10 An Evaluation of the Stability and Pharmacokinetics of *R*-Lipoic Acid and *R*-Dihydrolipoic Acid Dosage Forms in Human Plasma from Healthy Subjects

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INTRODUCTION: STRUCTURE, STEREOCHEMISTRY, AND MECHANISMS OF ACTION OF ALPHA LIPOIC ACID

Alpha lipoic acid (ALA) is a medium chain (C8) fatty acid with vicinal sulfur atoms at C6 and C8 existing either as free sulfhydryls (DHLA) or linked via an intramolecular disulfide (ALA). The C6 atom (alternatively designated as C3 of the dithiolane ring) of the octanoic acid chain is chiral and the molecules exist as four enantiomers (*R*-(+)-LA, *S*-(−)-LA, *R*-(−)-DHLA, and *S*-(+)-DHLA) (Figure 10.1) and two racemic mixtures (rac-ALA = +/−ALA = (*RS*)-ALA and rac-DHLA = (+/−)-ALA = *RS*-DHLA). These six forms of lipoic acid have

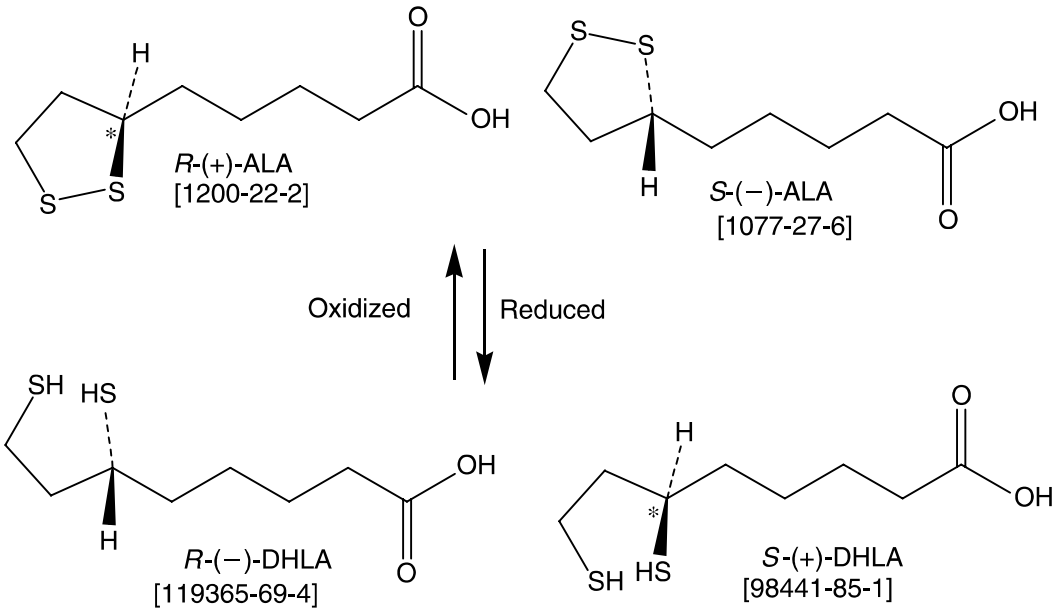


FIGURE 10.1 Three dimensional molecular diagrams depicting both the absolute configurations (*R* and *S*) which are mirror images and the change in specific rotations from (+) to (−) when RLA is reduced to *R*-DHLA of (−) to (+) when SLA is reduced to *S*-DHLA. The diagrams are useful in conceptualizing how one enantiomer can react differently than its mirror image with a receptor, transporter, signaling molecule, protein or enzyme in vivo. Only RLA is naturally occurring and proposed to be the eutomeric form of ALA.

biological similarities and differences in their mechanisms of action and should be considered pharmacologically distinct. RLA and *R*-DHLA are the naturally occurring enantiomers. The complete characterization of the pharmacological similarities and differences between the six forms is in its infancy but there are indications that the *R*-enantiomers are the eutomeric forms of ALA (Jacob et al. 1999; Packer et al. 2001). Stereochemical and non-stereochemical mechanisms of action of ALA have been identified and comprehensively reviewed (Carlson et al. 2007b). It is a well-known principle of enantioselective pharmacology that a given enantiomer may either be active (contributing to the PD), inactive (isomeric ballast), or detrimental (antimetabolite or competitive inhibitor, opposing the action of the preferred enantiomer) (Ariëns 1984; Jamali et al. 1989). Both in vitro and in vivo assays have revealed SLA may competitively inhibit the actions of RLA, which therefore becomes the limiting criteria therapeutically (Artwohl et al. 2000; Ulrich et al. 2001; May et al. 2006). Fundamental and yet currently unanswered questions are whether or not there is a single primary mechanism of action of ALA (or a summation of multiple factors) responsible for the therapeutic effects. It is unclear whether or not the therapeutic efficacy is primarily stereospecific or non-stereospecific or dose-concentration and time dependent.

The first description of the antioxidant properties of ALA was in 1959 (Rosenberg and Culik, 1959). In the late 1980s, rac-ALA and rac-DHLA were labeled as antioxidants due to the in vitro capacity to neutralize a variety of ROS, RNS, and RSS. By the early 1990s, ALA became available as a nutritional supplement (Packer et al. 1996) but the complex stereospecific and pleiotropic mechanisms of action in vivo have not been fully characterized (Pershadsingh 2007; Zhang et al. 2007). This is partially due to the fact that the bulk of 2320 papers published utilized only the racemic mixture and approximately 1% tested the enantiomers (Pub Med as of 3/21/07). Surprisingly, one study indicated that RLA was a better chelator than SLA and antioxidant (Ou et al. 1995). Recently, it was demonstrated that RLA was more effectively transported by the MCFA transporter in a human endothelial cell line than rac-ALA, indicating competitive inhibition by SLA (May et al. 2006). Recent studies attempting to elucidate the mechanisms of action have shifted primary emphasis away from direct scavenging of dangerous free radical species, single electron reductions of vitamin C, Vitamin E, and CoQ10 radicals, chelation of heavy and redox active transition metals due to the measurement of low micromolar concentrations in vivo (versus millimolar intracellular concentrations of GSH and ascorbate) and rapid disappearance from plasma and tissues subsequent to exogenous administration. These properties which undoubtedly have some effect in vivo must be reconciled with the short plasma half-lives of ALA, DHLA (all forms), and of the metabolites (Smith et al. 2004).

The new paradigm is founded on the ability of ALA to alter signal transduction and gene transcription by functioning as a metabolic and redox stressor and which may be mediated by either a pro-oxidant, reductive or redox effect via activation of early response genes (Packer et al. 1997; Dicter et al. 2002; Konrad 2005; Ogborne et al. 2005; Linnane et al. 2006). RLA has been shown to increase ATP production stereospecifically in the heart while SLA decreased it

(Hagen et al. 1999). Redox modulation may be both stereospecific and non-stereospecific and may result in increased expression of thioredoxin and other redox-sensitive genes (Lee et al. 2004; Larghero et al. 2006). RLA improved synthesis of GSH stereospecifically in the lens and may reverse age-related losses in GSH in the brain (Maitra et al. 1996; Suh et al. 2001; Bharat et al. 2002; Suh et al. 2004a). RLA binds to the IR and induces alterations in patterns of phosphorylation/dephosphorylation of various tyrosine kinases (Diesel et al. 2007) and phosphatases (Sommer et al. 2000; Cho et al. 2003), modulates ion transport (Sen 2000; Bishara et al. 2002), Na^+, K^+, ATPases (Arivazhagan and Panneerselvam 2004), plasma membrane redox system (Bera et al. 2005), the plasma redox status by lowering plasma L-Cys (Nolin et al. 2007), altering the free thiol/disulfide ratios (Gregus et al. 1992), signal transduction pathways and gene transcription associated with metabolic control genes (nutrigenes) associated with glucose and fat metabolism; peroxisome proliferator activated receptor-α (PPAR-α) and peroxisome proliferator activated receptor-γ (PPAR)-γ, (Pershadsingh et al. 2005; El Modaoui et al. 2006; Pershadsingh 2007), SREBP-1 (Moreau et al. 2007) leptin (Lee et al. 2006); downregulation of acute phase proteins such as CRP (Sola et al. 2005), IL-6 (Mantovani et al. 2003a; Sola et al. 2005), PAI (Sola et al. 2005), TNF-α, and IL-1β (Zhang and Frei 2001; Kiemer et al. 2002; Byun et al. 2005; Dulundu et al. 2007), transforming growth factor-β (TGF-β) (Oksala et al. 2007), adhesion molecules (ICAM-1,VCAM, MMP-9) (Marracci et al. 2004; Cantin et al. 2005; Yadav et al. 2005; Chaudhary et al. 2006; Kim et al. 2007b; Zhang et al. 2007), RAGE (receptor for advanced glycation end products) (Vincent et al. 2007), mediators of inflammation such as IFN-γ (Khanna et al. 1999), sPLA2 (Jameel et al. 2006), IL-18 (Lee et al. 2006), COX-2, PGE2, RANKL (receptor activator of NF-κB ligand), IL-1 (Ha et al. 2006), LOX-15 (Lapenna et al. 2003), iNOS (Demarco et al. 2004; Powell et al. 2004; Hurdag et al. 2005), induction or increased DNA binding of heat shock proteins (HSF-1, HSP-60, HSP-70) (Oksala et al. 2006; Oksala et al. 2007), regulation of apoptotic/antiapoptotic proteins such as caspase-3, BAX (Diesel et al. 2007), upregulation of the antiapoptotic protein Bcl_2 (Marsh et al. 2005) and phosphorylation of the apoptotic protein BAD (Kulhanek-Heinz 2004) inhibiting apoptosis, upregulation of eNOS (Visioli et al. 2002; Smith et al. 2003), and phase two detoxification enzymes via Nr-f2 and the ARE; SOD, (Balachandar et al. 2003), glutathione peroxidase (GPx), glutathione *S*-transferases [GST], NAD (P)H: quinone oxidoreductase 1 (Flier et al. 2002; Mantovani et al. 2003), γ-glutamylcysteine ligase (GCL) the rate limiting enzyme in the synthesis of GSH, hemi-oxygenase [HO-1] (Ogborne et al. 2005), increased B-lymphocytes (Wessner et al. 2006).

Many of these functions may be mediated via modulation of NF-κB, and activator protein-1 (AP-1), which was not stereospecific in inhibiting NF-κB in human aortic endothelial cells (HAEC) as SLA was equally effective (Packer 1998; Zhang and Frei 2001; Lee and Hughes 2002; Kim et al. 2007). The enantioselective preferences in modulation or expression of adenosine monophosphate protein kinase (AMPK) and PGC-1a (Kim et al. 2004; Lee et al. 2005b; Kim et al. 2007), Nr-f2 (Suh et al. 2001; Suh et al. 2004; Ogborne et al. 2005;

Suh et al. 2005) have not been tested but P38 MAPk and PI3K/Akt are critical proteins involved in the mechanisms of action of rac-ALA and there are indications of stereochemical preferences for RLA (Estrada et al. 1996). Recently, Zhang showed rac-ALA activated PI3K and P38 MAPk, which reduced the expression of LPS-induced inflammatory molecules but only rac-ALA was used (Abdul et al. 2007; Zhang et al. 2007). Some, but not all, of these proteins and signaling pathways have demonstrated stereospecific preferences for RLA but most of them have not yet been tested.

