NEW METABOLIC PATHWAYS OF α-LIPOIC ACID

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(Received October 31, 2000; accepted February 15, 2001)

This paper is available online at http://dmd.aspetjournals.org

ABSTRACT:
The excretion and biotransformation of rac-α-lipoic acid (LA), which is used for the symptomatic treatment of diabetic polyneuropathy, were investigated following single oral dosing of [14C]LA to mice (30 mg/kg), rats (30 mg/kg), dogs (10 mg/kg), and unlabeled LA to humans (600 mg). More than 80% of the radioactivity given was renally excreted. Metabolite profiles obtained by radiometric high-performance liquid chromatography revealed that LA was extensively metabolized irrespective of the species. Based on a new on-line liquid chromatography/tandem mass spectroscopy assay developed for negative ions, LA and a total of 12 metabolites were identified. Mitochondrial β-oxidation played the paramount role in the metabolism of LA. Simultaneously, the circulating metabolites were subjected to reduction of the 1,2-dithiolane ring and subsequent S-methylation. In addition, evidence is given for the first time that the methyl sulfides formed were partly oxidized to give sulfoxides, predominantly in dogs. The disulfide of 2,4-bismethylmercapto-butanoic acid, the most polar metabolite identified, was the major metabolite in dogs. Furthermore, new data are presented that suggest conjugation with glycine occurred as a separate metabolic pathway in competition with β-oxidation, predominantly in mice.

rac-α-Lipoic acid [R,S-5-(1-dithiolane-3-yl)pentanoic acid, CAS 62-46-4] in the following abbreviated as LA along with its reduced form, dihydrolipoic acid (Biewenga et al., 1997a), has attracted increasing interest as an antioxidant and is widely used for the treatment of diabetic polyneuropathy in humans (Kleemann et al., 1989). R-Lipoate is the naturally occurring form of LA and is an essential cofactor of the pyruvate dehydrogenase multienzyme complex.

Although in recent years the enantioselective pharmacokinetics of the parent drug has been extensively investigated in humans (Hermann et al., 1996, 1998; Hermann and Niebch, 1997), relatively little is known regarding the metabolism of LA. Previous studies on the metabolism in rats and Pseudomonas putida LP have shown that LA is subject to extensive β-oxidation (Harrison and McCormick, 1974; Furr et al., 1978). Major metabolites identified were bisnorlipoic acid, tetrarnorlipoic acid, and β-hydroxy-bisnorlipoic acid. After oral administration of LA to healthy volunteers, dimethylated products following β-oxidation, such as 4,6-bismethylmercapto-hexanoic acid and 2,4-bismethylmercapto-butanoic acid, were identified (Locher et al., 1995). Recently, 3-keto-lipoic acid, an intermediate in the course of β-oxidation, was detected in human plasma after oral dosing with 1 g of the R-enantiomer of LA to a healthy volunteer (Biewenga et al., 1997b). Although the formation of thiosulfonates, as well as thiosulfonates, was suggested following reaction with singlet oxygen (Stary et al., 1975) or hypochloric acid (Biewenga et al., 1994), little is known about whether oxidized products are of importance in the metabolism of LA. Thus, the present study was undertaken to complete the knowledge of the metabolism of LA in different species and to answer the question whether species differences play a role in it. Because the LA-related radioactivity is predominantly renally excreted (Locher et al., 1995), urine samples from mice, rats, and dogs following oral administration of [14C]LA (Fig. 1) were used for a species comparison. Human urine samples were taken from a clinical study in healthy volunteers orally dosed with 600 mg LA (Thioctacid, tablets). In addition, plasma samples from rats, dogs, and humans after oral administration were analyzed and in the case of dogs following intravenous dosing as well. Metabolites were identified by radiometric HPLC and on-line LC/MS/MS analysis. Structure assignments for metabolites were based on MS data obtained from reference substances. In some cases, reference compounds were oxidized by hydrogen peroxide, and the reaction mixtures obtained were analyzed.

DAUROGENESIS AND DISPOSITION

Dedicated to Professor Axel Kleemann’s 60th birthday.

1 Abbreviations used are: LA, rac-α-lipoic acid; HPLC, high-performance liquid chromatography; LC/MS/MS, liquid chromatography/tandem mass spectrometry; MS, mass spectrometry; LC/MS, liquid chromatography/mass spectrometry; LSC, liquid scintillation counting; dpm, disintegrations per minute; SPE, solid-phase extraction; ESI, electrospray ionization; CID, collision-induced dissociation.

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Fig. 1. Structural formula of rac-α-lipoic acid (LA).

