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NEW METABOLIC PATHWAYS OF α-LIPOIC ACID[1]

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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 [^{14}C]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 disulfoxide 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[2] 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, tetranorlipoic 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 thiolsulfinates, as well as thiolsulfonates, 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 [^{14}C]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.

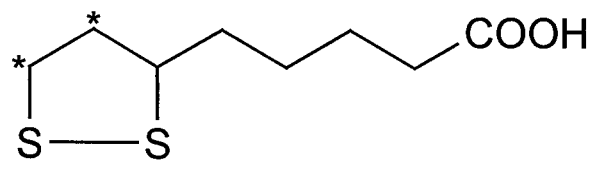

FIG. 1. *Structural formula of rac-α-lipoic acid (LA).* Asterisks denote the ^{14}C label.

[1] Dedicated to Professor Axel Kleemann's 60th birthday.

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

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Materials and Methods

Chemicals. *rac*-α-Lipoic acid was synthesized by ASTA Medica AG (Frankfurt, Germany). The potential metabolites bisnorlipoic acid (1,2-dithiolane-3-yl-propionic acid), tetranorlipoic acid (1,2-dithiolane-3-yl-formic acid), and 6,8-bismethylmercapto-octanoic acid, 4,6-bismethylmercapto-hexanoic acid, 2,4-bismethylmercapto-butanoic acid, and 6,8-dithiol-octanoic acid were kindly provided by Dr. Hübner, Degussa-Hüls and Dr. G. Laban, Arzneimittelwerk Dresden GmbH, Dresden, Germany. *N*-(2-mercaptopropionyl)-glycine was supplied by Fluka, Buchs, Switzerland. [7,8-^{14}C]*R*,*S*-5-(1,2-dithiolane-3-yl) pentanoic acid (Fig. 1), abbreviated as [^{14}C]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°C. All other chemical substances used came from commercial suppliers in analytical grade. Demineralized 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 [^{14}C]LA as a solution, representing 2.60 MBq/kg. The solution was prepared by adding the commercially available formulation Thioctacid T direkt to an aliquot of the [^{14}C]LA stock solution resulting in a final concentration of the test article of 3 mg/ml, representing 86.8 kBq/mg. Urine and feces were sampled in metabolic cages under dry ice cooling within the following intervals. Urine: 0 to 2 h, 2 to 4 h, 4 to 8 h, 8 to 24 h, 24 to 48 h, and 48 to 72 h postdose. Feces was collected in 24 h intervals up to 72 h postdose. At the end of the trial, urine samples were intermediately thawed at room temperature, and duplicate aliquots of 0.01 ml were taken for LSC. The residual samples were transferred into screw cap plastic bottles and stored at −20°C until analysis. Feces (triplicate aliquots of 120–160 mg) was combusted to $^{14}CO_2$ (OX 500, Zinsser, Frankfurt, Germany). The radioactivity formed was trapped with Oxisolve 400 (Zinsser) and measured by LSC. Wistar rats received [^{14}C]LA in the same dose as mice using the same test article. Urine and feces were sampled 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 subjected to centrifugation (2,000*g*, 10 min, Megafuge 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 oral and intravenous doses of 10 mg/kg of [^{14}C]LA, representing about 0.92 MBq/kg each. Specific radioactivity of the test article used was 93.6 kBq/mg and 95.5 kBq/mg for intravenous and oral dosing, respectively. The washout phase between both routes was 7 weeks. Radioactivity in plasma and urine reached the background level during this period. For intravenous dosing, aliquots of the [^{14}C]LA stock solution (Tris buffer) were added to the Thioctacid 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 (Thioctacid). 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 using containers cooled with dry ice in the morning of the fourth day at the following time intervals after the last dose: 0 to 2, 2 to 4, 4 to 8, 8 to 12, and 12 to 24 h. Blood samples were collected via a forearm vein catheter 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 (10,000*g*, 10 min). Plasma was separated from whole blood, transferred into labeled plastic tubes, and immediately frozen. All biological samples were kept at −30°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 10^5 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 a 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 Superspher 60 RP select B column (250 × 4 mm, 5 μm, with guard 4 × 4-mm i.d.; Merck). The flow rate was 0.5 ml/min. Mobile phase A was 20 mmol/l of KH_2PO_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 100% A; 6 to 16 min linear gradient from 0% B to 10% 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 (5 ml) 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_3PO_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 (Heidolph, Kehlheim, Germany) at a bath temperature of 40°C. The residue obtained was reconstituted in mobile phase A, 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 [^{14}C]LA. Frozen urine samples were thawed at room temperature, and aliquots were subjected to centrifugation for 10 min at 2,700*g*. Then, urine samples were diluted with water dependent on the content of radioactivity. Samples were transferred into borosilicate glass vials (Chromacol Ltd., Langenfeld, Germany) and placed in the autosampler, at a temperature of 4 to 8°C until radiometric HPLC analysis. Aliquots of thawed 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 10,600*g* 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 29.3 ± 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 radiometric 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 [^{14}C]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).