In a molecular modeling and energy-minimization study using lipoamide dehydrogenase, it was shown that only the active *R*-enantiomer is able to form direct contacts with the reactive thiol groups and imidazole base at the active site, whereas with the *S*-enantiomer the SH-group at C6 points away from the His450 base and functions as competitive inhibitor. This model may be useful to identify additional catalytic binding sites with enantioselective preferences for RLA or SLA in vivo (Raddatz et al. 1997).

STRUCTURE, PHYSICAL PROPERTIES, STABILITY, AND STEREOCHEMISTRY OF ALPHA LIPOIC ACID AND DIHYDROLIPOIC ACID

The first synthesis of rac-DHLA was reported by Stokstad's group at Lederle Laboratories and a number of successful synthetic procedures were developed and patented in the ensuing years (Bullock et al. 1957). Early investigators in the United States and Japan utilized rac-DHLA as an intermediate in the laboratory preparation of ALA, either by relying on spontaneous oxidation in solution, or more controllably with I_2, $FeCl_3$, or an alkyl nitrite to effect the desired transformation (Hornberger et al. 1952, 1953; Nakano and Sano 1956; Bullock et al. 1957; Deguchi 1960; Acker et al. 1963).

ALA exists as yellow crystalline needles or plates, depending on the solvents used and the temperature under which it forms but may also form polymeric chains of linear disulfides. The melting point of rac-ALA is 58°C–61°C and various melting points for RLA and SLA have been reported in the range of 46°C–51°C (Acker and Wayne 1957; Walton et al. 1995; Laban et al. 2007). The melting point of the enantiomers is lower, leading to reduced stability and increases the tendency to polymerize relative to rac-ALA. Interestingly, even RLA or SLA with high chemical and enantiomeric purities can either melt cleanly or sinter, which may be due to the presence of trace metals (Ames 2006).

The propensity of RLA to polymerize has a significant impact on the shelf life and bioavailability of derived dosage forms (Carlson 2007b). Subsequent to laboratory or industrial synthesis, the ALA polymers may be the predominant isolatable form. ALA can be separated from the polymer by repeated low temperature extraction with hydrocarbon solvents or by thermal depolymerization under high vacuum (Hornberger et al. 1953; Thomas and Reed, 1956). Zinc and HCl have been utilized to reduce the ALA polymer to DHLA with subsequent reoxidation in solution with I_2 to isolate crystalline ALA. Dilute (1 mM/ethanol)

alkaline solutions (pH 12) of DHLA were used to depolymerize the linear disulfide polymer of ALA by acting as either a direct reducing agent or via thiolate anions and the thiol-disulfide exchange reaction (Thomas and Reed, 1956).

R-(+)-α lipoic acid has a large positive specific rotation due to the ring strain of the dithiolane ring and *R*-DHLA has a small negative specific rotation due to opening of the ring. The highest specific rotation reported for RLA is +120 ($[\alpha]_D^{23} = +120$ c = 1 EtOH) (Carlson et al. 2007b). DHLA (all forms) occurs physically as a clear to lightly colored oil with a boiling point of 154°C–156°C (0.3 mm) (Reed and Niu 1954). Pure *R*-DHLA has a negative specific rotation ($[\alpha]_D^{23} = -14$ to -15 EtOH) and was first prepared and reported by Gunsalus. This group also reported distillation of *R*-(−)-DHLA at <0.1 mm/Hg caused partial racemization, because of the observed increase in the specific rotation from −14.7 [ee = 98%] to −8.9 [ee = 59.3%] (Gunsalus et al. 1956, 1957). Contrary to the original report, it was reported that thin-film distillation of *R*-DHLA yielded a clear product with a 99.6% ee value with trace amounts of RLA formed due to oxidation (Bringmann et al. 1999).

STABILITY OF DIHYDROLIPOIC ACID IN VITRO AND DURING SAMPLE PREPARATION

Racemic dihydrolipoic acid has been utilized in various in vitro models. Calibration solutions of DHLA can be prepared in 50% water/methanol and stored at −80°C to prevent oxidation to rac-ALA (Constantinescu et al. 1995; Haramaki et al. 1997a). Another study by Packer's group claimed that rac-DHLA is unstable and rapidly oxidizes to rac-ALA at room temperature. To quantify rac-DHLA, it had to be dissolved in mobile phase at pH 2.9, and stored at −80°C. It was also necessary to continuously sparge the solutions with helium, which caused evaporation of the acetonitrile (ACN) from the mobile phase, leading to gradual increases in the retention times (Han et al. 1995). This could be obviated by sparging the buffer solution and ACN separately and allowing the gradient pump to mix them while continuously sparging with helium or using an in-line degasser. Rac-DHLA from a sealed ampoule under nitrogen was stable for less than 20 min in an electrophoretic assay before being oxidized to ALA (Panak et al. 1996). In studies testing the binding affinities of rac-ALA and rac-DHLA with BSA-1, Shepkin found that DHLA dissolved in buffer solution (20 mM deuterium phosphate buffer with 150 mM NaCl) was oxidized to rac-ALA to the extent of 50% (Schepkin et al. 1994).

BASELINE LEVELS OF *R*-DIHYDROLIPOIC ACID IN HUMAN PLASMA

Originally microbiological assays were used to quantify serum levels of RLA at baseline and total ALA after administration of rac-ALA. Although the POF assay was sensitive to RLA and *R*-DHLA (the *S*-enantiomers were inactive) the assay did not differentiate RLA from *R*-DHLA (which would not survive the harsh

sample treatment, i.e., steam autoclaving at 120°C) utilized to remove baseline RLA from plasma proteins (Gunsalus et al. 1957; Guedes et al. 1965; Natraj et al. 1984). In more recent studies, baseline levels of RLA were detected. Teichert's group reported different values depending on the conditions of separating RLA from plasma proteins. Following acid hydrolysis, 12.3–43.1 ng/mL were detected. Enzymatic hydrolysis of plasma proteins yielded 1.4–11.6 ng/mL and <1–38.2 ng/mL using subtilisin and Alcalase, respectively. Baseline *R*-DHLA was detected in the range of 33–145 ng/mL after enzymatic hydrolysis with Alcalase (Teichert and Preiss 1992, 1995, 1997).

The Asta Medica group developed an enantioselective method utilizing a fluorescent adduct of *R*-DHLA or *S*-DHLA after $SnCl_2$ reduction of rac-ALA (Niebch et al. 1997). The method works equally well for derivatization in solvent, buffer, or plasma with either *R*-DHLA or *S*-DHLA subsequent to reduction of RLA and SLA standards, respectively, and can be used to determine the enantiomeric purities of the standards. No reports exist testing the reaction with preformed or baseline plasma *R*-DHLA. Since the authors claimed that DHLA was not stable in plasma, no attempt was made to split the sample in two portions to differentiate plasma RLA from *R*-DHLA and that formed during the ex vivo reduction step (Hermann and Niebch 1997). The method was utilized in several pharmacokinetic (PK) trials (Gleiter et al. 1999, 1996; Hermann et al. 1998; Breithaupt-Grogler et al. 1999; Krone 2002). The enantioselective method relies on reduction of ALA to DHLA and derivatization to an isoindole with two chiral centers by reaction with *o*-phthaldehyde (OPA) and D-phenylalanine (D-PA). The resulting diastereomers can be separated on a C_{18} column. Baseline levels of RLA were not detected (Niebch et al. 1997). Researchers at Degussa found endogenous levels of RLA to be 112 ± 67 ng/mL using essentially the same method, but neither group attempted to quantify baseline *R*-DHLA (which could be determined, if present by omitting the reduction step) due to the assumption that *R*-DHLA did not occur in plasma (Hermann et al. 1997; Bernkop-Schnurch et al. 2004).

In developing an high-performance liquid chromatography/electrochemical detection (HPLC/ECD) method for simultaneous detection of rac-ALA and rac-DHLA, it was impossible to prepare calibration solutions in human plasma due to rapid oxidation, but aqueous calibration solutions were used to quantify *R*-DHLA in human plasma. In some cases, when the entire workup was done under inert gas, DHLA could be quantified (Teichert and Preiss 1992, 1995, 1997). It was claimed that DHLA "as such" does not occur in plasma and therefore need not be considered as a possible interfering substance in the measurement of plasma ALA (Hermann and Niebch 1997). Later, Teichert and Preiss developed an HPLC method with integrated pulsed amperometric detection (IPAD) for simultaneous detection of ALA and its primary plasma and urinary metabolites and were unable to detect baseline levels of RLA, which they stated are in the range of 1 ng/mL (4.85 pmol/mL) with the LOQ = 0.022 nmol/mL [4.54 ng/mL] ALA. This is difficult to reconcile with the earlier range of measurements of baseline RLA (between 1 and 112 times greater, depending on the method). There was no report of attempts to quantify baseline levels of

R-DHLA (Teichert et al. 2002). Rac-DHLA could be recovered and detected immediately after being spiked into plasma (0.5 and 5.0 mg/mL; 70% recovery) but neither RLA nor *R*-DHLA was detected at baseline (Biewenga et al. 1999). *R*-DHLA could not be detected as a plasma metabolite after administration of 1 g of pure RLA utilizing an HPLC/ECD (coulometric) similar to the method reported by Sen (Sen et al. 1999). To test the stability in plasma, rac-DHLA was spiked into the sample (0.5 mg/mL) and stored overnight at −25°C. The following day, 94% had reverted to ALA. The authors commented that aqueous solutions of DHLA are more stable than DHLA in plasma since DHLA reduces plasma components faster than it is oxidized by (dissolved) air (Biewenga et al. 1999). If this were the case in vivo, a corresponding change or increases in RLA levels and higher concentrations of reduced amino thiols would be expected. High levels of hepatic and plasma thiols have been reported subsequent to 30 mg/kg intravenous (IV) injection (direct rather than infused) of rac-ALA into rats, suggesting reduction to rac-DHLA, which acts as the reducing agent (Gregus et al. 1992). In contrast, 100 mg/kg IP in rats oxidized plasma proteins but had no effect on plasma thiols (Cakatay and Kayali, 2005). Assuming linearity, extrapolation of Krone's data (from 20 mg/kg IV) indicates this dose would lead to a C_{max} of 103 μg/mL (0.5 mM) and an AUC of 11,676 ng × h/mL (Krone 2002). It has been shown that rac-DHLA increases plasma thiol concentrations and protects plasma proteins from oxidative stress (van der Vliet et al. 1995) but may be toxic in the range of 0.1–1 mM (20.83–208.33 μg/mL) (Kis et al. 1997; Kulhanek-Heinz 2004).

