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Review

Chromatographic analysis of lipoic acid and related compounds

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Abstract

The analysis of lipoic acid and related compounds, such as its reduced form dihydrolipoic acid, its amide form lipoamide and other analogues, in biological and food samples is important in biochemistry, nutritional and clinical chemistry. This review summarizes the chromatographic methods for the determination of lipoic acid and related compounds, and their applications to various samples such as bacteria, tissues, drugs and food. Gas chromatographic methods with flame ionization detection and flame photometric detection are commonly used for the quantification of lipoic acid present as its protein-bound form, after acid or base hydrolysis of these samples. High-performance liquid chromatographic methods with ultraviolet, fluorescence and electrochemical detection are mainly used for the determination of free lipoic acid and related compounds, such as dihydrolipoic acid, lipoamide and other analogues. Moreover, gas chromatography–mass spectrometry and capillary electrophoresis methods are also developed.

Keywords: Reviews; Lipoic acid; Dihydrolipoic acid; Lipoamide

Contents

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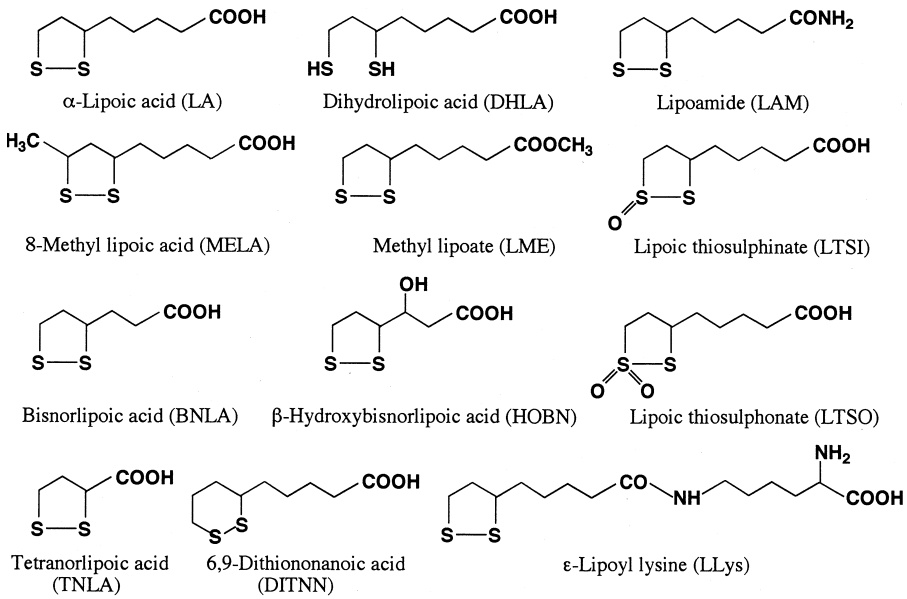

Fig. 1. Structures of lipoic acid and related compounds.

1. Introduction

α-Lipoic acid (LA, Fig. 1) is a natural disulphide-containing compound and is known as 6,8-thioctic acid, 1,2-dithiolane-3-pentanoic acid and 1,2-dithiolane-3-valeric acid. LA is a naturally occurring cofactor that has been reported to be present in a diverse group of microorganisms [1] and a variety of plant and animal tissues [2]. It is essential as an acyl carrier in the oxidative decarboxylation of the α-keto acids (pyruvate and α-ketoglutarate) [2–4] and it is an aminomethyl carrier in the glycine-cleavage enzyme systems [5,6]. In the functional protein-bound form of the cofactor, the carboxyl group of LA is covalently bound in an amide linkage to the ε-amino group of a lysyl residue [7–9]. LA was tentatively classified as a vitamin after its isolation, but it was later found to be synthesized by animals and humans [10]. However, the complete biosynthetic pathway that is responsible for de novo synthesis has not yet been elucidated. Isotope experiments have suggested that octanoic acid serves as the immediate precursor for the eight-carbon fatty acid chain, and cysteine appears to be the source of sulphur [11]. LA is also known to be metabolized to some major catabolites, *bisnor*-lipoic acid (BNLA), *tetranor*-lipoic acids (TNLA) and β-hydroxybisnorlipoic acid (HOBN) (Fig. 1), through β-oxidation in a bacterial strain [12] and in the rat [13]. On the other hand, LA is readily converted to its reduced form, dihydrolipoic acid (DHLA, Fig. 1), in mammalian cells by mitochondrial lipoamide dehydrogenase [14,15], and DHLA is able to permeate lipid bilayers [16]. Moreover, LA is proposed to oxidize to its thiosulphinate and thiosulphonate by electrophilic reagents [17,18].

Recently, LA and DHLA have been shown to provide potent antioxidant abilities against oxidative stress-induced processes [19–25]. In fact, LA dietary supplementation successfully prevents cerebral and myocardial damage induced by ischemia–reperfusion [26–28] and macromolecular alterations induced by high concentrations of glucose [29,30]. Preincubation of cultured human T cells with LA inhibits activation of NFκB, a transcription factor responsible for the activation of human immunodeficiency virus (HIV) [31,32]. In addition, LA and DHLA inhibit lipid peroxidation in vivo and in vitro, respectively [33–35], and inhibit skin inflammation induced by UVB radiation, xanthine/hypoxanthine, adriamycin and phorbol myristate acetate [36–38]. These injuries are mediated by free radicals, and in vitro experiments

have shown that both LA and DHLA are potent scavengers of reactive oxygen species, such as singlet oxygen [39], hydroxyl radical [40,41], hypochlorous acid [42], superoxide anion radical [40], peroxyl radical [43] and hydrogen peroxide [44]. On the other hand, several preliminary reports indicate that LA can elevate total glutathione (GSH) levels in some cell lines and tissues [45], and have demonstrated an interplay between LA and GSH in protection against lipid peroxidation and heavy metal toxicity [33,46–51]. A number of in vitro and in vivo studies also suggest that LA is able to recycle other natural antioxidants, such as ascorbic acid [35] and α-tocopherol [52,53], and a dietary supplement of LA can substitute for α-tocopherol in maintaining the lifespan of the mouse [53]. Thus, LA, DHLA and lipoamide (LAM) are used extensively in the treatment of various diseases [21,23,25], such as alcoholic liver disease [54,55], mushroom poisoning [56,57], heavy metal poisoning [48,58], diabetes [59–61], glaucoma [62], radiation injury [63], chagas disease [64] and neurodegenerative disorder [65].

Up to now, there has been little quantitative data on the LA content in biological and food samples, although most LA is considered to be present as a protein-bound form in physiological systems. Externally added LA is readily incorporated into cells and tissues, but it has not yet been determined whether protein-bound LA has antioxidant activity or whether it may act as a source of free LA. Moreover, the biological activity of exogenous LA probably involves both the oxidized and reduced forms [14], as well as some metabolic products [66], however, their pharmacological benefits are not well understood. Therefore, the determination of LA and related compounds in biological and food samples is important for studies on biochemical reactions involving LA, for nutritional and pharmacodynamic studies, or for the diagnosis of some kinds of disease. On the other hand, LA has been used as a racemic drug for the treatment of the various diseases mentioned above, but the naturally occurring *R*-enantiomer and the unnatural *S*-enantiomer exhibit different biological activities [38,67–69]. Therefore, a method for enantiomer separation and stereochemical analysis is also needed to study the pharmacokinetics and enantioselective metabolism of LA.