TABLE 1

Radioactive excretion of major metabolites in urine (0–24 h) following single oral administration of [^{14}C]LA to male mice (30 mg/kg, n = 15), male rats (30 mg/kg, n = 20), and male dogs (10 mg/kg, n = 3) expressed as a percentage of the dose (means)

Species	% Dose Excreted Total	% Dose Per Metabolite Fraction Identified								Sum of Others
		M1	M2	M3	M4	M5	M7	M9	M10	
Mouse	54.7 ± 12.7	2.9	1.3	1.0	—	4.8	5.2	9.9	1.5	28.1
Rat	71.8 ± 9.4	2.2	1.0	2.3	5.8	12.2	—	5.8	—	42.5
Dog	63.5 ± 7.3	9.1	5.2	11.1	—	1.4	—	1.9	—	34.8

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.

Results

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 samples by cochromatography with [^{14}C]LA (data not shown). After intravenous dosing of [^{14}C]LA to dogs, less than 5% of the radioactive dose was found in feces, indicating the predominance of the renal elimination of the drug-related radioactivity. However, LA was not detectable by radiometric HPLC in any urine samples obtained from animals (Fig. 2). Only plasma samples at early time points (Table 2) revealed LA after intravenous administration to dogs (Fig. 3). The radiometric metabolite profiles of plasma and urine samples showed that LA is extensively metabolized (Figs. 2 and 3; Tables 1 and 2). But, the low ^{14}C recoveries obtained from later plasma samples, as mentioned previously, have an effect on data presented in Table 2.

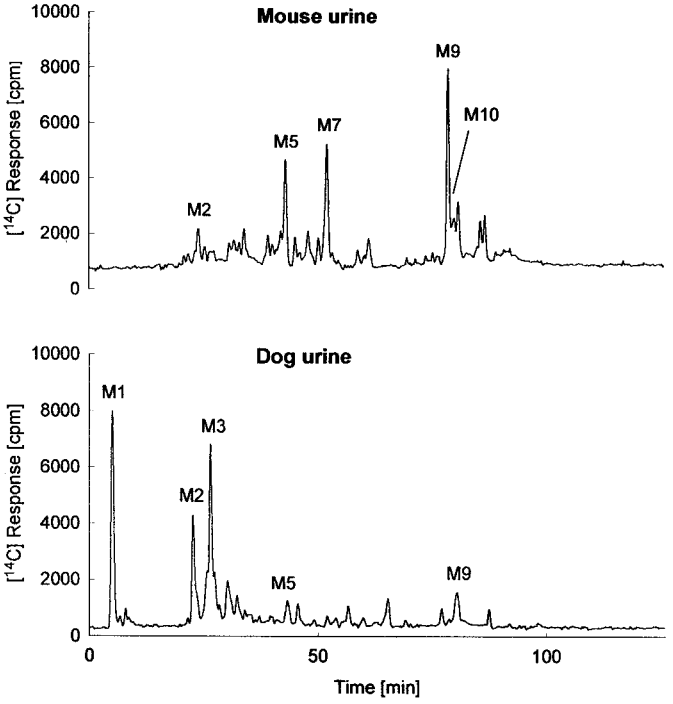

FIG. 2. *Representative radiochromatograms.*

Top, metabolite profiles obtained from mouse urine, collecting interval 0 to 2 h after oral administration of 30 mg/kg of [^{14}C]LA, representing 2.605 MBq/kg (70.4 μCi/kg). Bottom, metabolite profiles obtained from urine of the dog 3M, collecting interval 2 to 4 h after oral administration of 10 mg/kg of [^{14}C]LA, representing 0.925 MBq/kg (25 μCi/kg).

