue, we find it a more practical method for studying the pharmacokinetics of exogenous LA and DHLA in biological systems.

The conditions of the derivatization reaction (Fig. 1) were examined using 50 n*M* to 5 μ*M* solutions of LA and DHLA. Constant peak heights were obtained using a molar ratio of as low as 1.2 of the reagent to total LA and DHLA present. Representative calibration curves for both LA and DHLA show the linearity of the method over a wide range of concentrations (Fig. 2).

The effects of temperature on the yield and duration of the derivatization reaction were also studied (Fig. 3). We found that the reaction carried out at 60°C for 30 min resulted in the most satisfactory results. Although increasing the temperature to 90°C brings the derivatization reaction to completion within a shorter period of time (e.g., 15–20 min), such an increase in temperature was also found to result in the formation of unidentified peaks, especially with DHLA. In the latter case, hydrolysis of one or both of the *S*-carboxyethyl ester linkages might have occurred under such conditions.

Amongst the various elution conditions tested (e.g., addition to the mobile phase of various ion-pairing agents and organic modifiers), we found that a simple isocratic elution with acetonitrile–water (80:20) at a flow-rate of 1 ml/min gave the best separation within 10 min. The use of buffers (e.g., acetate, phosphate, trialkylammonium salts) with acetonitrile (or with other solvents such as methanol, ethanol or tetrahydrofuran) does not offer any practical or analytical advantage.

Fig. 4 shows typical chromatograms obtained after processing 1 ml of plasma spiked with 10 ng of LA (Fig. 4A), after processing plasma from a volunteer orally dosed with commercial capsules of LA (Fig. 4B), and the level of unchanged LA in urine from the same volunteer (Fig. 4C). The structural identity of LA and DHLA in both plasma and urine (as the reagent-derivatives) was confirmed by mass spectrometry after preparative liquid chromatography. Similar results were obtained with samples from diabetic patients treated with LA for polyneuropathy accompanying advanced disease. These chromatograms clearly demonstrate the applicability of the HPLC method suggested here as compared with other currently available methods (all of which have been tested by us, including the electrochemical method). The superiority of our method lies in its being the first HPLC assay with fluorimetric detection with which the reduced (DHLA) and oxidized (LA) forms of lipoate can be determined simultaneously in biological samples after supplementation with exogenous LA. The determination of endogenous, protein-bound LA and DHLA was not attempted by us since, because of increased variability and reduced reproducibility, our preliminary experiments showed that, like other investigators [30,31], such measurements may not be clinically useful.

As depicted in Fig. 4 the amide derivatives of both LA and DHLA were well separated from each other and from unreacted fluorescent amine using a standard column with simple isocratic elution. The presence of other endogenous sulfhydryls such as glutathione and cysteine does not interfere with the detection since their recovery, with the extraction method applied here, is extremely low. In fact, except for unreacted reagent, the chromatogram obtained following extraction of plasma from individuals orally dosed with LA shows only the presence of derivatized LA and DHLA with the internal standard (Fig. 4B). The urine analysis (Fig. 4C) shows only the presence of the unchanged drug LA, but not of its reduced form DHLA (the large peak at 4.8 min represents excess reagent). As far as we know this is the first documentation of unchanged LA in human urine. The fact that only LA, but not DHLA, is detected in urine may be indicative of the possible different pathways through which LA and DHLA are cleared from the body (renal versus hepatic clearance).

The recoveries of LA and DHLA following dichloromethane extraction of spiked plasma were 87 ± 5 and 76 ± 8, respectively ($n = 6$). Similar recoveries were also found from urine (83 ± 5 and 71 ± 7 for LA and DHLA, respectively). Generally speaking, most single organic solvent extractions (or a combination of solvents) yielded higher recovery and reproducibility for LA than for DHLA. Higher recoveries of both acids were obtained with ethyl