In the past, the determination of LA has been carried out by microbiological assay, colorimetric assay, gas chromatography (GC), GC–mass spectrometry (GC–MS), high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), enzyme immunoassay and enzyme cycling assay. The microbiological assay has already been reviewed by Stokstad et al. [93] and by Herbert and Guest [94,95]. Other non-chromatographic methods, such as colorimetric assays [96–99], enzyme immunoassay [100,101] and enzyme cycling assay [102], are also not covered in this review. The chromatographic methods reported are listed in Table 1. This review is concerned with the utilization of chromatographic methods for the determination of LA and related compounds in biological materials, and in drugs and food samples. The review consists of the following two main parts. In the first part (Section 2), general aspects of the analytical methods for the determination of LA and related compounds are surveyed, according to the instrument type. In the second part (Section 3), applications of these methods to the various samples are considered, according to the matrix type.

2. Analytical methods for the determination of lipoic acid and related compounds

2.1. Gas chromatography

GC has been used widely for the analysis of volatile compounds due to its inherent advantages of simplicity, high resolving power, high sensitivity and low cost. Therefore, GC is one of the most popular analytical techniques for LA and related compounds and this is at the top of the list of available methods shown in Table 1. Iguchi et al. [70] reported that a mixture containing LA and LAM could be separated and determined directly without derivatization by GC with flame ionization detection (FID) using a 15% diethyleneglycol succinate polyester packed column (75 cm×3 mm I.D.). However, these polar compounds tended to elute as broad and tailing peaks, due to strong adsorption to the column and injector during GC analysis. Therefore, they could not be detected in low concentrations without derivatization. Thus, several derivatives, such as methyl ester [71–74], *S*,*S*-dibenzyl (DBZ) methyl ester [75], *S*,*S*-

Table 1
Analytical methods for lipoic acid and related compounds

Method	Detection[a]	Analyte	Derivatization reagent[b]	Detection limit	Reference
GC	FID	LA, LAM			[70]
	FID	LA	CH_2N_2		[71,72]
	FID	LA (enantiomer)	CH_2N_2		[73]
	FID	LA	HCl–MeOH		[59]
	FID	LA	BF_3–MeOH	20 ng	[74]
	FID	LA	BZ-Cl–CH_2N_2	50 ng	[75]
	FPD	LA	ECF–HCl–MeOH	50 pg	[76,77]
	FID	LAM	KCN		[78]
GC–MS	EI	LA	CH_2N_2		[79,80]
	EI	LA	BZ-Cl–CH_2N_2		[81]
	CI	LA	MTBSTFA	10 pg	[82]
	EI	LTSI, LTSO	BSTFA–TMCS		[83]
HPLC	UV	LA, LAM, LME, BNLA, TNLA, DHLA, MELA, HOBN, DITNN		LA:15 mg, LAM:16 mg	[84]
	FL	LA	Br-AMN		[85]
	FL	LA (enantiomer)	OPA–D-Phe	3 ng/ml	[86]
	EC	LA, LAM		LA: 0.05 ng LAM: 0.1 ng	[87]
	EC	LA, DHLA		LA: 1 ng/ml, DHLA: 5 ng/ml	[88–90]
	EC	LA, DHLA		LA: 10 ng, DHLA: 2 ng	[91]
CE	UV	LA, LAM			[92]

[a]FID, flame ionization detection; FPD, flame photometric detection; EI, electron ionization; CI, chemical ionization; UV, ultraviolet; FL, fluorescence; EC, electrochemical.
[b]CH_2N_2, diazomethane; HCl–MeOH, hydrogen chloride–methanol; BF_3–MeOH, boron trifluoride–methanol; BZ-Cl, benzyl chloride; ECF, ethyl chloroformate; KCN, potassium cyanide; MTBSTFA, *N*-methyl-*N*-(*tert*.-butyldimethylsilyl)-trifluoroacetamide; BSTFA: *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide; TMS: trimethylchlorosilane; Br-AMN: 2-bromoacetyl-6-methoxynaphthalene; OPA: *o*-phthalaldehyde; D-Phe: D-phenylalanine.

diethoxycarbonyl (DEOC) methyl ester [76,77] and thietanevaleramide [78] have been tested for the GC analysis of LA and related compounds.

Shih and Steinsberger [71] reported that LA and its metabolites, BNLA and TBLA, and their reduced forms, could be distinctly separated and determined by GC–FID using a 3% Poly A-103 packed column (1 m long) after methylation with diazomethane (Fig. 2). Natraj et al. [59] and Liaud et al. [74] used hydrogen chloride–methanol (HCl–MeOH) and boron trifluoride–methanol (BF_3–MeOH), respectively, as a methylation reagent for LA and DHLA, and analyzed them by GC–FID using a 3% SP-1000 packed column and a DB-1 capillary column (30 m×0.25 mm I.D.; 0.2 μm film thickness), respectively. Although methyl LA gave a single peak, methyl DHLA gave two peaks, due to the partial oxidation of DHLA to LA during GC analysis. The detection limit for LA was 20 ng [74]. In contrast, Konig et al. [73] reported that the *R*- and *S*-LA enantiomers could be easily separated as their methyl esters on a heptakis(3-*O*-acetyl-2,6-di-*O*-phenyl)-β-cyclodextrin (Lipodex D) capillary column (40 m long).

As mentioned above, standard LA can be easily gas chromatographed as its methyl ester derivative, but it is difficult to detect the LA in biological and food samples as its derivatives because a part of LA forms mixed disulphides [103] and disulphide polymers [104] in these samples. Therefore, these sulphides must be reduced to thiols and then derivatized

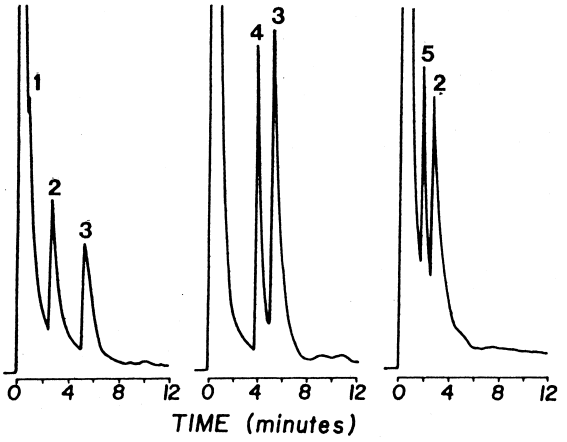

Fig. 2. Gas chromatograms of methyl esters of lipoic acid and related compounds. GC conditions: Column, 3% Poly A-103 on Gas Chrome Q (1 m long); column temperature, programmed at 10°C/min from 180 to 260°C; nitrogen flow-rate, 40 ml/min; FID detection. Peaks: 1=TNLA, 2=BNLA, 3=LA, 4=DHLA, 5= reduced BNLA. (Reproduced from Ref. [71], with permission).

with appropriate reagents for GC analysis. White [75] determined LA as its *S,S*-DBZ methyl ester derivative after reduction to DHLA using sodium borohydride ($NaBH_4$) by GC–FID using a 3% OV-1 packed column (122 cm×3.2 mm I.D.). Calibration curves using C_7 and C_9 homologues as internal standards were linear in the range 0–20 μg. Furthermore, increased sensitivity should be possible by using an electron capture detector and/or using pentafluorobenzyl chloride in the derivatization step instead of benzyl chloride (BZ-Cl). Recently, Kataoka et al. [76,77] developed a selective and sensitive method for the determination of LA by GC with flame photometric detection (FPD). LA was converted into its *S,S*-DEOC methyl ester derivative after reduction with $NaBH_4$ (Fig. 3). As shown in Fig. 4, LA was eluted as a single and symmetrical peak within 6 min using a DB-210 capillary column (15 m×0.53 mm I.D., 1.0 μm film thickness). The detection limit was ca. 50 pg injected and the calibration curve was linear in the range 20–500 ng.

