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Short communication

Determination of lipoic acid in human plasma by high-performance liquid chromatography with electrochemical detection

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Abstract

A selective and sensitive method for the determination of lipoic acid in human plasma samples has been developed. After enzymatic hydrolysis of the sample, the liberated lipoic acid was extracted by a solid-phase cartridge and measured by HPLC using electrochemical detection. The detection limit was 1 ng/ml lipoic acid in plasma. The calibration curve was non-linear in the range 0.01–50 μg/ml but could be described by a power function. The average extraction recoveries were 82.5 and 85.1% at the 25 and 2500 ng/ml levels, respectively. Coefficients of variation for both within-day and day-to-day analysis were between 2.1 and 9.4%. The assay method is sensitive, reproducible and suitable for disposition studies of lipoic acid in humans.

1. Introduction

α-Lipoic acid (thioctic acid, 1,2-dithiolane-3-valeric acid) is a naturally occurring cofactor which functions in α-keto acid dehydrogenase complexes in most cells and which is used extensively for the treatment of various diseases and symptoms, e.g. diabetic polyneuropathy, liver cirrhosis and intoxications with metal ions and amatoxine. Lipoic acid can be converted into dihydrolipoic acid by chemical or enzymatic reduction. The redox potential of the lipoic–dihydrolipoic acid system is very low (about −0.32 V) [below the redox potential of pairs such as glutathione (GSH–GSSG) and cystine–cysteine] and therefore able to reduce the oxidized form of these pairs.

Many methods are currently available for the analysis of lipoic acid (TLC, polarography, microbiological and colorimetric assay, GC, GC–MS, HPLC). Most of these methods are not satisfactory due to their non-specificity, lack of sensitivity, expensive sample preparation and need for expensive analytical equipment. Especially for clinical practice it is absolutely necessary to evaluate the pharmacokinetics of lipoic acid. For that purpose an adequate method has to be developed.

In a previous work we described, for the first time, the determination of lipoic acid in human plasma by HPLC and electrochemical detection after acid hydrolysis and liquid–liquid extraction [1]. In 1990, Kamata and Akiyama [2] described

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this detection method for the determination of lipoic acid in methanolic solution. The recovery after acid hydrolysis was only about 30% but a newly developed method using enzymatic hydrolysis by a thermophilic protease followed by extraction with benzene–dichloromethane yielded recoveries of about 70%. The thermophilic protease was no longer available and had to be replaced by Alcalase which showed the best recoveries (above 80%) [3], although Alcalase was not able to release lipoic acid from ϵ-lipoyllysine synthesized as model compound [4]. If all preparations were carried out under inert gas, dihydrolipoic acid could be detected in some cases. Calibration of dihydrolipoic acid in plasma samples was not possible because of its rapid oxidation. Investigations of detection in methanolic solutions of both lipoic and dihydrolipoic acid at a potential of +1.1 V showed a four to five times higher sensitivity for the determination of lipoic acid [3]. Dihydrolipoic acid was oxidized to lipoic acid and further oxidation was not probable under these conditions. The higher electron transfer during the oxidation of lipoic acid was explained by superoxidation [5]. The very specific HPLC analysis of thiols and disulfides can be accomplished by a thin-layer dual mercury amalgam electrode [6]. Handelmann et al. [7] reported the first adaptation of the dual Hg/Au electrode electrochemical detection to the HPLC analysis of lipoic acid and dihydrolipoic acid in tissue cultures.

To study the pharmacokinetics of lipoic acid in human plasma, we have developed a selective, sensitive and reproducible HPLC method. This paper describes the assay methodology and its application to the determination of lipoic acid in human plasma.

2. Experimental

2.1. Chemicals

RS-α-Lipoic acid and dihydrolipoic acid were obtained from Asta Medica (Frankfurt/Main, Germany). Alcalase, water for chromatography and all salts used in this study were purchased from Merck (Darmstadt, Germany). Methanol and acetonitrile were of HPLC grade (J.T. Baker, Deventer, Netherlands).

2.2. Standard solutions

Lipoic acid was dissolved in an equimolar solution of sodium hydroxide in ultrapure water to obtain a stock solution of 0.2 mg/ml. Calibration standards in the concentration range of 0.01–50 μg/ml were prepared by appropriate dilution of the stock solution in water.