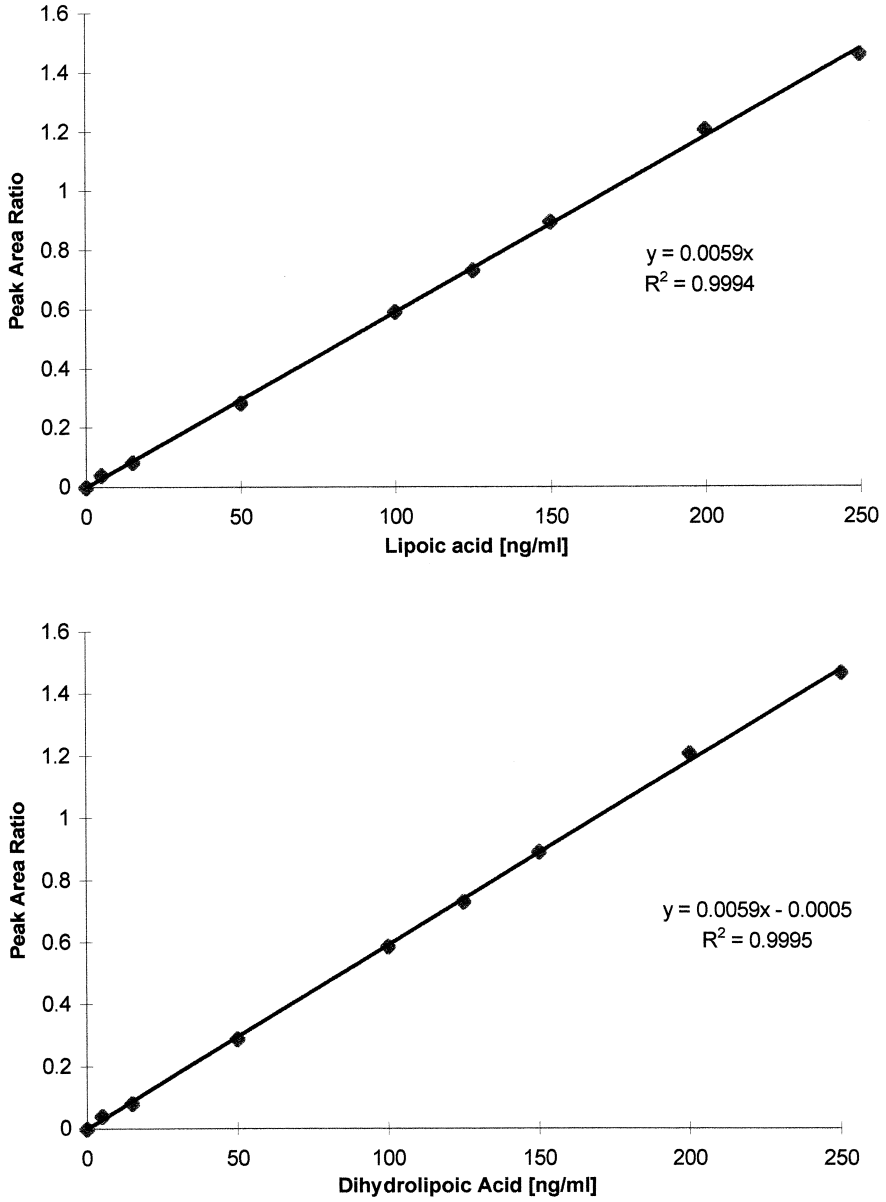

Fig. 2. Representative concentration–response curves for LA and DHLA. Aliquots of stock solutions of each acid were spiked into human plasma to obtain the indicated concentration in the sample. The symbols represent the mean of six independent determinations.

acetate or diethyl ether, alone or in combination with protic solvents. However, this was also accompanied by increased recoveries of materials yielding unidentified peaks following derivatization. In addition, the calibration graphs following extraction with a single solvent or combinations of solvents other than with dichloromethane alone were less reproducible. The within-day reproducibility of the assay is 97.4% and the day-to-day (calculated as the average of the mean individual response values at each concentration compared with day one of analysis) reproducibility is 96.8%.