Asterisks denote the 14C label.
Materials and Methods

Chemicals. rac-$\alpha$-Lipoic acid was synthesized by ASTA Medica AG (Frankfurt, Germany). The potential metabolites bisnorlipoic acid (1,2-dithio-
lane-3-yl-propionic acid), tetranorlipoic acid (1,2-dithiolane-3-yl-formic acid), and 6,8-bismethylmercaptopo-ctanoic acid, 4,6-bismethylmercaptopo-hexanoic acid, 2,4-bismethylmercaptopo-butanoic acid, and 6,8-dithiol-ocatic acid were kindly provided by Dr. Hübner, Degussa-Hüls and Dr. G. Laban, Arzneimittelwerk Dresden GmbH, Dresden, Germany. N-(2-mercaptopropionyloxy)glycine was supplied by Fluka, Buchs, Switzerland. [7,8-$^{14}$C]RJ,S-5-[1,2-dithiolane-3-
yl] pentanoic acid (Fig. 1), abbreviated as [14C]LA, was synthesized by Amersham International plc (Buckinghamshire, UK), on behalf of ASTA Medica AG. Its radiochemical purity was 98.7% (thin layer chromatography) and specific activity 2.52 GBq/mmol. The radiolabeled substance was supplied by Amersham as a solution in 35 mmol/l of Tris buffer and was stored in the dark at $-20^\circ$C. All other chemical substances used came from commercial suppliers in analytical grade. Deionized water (Milli-Q water purification system, Millipore Waters, Eschborn, Germany) was used for the preparation of aqueous solutions.

Animal Studies. Urine and feces samples were taken from an excretion balance study in NMRI mice following a single dose by oral gavage of 30 mg/kg of [14C]LA as a solution, representing 2.60 MBq/kg. The solution was prepared by adding the commercially available formulation Thiocidac T direkt solution. A target volume of 0.83 ml/kg of this solution was administered via gastric gavage. Blood was sampled at 0.08, 0.25, 0.5, 1, 2, 4, 6, 8, 24, 30, 48, 72, 96, 168, 264, and 336 h postdose. Feces samples were collected up to 48 h postdose in the same manner as described for mice. For plasma sampling, rats were sacrificed under deep ether anesthesia by cardiac puncture at 1 and 3 h postdose. Heparinized blood was subsequently centrifuged at 1 and 3 h postdose. Heparinized blood was subsequently subjected to centrifugation (2,000 g, 10 min, Megaufuge 1.0 R, Heraeus, Hanau, Germany). Radioactivity was determined in duplicate (aliquots 300–500 µl) as described previously. In a crossover study starting with the oral dose, beagle dogs received an oral and intravenous doses of 10 mg/kg of [14C]LA, representing 2.60 MBq/kg. The solution was prepared by adding the commercially available formulation Thiocidac T direkt solution. A target volume of 0.83 ml/kg of this solution was administered as a bolus over an approximately 30-s period via a cephalic vein. For oral administration, the same test article was diluted with water to a target concentration of 2 mg/ml. A target volume of 5 ml/kg was orally administered by gastric gavage. Blood was sampled at 0.08, 0.25, 0.5, 1, 2, 4, 6, 8, 24, 30, 48, 72, 96, 168, 264, and 336 h postdose. Feces samples were homogenized with an ultra-turrax (TP 18-10, IKA, Staufen, Germany) after adding about 1.5 times their weight in water to get a more fluid consistency. Aliquots for combustion and LSC were taken before the samples were deep frozen.

Human Studies. Human plasma and urine were taken from pharmacokinetic or excretion studies in healthy volunteers, following either a single administration of 600 mg as oral solution (reference) or after multiple doses (600 mg 3 times a day over 3 days) as tablet (Thiocidac). Approval for the studies was obtained from the independent ethical committee of the general medical council of Hesse, Germany. Written consent was obtained from each subject before enrollment. Physical examinations were performed, and medical histories, routine laboratory tests, electrocardiograms, and vital signs were recorded before and after the course of drug treatment. During the study, no concomitant medications were allowed. Urine was collected containing aliquots of urine samples were collected via a forearm vein catheterer at 10, 20, 30, and 40 min predose, and at 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, and 8 h postdose using 7.5-ml heparinized tubes (Monovette, Sarstedt, Germany). Samples were centrifuged within 30 min of the withdrawal of the blood (10,000g, 10 min). Plasma was separated from whole blood, transferred into labeled plastic tubes, and immediately frozen. All biological samples were kept at $-30^\circ$C until analysis.