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 C_{18} 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 (H_2S) 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 CH_3S^- at *m/z* 47 that corresponds to the first loss of mercaptomethane (48 Da) from $[M - H]^-$. The tetranorlipoic 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),

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)

Species	Sampling Point (h)	^{14}C Recovery % of the Dose[a]	Relative Amounts % of Radioactivity[b]							Sum of Others
			M3	M6	M8	M10	M11	M12	LA	
						%				
Rat p.o.	1.00	2.9	—	—	27.0	—	29.7	10.9	—	32.4
Rat p.o.	3.00	1.7	—	—	29.1	—	34.4	6.2	—	30.3
Dog p.o.	1.00	4.8	—	13.9	19.3	19.7	—	—	—	47.1
Dog p.o.	2.00	6.9	7.6	3.0	20.5	24.7	—	—	—	44.2
Dog p.o.	4.00	3.9	20.7	—	18.1	13.8	—	—	—	47.4
Dog i.v.	0.08	17.4	—	23.5	—	—	—	—	23.3	53.2
Dog i.v.	0.25	12.2	—	44.6	—	—	—	—	—	55.4
Dog i.v.	0.50	11.2	—	16.9	10.5	9.0	—	—	—	63.6
Dog i.v.	1.00	10.2	4.3	12.8	12.6	15.4	—	—	—	54.9
Dog i.v.	2.00	7.8	6.7	4.8	20.7	23.1	—	—	—	44.7
Dog i.v.	4.00	4.3	8.0	—	21.9	19.8	—	—	—	50.3

[a] 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).

[b] 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%.

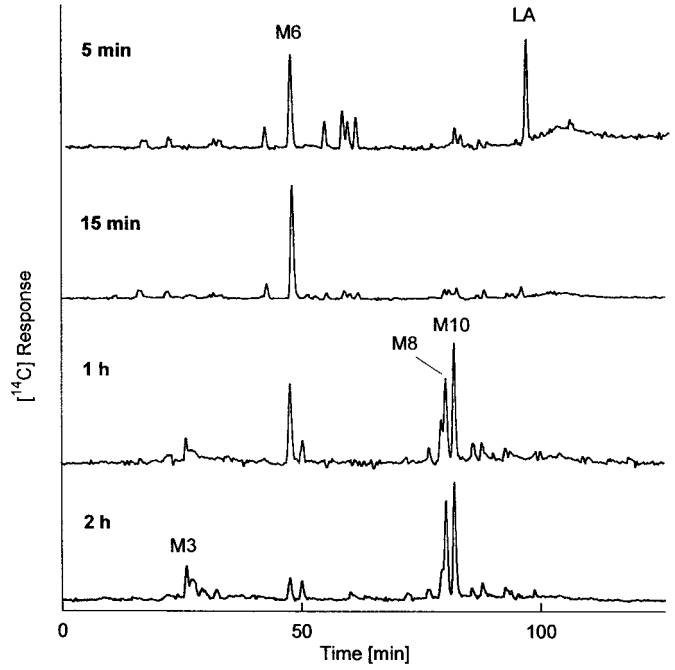

FIG. 3. *Metabolite profile from dog plasma after intravenous administration of 10 mg/kg of [^{14}C]LA, representing 0.925 MBq/kg (25 μCi/kg), obtained by radiometric HPLC following sample preparation by SPE.*

glycine conjugates of carboxylic acids can be detected by the abundant ion at *m/z* 74 ($NH_2CH_2COO^-$) 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 disulfoxide, characterized by the abundant ion at *m/z* 63, which revealed an oxygen-bearing methylated sulfur atom ($CH_3—S═O^-$). 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 CO_2 (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 disulfoxide 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