Ogawa et al. [78] developed a new derivatization method for LAM based on the formation of 2-thietanevaleramide with potassium cyanide (Fig. 5). The derivative was analyzed by GC–FID using a 3% 1,4-butanediol succinate packed column (75 cm×4 mm I.D.). LAM was eluted as a single and tailing

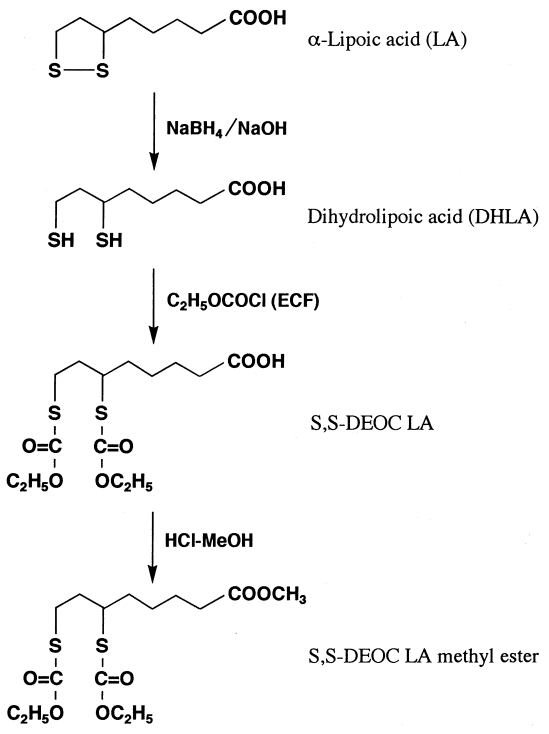

Fig. 3. Reduction and conversion of lipoic acid to its *S,S*-DEOC methyl ester derivative.

peak within 5 min. This method was highly specific for LAM, but the detection sensitivity was low.

2.2. Gas chromatography–mass spectrometry

GC–MS can be usually operated in two modes, total ion monitoring and selected ion monitoring (SIM). For SIM, only the base peaks are chosen to obtained the highest possible sensitivity. A magnetic sector instrument is used for positive ion electron ionization (EI)–MS, which yields excellent fragmentation patterns, with further confirmation being achieved using chemical ionization (CI) of the sample with a quadruple instrument. Since CI–MS is a much softer ionization method, it has the advantage of producing far less fragmentation of the compound and so allows a greater chance of the molecular ion being present, which can aid interpretation. Thus, GC–MS is one of the best on-line identification systems because of selectivity and sensitivity, but it

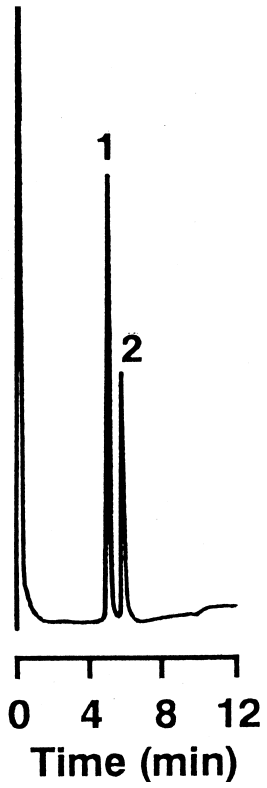

Fig. 4. Gas chromatogram of lipoic acid as its *S,S*-DEOC methyl ester. GC conditions: column, DB-210 (15 m×0.53 mm I.D., 1.0 μm film thickness); column temperature, programmed at 5°C/min from 200 to 250°C; injection and detector temperature, 260°C; nitrogen flow-rate, 10 ml/min; FPD detection. Peaks: 1=*S,S*-dimethoxycarbonyllipoic acid (internal standard), 2=LA.

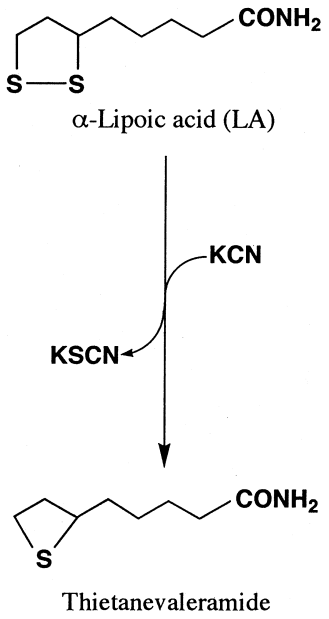

Fig. 5. Derivatization of lipoamide to 2-thietanevaleramide.

requires conversion into volatile derivatives before analysis.

Few GC–MS data on the analysis of LA and related compounds using EI and CI after derivatization are available. Pratt et al. [79] and Jackman et al. [80] reported that methyl LA could be identified by monitoring the presence of the fragment ion (*m/z* 123) and the molecular ion (*m/z* 220). White [81] developed a method for the identification of LA as its *S,S*-DBZ methyl ester derivative by EI–MS. This derivative gave a strong mass ion at *m/z* 311 (M^+- 91) and a weak ion at *m/z* 137, which resulted from cleavage of the C_7 and C_8 bond of LA. He also tested some reagents, such as dinitrofluorobenzene, iodoacetate and 2,2-dimethoxypropane, for the derivatization of reduced LA, but the derivatives prepared by these reagents proved unacceptable for the GC–MS analysis of LA due to either nonvolatility, instability or difficulty in preparation. Mattulat and Baltes [82] developed a sensitive method for the determination of LA by CI–MS after conversion into its *tert.*-butyldimethylsilyl (*tert.*-BDMS) derivative with *N*-methyl-*N*-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA), as shown in Fig. 6. The detection limit was about 10 pg/μl of LA derivative and the calibration curve was linear from 10 pg/μl to 20 ng/μl using the base peak *m/z*=189 for quantification. On the other hand, Biewenga and Bast [83] characterized possible oxidation products of LA, lipoic thiosulphinate (LTSI, β-lipoic acid) and lipoic thiosulphonate (LTSO), as their trimethylsilyl (TMS) derivatives by EI–MS.