ENZYMATIC REDUCTION OF ALPHA LIPOIC ACID: IS DIHYDROLIPOIC ACID AN IN VIVO PLASMA METABOLITE OF ALPHA LIPOIC ACID?

Endogenous *R*-DHLA is bound to the E2 subunits of the mitochondrial 2-oxo acid dehydrogenase complexes where it stereospecifically functions as an acetyltransferase (Gunsalus et al. 1956). Watanabe proposed *R*-DHLA as the physiological substrate for $mPGES_2$, indicating a role in modulation of inflammation pathways and serving the first function for the endogenously produced dithiol outside the mitochondrial 2-oxo acid dehydrogenase complexes (Watanabe et al. 2003).

In the mid-1990s, several papers appeared reporting the cytosolic and mitochondrial enzymes responsible for reduction of rac-ALA, RLA, or SLA to the corresponding reduced forms (Bunik et al. 1995; Biewenga et al. 1996; Haramaki et al. 1997). Glutathione reductase was first believed to be the primary NADPH-dependent enzyme for cytosolic reduction, later it was determined that all or most of the reduction should be attributed to thioredoxin reductase [Trx-1] (Arner et al. 1996; Jones et al. 2002; Biaglow et al. 2003), which has a preference for RLA in mammalian mitochondria [Trx-2] (Bunik et al. 1995). Pig heart PDH also has a preference for RLA (Loffelhardt et al. 1995) and RLA increases ATP production in the heart whereas SLA diminishes it (Zimmer et al. 1995; Hagen et al. 1999).

It is widely believed that most of the antioxidant properties and therapeutic potential of rac-ALA and RLA are due to the in vivo reduction to rac-DHLA and *R*-DHLA (Packer et al. 1995) although recently it was determined that the disulfide radical cation should be considered as a candidate for the pharmacologically active species (Bucher et al. 2006). Most of the evidence to date that DHLA is the more physiologically active entity of the ALA/DHLA redox couple comes from extrapolation of high concentration (0.5–10 mM) in vitro assays using various cell lines where ALA and DHLA can be maintained for up to 72 h without significant metabolic transformation (except for redox cycling of the dithiolane ring and small amount of B-oxidized products) (Constantinescu et al. 1995; Roy et al. 1997; Han 2007). In vivo, DHLA is difficult to detect because of its rapid side chain oxidation and S,S-dimethylation. BMHA & BMBA (the 6 & 4 carbon metabolites) appear rapidly in plasma and have longer half-lives than the parent compound, rac-ALA (Schupke et al. 2001; Teichert et al. 2002).

Any attempt to characterize the in vivo mechanisms of action of ALA or DHLA must take into account the short plasma half-life of ALA, its rapid transformation into five primary metabolites, the lack of accumulation of the parent compound or its metabolites, and the rapid loss of PD activity by S-methylation of the dithiolane ring such that several sensitive assays have been unable to detect even low levels of DHLA in plasma.

Despite the pharmacological limitations of DHLA, rac-ALA and RLA possess therapeutic efficacy in humans (IV and PO), demonstrated by a reduction in the dose necessary to produce a similar benefit as determined by the glucose challenge and insulin clamp tests (DeFronzo et al. 1979; Jacob et al. 1999). Intravenous load doses of rac-ALA beginning at 600–1000 mg for 10 days could be reduced to 500 mg to achieve the same result (improved glucose metabolic clearance rate and improved insulin sensitivity index [ISI]) as per oral dosing of 600–1200 mg daily for 4 weeks. These treatments led to similar reductions in the effective doses. In two PK trials, one using 600 mg rac-ALA and the second 600 mg RLA-Tris salt (based on the weight of RLA), RLA-Tris produced average C_{max} plasma levels 2.3 times higher and the bioavailability (AUC) 1.9 times greater than rac-ALA (Krone 2002, pp. 163).

It is still unclear whether or not these effects are produced by an initial prooxidative or antioxidant signal, although evidence is mounting that the primary stimulus is oxidative which results in the induction of genes that improves the overall antioxidant status (Konrad 2005; Dicter et al. 2004; Cheng et al. 2006; Linnane et al. 2006). RLA is insulin mimetic and like insulin activates the insulin signaling pathway in 3T3-L1 adipocytes via hydrogen peroxide generation (Moini et al. 2002; Cho et al. 2003).

To fully define the in vivo mechanisms of action of rac-ALA and RLA it is essential to evaluate PK data in humans of RLA and its metabolites. It is an established principle in enantioselective PK that a racemic compound will not react in the same manner as a single enantiomer in a chiral environment due to differences in dissolution, disintegration, or any of the PK parameters such as absorption, distribution, metabolism, and elimination. Differences in these PK

parameters will present different concentrations to any stereospecific target such as receptor sites, transporters, and enzymes and may differentially affect signal transduction and gene transcription.

Racemic dihydrolipoic acid was not detected even as a low-level plasma metabolite in four animal species (dog, mouse, rat, and human) after administration of rac-ALA, but was a likely, albeit transient intermediate in the formation of three ALA metabolites, BMOA, BMHA, and BMBA (Schupke et al. 2001; Teichert et al. 2002) (Figure 10.2). Krone discovered enantiose-

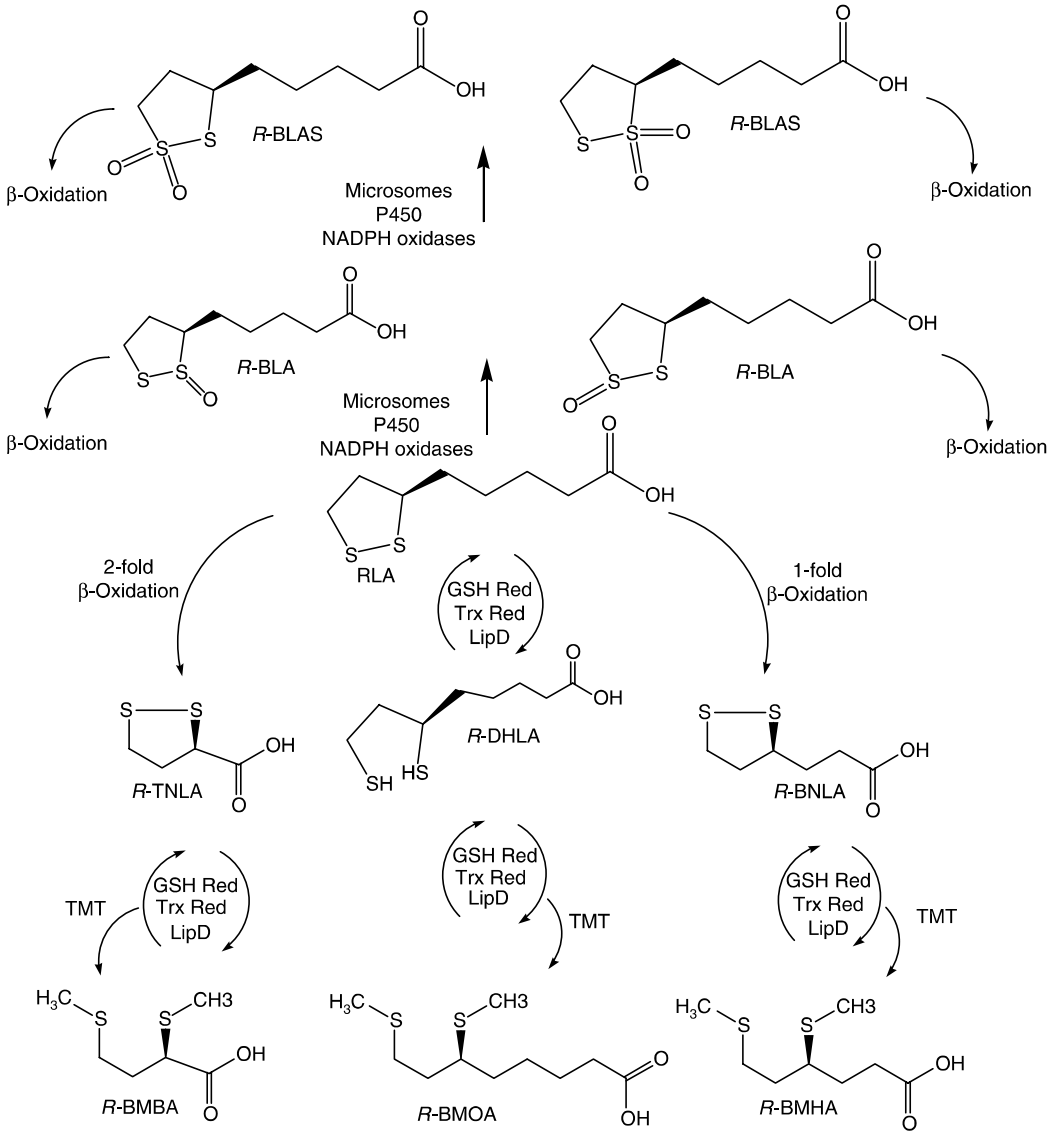


FIGURE 10.2 The metabolites of *R*-lipoic acid, *R*-tetranorlipoic acid (R-TNLA), *R*-bisnorlipoic acid (*R*-BNLA), and *R*-β-lipoic acid, most likely contribute to the pharmacodynamics of *R*-lipoic acid. The presence of *R*-BNLA and *R*-TNLA in plasma in higher concentrations than rac-BNLA and rac-TNLA indicates concentration effects or enantioselective metabolism and possible in vivo differences between *R*-lipoic acid and rac-α lipoic acid (Krone 2002). Methylation and oxidation of the sulfhydryls presumably causes a loss of activity and enhances renal elimination. (Lang 1992, Schupke et al. 2001, Krone 2002, Teichert et al. 2002).

lective differences in the plasma PK and metabolism between rac-ALA and RLA in humans but did not identify *R*-DHLA after the administration of 600 mg of RLA-Tris salt. The metabolite profile of RLA revealed substantially higher levels of *R*-bisnorlipoic acid (*R*-BNLA) whereas this metabolite was found in low levels or was absent after administration of rac-ALA to healthy volunteers (Schupke et al. 2001; Krone 2002; Teichert et al. 2002).There are three possible metabolic pathways for the formation of these primary metabolites from the parent compound (Krone 2002). The first pathway involves the metabolism of RLA to the metabolite BMOA by opening of the dithiolane ring to *R*-DHLA, followed by S,S-dimethylation and onefold or twofold β-oxidation reactions, yielding the metabolites BMHA and BMBA. The second possible pathway involves the metabolism of RLA via β-oxidation to the metabolite BNLA, followed by S,S-dimethylation of the dithiolane ring, which precludes the presence of *R*-DHLA as an intermediate metabolite. The third and last possible pathway for the formation of the metabolite *R*-tetranorlipoic acid (*R*-TNLA) from RLA is by a twofold β-oxidation, followed by S,S-dimethylation, which will produce the metabolite *R*-BMBA. This potential pathway also severely limits the mean residence time of *R*-DHLA in plasma, as a potential metabolite and as the agent responsible for the PD effects of rac-ALA or RLA. The formation-rate of the metabolite *R*-TNLA is not dependent on the concentrations of the metabolite *R*-BNLA in the plasma (Krone 2002). The enzymes responsible for S-methylation, the *S*-methyl-transferases are found in the intestinal mucosa, especially in the cecum and the colon, liver, kidneys, erythrocytes, and the lymphocytes (Stevens et al. 1990; Creveling 2002).