TABLE 3

LC/MS/MS analysis (CID product ion spectra) of [M − H]⁻ ions of reference compounds and oxidation products

Reference Compounds	[M − H]⁻ Ion *m/z*	Observed Product Ions (Relative Abundance as Percentage in Parentheses)							
		m/z							
LA	205 (100)	171 (18)	159 (3)	127 (3)	93 (6)	65 (27)	64 (22)		33 (3)
Dihydrolipoic acid	207 (100)	173 (13)	139 (2)	129 (3)			59 (2)		33 (93)
Bisnorlipoic acid	177 (100)	143 (9)	131 (2)	99 (26)	87 (7)	65 (4)	64 (5)		33 (4)
Tetranorlipoic acid	149 (100)	105 (6)					59 (33)		33 (2)
6,8-Bismethylmercapto-octanoic acid	235 (100)	187 (15)	139 (4)				59 (3)	47 (37)	
4,6-Bismethylmercapto-hexanoic acid	207 (76)	159 (5)	111 (3)	115 (8)		67 (11)		47 (100)	
2,4-Bismethylmercapto-butanoic acid	179 (100)	135 (1)						47 (59)	
N-(2-Mercapto-propionyl)-glycine	162 (100)				87 (6)	84 (6)	74 (16)		33 (10)
Oxidation products[a]									
2,4-Bismethylmercapto-butanoic acid									
+1 × oxygen	195 (100)	151 (5)			77 (7)	63 (31)		47 (26)	
+2 × oxygen	211 (61)	167 (8)			77 (11)	63 (100)			
4,6-Bismethylmercapto-hexanoic acid									
+1 × oxygen	223 (100)	175 (9)	131 (5)	111 (18)	77 (15)	67 (39)	63 (40)	47 (41)	
+2 × oxygen	239 (94)	175 (10)	131 (10)	111 (29)	79 (18)	67 (67)	63 (100)		

[a] Generation of the oxidized products and their LC/MS/MS analysis are described under *Materials and Methods.*

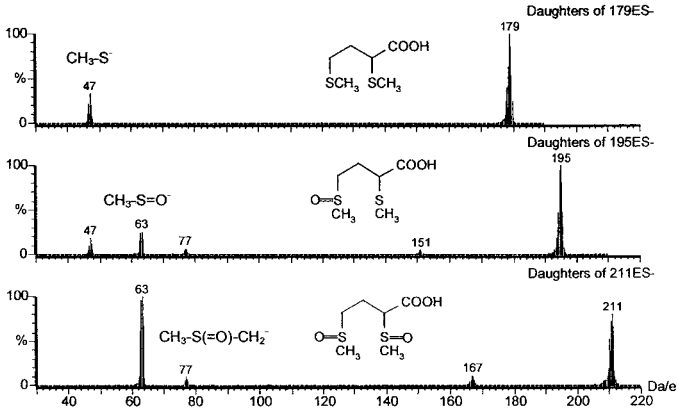

FIG. 4. *Product ion spectrum of the [M − H]⁻ ion at m/z 179 of 2,4-bismethylmercapto-butanoic acid (top) and from reaction products of oxidation with hydrogen peroxide after 30 min reaction time (middle) at m/z 195 and after 58 min at m/z 211 (bottom) obtained after flow injection analysis.*