2.3. High-performance liquid chromatography

HPLC is one of the most popular analytical techniques and can be used in combination with various detection systems such as ultraviolet (UV), fluorescence (FL) and electrochemical (EC) detectors. However, little work has been done on the determination of LA and related compounds by HPLC. Howard and McCormick [84] reported that

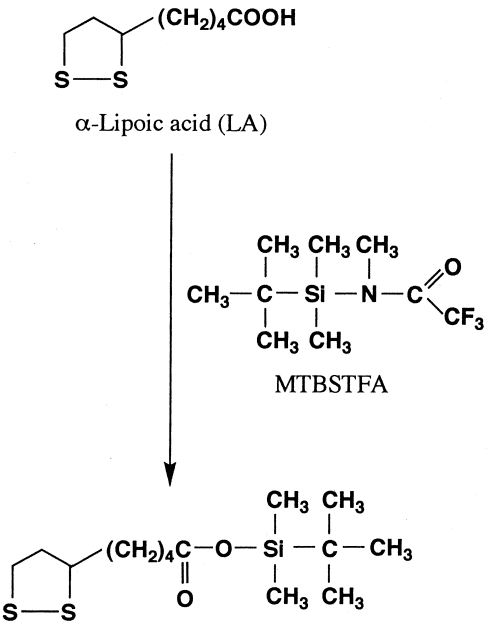

Fig. 6. Derivatization of lipoic acid with MTBSTFA.

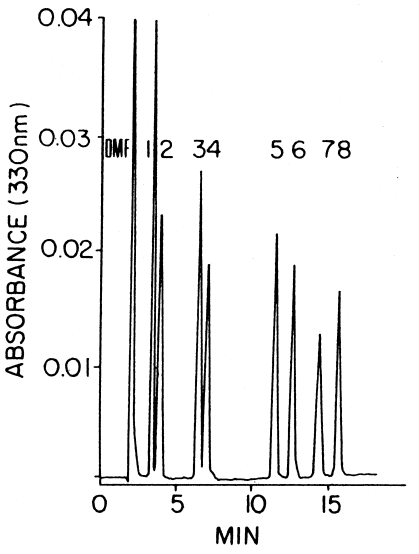

Fig. 7. HPLC elution pattern for lipoic acid and analogues. HPLC conditions: Column, μBondapak C_{18} (30 cm×3.9 mm I.D.); elution, gradient from 40% methanol containing 0.04% acetic acid to 70% methanol containing 0.02% acetic acid; flow-rate, 2 ml/min; detection, UV at 330 nm. Peaks: 1=TNLA, 2=HOBN, 3=BNLA, 4=LAM, 5=LA, 6=DITNN, 7=MELA, 8=LME. (Reproduced from Ref. [84], with permission).

LA and some of its analogues could be separated by reversed-phase HPLC on a μBondapak C_{18} column (30 cm×3.9 mm I.D.) using UV detection at 330 nm (absorption maximum of the dithiolane ring) with a gradient elution system. As shown in Fig. 7, eight compounds could be separated within 16 min, but the detection sensitivities of these compounds were very low, due to the lack of a strong chromophore [3,105], which is needed for conventional UV or FL detection.

Few derivatization reagents have been used for the determination of LA by FL detection. Gatti et al. [85] used 2-bromoacetyl-6-methoxynaphthalene (Br-AMN) as a fluorogenic labelling reagent (Fig. 8). LA was separated using a Hypersil 5 ODS column (25 cm×4.5 mm I.D.; 5 μm particle size) under isocratic conditions and detection was at an excitation wavelength of 300 nm and an emission wavelength of 460 nm. Recently, Niebch et al. [86] developed a stereoselective and sensitive method for the HPLC analysis of LA enantiomers. (+)*R*- and (−)*S*-LA were derivatized with *o*-phthalaldehyde (OPA) in the presence of D-phenylalanine (D-Phe) after reduction to the dithiol enantiomers (Fig. 9) and the two diastereomeric derivatives were separated by re-

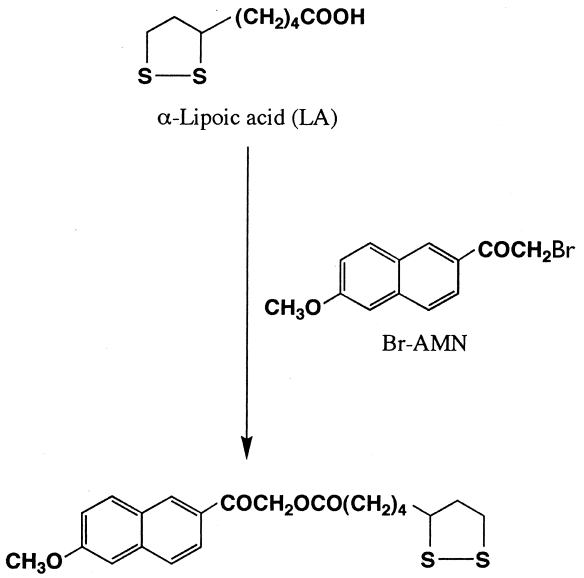

Fig. 8. Fluorogenic derivatization of lipoic acid with Br-AMN.

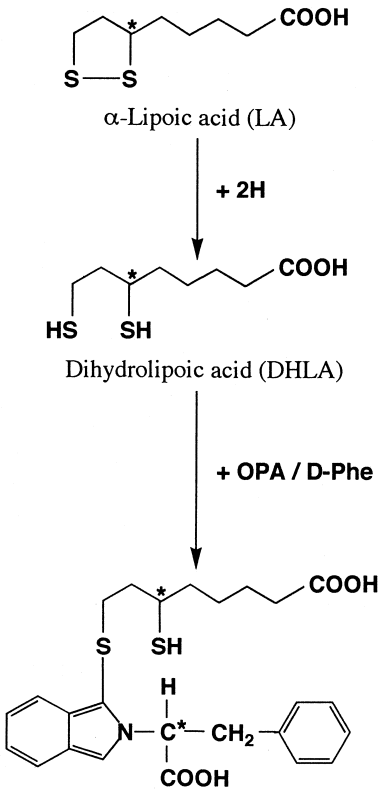

Fig. 9. Derivatization process for lipoic acid enantiomers with OPA/D-Phe.

versed-phase HPLC on a LiChrospher 60 SelectB column (25 cm×4 mm I.D.; 5 μm particle size) using FL detection with an excitation wavelength of 230 nm and an emission filter of >418 nm. The detection limit was about 3 ng/ml and the working range of the assay was 15–1000 ng/ml for either enantiomer.