On the basis of the rapid appearance of *R*-BMHA in plasma after consumption of Na-RALA, K-RALA (5 min), or RLA-Tris (30–45 min), it is probable that a significant portion of the reduction and S-methylation occurring in the intestines is released into the mesenteric circulation and transported to the liver for uptake and further transformation. The metabolite *R*-TNLA was measured in the plasma in higher concentrations than *R*-BNLA after administration of 600 mg RLA-Tris salt and in higher concentrations in muscle than in plasma, indicating tissue-specific metabolism. It can be assumed that in humans the twofold β-oxidation (loss of four carbons from the side chain) is the favored pathway. Interestingly, *R*-TNLA was not identified after administration of 1 g RLA (yielding a C_{max} of one-seventh to one-tenth that of 600 mg RLA-Tris) indicating the possibility of dose-concentration dependent differences in the metabolite profiles of RLA (Biewenga et al. 1999; Krone 2002).

The results of ALA PK studies suggest that if untransformed *R*-DHLA can be detected as a plasma component, it will be found in low concentrations by escaping the highly efficient *S*-methyl transferases and by hydrophobic or covalent binding (SH/S–S) to plasma and tissue proteins. Additionally, any potential antioxidant property or therapeutic effect of *R*-DHLA or rac-DHLA in vivo is likely via a rapid, catalytic activation or modulation of specific receptors or signaling pathways since it is rapidly metabolized. The pharmacodynamic

effect of RLA/*R*-DHLA is correlated to the redox properties of the dithiolane ring, but this is lost upon S-methylation and subsequent S-oxidation (Krone 2002). Alternatively, whereas DHLA in vitro is rapidly exported from the cell it is possible that DHLA like other thiols binds in vivo via thiol-disulfide exchange reactions (Smith et al. 2004). This may account for the low (12.4%) 24 h urinary excretion of RLA and its primary metabolites in humans (Teichert et al. 2003). Interestingly, even though baseline levels of RLA have been reported in the 80 nM–545 nM (16.5–112 ng/mL) range, it may have a significant role in maintaining the plasma redox status as it constitutes the second anodic wave as determined by cyclic voltammetry (Chevion et al. 1997; Takenouchi et al. 1960; Bernkop-Schnurch et al. 2004).

ANIMAL AND HUMAN PLASMA PHARMACOKINETICS OF RAC-DIHYDROLIPOIC ACID

Donatelli provided the first report of rac-ALA PK (as Na-ALA). Rabbits administered a 50 mg/kg IV dose of rac-ALA reached C_{max} in plasma of 76 μg/mL within 5 min and IM injections of 100 mg/kg gave C_{max} of 16 μg/mL within 20–30 min, which were completely in the disulfide form. There was no evidence of circulating rac-DHLA from either route of administration (Donatelli 1955).

In contrast, Riedel found rac-DHLA present in febrile and afebrile rabbit plasma after injection of 80 mg/kg rac-ALA, utilizing a modification of Haj-Yehia's assay (the sulfhydryls were blocked as the *S*,*S*-DEOC derivatives, and the fluorescent amide was made with panacyl bromide rather than APMB) by exposing rabbits to LPS to induce fever (Riedel et al. 2003). In afebrile rabbits, this IV dose yielded maximum plasma concentrations of $96.5 +/- 10.7$ μg/mL ($467 +/- 51.86$ μM) rac-ALA and $2.2 +/- 0.26$ μg/mL ($10.68 +/- 1.3$ μM) DHLA increasing to $8.6 +/- 2.28$ μg/mL ($41.7 +/- 1.1$ μM) within 30 min of injection. In febrile rabbits, injection of 80 mg/kg rac-ALA yielded similar plasma concentrations of rac-ALA but DHLA concentrations decreased from $2.12 +/- 0.3$ μg/mL ($10.29 +/- 1.45$ μM) to $0.84 +/- 0.22$ μg/mL ($4.07 +/- 1.07$ μM) within 45 min, following the injection of LPS. The important findings of this study were that rac-DHLA could be measured in a quantity 2%–11% the amount of administered rac-ALA, was sufficiently stable to measure over time, and was found in a lower quantity (0.8% the amount of ALA) when the animal was subjected to oxidative stress subsequent to injection of LPS due to oxidation to rac-ALA. Riedel demonstrated that LPS-induced fever was dependent on the induction of systemic oxidative stress, which activated the NMDA receptor via oxidation of receptor thiols.

Hill measured rac-ALA and rac-DHLA (but no other metabolites) in dogs and cats using a modification (sample prep) of an HPLC/ECD procedure (Sen et al. 1999). The studies showed significant differences in metabolism of rac-ALA between the two species but both demonstrated cyclic patterns of

excretion of both rac-ALA and rac-DHLA in urine, detectable up to 10 days after administration, suggestive of tight plasma protein binding (Hill et al. 2002, 2004, 2005), and inefficient *S*-methyltransferases. No rac-DHLA was detected in dog plasma after PO or IV administration but was found in urine in equal or larger amounts than rac-ALA after IV injection of 25 mg/kg rac-ALA; suggesting reduction in the periphery. Rac-DHLA could be detected in dog urine on day 1; reached highest levels on day 3; and was still detectable on days 4, 7, and 10 after administration. Cats fed 15 mg/kg had equal or greater concentrations of rac-DHLA in plasma and urine than rac-ALA; reaching levels of 1.7 μM (0.35 μg/mL), at $T = 10$ h, whereas rac-ALA reached levels of 0.3 μM (0.062 μg/mL). Rac-DHLA could be detected up to 10 days post-administration, but reached peak levels on days 2 and 5. These findings are not consistent with those of Schupke who was unable to detect [7,8-^{14}C] rac-DHLA as a metabolite in dog urine up to 72 h post-dosing (Schupke et al. 2001).

Racemic dihydrolipoic acid was first identified in human plasma and urine (HPLC with fluorescence detection) after a volunteer consumed an undisclosed amount of commercial rac-ALA but the concentrations were not reported and the chromatograms were apparently not drawn to scale (Haj-Yehia et al. 2000). As reported above, *R*-DHLA was not detected after administration of 1 g RLA in a human volunteer although the LOD of rac-DHLA in plasma was reported to be sufficient for low level detection (12.9 ng/mL) (Biewenga et al. 1999).

Taken together, this suggested the possibility of reduction of rac-ALA/RLA to rac-DHLA/*R*-DHLA in the complex milieu of the GI tract or in the blood and becoming tightly bound to plasma proteins. An alternative explanation for detectable DHLA would be that rac-ALA/RLA was transported into peripheral cells after escape of the hepatic first pass effect, reduced and a small portion of the effluxed DHLA escaped S-methylation and became bound to plasma proteins. It was also possible that several studies could not detect DHLA because the methods used to remove rac-ALA or RLA from plasma proteins were inadequate for removal of rac-DHLA or *R*-DHLA, due to possible covalent binding to Cys-34 of albumin or other free plasma thiols (Hortin et al. 2006; Gregus et al. 1992). Regardless of the mechanisms, concentrations were expected to be low due to which may be some researchers identified DHLA as a plasma component and others did not.

Early Japanese studies revealed disulfide reductase activity in the gut since the mixed disulfide bond between thiamine and 6-acetyl DHLA (thiamine-8-(methyl-6-acetyl-dihydrothioctate)-disulfide) (TATD) was reduced during GI absorption so that the each of the free thiols could be measured in plasma (Sugiyama and Yoshiga 1963; Matsuda 1968) (Figure 10.3). This suggests the intramolecular disulfide of ALA could be similarly reduced.

In an often cited paper concerning the extent of ALA absorption from the gut there is no mention of rac-DHLA as a metabolite of rac-ALA or [7,8-^{14}C] rac-α-lipoic acid (Peter and Borbe 1995).

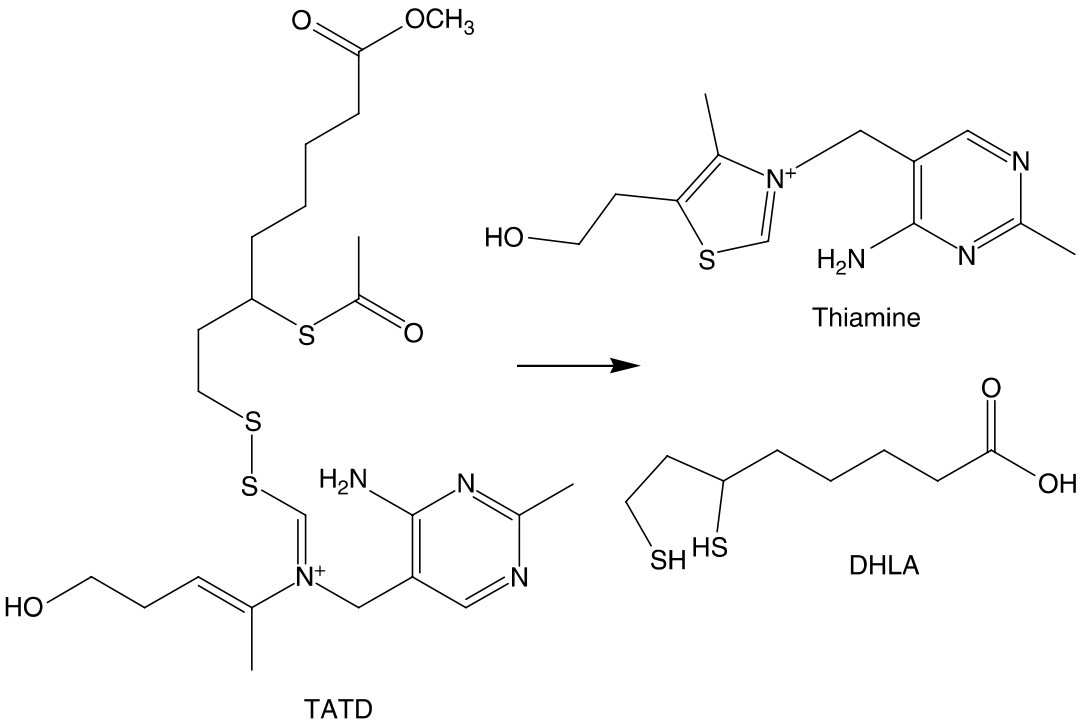


FIGURE 10.3 Thiamine-8-(methyl-6-acetyl-dihydrothioctate)-disulfide (TATD) is reduced by disulfide reductases releasing the free thiols into mesenteric venous circulation.