Determination of Radioactivity. Samples were counted after dark adaption in a liquid scintillation counter (Rackbeta 1219, Pharmacia-LKB, Freiburg, Germany) for 10 min or to reach $10^4$ counts, whichever came first. The observed counts per minute were converted to dpm using previously prepared quench curves. The dpm data were used to calculate concentrations (microgram equivalents/gram or milliliter) and the percentage of dose recovered in the samples. HPLC. The HPLC system used to profile radioactivity was the KONTRON system 450-MT2 (KONTRON Instruments, Neufahrn, Germany) consisting of two pumps 420, gradient mixer M 800, autosampler 465, mixer M 800, and an ultraviolet detector 432 set at 210 nm, range 0.5, and at a response time of 2 s, connected in series with a radiomonitor LB 507 B (set at a peak half-width of 30 s, response time of 1 s, and a range, 10,000 dpm), equipped with a solid scintillator flow cell YG-150 U4D (Berthold, Bad Wildbad, Germany). The lower limit of detection was 50 dpm. Separation was performed on a Supersep 60 RP select B column (250 x 4 mm, 5 µm, with guard 4 x 4 mm i.d.; Merck). The flow rate was 0.5 ml/min. Mobile phase A was 20 mmol/l of KH$_2$PO$_4$ adjusted with phosphoric acid to pH 2.7, and mobile phase B was 90% acetonitrile/10% water adjusted with phosphoric acid to pH 2.7. The gradient was as follows: 0 to 6 min at A: 6 to 16 min linear gradient from 90% B to 100% B; 16 to 61 min linear gradient from 10% B to 20% B, 61 to 91 min linear gradient from 20% B to 50% B; 91 to 101 min linear increase from 50% B to 80% B followed by a linear increase to 100% B during 10 min and holding that until 121 min.

Sample Preparation. Solid-phase extraction (SPE) with the aid of OASIS, HLB 3-ml cartridges (Waters Corp., Milford, MA) gave the best recoveries for plasma preparation. The procedure was as follows. The columns fitted on a Baker station (Baker Inc., Phillipsburg, NJ) were equilibrated with methanol (5 ml), followed by 5 ml of water. Plasma samples were diluted 1+1 (v/v) with water containing 2% phosphoric acid and drawn through the columns with vacuum. After washing with 400 µl of acidified water (2% H$_3$PO$_4$), air was drawn through the columns for about 1 min. Then, they were eluted with 2×2 ml of methanol. Solvent was removed by a rotary evaporator VV2000 (Heidelberg, Kehlheim, Germany) at a bath temperature of 40°C. The residue obtained was reconstituted in mobile phase B, centrifuged at 7,500 g, and aliquots of 100 to 250 µl were injected onto the HPLC column. $^{14}$C recovery from dog plasma samples till 3 h after dosing was between 40 and 70% and about 75% for blank plasma spiked with [14C]LA. Frozen urine samples were thawed at room temperature, and aliquots were subjected to centrifugation for 10 min at 2,700g. Then, urine samples were diluted with water dependent on the content of radioactivity. Samples were transferred into borosilicate glass vials (Chromaco Ltd., Langenfeld, Germany) and placed in the autosampler, at a temperature of 4 to 8°C until radiometric HPLC analysis. Aliquots of urine and feces homogenates (about 10 g) were subsequently extracted 5 times with methanol (5 ml) containing 1% formic acid with the aid of an ultra-turrax for 3×30 s (approximately 10,000 rpm). After centrifugation of the combined extracts for 5 min at 10,600g the supernatants obtained were evaporated to dryness under vacuum at 40°C by a rotary evaporator. Residue was reconstituted with 2×250 µl of methanol, centrifuged once more, filtered, using a Minisart GF filter (Sartorius AG, Göttingen, Germany), and injected onto the HPLC column. Using this procedure, the following $^{14}$C recoveries from feces were obtained: mouse 29.5 ± 7.5% (n = 4), rat 11.5 ± 0.7% (n = 4), and dog 22.2 ± 1.5% (n = 8).

Sample Stability. The stability of samples was checked by repeated radiochromatographic HPLC analysis of the same samples. Keeping urine samples up to 8 h at 4 to 8°C in the autosampler did not cause any detectable change in the radiometric chromatogram. Fecal extracts and plasma samples were analyzed immediately after preparation. Furthermore, blank samples from rat urine, dog urine, and dog plasma were spiked with [14C]LA when running the in vivo parts and stored together with the samples. Analysis of these samples showed, besides the parent compound, only marginal levels of further radioactive peaks that did not correlate to metabolite fractions were found (data not shown).
Excretion Data and Metabolite Profiling in Animals. Following oral administration, the drug-related radioactivity was rapidly excreted in urine, in fact more than half of the dose during the first 24 h, in all animals (Table 1).

In the same time period, only a minor part of the dose was fecally excreted, namely 14% for mice, 17% for rats, and 11% for dogs after oral administration. LA was identified as the major fraction in fecal excreted, namely 14% for mice, 17% for rats, and 11% for dogs after oral administration. The drug-related radioactivity was rapidly excreted in urine, in fact more than half of the dose during the first 24 h. Aliquots were taken at several time points after starting the reaction and analyzed directly by LC/MS/MS.