LA and DHLA can be readily interconverted by application of an electric potential and, therefore, these compounds are suitable for measurement by an EC detector. The choice of instruments and electrode materials, and the composition of the mobile phase, are factors that influence both the sensitivity and selectivity of the EC analysis. Isocratic elution is usually used in the combination of HPLC and electrochemistry, because gradient elution cannot be used in a high sensitivity range with this detector. Kamata and Akiyama [87] reported that LA and LAM could be simultaneously analyzed by HPLC on a Shim-pack CLC ODS column (15 cm×6 mm I.D.; 5 μm particle size) using a Ag–AgCl EC detector and 50% acetonitrile in 0.05 *M* potassium dihydrogenphosphate (pH 2) as the mobile phase. The detection limits of LA and LAM were 50 and 100 pg, respectively, and the calibration curves for these compounds were linear over the range 0.5–5 ng. Teichert and Preiß [88–90] measured LA and DHLA using a glassy-carbon electrode system at the high oxidation potential of 1.1 V. HPLC separation was achieved using a Nucleosil 120 C_{18} column (25 cm×4 mm I.D.; 5 μm particle size) and 28.5% acetonitrile in 0.05 *M* potassium dihydrogenphosphate (pH 2.5) as the mobile phase. The detection limit for LA was 1 ng/ml and the calibration curve was non-linear in the range 0.01–50 μg/ml, but could be described by a power function. Handelman et al. [14] and Han et al. [91] used an alternative EC system, a dual Hg–Au electrode, for the determination of LA and DHLA. In this dual-electrode system, one electrode acts as the generator and the second electrode acts as a detector (Fig. 10). LA was reduced to DHLA on the high negative potential generator electrode and then detected as DHLA on the downstream electrode. LA and DHLA were separated within 8 min using a Microsorb C_{18} column (10 cm×4.6 mm I.D.; 3 μm particle size) and 50% 0.2 *M* monochloroacetic acid (pH 2.9)–30% methanol–20% acetonitrile as the mobile phase (Fig. 11). The detection limits for LA and DHLA were 10 and 2 ng, respectively. The mercury surface-

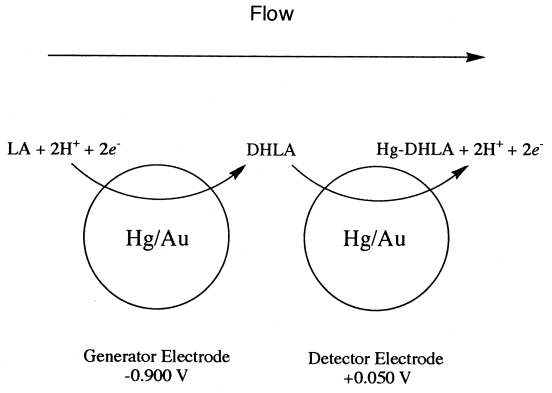

Fig. 10. Schematic diagram of the dual Hg–Au electrode, and the reaction with lipoic acid and dihydrolipoic acid. (Reproduced from Ref. [91], with permission).

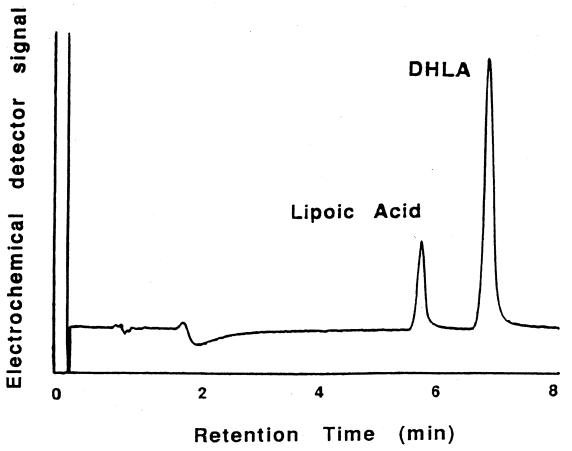

Fig. 11. HPLC pattern of lipoic acid and dihydrolipoic acid. HPLC conditions: Column, Microsorb C_{18} (10 cm×4.6 mm I.D., 3 μm particle size); mobile phase, 50% 0.2 *M* monochloroacetic acid–30% methanol–20% acetonitrile; flow-rate, 1 ml/min; detection, EC detector with a dual Hg–Au electrode. (Reproduced from Ref. [91], with permission).

specific reaction is much more specific than the reaction on the glassy-carbon surface and, therefore, the former is commonly used for the measurement of disulphides and thiols [106–108]. Although both LA and DHLA can be measured in their free form by these EC methods, bound forms of these compounds cannot be detected.

2.4. Capillary electrophoresis

CE is capable of achieving higher separation efficiency, uses less organic solvents and requires small amounts of samples in comparison with HPLC. The migration behaviour of ionized compounds is dependent on various factors, such as buffer pH, organic modifier, concentration of buffer solution, temperature of the capillary tubing and the electric field strength. A CE method for the determination of LA and LAM was recently developed by Panak et al. [92]. As shown in Fig. 12, these compounds and GSH could be successfully analyzed using an uncoated capillary column (30 cm effective length, 50 mm I.D.) with 50 m*M* Tris(hydroxymethyl)aminomethane–hydrochloric acid (Tris–HCl)–30 m*M* sodium dodecyl sulphate (pH 7.0) as the running buffer, at 20 kV. A detection limit in the

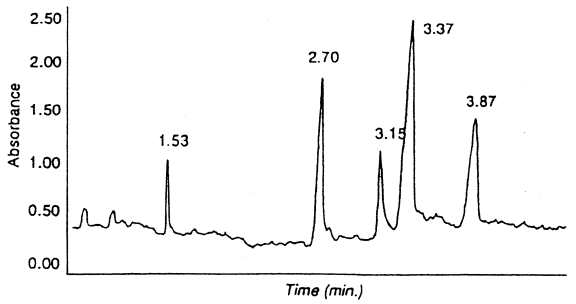

Fig. 12. Capillary electrophoresis migration pattern of lipoic acid and related compounds. Electrophoretic conditions: Capillary, uncoated silica (30 cm effective length); running buffer, 50 m*M* Tris–HCl–30 m*M* sodium dodecyl sulphate (pH 7.0); voltage, 20 kV; detection, UV at 214 nm. The migration times of GSH, LAM, GSSG and LA were 2.70, 3.15, 3.37 and 3.87 min, respectively. (Reproduced from Ref. [92], with permission).

femtomolar range was achieved by monitoring the UV absorbance at 214 nm.

3. Applications

3.1. General techniques for sample preparation

Because the majority of LA in biological and food samples is present as a protein-bound form with an amide linkage to the ε-amino group of a lysine residue [3,7–9], the release of LA by hydrolysis of the samples is necessary. For this purpose, several procedures, such as acid, base and enzymatic hydrolysis have been reported.

White [75,81], Pratt et al. [79] and Liaud et al. [74] hydrolyzed sample using 6 *M* HCl at 120°C for 2–4 h, and the released LA was easily extracted into dichloromethane. The release of LA from its protein-bound form under these hydrolysis conditions was >95%, but LA tended to become oxidized to its thiosulphinate or thiosulphonate form during hydrolysis. The recovery of LA from plasma by hydrolysis in 6 *M* HCl at 120°C for 6 h was about 30% [90]. Moreover, Mattulat and Baltes [82] reported that the recoveries of LA from the synthesized model compound ε-lipoyl lysine (LLys) by hydrolysis with 1 and 6 *M* HCl at 100°C for 3 h were about 80 and 25%, respectively. The addition of several thiol-protecting agents, such as mercaptoacetic acid, mer-

captoethanol or ethanethiol, to the hydrolysis mixture did not effect for the oxidation of LA [81]. Therefore, [8,8-$^{2}H_{2}$]LA [81] and C_{7} and C_{8} homologues of LA [75] were added to the sample as internal standards before hydrolysis. As an alternative acid hydrolysis method, Shih and Steinsberger [71] hydrolyzed sample using 6 *M* sulphuric acid at 125°C for 6 h, and LA in hydrolysate was extracted into benzene. By using [7,8-^{14}C]LA, the recovery of LA during hydrolysis and extraction was found to be 34%. Moreover, Mattulat and Baltes [82] reported that the recoveries of LA from LLys by hydrolysis with 1 and 4 *M* sulphuric acid at 100°C for 3 h were 80–85%. For food samples, optimum hydrolysis conditions were heating at 120°C for 7 h in 2 *M* sulphuric acid and the recoveries of LA from sample were about 60–70%. In addition, the use of organic acids, such as *p*-toluenesulphonic acid [8] and methanesulphonic acids [81,109], have also been described for the release of LA from the protein-bound form.