The buffer used for enzymatic hydrolysis was prepared as follows: 147.4 mmol of sodium chloride, 5.6 mmol of potassium chloride, 5.48 mmol of disodium phosphate dibasic dihydrate and 0.32 mmol of sodium phosphate monobasic monohydrate were dissolved in 1 l of ultrapure water (pH 7.4). A 50-μl aliquot of Alcalase was dissolved in 12.5 ml of buffer and diluted with water to a total volume of 25 ml.

2.3. Preparation of samples

For calibration, plasma samples were spiked with the diluted stock solution and frozen in 1-ml aliquots. Twelve concentrations were measured over the whole calibration range. Trisodium citrate dihydrate was used as anticoagulant. After thawing, 1 ml of plasma was diluted with 0.5 ml of enzyme solution and hydrolyzed at 37°C for 30 min. The reaction mixture was shaken slightly and 1 ml of water and 1 ml of 0.2 *M* trichloroacetic acid were added.

2.4. Extraction procedure

Baker SPE phenyl cartridges (1 ml) were preconditioned with 3 ml of methanol and 3 ml of water. The mixture was applied to the cartridge, washed with 5 ml of water and dried under full vacuum for 5 min. The samples were eluted by 1 ml of methanol and evaporated to dryness at 40°C (nitrogen). The residue was dissolved in 200 μl of the mobile phase and injected into the HPLC system.

2.5. Chromatography

The HPLC system consisted of a pump (Model 64; Knauer, Berlin, Germany), a Knauer manual loop sample injector with a 20-μl sample loop, an electrochemical detector (Model 656; Metrohm, Herisau, Switzerland) equipped with a glassy carbon electrode, and a Knauer HPLC software package version 2.11 for integration of peaks. Chromatographic separations were performed with a Nucleosil 120-C_{18} 5-μm column (250 mm × 4 mm I.D.). A precolumn (30 mm × 4 mm I.D.) with an integrated guard column (5 mm × 4 mm I.D.) packed with 5-μm Nucleosil C_{18} was placed in front of the analytical column. The guard column was replaced frequently to maintain a good column efficiency. The mobile phase was 28.5% acetonitrile in 0.05 *M* potassium dihydrogenphosphate, which was adjusted to pH 2.5 with phosphoric acid. The flow-rate was 1 ml/min. The electrode was set at a potential of +1.1 V.

2.6. Extraction recovery and assay reproducibility

The recovery of lipoic acid from plasma was determined at three different concentrations. Known amounts of lipoic acid were added to drug-free plasma, and the area responses of lipoic acid in the extracted spiked samples were compared to those obtained by direct injection of standard solutions containing equivalent amounts of lipoic acid.

Assay reproducibility was assessed at low, medium and high concentrations of lipoic acid.