PLASMA PHARMACOKINETICS OF *R*-(+)-α LIPOIC ACID IN HUMANS

Reports of human plasma levels of RLA (after administration of the single enantiomer) are scarce but were mentioned in a book chapter, a paper, and a patent by a Dutch group. A 1 g dose of RLA generated a $C_{max} = 400$ ng/mL and later 1154 ng/mL but the AUC and other PK parameters were not reported (Biewenga et al. 1999; Biewenga, 1997; Biewenga et al. 1997). Adjusted for weight, and assuming linearity (Hermann et al. 1997), rac-ALA reaches peak plasma concentrations 16.45–31.17 times higher than pure RLA (based on the lower value), and 5.71–10.8 (based on the higher value) since 600 mg of rac-ALA has been reported to give average C_{max} 3949 ng/mL (Krone 2002) up to 7467 ng/mL (Teichert et al. 2003).

Owing to the unequal distribution of the enantiomers in plasma [RLA: SLA = 1.6–2.0:1] (Hermann et al. 1997; Niebch et al. 1997), the RLA content of 1 g rac-ALA would give a plasma C_{max} of 4114 ng/mL, 3.56–10-fold higher than the level achieved by administration of pure RLA. This limits the therapeutic potential of RLA since 4–10 g would have to be consumed to achieve therapeutic concentrations. Also reported was the C_{max} of two RLA metabolites; 3-keto-RLA (2092 ng/mL) and *R*-BNLA (704 ng/mL), and a partial plasma concentration versus time curve for RLA and the two metabolites was presented (Biewenga 1997b, Biewenga et al. 1999). In a recent clinical trial with rac-ALA in MS patients,

the greatest therapeutic benefit was correlated to C_{max} (Yadav et al. 2005). The highest plasma concentration in this study (18 μg/mL) is unobtainable with pure RLA. On the basis of the work of Krone with RLA-Tris salt and the comments of Locher (who claimed that concentrations of 50 μM, 10.3 μg/mL could be reached with 600 mg RLA-Tris), the only way to achieve therapeutic levels (10 μg/mL) of RLA was to use a stable, salt form (Walgren et al. 2004) that is water soluble and not as prone to polymerization as pure RLA. Doses of RLA salts reached levels higher than an equal weight of rac-ALA, which was significantly higher than pure RLA (Krone 2002). For example, 600 mg PO rac-ALA gave an average ($n = 9$; 6 males and 3 females) C_{max} of 3949 ng/mL (range 3065–5087 ng/mL) and an AUC of 3098 ng × h/mL (range 2513–3817 ng × h/mL) whereas 600 mg RLA-Tris gave an average ($n = 12$ males) C_{max} of 7235 ng/mL (range 5,099–10,267 ng/mL) and ($n = 12$ females) 11,357 ng/mL (range 7,331–11,209 ng/mL] and an AUC of 4690 males ng × h/mL (range 3956–5560) and 7304 females ng × h/mL (range 6146–8680) (Krone 2002).

MATERIALS

All of the test compounds and reference standards were synthesized, purified, identified, characterized, and validated by GeroNova Research, Inc. using NMR, GC–MS, polarimetry and a validated chiral HPLC assay (Carlson et al. 2007b). Rac-ALA, *R*-(+)-LA, rac-DHLA, *R*-(−)-DHLA had chemical purities >99% and the chiral compounds had enantiomeric excesses >99%. *R*-(+)-LA $[\alpha]_D^{23} = +120°$; *R*-(−)-DHLA $[\alpha]_D^{23} = -14.8°$. The RLA and *R*-DHLA mixture containing 30%–40% of the dimeric compound had an $[\alpha]_D^{23} = +41.4°–52°$, depending on the dimer content and the conditions under which it was formed. Higher temperatures and longer reaction times generally reduced the specific rotation. *R*-DHLA (as well as rac-DHLA and *S*-DHLA) can be readily identified and differentiated from the corresponding oxidized forms using the USP method for rac-ALA. The method is non-enantioselective but works equally well for all forms and can be quantified as total ALA and total DHLA. Standards must be made separately to prevent reaction between ALA and DHLA.

High-performance liquid chromatography grade solvents and reagent grade chemicals were purchased from GFS, Alfa Aesar, and TCI. APMB was purchased from City Chemical and purified by base-acid, 2 × recrystallizations from $CHCl_3$, and column chromatography.

ANALYTICAL EQUIPMENT AND METHODS

Specific rotations of the test compounds were measured on a Rudolph Research Autopol III Polarimeter. All specific rotations were run at room temperature (23°C) with $c = 1$ in EtOH. Comparative tests in benzene, acetonitrile, and methanol gave essentially the same results as long as the RLA did not polymerize upon dissolution and the resulting solutions remained clear.

High-performance liquid chromatography equipment: For comparison of published methods, two HPLC systems and three methods were used for plasma analysis of RLA and *R*-DHLA.

Method 1, System 1 was comprised of a Hewlett-Packard (HP) 1090 HPLC with an HP Model 1046A Programmable Fluorescence Detector (Haj-Yehia et al. 2000). A 250 μL injection loop was used. Separation of the fluorescent amides of *S*,*S*-DEOC-(*R*)-DHLA-APMB and RLA-APMB was attempted on an Agilent Zorbax Eclipse × DB-C8 column (4.6 × 15 mm, 5 μm) and detected at excitation 343 nm and emission 423 nm.

Method 2, System 1 was comprised of a Hewlett-Packard (HP) 1090 HPLC with an HP Model 1046A Programmable Fluorescence Detector. A 250 μL injection loop was used. Separation of the diastereomers formed by reaction of *R*-DHLA with OPA and DPA is accomplished by reverse phase HPLC on a Merck, LiChrospher 60 SelectB or Phenomenex Gemini column (25 cm × 4 mm I.D.: 5 μm particle size at 35°C) utilizing fluorescence detection with an excitation wavelength of 230 nm and an emission wavelength of 418 nm (Niebch et al. 1997; Bernkop-Schnurch et al. 2004). The standards and samples are eluted with a mixture of 55% 0.2 M K_2HPO_4 or Na_2HPO_4 (pH 5.8) and 45% acetonitrile/methanol (1:1) as the mobile phase (1.7 mL/min). Identification and quantification are performed by both the external standard and internal standard methods.

The original methods were modified to differentiate baseline or plasma *R*-DHLA from baseline or plasma RLA by splitting the plasma sample in two portions (Niebch et al. 1997; Bernkop-Schnurch et al. 2004). The first portion is reacted directly with the derivatizing reagents to detect *R*-DHLA. The second portion containing RLA and *R*-DHLA was reduced with $SnCl_2$ and derivatized in an identical manner as the first. In this way it was possible to quantify RLA and *R*-DHLA by difference.

Method 3, System 2 was comprised of a Hewlett-Packard (HP) 1050 HPLC and an ESA Coulochem II multielectrode detector, fitted with ESA 5010 Analytical Cell (electrode 1), a 5011A high sensitivity analytical cell (electrode 2), and an ESA 5020 guard cell (Sen et al. 1999). The electrodes were set at 0.4, 0.85, and 0.9 V, respectively. Separation was achieved with a Phenomenex C18 Gemini Column (250 × 4.6 mm; 5 μm). The mobile phase consisted of 50% of 50 mM NaH2PO4 (pH 2.7), 30% ACN, and 20% MeOH, which were filtered separately through a 0.45 μm filter and mixed with the gradient pump. The system was continuously sparged with helium. The flow rate was set at 1 mL/min.

PLASMA COLLECTION AND COMPARISON OF ANTICOAGULANTS

Each participant gave their informed and written consent before their inclusion in the study. Neither volunteer were taking prescription drugs or being treated for any known medical conditions. Pretrial screening for blood pressure, blood lipid profiles, homocysteine, and CRP were all within the reference ranges. The volunteers were regular users of RLA and *R*-DHLA, as well as followers of

complex daily vitamin regimens. On the basis of a previous trial showing that rac-ALA does not accumulate or modify subsequent PK profiles, a 3 day wash out for RLA/*R*-DHLA was considered adequate (Teichert et al. 2003). Volunteers were instructed to avoid consumption of any alcohol or nutritional supplements for at least 3 days before the trial. Blood glucose was checked at each time point in the Na-RALA trials using an Onetouch Ultra monitor.

Whole blood was drawn from fasted subjects (10–12 h) by our in-house licensed phlebotomist (S.J. Fischer) into 4.5–9 mL evacuated tubes and chilled for 5 min in an ice–water bath, which reduced hemolysis. Blood was collected from the medial antibrachial or medial cubital veins (with applied tourniquet) and an indwelling 22 gauge catheter at $T=$ 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 min for RLA salts and $T=$ 0, 30, 60, 90, 120, 150, 180, 210, and 240 min for RLA and *R*-DHLA into prechilled, acidic citrate (Biopool Stabilyte pH 4.5) or Li Heparin tubes, with modifications of previously published procedures. All of the common anticoagulants, Na, Li, NH_4 heparin, EDTA, and citrate, have been utilized in previous PK trials of ALA. Since one of our primary objectives was to attempt the detection of baseline as well as post-dosing levels of RLA and *R*-DHLA, we faced the same challenges as investigators attempting to measure the true in vivo plasma thiol redox status (Kleinman et al. 2000). DHLA, like plasma amino thiols, is prone to rapid oxidation so we found it necessary to test the stability of *R*-DHLA in the various anticoagulants. We selected acidic citrate (Biopool, Stabilyte) based on the report that plasma amino thiols were more stable in this anticoagulant than in heparin or EDTA (Williams et al. 2001). Additionally, we were able unable to detect *R*-DHLA in our preliminary PK measurements on the mixture of RLA and *R*-DHLA using EDTA or heparin tubes. Two tubes were used for the baseline blood draws to have sufficient blank plasma for calibration curves. RLA and *R*-DHLA were separated from plasma proteins using the method of Chen (Chen et al. 2005). Although recoveries of >90% were reported, 52%–63% of recoveries were in our hands and the calculations of plasma concentrations were adjusted based on the percentage recoveries of RLA or *R*-DHLA from each volunteer's plasma, spiked at different concentrations above and below C_{max}. This was done to adjust for interindividual differences in plasma protein binding. In a later study involving 12 healthy subjects we were able to improve the percent recoveries from 84% to 93% (Carlson et al. 2007a).