Kataoka et al. [76,77] used a base hydrolysis to release LA from sample, because the oxidation of LA during hydrolysis was far more resistant to base hydrolysis than to acid hydrolysis. Optimum hydrolysis conditions were heating at 110°C for 3 h in 2 *M* potassium hydroxide containing 4% bovine serum albumin. The degradation of LA during hydrolysis could be reduced by adding bovine serum albumin. The released LA could be easily extracted into dichloromethane and directly derivatized to its *S*,*S*-DEOC methyl ester. The recoveries of LA from biological samples during hydrolysis and extraction were 50–60%.

Suzuki and Reed [110] used a specific enzyme lipoamidase to release LA from the ε-amino group of a lysine residue, but it has never been applied directly to tissue homogenates. Recently, Teichert and Preiß [88–90] reported a mild enzymatic hydrolysis using several proteases to release LA from plasma sample. The recoveries of LA from plasma by hydrolyses with thermophilic protease, Alcalase and subtilisin were about 70, 80 and 82%, respectively. The released LA in hydrolysate was extracted by a solid-phase extraction cartridge. In this method, if all preparations were carried out under inert gas, DHLA could also be detected in some cases.

The contents of LA and related compounds obtained from biological, drug and food samples by various analytical methods described above are listed in Table 2.

3.2. Bacteria

LA has been identified in bacterial cells by GC–MS. White [81] detected LA in *Escherichia coli* (*E. coli*) by 6 *M* HCl hydrolysis, extraction into dichloromethane, subsequent derivatization with BZ-Cl and GC–MS analysis. Pratt et al. [79] detected LA in *E. coli* and *Halobacterium halobium* by a modified GC–MS method. They identified the released LA from the protein-bound form as its methyl ester. In addition, White [75] determined the LA content in acid hydrolysates of some bacterial samples by GC–FID analysis, based on preparation of the *S*,*S*-DBZ methyl ester derivative, and showed that the LA content of *E. coli* depended on the carbon source used for its growth. Kozma-Kavacs et al. [72] determined the LA content in *Saccharomyces cerevisiae* by GC–FID analysis of its methyl ester. The recovery of LA from sample during sulphuric acid hydrolysis and dichloromethane extraction was about 30%. On the other hand, Kataoka et al. [76] determined the LA content in several bacterial cells by GC–FPD based on the preparation of its *S*,*S*-DEOC methyl ester derivative. They used a base hydrolysis to release LA from sample. As shown in Fig. 13A and C, it was difficult to determine the content of LA by GC–FID due to the interfering peaks and low sensitivity, but the LA in the sample could be analyzed by GC–FPD without any such interference from matrix substances. The analytical data in Table 2 represent the total content of free and bound forms of LA and DHLA in these samples, as the samples were hydrolyzed with potassium hydroxide and reduced with $NaBH_{4}$. The detection limit of LA in these samples was ca. 10 ng/g. The contents of bacterial LA summarized in Table 2 are in good agreement with data previously reported based on the microbiological assay [97].

3.3. Animal tissues

Shih and Steinsberger [71] determined the LA content in rat tissues and other biological materials by GC–FID analysis based on delipidation, sulphuric

Table 2
Lipoic acid contents in bacteria, tissue and food samples

Sample	Method	Content (μg/g)	Reference	Sample	Method	Content (μg/g)	Reference
Escherichia coli B	GC–FID	2.3–11.8	[75]	Bovine heart	GC–MS	0.7–1.0	[82]
Escherichia coli	GC–FPD	23.45	[76]	liver	GC–MS	0.6–1.1	[82]
Bacteroides fragilis	GC–FID	2.4	[75]	kidney	GC–MS	0.9–1.3	[82]
Aerobacter aerogenes	GC–FPD	10.62	[76]	muscle	GC–MS	0.2–0.4	[82]
Aerobacter vinelandii	GC–FPD	34.96	[76]	Calf heart	GC–MS	0.5–0.7	[82]
Bacillus subtilis	GC–FPD	17.65	[76]	liver	GC–MS	0.3–0.5	[82]
Clostridium perfringens	GC–FPD	ND[a]	[76]	kidney	GC–MS	0.5–0.7	[82]
Micrococcus lysodeikticus	GC–FPD	4.34	[76]	muscle	GC–MS	0.07–0.15	[82]
Pseudomonas fluorescens	GC–FPD	16.25	[76]	Lamb heart	GC–MS	0.5–0.7	[82]
Saccharomyces cerevisiae	GC–FPD	1.54	[76]	liver	GC–MS	0.7–0.8	[82]
Saccharomyces cerevisiae	GC–FID	3.3–8.3	[73]	kidney	GC–MS	0.5–0.7	[82]
Human plasma	HPLC–EC	1–25[b]	[88]	muscle	GC–MS	0.2–0.4	[82]
Rat liver	GC–FID	8.0–13.1	[59]	Pig heart	GC–MS	1.1–1.6	[82]
liver	GC–FID	1.2	[75]	liver	GC–MS	0.6–0.8	[82]
kidney	GC–FID	1.2	[75]	kidney	GC–MS	0.4–0.7	[82]
Mouse brain	GC–FPD	0.83	[76]	muscle	GC–MS	0.15–0.3	[82]
heart	GC–FPD	2.03	[76]	Chicken	GC–FPD	0.91	[77]
lung	GC–FPD	0.80	[76]	Pork	GC–FPD	1.07	[77]
liver	GC–FPD	1.23	[76]	Beef	GC–FPD	2.36	[77]
spleen	GC–FPD	0.52	[76]	Cow's milk	GC–FPD	ND	[77]
pancreas	GC–FPD	0.84	[76]	Egg white	GC–FPD	ND	[77]
kidney	GC–FPD	1.54	[76]	Egg yolk	GC–FPD	1.24	[77]
testis	GC–FPD	1.06	[76]	Yellowtail	GC–FPD	0.75	[77]
muscle	GC–FPD	0.78	[76]	Cuttlefish	GC–FPD	0.55	[77]
Chicken liver	GC–FID	5–10	[71]	Wheat grain	GC–FID	0.1	[74]

[a]Not detectable.
[b]ng/ml.