Mass Spectroscopy. A tandem quadrupole mass spectrometer (VG Quattro, Micromass, Manchester, UK) was used with a Q1-X-Q2 configuration, where Q represents a quadrupole analyzer, and X is a hexapole collision cell, equipped with MassLynx software in electrospray ionization (ESI) mode. This mass spectrometer equipped with a Mega Flow electrospray ion source was used in MS 1 and MS/MS mode. ESI and MS/MS parameters were optimized using solutions of reference compounds (Table 3). Unlike the previously described HPLC conditions, for on-line LC/MS/MS the phosphate buffer system was substituted by a buffer-free system acidified with formic acid: mobile phase A: 0.05% formic acid in water, pH 3.0, mobile phase B: 90% acetonitrile/10% water (v/v) with 0.05% (v/v) formic acid, pH 3.6. For pH shifting to support the negative electrospray ionization, a make-up flow of 60 to 80 μl/min of 0.25% ammonia in acetonitrile/water 50/50 (v/v) was added post column between the ultraviolet and radiometric detector. This resulted in a pH level of the eluent flow of 7.5 and no increase in salt concentration, which would have decreased the electrospray sensitivity. Following downstream radiometric detection, the eluent flow (0.5 ml/min) was split resulting in a flow rate of 35 μl/min that was transferred to the electrospray needle. Nitrogen was used as a nebulizer and drying gas. The ion source was heated constantly to 80°C. Typical ESI parameters for the negative ion mode were: capillary voltage 2.2 kV and counter electrode voltage 0.2 kV. A standard cone voltage of 18 V (for up-front fragmentation 18 V, 25 V, and 35 V) and a constant skimmer offset of 5 V was used. The MS1 scan range was from 120 to 500 m/z (full scan analysis) in 2 s. All MS data were acquired as profile data with 4 points per Dalton. The MS/MS (collision-induced dissociation; CID) experiments were performed with a scan rate of 200 units in 1.5 s, with argon as collision gas at 2.5 × 10^-5 mb and a collision energy of 30 eV.

Results

Oxidation of LA with Hydrogen Peroxide. Reference compounds were dissolved in mobile phases A/B 80/20 (v/v), as described previously, at concentrations of 20 to 30 μg/ml at room temperature. The pH level of the solution was adjusted to 8.0 to 8.5 by addition of 150 μl/ml of 0.25% ammonia in acetonitrile/water (1:1, v/v). The reaction was started at room temperature by addition of 30% hydrogen peroxide water (Merck) to achieve a final concentration of 90 to 100 mmol/l. Aliquots were taken at several time points after starting the reaction and analyzed directly by LC/MS/MS.

Mass Spectroscopy. A tandem quadrupole mass spectrometer (VG Quattro, Micromass, Manchester, UK) was used with a Q1-X-Q2 configuration, where Q represents a quadrupole analyzer, and X is a hexapole collision cell, equipped with MassLynx software in electrospray ionization (ESI) mode. This mass spectrometer equipped with a Mega Flow electrospray ion source was used in MS 1 and MS/MS mode. ESI and MS/MS parameters were optimized using solutions of reference compounds (Table 3). Unlike the previously described HPLC conditions, for on-line LC/MS/MS the phosphate buffer system was substituted by a buffer-free system acidified with formic acid: mobile phase A: 0.05% formic acid in water, pH 3.0, mobile phase B: 90% acetonitrile/10% water (v/v) with 0.05% (v/v) formic acid, pH 3.6. For pH shifting to support the negative electrospray ionization, a make-up flow of 60 to 80 μl/min of 0.25% ammonia in acetonitrile/water 50/50 (v/v) was added post column between the ultraviolet and radiometric detector. This resulted in a pH level of the eluent flow of 7.5 and no increase in salt concentration, which would have decreased the electrospray sensitivity. Following downstream radiometric detection, the eluent flow (0.5 ml/min) was split resulting in a flow rate of 35 μl/min that was transferred to the electrospray needle. Nitrogen was used as a nebulizer and drying gas. The ion source was heated constantly to 80°C. Typical ESI parameters for the negative ion mode were: capillary voltage 2.2 kV and counter electrode voltage 0.2 kV. A standard cone voltage of 18 V (for up-front fragmentation 18 V, 25 V, and 35 V) and a constant skimmer offset of 5 V was used. The MS1 scan range was from 120 to 500 m/z (full scan analysis) in 2 s. All MS data were acquired as profile data with 4 points per Dalton. The MS/MS (collision-induced dissociation; CID) experiments were performed with a scan rate of 200 units in 1.5 s, with argon as collision gas at 2.5 × 10^-5 mb and a collision energy of 30 eV.

This is most likely due to the loss of some parts of highly polar components during the process of sample preparation by SPE.

Metabolite patterns in urine revealed a clear species dependence in the metabolism of LA, which is demonstrated by the increasing polarity of the major metabolites from mouse to rat to dog (Table 1, Fig. 2). The most polar metabolite M1, the major one in dogs, was eluted from the C18 column already after 5 min only with the aid of the pure mobile phase A.

LC/MS/MS Characterization of Reference Compounds and Oxidized Products. Data of LC/MS/MS analysis of LA and some derivatives are shown in Table 3. The intact 1,2-dithiolane ring is characterized by the less abundant HS^- fragment at m/z 33 in the CID product ion spectrum. This is in accordance with the first loss of 34 Da (H2S) from the molecular anion [M-H]^-. The abundance of the sulfide ion (m/z 33) is strongly increased when a thiole function is already preformed in the structure, such as in the dihydrolipoic acid.