COMPARISONS OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHODS OF ANALYSIS

The literature was searched for methods reported to allow either simultaneous quantification of RLA and *R*-DHLA (with or without enantioselective considerations) or that could be differentiated by splitting the samples into two fractions and measuring the RLA and *R*-DHLA separately (Teichert and Preiss 1992, 1995, 2002; Niebch et al. 1997; Witt and Rustow 1998; Biewenga et al. 1999; Sen et al. 1999; Haj-Yehia et al. 2000; Satoh et al. 2007). Interestingly, of all the methods allowing simultaneous detection of ALA and DHLA, only Teichert's and

Bernkop-Schnurch's groups detected baseline RLA, and only Teichert detected baseline *R*-DHLA. Although Haj-Yehia's group claimed the method was used in a PK trial, no data was reported and Witt's group claimed the method could be used for quantifying DHLA in plasma, but reported values only for spiked plasma and no PK data.

The first method under consideration was from the group first identifying rac-DHLA in human plasma and urine subsequent to consumption of rac-ALA (Haj-Yehia et al. 2000). Blocking the free thiols was readily accomplished as long as fresh bottles of ECF were used. Old reagents gave incomplete reactions due to hydrolysis of the ECF. The biggest problem encountered was the coupling of APMB with RLA and *S*,*S*-DEOC-(*R*)-DHLA even with a variety of modifications of the original procedure (Assaf 2005). At times, the fluorophore of *S*,*S*-DEOC-(*R*)-DHLA-APMB amide was detected in solvent and at other times it would completely disappear. Attempts at detection in plasma using heparin or EDTA gave poor results despite the fact that the original procedure utilized EDTA. The RLA-APMB amide was more readily detected by UV detection. Unfortunately, after considerable effort to perfect and optimize the procedure, we were unable to obtain consistent results and it was abandoned. Even if we were able to ultimately perfect the method, it was believed to be too time consuming and labor intensive for routine high-throughput analysis of ALA and DHLA in human PK trials with multiple volunteers.

A more rapid procedure was desired and the coulometric methods developed by Sen and Biewenga (apparently independently of one another), which Hill utilized for detection of rac-ALA and rac-DHLA in dog and cat plasma, urine, and feces, was selected. Although the method has been reported to have a sufficiently low LOD for DHLA (1–5 pmol/0.206–1.03 ng) in plasma, in preparation, we were unable to use it for detecting baseline or low levels of *R*-DHLA. The method proved to be the method of choice for rapid quantification of RLA in PK trials of RLA dosage forms. In some samples, detection of baseline levels of RLA was possible, including those from purchased and pooled plasma.

RESULTS AND DISCUSSION: A REEVALUATION OF THE STABILITY OF *R*-DIHYDROLIPOIC ACID

We have worked extensively with *R*-DHLA for several years and have tested its stability, neat, in solution, and, recently, in plasma. We herein report our findings regarding the stability of *R*-DHLA under various conditions and its contribution to the stability and bioavailability of RLA. While developing industrial-scale preparations of RLA and *R*-DHLA, we discovered that *R*-DHLA is quite stable in air when dry and free of trace metals. A vial of pure, neat *R*-DHLA open to the air had oxidized (to RLA) by ~14% (USP method for rac-ALA, HPLC/UV) in one year, although a solution left drying overnight over molecular sieves had mostly reverted to RLA by the next morning.

In agreement with Bringmann, we discovered that *R*-DHLA can be distilled under inert gas with formation of 1%–3% RLA and with no evidence of racemization as had been previously reported by Gunsalus. If *R*-DHLA is exposed to high temperatures and strong reducing conditions or is improperly distilled, several impurities form with extremely obnoxious odors. We have identified these impurities by LC/MS. The major impurity is the seven-membered thiolactone but reductive desulfurization also occurs, generating small amounts of alkenes, thietanes, and thiophenes (Figure 10.4).

Our initial interest in *R*-DHLA was not as a dosage form but to see if we could expand on Thomas and Reed's initial report and use it to depolymerize RLA polysulfides, neat and on a preparative scale. RLA is more prone to polymerization (which may occur instantly at its sintering point [46°C –47°C]) than rac-ALA

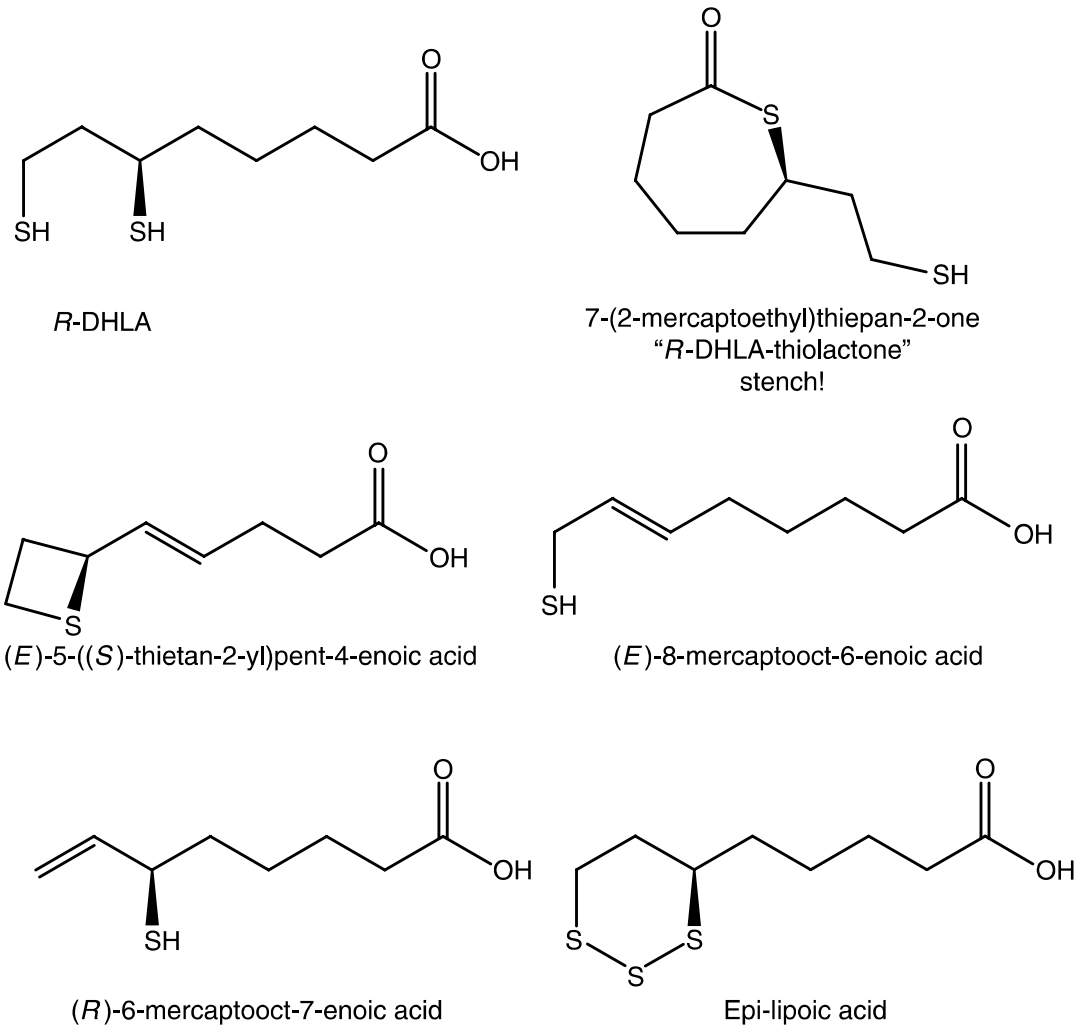


FIGURE 10.4 Impurities identified by LC/MS in commercial *R*-dihydrolipoic acid (*R*-DHLA). The primary impurity is *R*-DHLA-thiolactone. Epi-lipoic acid (epi-LA) is a trisulfide impurity found in commercial rac-lipoic acid (0.1%–3%) and in lower quantities in *R*-lipoic acid (<0.1%).

(Thomas and Reed 1956). To our surprise, we discovered that equal weights of RLA and *R*-DHLA can be mixed at room temperature or heated together on a cover slip using a digital micro-block apparatus, with no evidence of the sticky disulfide polymer or oxidation. The mixture was soluble in organic solvents or dilute base and both compounds could still be separated by HPLC and detected by UV (215 nm). We tested the stability of *R*-DHLA by heating it together with RLA at temperatures up to 160°C for 4 h with both compounds still detectable by UV and no evidence of decomposition or racemization.

At first, we were unaware of the formation of a new stable dimeric compound due to the observation of the chromatogram that displayed RLA and *R*-DHLA still present in the same ratios with the same peak areas as the standard solution, which had been made by mixing RLA and *R*-DHLA together in solvent. It was only when we ran the standards separately that we observed a 30%–40% reduction in the peak areas of both RLA and *R*-DHLA. When the mixture was reduced with alkaline $NaBH_4$, the theoretical amount of *R*-DHLA was recovered. The mixture was titrated with 0.1 N NaOH to 98% of the theoretical amount indicating the carboxyl groups were both free. This led us to believe that we were looking at a stable thiol–disulfide intermediate (Figure 10.5). The unexpected air and heat stability and the formation of the newly identified dimer led us to speculate that *R*-DHLA alone or in the presence of RLA might also be more stable in plasma than previously suggested (at a particular concentration and if absorption kinetics were the same for RLA and *R*-DHLA). Alternatively, we thought *R*-DHLA or the dimer could serve as a pro-drug or delivery system to improve the bioavailability of RLA and extend its residence time in plasma or tissues. Investigations of the effect of the dimer on the PK of RLA and *R*-DHLA are underway.

Even transient stability in plasma could have a significant biological or therapeutic impact that may be different than RLA itself, since rac-DHLA effluxed from the cell in vitro was rapidly converted to rac-ALA but was stable long enough to be able to significantly increase the reduction of cystine and increase intracellular uptake of cysteine (Han et al. 1997; Kis et al. 1997).

R-(+)-α lipoic acid is a difficult compound to manufacture, isolate (on an industrial scale), handle, store, and convert into dosage forms because of its propensity to polymerize. Commercial dosage forms of RLA have extremely poor shelf life, low GI absorption, and bioavailability. It was difficult to reconcile some of the existing reports of the alleged instability of DHLA in vitro with therapeutic claims. Despite many claims to the inherent instability of DHLA, it has been utilized in a number of experimental models and has been suggested for therapeutic applications (Lee et al. 2005; Ho et al. 2007; Holmquist et al. 2007).