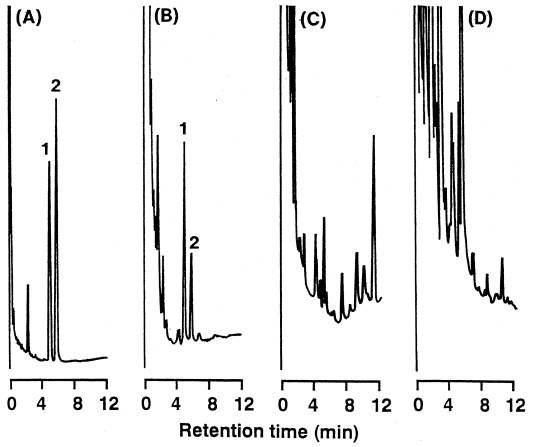

Fig. 13. Gas chromatograms obtained from (A) *E. coli* (5 mg), (B and D) mouse liver (50 mg) and (C) *E. coli* (50 mg). The derivatized samples were analyzed by either GC–FPD (A and B) or GC–FID (C and D). GC conditions and peak numbers as in Fig. 4.

acid hydrolysis, benzene extraction and derivatization with CH_2N_2. As shown in Fig. 14, when the benzene extracts were reduced with $NaBH_4$, peak 2 (LA) disappeared and a new peak 1 (DHLA) was found. They also reported that the LA level in the chicken egg increased with incubation time or during embryo development. White [75] and Natraj et al. [59] determined the content of LA in rat tissues by GC–FID as its *S,S*-DBZ methyl ester and methyl ester derivatives, respectively. In addition, Kataoka et al. [76] reported total LA contents for several tissues of mouse. The LA in the tissues was selectively determined as its *S,S*-DEOC methyl ester derivative by GC–FPD (Fig. 13 B). On the other hand, Han et al. [91] determined free LA and DHLA in Jurkat cells and fibroblasts by HPLC–EC analysis. As shown in Fig. 15, after a 30-min incubation of Jurkat cell culture with LA, both LA and DHLA were detected in the pellet and the culture medium.

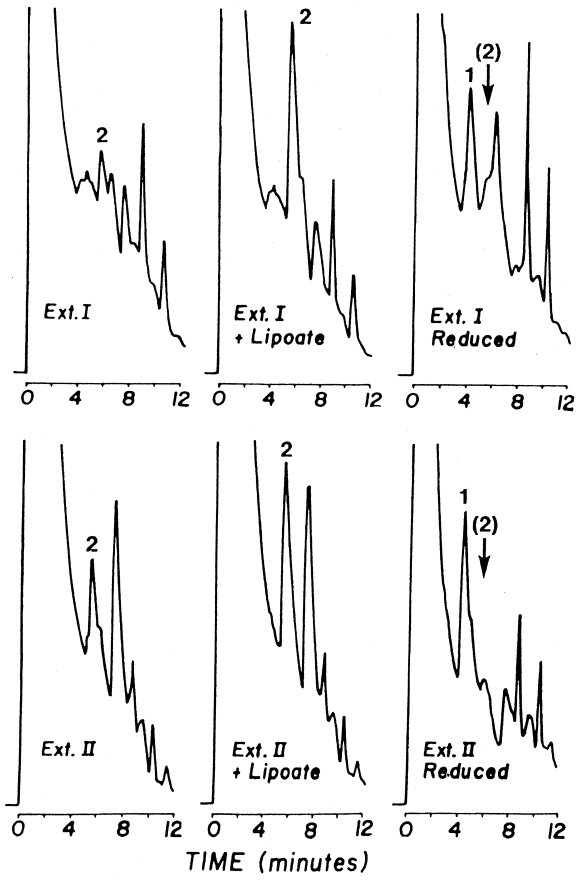

Fig. 14. Gas chromatograms of two independent chick liver extracts (I and II). GC conditions as in Fig. 2. Peaks: 1=DHLA, 2=LA. (Reproduced from Ref. [71], with permission).

These results indicate that LA is converted to DHLA in the cell. Moreover, Teichert and Preiß [88–90] developed a selective and sensitive method for the determination of LA and DHLA in human plasma. This method consists of enzymatic hydrolysis to release the protein-bound LA, solid-phase extraction, and HPLC–EC analysis. The detection limit of LA in plasma for this method was 1 ng/ml, and the basic levels of LA and DHLA in plasma from six healthy volunteers were 1–25 and 33–145 ng/ml, respectively.

3.4. Drug

LA and LAM have been used for many years as a drug for the treatment of the various diseases described in Section 1, and a specific assay for *R*- and *S*-LA was required recently for investigation of the pharmacokinetics of these enantiomers because they exhibit different biological activities in vitro and in vivo. Kamata and Akiyama [87] developed a HPLC–EC method for the determination of LA and LAM and reported that these compounds could be quantitatively recovered from a spiked placebo formulation. Gatti et al. [85] determined LA and LAM in a commercial formulation of the cited acidic drug by a HPLC–FL method involving pre-column derivatization with Br-AMN. On the other hand, Niebch et al. [86] developed a HPLC–FL method for the determination of LA enantiomers and applied it to pharmacokinetic studies. As shown in Fig. 16, *R*- and *S*-LA could be separated and determined in a plasma sample from a volunteer after an intravenous injection of racemic LA drug.

3.5. Food

Mattulat and Baltes [82] determined LA in meat of commercial quality by a sensitive CI–GC–MS method based on sulphuric acid hydrolysis, diethyl ether extraction, derivatization with MTBSTFA and SIM using the base peak m/z 189 for quantification. The recoveries of LA in meat with this method were about 60–70%. Liaud et al. [74] determined LA in vegetables by GC–FID. LA was found to be present at low levels in wheat germ (ca. 0.1 ppm), but was not detected in flour or semolina. Moreover, Kataoka et al. [77] determined LA in various food samples, and showed that LA contents were high in foods derived from animal but were low or undetectable in vegetables.

4. Conclusions

The determination of LA and related compounds in biological and food samples is important for biochemical, nutritional and pharmacodynamic studies. LA and related compounds exist as their free and protein-bound forms, or as reduced and oxidized forms in the samples. Therefore, practical and reliable methods for determining these compounds should be established. For this purpose, various

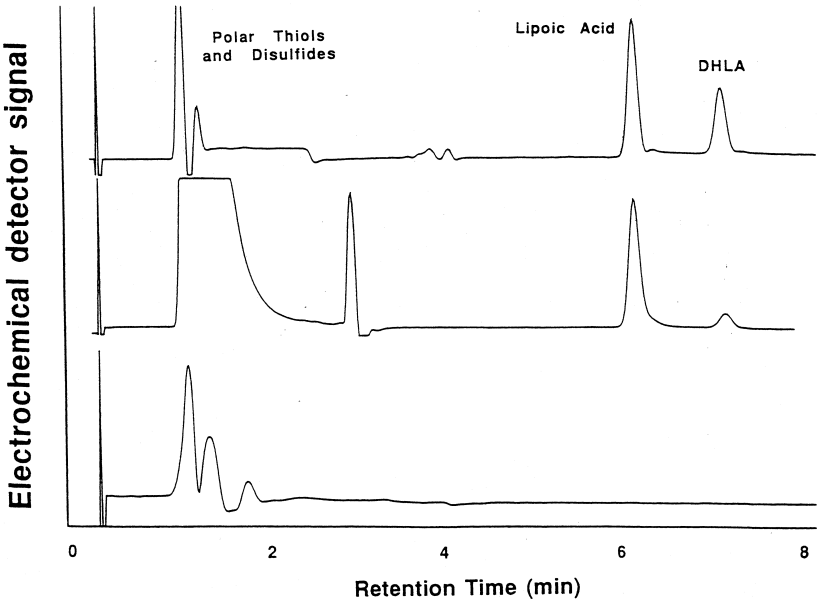

Fig. 15. HPLC patterns demonstrating cellular conversion of lipoic acid to dihydrolipoic acid in Jurkat cells. The bottom chromatogram shows an analysis of untreated Jurkat cells that contain no free LA and DHLA. The middle chromatogram shows the medium obtained from Jurkat cells after incubation with 2 m*M* LA for 30 min. The top chromatogram is the analysis of the Jurkat cell pellet after the addition of LA. HPLC conditions as in Fig. 11. (Reproduced from Ref. [91], with permission).