Accordingly, S-methylated derivatives indicate the anion CH3S^- at m/z 47 that corresponds to the first loss of mercaptomethane (48 Da) from [M-H]^-. The tetrathiolipoic acid and its derivatives make an exception to this rule. Here, carbon dioxide (44 Da) is always first cleaved from the molecular anion. As described by Ito et al. (1995),
glycine conjugates of carboxylic acids can be detected by the abundant ion at \([M+Na]^+\) in the product ion spectrum. We could confirm this with N-(2-mercaptopropionyl)-glycine (Table 3) by use of our LC/MS/MS assay as described previously. The formation of sulfoxide structures starting from methyl sulfides is suggested on the basis of oxidation experiments using hydrogen peroxide. Figure 4 illustrates the course of such an oxidation using 2,4-bismethylmercapto-butanoic acid as an example.

The increase of the [M – H]− from \(m/z\) 179 to \(m/z\) 211 provides clear proof that two atoms of oxygen were incorporated. In accordance with that, the MS feature of the sulfur atoms was changed, from the \(CH_3S^+\) ion at \(m/z\) 47 of the dimethylated compound over a period of about 1 h to the disulfide, characterized by the abundant ion at \(m/z\) 63, which revealed an oxygen-bearing methylated sulfur atom (\(CH_3S=O^-\)). The intermediate (M – H− at \(m/z\) 195) clearly revealed the simultaneous appearance of the typical ions for methyl sulfide (\(m/z\) 47) and methyl sulfoxide (\(m/z\) 63). The methylated derivatives of bisnorlipoic acid or lipoic acid demonstrated after about 75 min the incorporation of four oxygen atoms indicated by the identified molecular anions [M – H]− at \(m/z\) 271 and 299, respectively (data not shown). These [M – H]− ions used as precursor ions revealed a base peak at \(m/z\) 79 in the CID product ion spectra. We suggest that this is represented by the methyl sulfone structure \(CH_3-SO_2\). Based on this data, we suggest that the product ions \(m/z\) 63 and \(m/z\) 79 characterize methylsulfoxide and methylsulfone, respectively, and are unrelated to side chain hydroxylated products that appear as intermediates of β-oxidation.

The MS/MS information obtained from reference compounds, as well as oxidized references, was used as diagnostic data for substructure identification of metabolites in biological samples, as subsequently described.

**Identification of Metabolites.** Metabolites identified were encoded in the order of their elution from the radiometric HPLC column. LC/MS/MS data obtained from reference substances (Table 3) and major metabolites in \(^{14}C\) samples of animals were used to analyze human samples by LC/MS/MS after administration of unlabeled LA. In this way, metabolites identified in human samples obtained the same assignment as those found at the same retention time in animal samples after dosing of \(^{14}C\)LA.

**Metabolite M1.** The loss of CO₂ (44 Da) from [M – H]− in the CID spectrum yielding \(m/z\) 167 indicates the presence of the carboxyl group (Fig. 5).

The detected molecular anion [M – H]− of \(m/z\) 211 suggested the incorporation of two oxygen atoms referred to 2,4-bismethylmercapto-butanoic acid. The comparison with Fig. 4 (bottom trace) revealed a high degree of correspondence. Therefore, we suggest the presence of the disulfide of 2,4-bismethylmercapto-butanoic acid as shown in Fig. 10.

**Metabolites M2 and M3.** The MS information from both are very similar (Fig. 6) suggesting isomers. The molecular anion [M – H]− found at \(m/z\) 195 could indicate that one oxygen atom was incorporated in 2,4-bismethylmercapto-butanoic acid. This is supported by the typical fragmentation pattern already observed in Fig. 4 (middle trace) illustrating the course of oxidation of 2,4-bismethylmercapto-butanoic acid.

Based on this fragmentation pattern, we propose for M2/M3 isomeric sulfoxide structures of 2,4-bismethylmercapto-butanoic

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**TABLE 2**

*Means of \(^{14}C\) recovery of LA and major metabolites in whole-body plasma following single oral administration of \(^{14}C\)LA to male rats (30 mg/kg, \(n = 2\)) and male dogs (10 mg/kg, \(n = 3\)) and intravenous administration of \(^{14}C\)LA to male dogs (10 mg/kg, \(n = 3\)).*