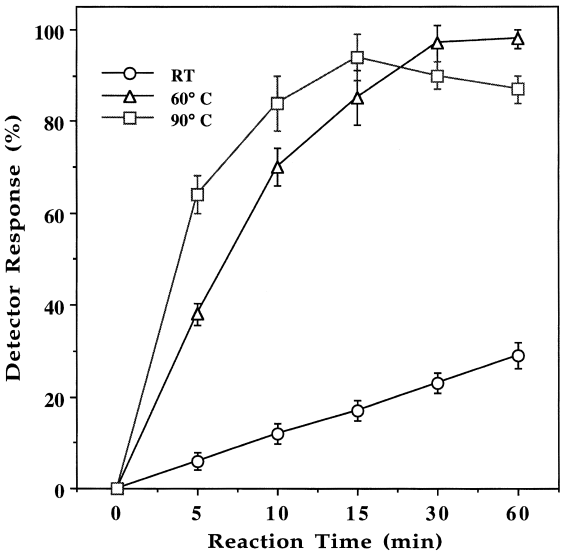

Fig. 3. Dependence of the detector response and the time for completion of the derivatization reaction for DHLA on temperature. Reactions were carried out at the specified temperature with 50 ng/ml of DHLA as described in the text. Aliquots of the reaction mixture were taken at different time points and injected directly into the chromatograph. Each point represents the mean±SD of six experiments. RT=room temperature.

4. Conclusions

A simple, sensitive, and reproducible HPLC method for the fluorimetric determination of both LA and DHLA is reported. The detection limit of this method with a signal-to-noise ratio of 3 is 0.1 and 0.5 ng/ml for DHLA and LA, respectively. Baseline separation of LA and protected DHLA is easily achieved on a C_8 column. The analysis is rapid (~15 min/run) and uses simple HPLC apparatus with isocratic elution. The satisfactory stability of the amide derivatives of both acids facilitates automatization of the procedure. This method may provide valuable information concerning the effects of LA and DHLA on the redox status in vitro, and concerning the cellular location and tissue distribution of both acids in vivo following supplementation with exogenous LA.

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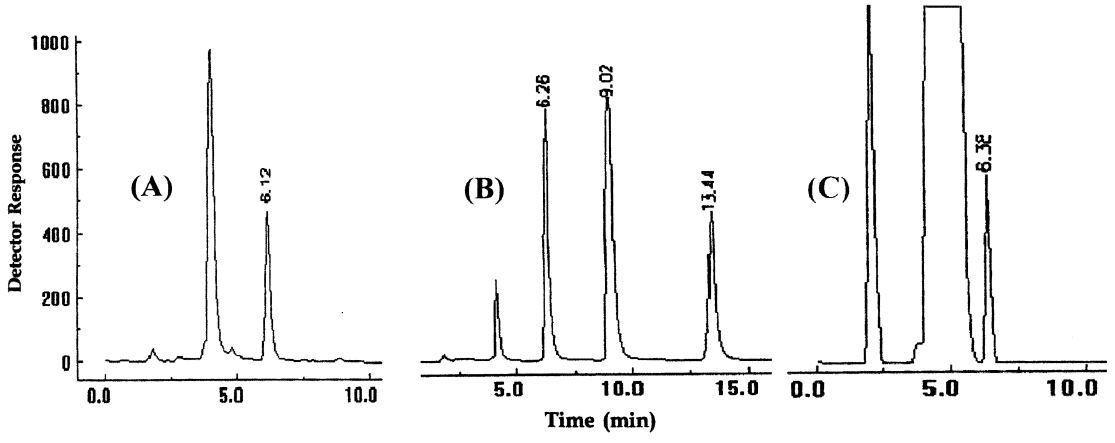

Fig. 4. Chromatograms showing the fluorescent amide derivatives of LA (t_R = 6.12 min) after extraction from LA-spiked plasma (A), and LA (t_R = 6.26 min) and DHLA (t_R = 9.02 min) with the internal standard (decanoic acid, t_R = 13.44 min) after extraction from the plasma of a volunteer orally dosed with commercial capsules of LA (B), and the presence of LA (t_R = 6.38 min) in urine from the same volunteer (C).

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