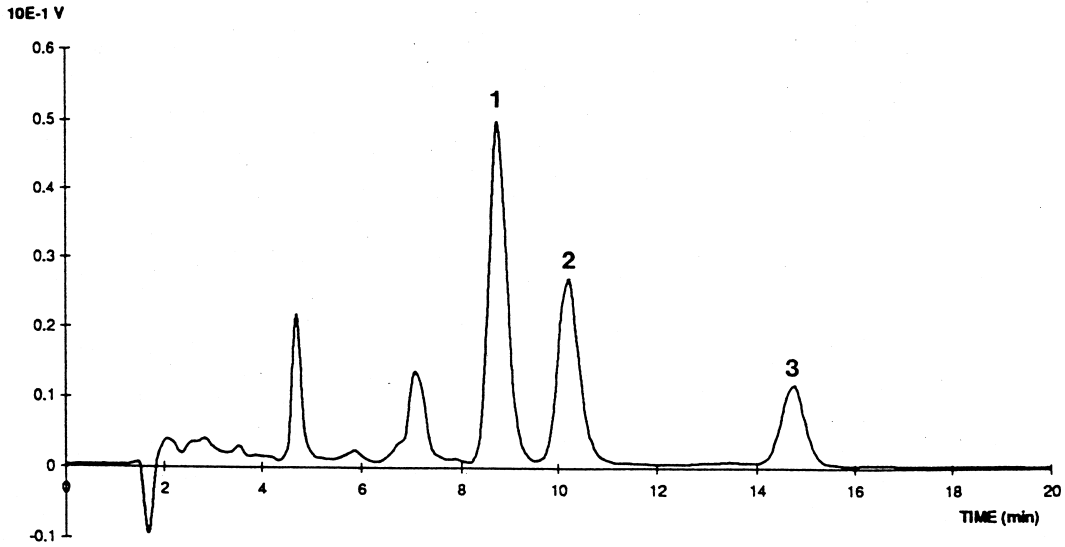

Fig. 16. HPLC profile of a plasma sample from a volunteer after intravenous infusion of racemic lipoic acid. HPLC conditions: Column, LiChrospher 60 SelectB (25 cm×4 mm I.D., 5 μm particle size); column oven temperature, 35°C; mobile phase, 55% (v/v) 0.02 *M* dipotassium hydrogenphosphate (pH 5.8)+45% (v/v) acetonitrile–methanol (1:1, v/v); flow-rate, 1.7 ml/min; FL detection, excitation at 230 nm and emission at >418 nm. Peaks: 1=(+)*R*-LA (1001.30 ng/ml), 2=(−)*S*-LA (598.47 ng/ml), 3=10-mercaptodecanoic acid (internal standard). (Reproduced from Ref. [86], with permission).

chromatographic methods have been developed. GC methods based on prior derivatization are the most useful techniques for the determination of protein-bound LA in biological samples. Detection with FPD is highly sensitive and selective for LA in comparison with FID. Although several hydrolysis methods with acid, base or enzyme are used to release LA from sample, the release of LA is partial and the recovery is generally low. Therefore, LA homologues are used as an internal standard. Protein-bound DHLA in biological sample cannot be determined by these methods, because it is easily oxidized or decomposed during hydrolysis. The GC–MS method, which is capable of simultaneously measuring the retention time and the molecular mass, is a powerful technique for the identification and quantification of LA in complex matrix samples. Although this technique is selective and sensitive, it requires sophisticated and expensive equipment that is beyond the reach of many laboratories. HPLC methods are also useful techniques for the simultaneous determination of free LA and related compounds. Although the sensitivity of the HPLC–UV method for these compounds is low, due to lack a strong chromophore, the compounds can be sensitively detected by HPLC–FL based on fluorogenic labelling. The HPLC–EC method is selective and sensitive for the simultaneous determination of free LA and DHLA without prior derivatization, but it cannot be applied to their protein-bound forms. The CE method with UV detection is capable of achieving higher separation efficiency, but has not been applied to the analysis of biological samples. All of the chromatographic methods described above have advantages and disadvantages and, so far, there are no all-powerful analytical methods. The choice of analytical method depends on the presence of LA and related compounds as various forms, such as free and protein-bound forms and redox forms, at low concentration levels in the samples. Therefore, the investigators must select a suitable method for the purpose. In my personal view, HPLC–EC methods are suitable for the determination of free LA and DHLA, and GC–FPD and GC–CI–MS methods are suitable for the analysis of total LA. Last, I hope that this review will serve as a guide to achieve the analysis of LA and related compounds in samples.

5. List of abbreviations

BF_3–MeOH	Boron trifluoride–methanol
tert.-BDMS	*tert*.-Butyldimethylsilyl
BNLA	Bisnorlipoic acid
Br-AMN	2-Bromoacetyl-6-methoxynaphthalene
BSTFA	*N*,*O*-Bis(trimethylsilyl)trifluoroacetamide
BZ-Cl	Benzyl chloride
CE	Capillary electrophoresis
CH_2N_2	Diazomethane
CI	Chemical ionization
DBZ	Dibenzyl
DEOC	Diethoxycarbonyl
DHLA	Dihydrolipoic acid
DITNN	6,9-Dithiononanoic acid
EC	Electrochemical
ECF	Ethyl chloroformate
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
FID	Flame ionization detection
FL	Fluorescence
FPD	Flame photometric detection
GC	Gas chromatography
GC–MS	Gas chromatography–mass spectrometry
GSH	Glutathione
HCl–MeOH	Hydrogen chloride–methanol
HOBN	β-Hydroxybisnorlipoic acid
HPLC	High-performance liquid chromatography
LA	α-Lipoic acid
LAM	Lipoamide
LLys	ε-Lipoyl lysine
LME	Methyl lipoic acid
LTSI	Lipoic thiosulphinate
LTSO	Lipoic thiosulphonate
MELA	8-Methyl lipoic acid
MTBSTFA	*N*-Methyl-*N*-(*tert*.-butyldimethylsilyl)-trifluoroacetamide
$NaBH_4$	Sodium borohydride
OPA	*o*-Phthalaldehyde
SIM	Selected ion monitoring
TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl
TNLA	Tetranorlipoic acid

Tris–HCl	Tris(hydroxymethyl)aminomethane–hydrochloric acid
UV	Ultraviolet

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