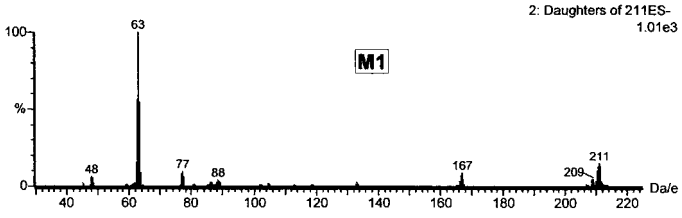

FIG. 5. *Product ion spectrum of the [M − H]⁻ ion at m/z 211 of metabolite **M1** analyzed in urine from dog 3M collected 2 to 4 h following oral administration of 10 mg/kg of [¹⁴C]LA.*

acid as shown in Fig. 10. For steric reasons, the oxidation of the CH_3-S function at the C-4 position (**M2**) should result in higher polarity than S-oxidation at the C-2 position. Therefore, the structure with the lower retention time (Table 4) was encoded with **M2**. At the same time, sulfoxide formation resulted in formation of a new asymmetric center in the molecule. Therefore, metabolites **M2**/**M3** could also represent diastereomers. However, in the frame of the present study, it could be not distinguished between positional isomers and diastereomers. In the following only the formulas of positional isomers are used.

Metabolites M4 and **M5**. The largest mass showing up was the molecular anion [M − H]⁻ at *m/z* 223. Very abundant daughter ions at *m/z* 47 and *m/z* 63 indicate the simultaneous presence of CH_3S— and $CH_3S(O)$— groups, respectively. The MS information of **M4** and **M5** (data not shown) was very similar, as described in the cases of **M2** and **M3**. Here, also, we were able to produce almost identical CID product ion spectra following peroxide oxidation of 4,6-bismethylmercapto-hexanoic acid (Table 3). Thus, we assume the presence of isomeric sulfoxides of 4,6-bismethylmercapto-hexanoic acid as shown in Fig. 10, albeit diastereomers are also possible here, as already discussed for **M2**/**M3**. In addition, the order of assignment is also based on the same considerations as described for **M2**/**M3**.

Metabolite M6. The MS and MS/MS data (Table 3), as well as the retention time obtained (Table 4), corresponded exactly to those from the authentic compound tetranorlipoic acid. This indicates that **M6** is tetranorlipoic acid.

Metabolites M7 and **M9**. The CID product ion spectra (Fig. 7) of both metabolites are characterized by a very prominent anion at *m/z* 74. This is typical for the cleavage of the glycine moiety from the [M − H]⁻ ion as documented for glycine conjugates of carboxylic acids (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]⁻ 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-butanoic 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

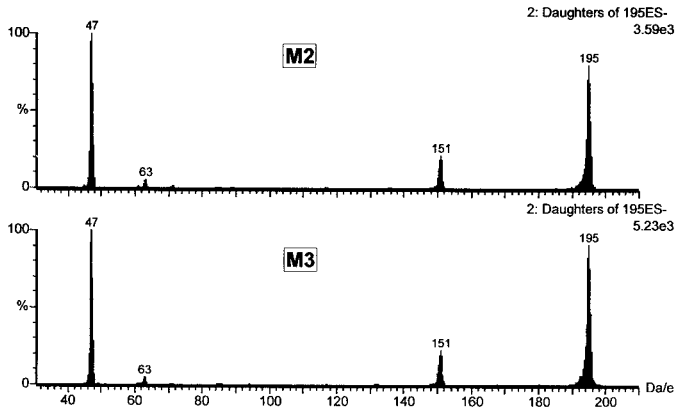

FIG. 6. *Product ion spectrum of the [M − H]⁻ ion at m/z 195 of metabolite **M2** (top) and metabolite **M3** (bottom) analyzed in urine from dog 3M, collected 2 to 4 h following oral administration of 10 mg/kg of [^{14}C]LA.*

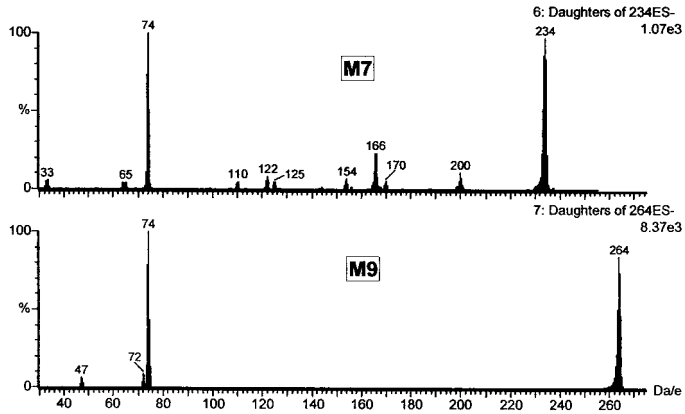

FIG. 7. *Product ion spectrum of the [M − H]⁻ ion at m/z 234 of metabolite **M7** (top) and **M9** (bottom) at m/z 264 analyzed in urine from mice, collected 0 to 2 h following oral administration of 30 mg/kg of [^{14}C]LA.*