<table>
<thead>
<tr>
<th>Species</th>
<th>Sampling Point (h)</th>
<th>(^{14}C) Recovery % of the Dose</th>
<th>Relative Amounts % of Radioactivity</th>
<th>Sum of Others</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>M3</td>
<td>M6</td>
</tr>
<tr>
<td>Rat p.o.</td>
<td>1.00</td>
<td>2.9</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Rat p.o.</td>
<td>3.00</td>
<td>1.7</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Dog p.o.</td>
<td>1.00</td>
<td>4.8</td>
<td>—</td>
<td>13.9</td>
</tr>
<tr>
<td>Dog p.o.</td>
<td>2.00</td>
<td>6.9</td>
<td>7.6</td>
<td>3.0</td>
</tr>
<tr>
<td>Dog p.o.</td>
<td>4.00</td>
<td>3.9</td>
<td>20.7</td>
<td>—</td>
</tr>
<tr>
<td>Dog i.v.</td>
<td>0.08</td>
<td>17.4</td>
<td>—</td>
<td>23.5</td>
</tr>
<tr>
<td>Dog i.v.</td>
<td>0.25</td>
<td>12.2</td>
<td>—</td>
<td>44.6</td>
</tr>
<tr>
<td>Dog i.v.</td>
<td>0.50</td>
<td>11.2</td>
<td>—</td>
<td>16.9</td>
</tr>
<tr>
<td>Dog i.v.</td>
<td>1.00</td>
<td>10.2</td>
<td>4.3</td>
<td>12.8</td>
</tr>
<tr>
<td>Dog i.v.</td>
<td>2.00</td>
<td>7.8</td>
<td>6.7</td>
<td>4.8</td>
</tr>
<tr>
<td>Dog i.v.</td>
<td>4.00</td>
<td>4.3</td>
<td>8.0</td>
<td>—</td>
</tr>
</tbody>
</table>

*In the case of rats, the percentage of the dose was calculated on the basis of weight percentage of the total body weight using 4.02% for plasma (Spector, 1956; Waynforth, 1980), whereas for dogs the total body plasma was calculated from total blood volume (84.5 ml/kg), along with animal weights and plasma/blood ratios (Morton et al., 1993).*  

*Values represent relative peak areas expressed as percentage (i.e., 100% equals the sum of all peak areas in the respective radiochromatogram). The \(^{14}C\) recoveries obtained by SPE ranged between 40 and 70%.*
based on the same considerations as described for M2 in Fig. 10, albeit diastereomers are also possible here, as already isomeric sulfoxides of 4,6-bismethylmercapto-hexanoic acid as shown in Fig. 10. For steric reasons, the oxidation of the CH$_3$S function at the C-4 position (Table 3). The molecular anions of M7 and M9 indicate a difference of 30 Da. M7 revealed a product ion at m/z 33, which was not obtained from the [M – H]$^-$ ion at m/z 264 of M9. It is replaced here with m/z 47. Thus, the increase by 30 Da to M7 is due to the S-methylation in M9. Based on that, we suggest the presence of the glycine conjugate of bisnorlipoic acid (M7), as well as its methylated derivative (M9) as shown in Fig. 10.

Metabolite M8. The abundant anion peak [M – H]$^-$ indicated a molecular weight of 192. In the CID product ion spectrum, the important ions m/z 33 and 47 appeared side by side. This suggests that, after the ring opening, only one sulfur atom was methylated. The MS/MS data provided no evidence for S-oxidation. We speculate that a partly S-methylated 2,3-en derivative of bisnorlipoic acid was present (see Fig. 10).

Metabolites M10 and M11. In both cases, data obtained from LC/MS/MS (data not shown) as well as the retention times from HPLC (Table 4) matched very well those of the authentic compounds 2,4-bismethylmercapto-butanonic acid and 4,6-bismethylmercapto-hexanoic acid (Table 3), respectively. This suggests that the structure of M10 and M11 is consistent with those of these reference compounds.

Metabolite M12. The structure proposed for M12 is based only on LC/MS data, because this metabolite fraction occurred as a shoulder of M11 in plasma samples of rats (Table 2) and humans (Fig. 8). The

### TABLE 3

<table>
<thead>
<tr>
<th>Reference Compounds</th>
<th>[M – H]$^-$ Ion m/z</th>
<th>Observed Product Ions (Relative Abundance as Percentage in Parentheses)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA</td>
<td>205 (100)</td>
<td>171 (18) 159 (3) 127 (3) 93 (6) 65 (27) 64 (22) 33 (3)</td>
</tr>
<tr>
<td>Dihydriolipoic acid</td>
<td>207 (100)</td>
<td>173 (13) 139 (2) 129 (3) 99 (26) 87 (7) 65 (4) 64 (5) 33 (4)</td>
</tr>
<tr>
<td>Bisnorlipoic acid</td>
<td>177 (100)</td>
<td>143 (9) 131 (2)</td>
</tr>
<tr>
<td>Tetranorlipoic acid</td>
<td>149 (100)</td>
<td>105 (6) 59 (33) 33 (2)</td>
</tr>
<tr>
<td>6,8-Bismethylmercapto-octanoic acid</td>
<td>235 (100)</td>
<td>187 (15) 139 (4) 59 (3) 47 (37)</td>
</tr>
<tr>
<td>4,6-Bismethylmercapto-hexanoic acid</td>
<td>207 (76)</td>
<td>159 (5) 111 (3) 115 (8) 67 (11) 47 (100)</td>
</tr>
<tr>
<td>2,4-Bismethylmercapto-butanolic acid</td>
<td>179 (100)</td>
<td>135 (1) 87 (6) 84 (6) 74 (16) 33 (10)</td>
</tr>
</tbody>
</table>

*Generation of the oxidized products and their LC/MS/MS analysis are described under Materials and Methods.*
peak intensity of the [M – H]⁻ ion at m/z 219 was not sufficient to obtain CID product ions from that. The appearance of a fragment ion at m/z 175 indicates the presence of the carboxylic group (loss of CO₂). The increase of the molecular anion by 14 Da, compared with LA, let us assume that 3-keto-lipoic acid is present (see Fig. 10).