PHARMACOKINETICS OF Na-RLA VERSUS *R*-(+)-α LIPOIC ACID AND *R*-DIHYDROLIPOIC ACID

Pure RLA has poor stability and bioavailability due to the propensity to polymerize. Plasma levels of from 1 g RLA have been reported to reach a C_{max} of

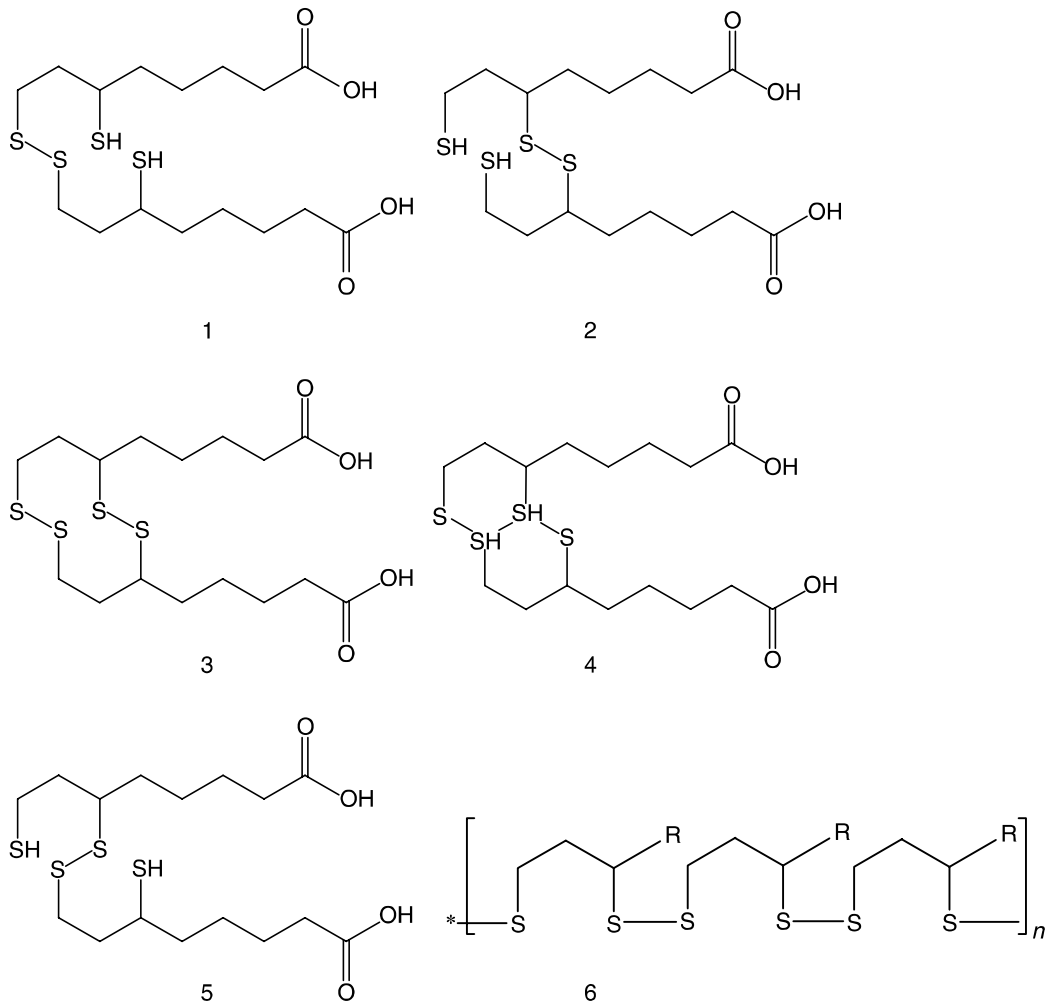


FIGURE 10.5 Racemic α-lipoic acid and to a greater extent *R*-lipoic acid form insoluble chains of linear disulfides (structure 6), which occur physically as sticky yellow masses (Thomas and Reed 1956). *R*-dihydrolipoic acid (*R*-DHLA) reduces the polymer to oil and stabilizes the structure to heat and further polymerization. Preliminary pharmacokinetic studies indicate the presence of *R*-DHLA increases the bioavailability of *R*-lipoic acid above (~2 fold) that of *R*-lipoic acid alone. Attempts to elucidate the structure are underway. Structures 1 and 3 are the most probable structures.

1.154 μg/mL, which argues against a therapeutic effect from low supplemental doses of RLA that yields concentrations barely distinguishable from baseline. This limitation is overcome by using 600–800 mg (based on RLA content) of a salt form of RLA; Na-RLA, K-RLA, and RLA-Tris that can reach plasma levels from 8–18 μg/mL. This falls into the proposed therapeutic range of 10–20 μg/mL (50–100 μM) (Anderwald et al. 2002; Krone 2002). A dose of 1200 mg Na-RLA (based on RLA content) reached $C_{max} = 45.1$ μg/mL (225 μM), which rivals the C_{max} of a 1200 mg infusion of rac-ALA (Hermann et al. 1997) and is suggested to be the upper limit for potential therapeutic action. Therefore, millimolar concentrations used in vitro or high-dose animal experiments have

little physiological relevance for humans. It is relevant that load oral doses in rats led to C_{max} in tissues of 150 μM (Smith et al. 2004; Hagen 2007).

The nonlinear response between the 600 and 1200 mg dose of Na-RALA in Subject 1 suggests the possibility of saturation of the first pass mechanisms thus allowing more RLA into circulation and the periphery. More volunteers are needed to see if this trend is maintained. The dose, 45.1 μg/mL (225 μM), is close to the upper limit of tolerability in humans since it produced transient nausea at $T = 20$ min (10 min after C_{max}) for 10 min duration and was not associated with a sudden drop in blood sugar. Subject 2 also experienced brief nausea at $T = 20$ min, 10 min after $C_{max} = 18$ μg/mL. This also was not associated with any changes in blood sugar. The IV infusion of 1200 mg rac-ALA over 35 min produced a similar concentration time curve and induced nausea in 33% of the volunteers (28 of 86 people; assuming 50–70 kg/volunteer = 17–24 mg/kg) (Biewenga 1997B) (Table 10.1).

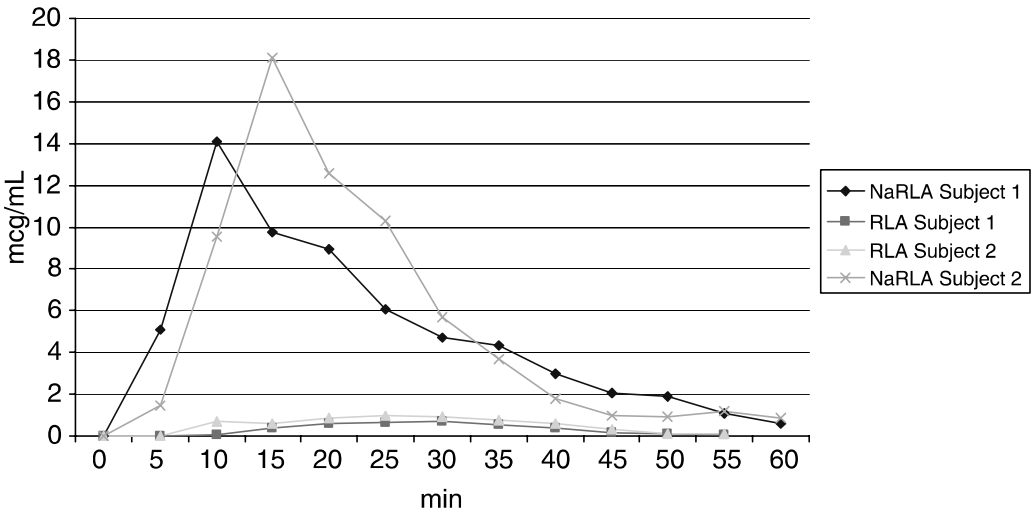
R-(−)-dihydrolipoic acid was sufficiently stable in plasma to be quantified (266 ng/mL) at baseline in Subject 2 but was undetected in Subject 1. It is unknown whether the 3 day wash out period was sufficient to clear *R*-DHLA from plasma (from daily consumption of Na-RALA) or whether it was from endogenous sources. Either way, it was sufficiently stable to be measured. Like the measurements in rabbit, it was possible to measure *R*-DHLA in human plasma at C_{max} of RLA in quantities 68 times lower. Our preliminary results show that 600 mg *R*-DHLA is absorbed slowly from the gut and reaches plasma levels 51 times lower than an equal weight of Na-RLA. Two unidentified metabolites appear in plasma and return to baseline within 4 h while *R*-DHLA is still quantified (223 ng/mL at $T = 240$ min), with no sign of decline. This suggests that *R*-DHLA may undergo slow thiol–disulfide reactions with gut and

TABLE 10.1

Comparisons of Pharmacokinetic Values between 600 mg *R*-Lipoic Acid and 600 mg *R*-Lipoic Acid (as Sodium *R*-Lipoate) in a Single Male (Subject 1) and Female Subject (Subject 2) Reveal Significant Increases in the C_{max} and AUC Values When *R*-Lipoic Acid Is Administered in the Form of Sodium *R*-Lipoate. An Unsuccessful Attempt Was Made to Determine the Pharmacokinetic Values for 600 mg *R*-Dihydrolipoic Acid (Subject 1)

Subject	Dosage (mg)	C_{max} (μg/mL)	T_{max} (min)	$T_{\frac{1}{2}}$ (min)	AUC 0–*t* (μg min/mL)	AUC 0–*t* (μg h/mL)
1	600 R-DHLA	0.26	120	ND	ND	ND
1	600 RLA	0.70	120.0	24.36	74.5	1.24
1	600 mg Na-RALA	14.10	10.0	5.34	311.1	5.185
2	600 RLA	1.01	70.0	31.67	130.0	2.17
2	600 Na-RALA	18.10	15.0	7.06	342.8	5.71

ND = Not determined.



NaRLA Subject 1; C_{max} = 14.1 µg/mL; AUC 5.19 µg h/mL (C_{max} = 20.1x, AUC = 4.2)
RLA; Subject 1; C_{max} = 0.7 µg/mL; AUC 1.24 µg h/mL
RLA; Subject 2; C_{max} = 1.01 µg/mL; AUC 2.17 µg h/mL
NaRLA Subject 2; C_{max} = 18.1 µg/mL; AUC 5.71 µg h/mL (C_{max} = 17.92, AUC = 2.6)

CHART 10.1 Comparisons of plasma concentration versus time curves of 600 mg R-lipoic acid administered as the free acid or an equivalent amount of sodium R-lipoate in subjects one and two.