TABLE 4

Summary of LA metabolites identified in different matrices of the species investigated

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

P, plasma; U, urine; F, feces.
[a] Radiomonitor.

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 CO_2). 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 [^{14}C]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-bismethylmercapto-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.

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 tetranorlipoic acid was subjected to S-methylation, shown by the concomitant increase of metabolite **M10**, identified as 2,4-bismethylmercapto-butanoic acid. Consequently, the 1,2-dithiolane moiety was reduced in the meantime, because only the thiols 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 [^{14}C]LA (data not shown) indicates that the ^{14}C 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 CH_3-S 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, tetranorlipoic 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 tetranorlipoic 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-

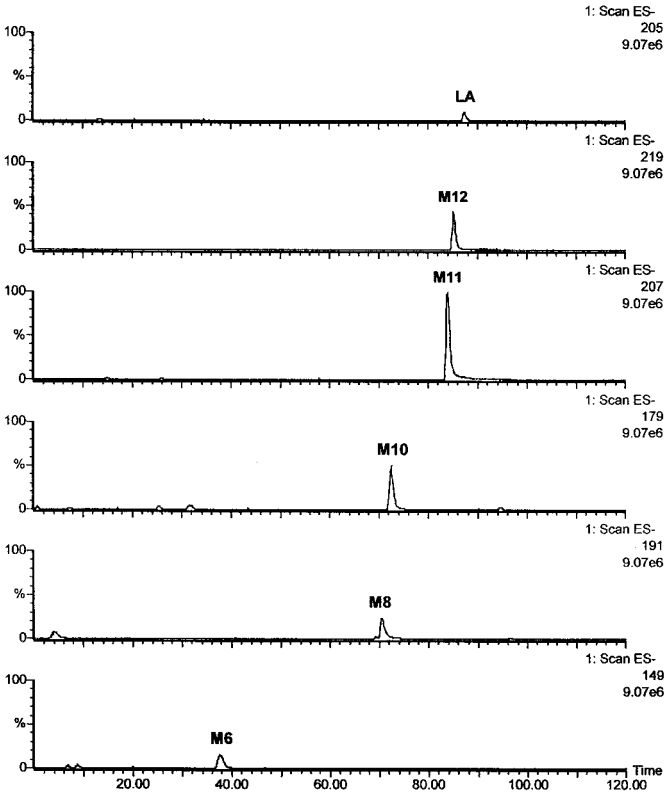

FIG. 8. *Reconstructed ion chromatograms from full scan LC/MS analysis of human plasma 1 h after oral administration of 600 mg of Thioctacid (solution), indicating the presence of five metabolites besides LA.*

Plasma samples were pooled from 12 subjects and prepared by SPE as described. The mass spectrometer was switched on-line 10 min after starting the chromatographic run. All ion traces are normalized on 9.07×10^6 counts corresponding to 100% relative intensity. The identification of **M6**, **M10**, and **M11** is based on LC/MS/MS data from reference compounds.