LA. The radiopeak of the parent drug at the retention time of 97 min could be enlarged by spiking with [¹⁴C]LA in fecal extracts from mice, rats, and dogs.

The LC/MS/MS results obtained, along with chromatographic data in comparison with those from an authentic sample (Table 3), provided clear evidence for the presence of LA in plasma samples from humans after oral administration (Fig. 8). Traces of LA were also discernible in some human urine samples (data not shown). Metabolites M8, M10, and M11 were found in plasma, as well as urine samples of humans (Figs. 8 and 9). This data (Fig. 8) confirms recent findings obtained by HPLC with electrochemical detection that 4,6-bis(methylmercapto-hexanoic acid (M11) is the major metabolite in human plasma (Teichert and Preiss, 2000). Metabolite M1 dominant in dog urine was not identified in human urine. The LC/MS/MS data presented here is not quantitative, because the ionization efficiency of each component is not exactly known due to the lack of pure authentic standards. However, since all metabolites contain the carboxylic acid function that is almost exclusively responsible for negative ionization efficiency, an approximate comparison of peak abundance should be possible.

### Table 4

<table>
<thead>
<tr>
<th>Metabolite Code</th>
<th>Retention Time a</th>
<th>Species/Biological Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>5</td>
<td>Mouse/U; rat/U; dog/U</td>
</tr>
<tr>
<td>M2</td>
<td>23</td>
<td>Mouse/U; rat/U; dog/U; human/U</td>
</tr>
<tr>
<td>M3</td>
<td>26</td>
<td>Mouse/U; rat/U; dog/U; human/U/P</td>
</tr>
<tr>
<td>M4</td>
<td>40</td>
<td>Rat/U; human/U</td>
</tr>
<tr>
<td>M5</td>
<td>43</td>
<td>Mouse/U; rat/U; dog/U; human/U/P</td>
</tr>
<tr>
<td>M6</td>
<td>48</td>
<td>Dog/P; human/P</td>
</tr>
<tr>
<td>M7</td>
<td>52</td>
<td>Mouse/U</td>
</tr>
<tr>
<td>M8</td>
<td>80</td>
<td>Rat/P; dog/P; human/U/P</td>
</tr>
<tr>
<td>M9</td>
<td>81</td>
<td>Mouse/U; rat/U; dog/U</td>
</tr>
<tr>
<td>M10</td>
<td>94</td>
<td>Mouse/U; dog/U; human/U/P</td>
</tr>
<tr>
<td>M11</td>
<td>95</td>
<td>Rat/P; human/U/P</td>
</tr>
<tr>
<td>M12</td>
<td>98</td>
<td>Rat/P; human/P</td>
</tr>
<tr>
<td>LA</td>
<td>97</td>
<td>Mouse/F; rat/F; dog/F; human/U/P</td>
</tr>
</tbody>
</table>

P, plasma; U, urine; F, feces.

### Discussion

Results of the present study confirm the paramount role of β-oxidation in the metabolism of LA resulting in a strong first pass effect. This is reflected by the calculated plasma data in Table 2 and illustrated for dog plasma in Fig. 3. Already 5 min after intravenous dosing in dogs, tetranorlipoic acid (M6), the final product of β-oxidation, appeared in plasma samples in approximately comparable concentration to LA (Fig. 3). Ten minutes later, the total of the drug-related radioactivity in the plasma of dogs was predominantly represented by tetranorlipoic acid (M6), and LA was conspicuously absent. Subsequently tetrornorlipoic acid was subjected to S-methylation, shown by the concomitant increase of metabolite M10, identified as 2,4-bis(methylmercapto-butanoic acid. Consequently, the 1,2-dithiolane moiety was reduced in the meantime, because only the thiol and not the disulfides are substrates for thiol methyltransferases (Handelman et al., 1994).