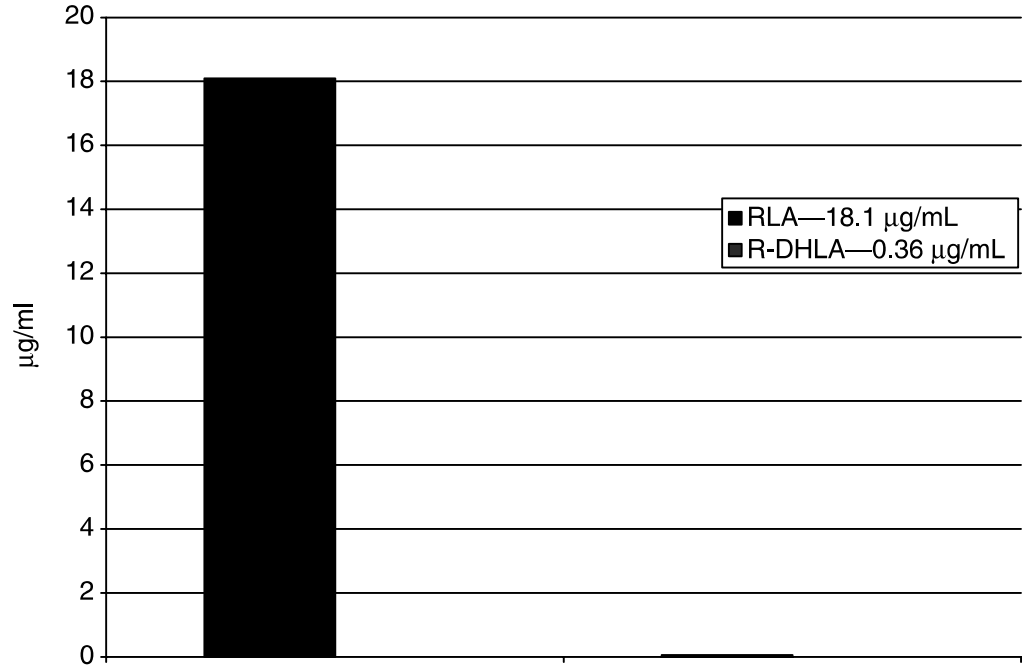


CHART 10.2 *R*-Dihydrolipoic acid at C_{max} 600 mg Na-*R*-lipoate Subject 2.

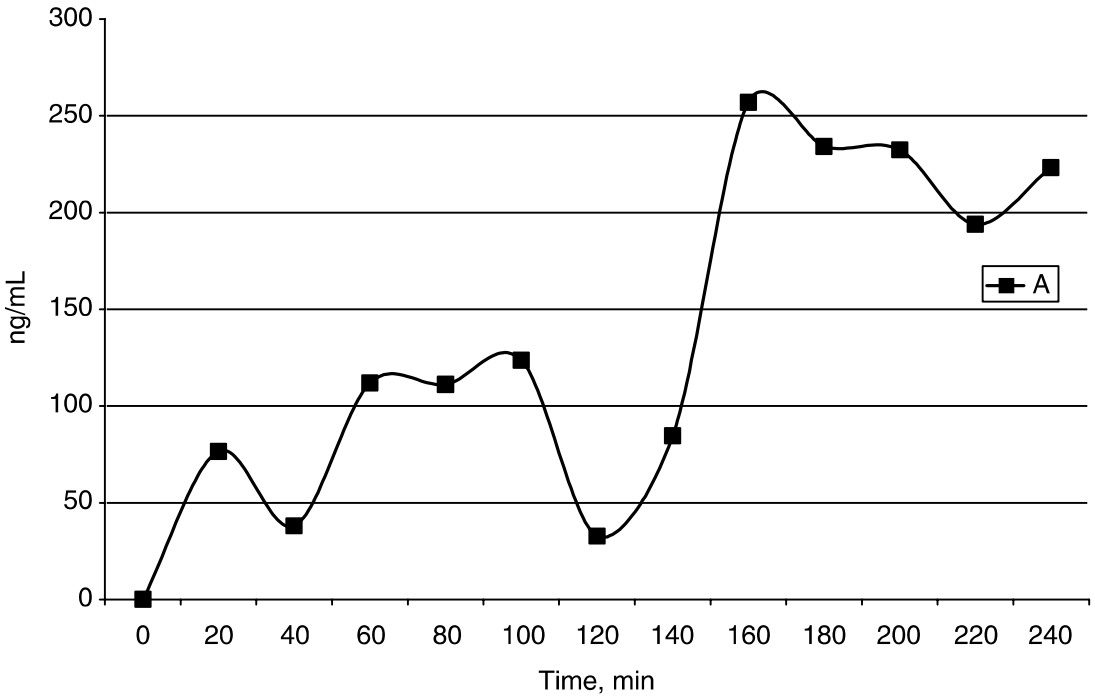


CHART 10.3 *R*-dihydrolipoic acid (*R*-DHLA) 600 mg concentration versus time curves for Subject 1.

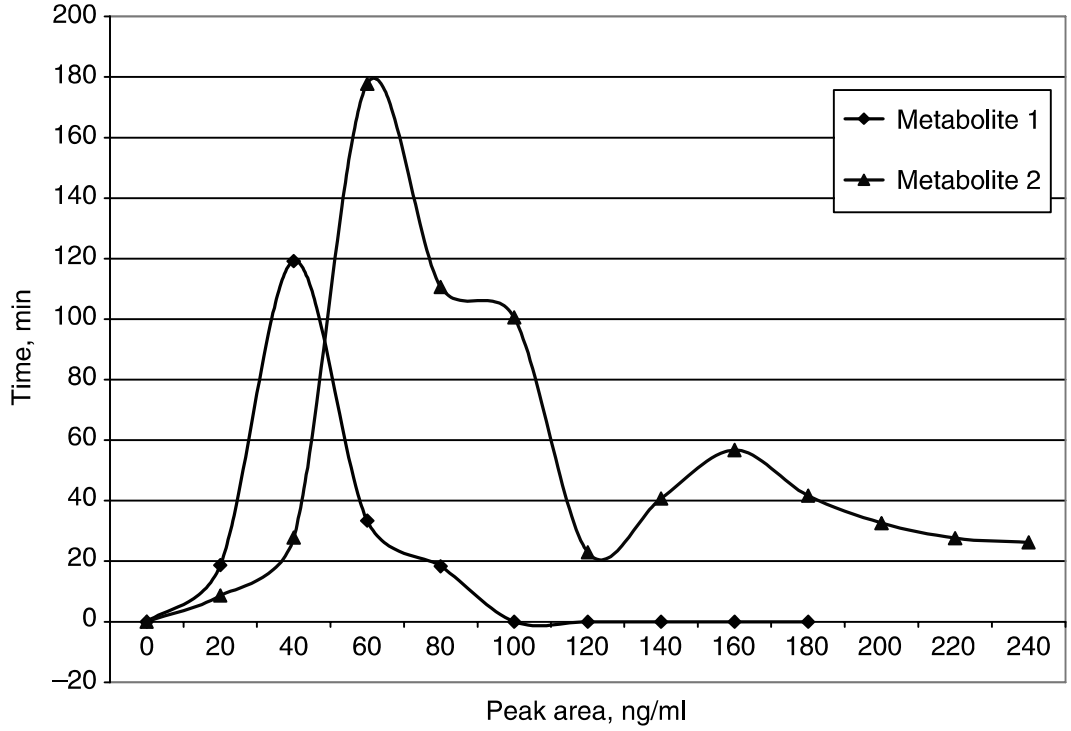


CHART 10.4 Unknown human plasma metabolites identified by derivatization and flourescence detection after consumption of 600 mg *R*-DHLA.

plasma thiols or bis-methylated. It was impossible to calculate the elimination half-life or the AUC. Blood should be collected in future studies out to at least 480 min (Charts 10.1 through 10.4).

Studies are in progress to systematically explore individual differences in protein binding, and expand the number of volunteers with Na-RALA, K-RALA, *R*-DHLA and the mixed dosage form containing RLA and *R*-DHLA and the new dimer.

ABBREVIATIONS

ACN = acetonitrile
AMPK = adenosine monophosphate protein kinase
AP-1 = activator protein-1
APMB = 2-(4-aminophenyl)-6-methylbenzothiazole
ARE = antioxidant response element
ATP = adenosine triphosphate
BAD = Bcl-xL/Bcl-2 associated death promoter
BAX = Bcl-2 associated x protein
Bcl2 = B-cell lymphoma 2
BMBA = bis(methylthio)butanoic acid
BMHA = bis(methylthio)hexanoic acid
BMOA = bis(methylthio)octanoic acid
BNLA = bisnorlipoic acid
BSA-1 = Bovine serum albumin-1
COX-2 = cyclooxygenase-2
CRP = C-Reactive Protein
D-PA = D-phenylalanine
ECF = ethyl chloroformate
ee = enantiomeric excess
eNOS = endothelial nitric oxide synthase
GCL = γ-glutamylcysteine ligase
GPx = glutathione peroxidase
GSH = reduced glutathione
GSH Red = glutathione reductase
GST = glutathione *S*-transferases
HAEC = human aortic endothelial cells
HO-1 = hemi-oxygenase-1
HSF-1 = heat shock factor-1
HSP-60 = heat shock protein-60
HSP-70 = heat shock protein-70
ICAM-1 = intercellular adhesion molecule-1
IFN-γ = interferon-γ
IL-1 = interleukin-1
IL-6 = interleukin-6
IL-1β = interleukin-1β
IL-18 = interleukin-18

iNOS = inducible nitric oxide synthase
IPAD = integrated pulsed amperometric detection
IR = insulin receptor
L-Cys = L-cysteine
LipD = lipoamide dehydrogenase
LOD = limit of detection
LOQ = limit of quantification
LOX-15 = lipoxygenase-15
MCFA = medium chain fatty acid
MMP-9 = matrix metalloproteinase-9
mPGES2 = membrane-associated prostaglandin E2 synthase
NF-κB = nuclear factor κβ
NQOR = NAD(P)H: quinone oxidoreductase-1
Nr-f2 = nuclear factor E2 p45-related factor-2
OPA = *o*-phthaldehyde
PI3K = phosphatidylinositol-3-kinase
P38 MAPk = P38 mitogen-activated protein kinase
P450 = cytochrome P450
PAI = plasminogen activator inhibitor-1
PD = pharmacodynamic
PGC-1α = peroxisome proliferator activator receptor gamma-coactivator 1α
PGE2 = prostaglandin E2
PK = pharmacokinetic
POF = Pyruvate oxidation factor
PPAR-α = peroxisome proliferator activated receptor-α
PPAR-γ = peroxisome proliferator activated receptor-γ
rac-ALA = racemic α-lipoic acid
rac-DHLA = racemic dihydrolipoic acid
RAGE = receptor for advanced glycation end products
RANKL = receptor activator of NF-κB ligand
R-BLA = *R*-β-lipoic acid
R-BLAS = *R*-β-lipoic acid sulfoxide
R-DHLA = *R*-(−)-dihydrolipoic acid
RLA = *R*-(+)-α lipoic acid
S-DHLA = *S*-(+)-dihydrolipoic acid
SLA = *S*-(−)-α lipoic acid
SOD = superoxide dismutase
sPLA2 = phospholipase A-2
SREBP-1 = sterol-regulatory element binding protein-1
TGF-β = transforming growth factor-β
TMT = thiol methyltransferase
TNF-α = tumor necrosis factor-α
TNLA = tetranorlipoic acid
Trx Red = thioredoxin reductase
VCAM = vascular cell adhesion molecule

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