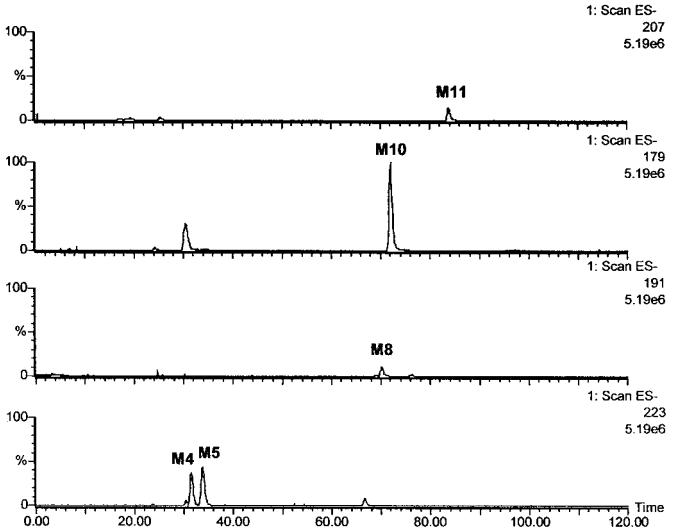

FIG. 9. *Reconstructed ion chromatograms from full scan LC/MS analysis of human urine (subject 2) collecting interval 4 to 8 h after oral administration of 600 mg of Thioctacid (tablets, three times a day, over 3 days), indicating the presence of five metabolites.*

The mass spectrometer was switched on-line 10 min after starting the chromatographic run. All ion traces are normalized on 5.19×10^6 counts corresponding to 100% relative intensity.

lism 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 tetranorlipoic 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 higher 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/MS data indicate that the metabolism in humans mainly resembles that 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 [^{14}C]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 ^{14}C 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.

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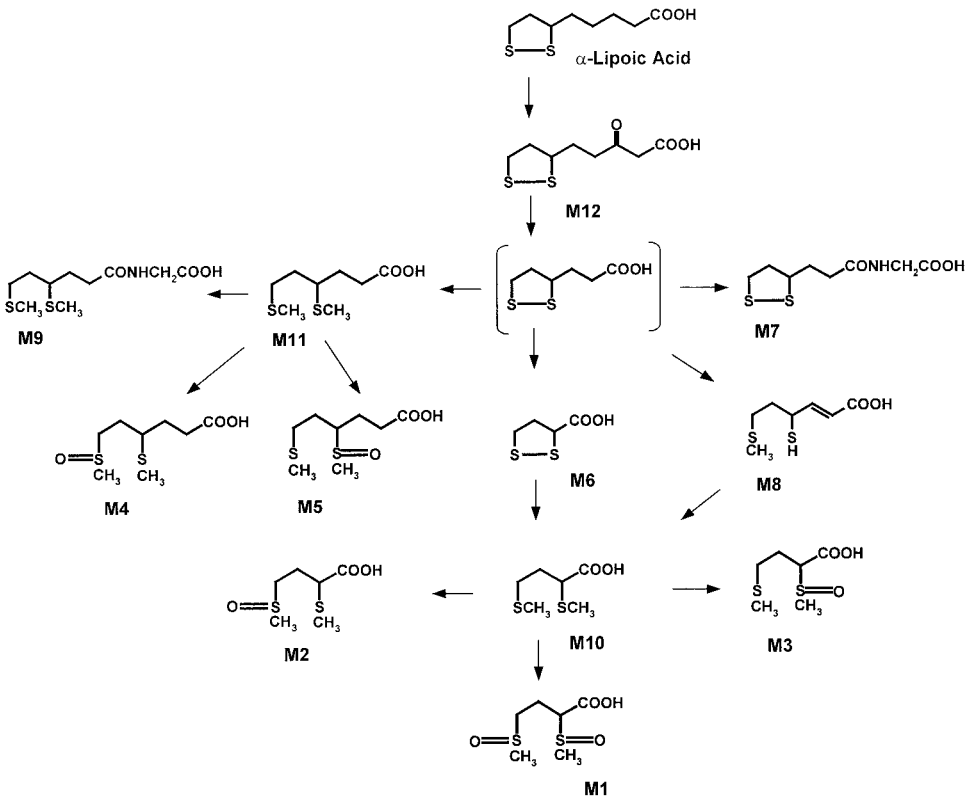

FIG. 10. *Biotransformation of LA.*

Arrows indicate potential metabolic pathways. Bisnorlipoic acid, marked by brackets, could not be identified as metabolite. The occurrence of metabolites with respect to matrices and species investigated is shown in Table 4.

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