LC/MS/MS data provide evidence that M8 (Fig. 3, third trace) represents a partly S-methylated derivative of bisnorlipoic acid. The structure of the 2,3-dehydrogenated derivative would be conceivable as the intermediate in the course of β-oxidation from bisnor- to tetranorlipoic acid derivatives (Fig. 10). But, conversely, M8 cannot metabolically emerge from tetranorlipoic acid (M6), which was solely circulating before (Fig. 3, second trace). Therefore, we suggest that M8 is formed in deeper compartments and is subsequently excreted back into plasma. The great volume of distribution of total radioactivity that was determined to be 12.3 l/kg for rats following intravenous administration of 10 mg/kg [¹⁴C]LA (data not shown) indicates that the [¹⁴C] label is widely distributed within the organism. This corresponds to the affinity of LA to proteins (Teichert and Preiss, 1995; Biewenga et al., 1997a). In the further course of metabolism, the CH₃S group is oxidized as demonstrated by the appearance of metabolite M3 in dog plasma (Fig. 3, fourth trace). In rat plasma, only derivatives of the bisnorlipoic acid (M11 and M8) were found, and a lower amount of the 3-keto derivative of LA (M12) could be identified. However, tetrnorlipoic acid (M6) as well as its methylated (M10) and oxidized derivatives (M3) that predominantly appeared in dog plasma (Fig. 3) are missing in plasma samples of rats (Table 2). Thus, compared with mice and rats, dogs seem to have a more strongly pronounced ability to perform a sequential β-oxidation of the hexanoic acid derivative to form tetrnorlipoic acid (M6).

The rapid appearance of these metabolites in plasma highlights the fact that the β-oxidation of LA proceeds very effectively, especially in the liver mitochondria of dogs (Lang, 1992). These results suggest that after intravenous administration the metabo-
LC/MS/MS data from reference compounds. The identification of metabolites of LA provides the proof that metabolites of LA are substrates of glycine conjugation. The identification of depletion of the hepatic coenzyme A. The present results support the use in the mitochondrial matrix of the liver and kidney. Recently, Gregus and coworkers (1996) were able to show that LA affects the metabolism of the 1,2-dithiolane moiety already partly starts in the circulating system. In fact, it can be mediated by a methyltransferase following glutathione reductase in the erythrocytes (Constantinescu et al., 1995; Haramaki et al., 1997). But the enzymatic reduction by mitochondrial lipoamide dehydrogenase in tissues like muscle, nerve, and liver should be more effective (Biewenga et al., 1996). The vast tissue distribution of the reductive enzymes contributes also to the high clearance of LA. In an additional pathway, bisnorlipoic acid and its methylated derivative undergo conjugation with glycine, preferably in mice. This conjugation process can be considered as a competitive reaction to β-oxidation, because the same activated intermediate (coenzyme A thioester) is used in the mitochondrial matrix of the liver and kidney. Recently, Gregus and coworkers (1996) were able to show that LA affects the glycine conjugation of benzoic acid in rats, among others by depletion of the hepatic coenzyme A. The present results support this. The identification of M7 (mice) and M9 (mice, rats, and dogs) provides the proof that metabolites of LA are substrates of glycine N-acyltransferase. The conjugates formed cannot be substrates for β-oxidation. The tendency to form glycine conjugates decreases in the order from mice to rats to dogs (Table 1). A comparison of the metabolite structures identified in animals (Fig. 10, Table 4) indicates similarities in metabolism between mice and rats, but reflects differences from that in dogs. Whereas in mice and rats derivatives of bisnorlipoic acid are predominantly formed, in dogs the majority of metabolites are related to tetrnorlipoic acid. It is interesting to note that the free bisnorlipoic acid did not appear as a metabolite.

Metabolites containing sulfur oxidized as well as the intact 1,2-dithiolane ring were not found either. With the exception of the glycine conjugate M7 (mouse), only ring-opened metabolites were identified in urine. Among the species compared here, the dog showed the highest amounts of sulfoxides. Only dog urine (Fig. 2) contained the 2-fold S-oxidized structure M1 in considerable amounts, although M2 and M3, the two starting products for M1, appeared in urine samples of all species.

The present results from human samples based on LC/MS data indicate that the metabolism in humans mainly resembles the observed in mice and rats. The pronounced formation of oxidized structures related to tetranorlipoic acid, as found in samples of dogs, had no equivalent in humans. In accordance with this, 3-keto lipoic acid, an intermediate in the course of β-oxidation of LA, was found in plasma samples from rats as well as from humans, but not in those from dogs. However, unlike animals, glycine conjugates were not identified in human samples. Due to the endogenous occurrence of LA and its tight protein binding, the use of [14C]LA in humans is not permissible. Thus, a direct comparison between the metabolism in animals and humans is not possible on the basis of the [14C] data.

To our knowledge this study shows for the first time that, besides β-oxidation of the side chain and methylation of the reduced 1,2-dithiolane moiety, two further metabolic pathways exist in the metabolism of LA. First, methyl sulfides are oxidized to methyl sulfoxides that are formed in all investigated species, including humans, but are most pronounced in dogs. Second, the glycine conjugation is a competitive metabolic pathway to β-oxidation, predominantly in mice, but not in humans.

Acknowledgments. We thank S. Emmel, B. Fühler, M. Meckel, G. Motzheim, H. Pöthig, and A Scherf for their excellent and skilful technical assistance. Furthermore, we are grateful to Dr. G. Laban and Dr. C. Rundfeldt for helpful discussions. The in vivo part of the studies in dogs was performed by Inveresk Research (Tranent, Scotland). We also thank Drs. N. McCraken, S. Cameron, and C. Young for their excellent work and cooperation.
Fig. 10. Biotransformation of LA.

References