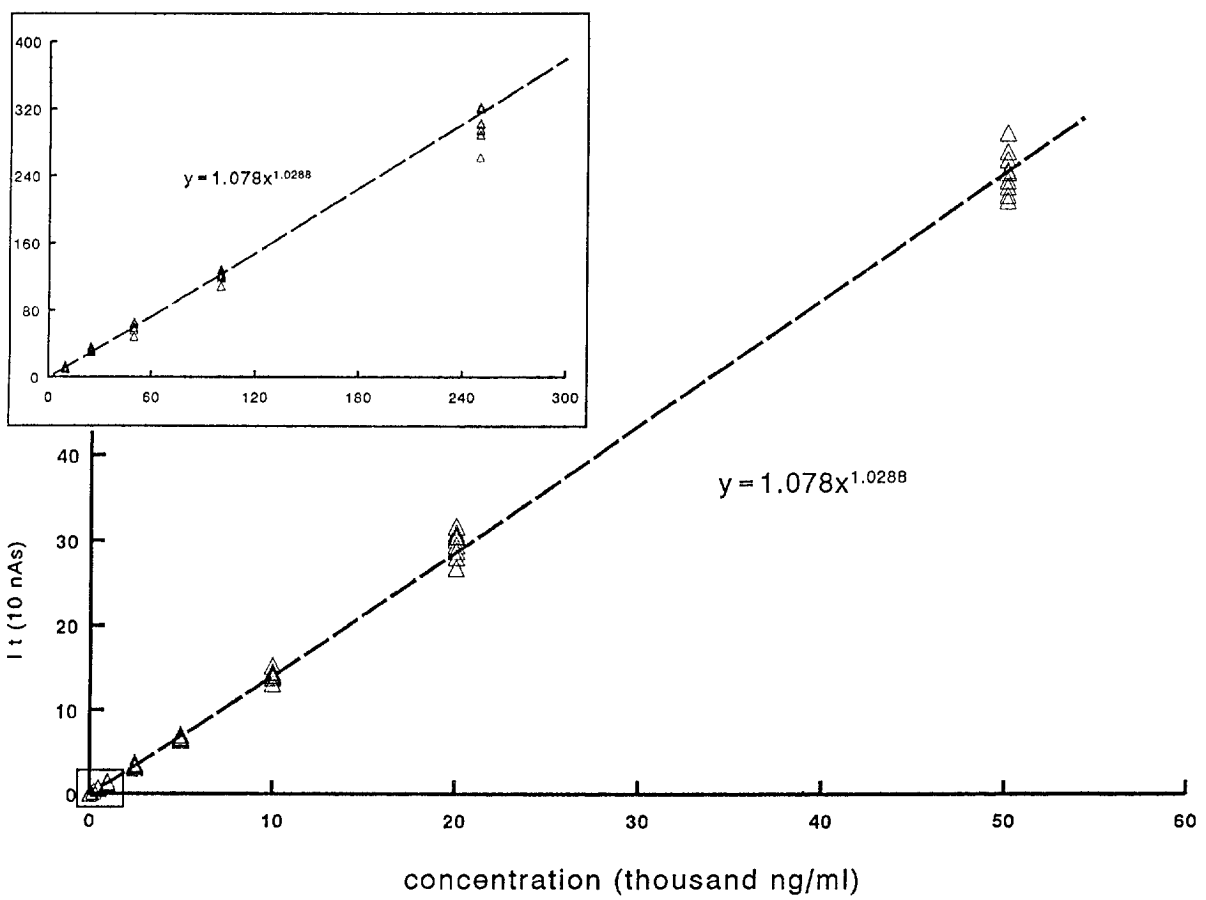

Fig. 1. Calibration curve of lipoic acid. Inset: curve segment up to 250 ng/ml.

Within-day reproducibility was calculated from six replicate analyses. Day-to-day reproducibility was determined from analyses on six different days.

2.7. Stability of lipoic acid in plasma

Plasma samples spiked with lipoic acid were stored at −70°C and analyzed at different times over a six-month period. Areas of lipoic acid in these samples were compared to those from freshly prepared controls.

2.8. Application of the method

After intravenous and oral administration of lipoic acid, fifteen blood samples were collected from each volunteer over a period of 10 h. Twenty-four volunteers participated. Plasma was harvested after centrifugation and analyzed for lipoic acid concentration using the method described in this report.

3. Results and discussion

The present assay for measuring lipoic acid, specifically in plasma, is simple, robust and easy to automate. Some endogenous compounds seem to be co-extracted, but none of these interfere significantly with the detection of lipoic acid. The mean retention time of lipoic acid was 14.3 min. Calibration was carried out using an external standard. The procedure described can be further improved by employment of an internal standard; however, the accuracy and reproducibility of the method were sufficient for the elucidation of pharmacokinetics. Three quality control standards were co-analyzed daily. Before starting with the measurements, a system aptitude test was carried out every day.

There was a change of slope of the curve with increasing lipoic acid concentration caused by saturation of the protein binding. This corresponds with the results of Schepkin et al. [8] who measured the average binding ratio of lipoic acid to bovine serum albumin to be 10 mol/mol. Therefore a non-linear calibration curve ($y = 1.078x^{1.0288}$; coefficient of correlation 0.9999) over the whole range of 0.01–50 μg/ml was used for the calculation of concentrations (Fig. 1). The average inaccuracy of the recalculated concentrations was 5.2% for the non-linear curve.

The within-day coefficients of variation at 25, 250 and 2500 ng/ml were 9.4, 4.6 and 7.7%, respectively ($n = 6$). The corresponding values for between-day analysis ($n = 6$) were 8.2, 5.4, and 2.1%, respectively. The recoveries (mean ± S.D.) at the same concentrations were 82.5 ± 8.0, 78.4 ± 3.7 and 85.1 ± 6.8%, respectively. We did not observe a decrease in lipoic acid levels with time. The average recovery from six-month spiked plasma samples was 103.6% at the 25

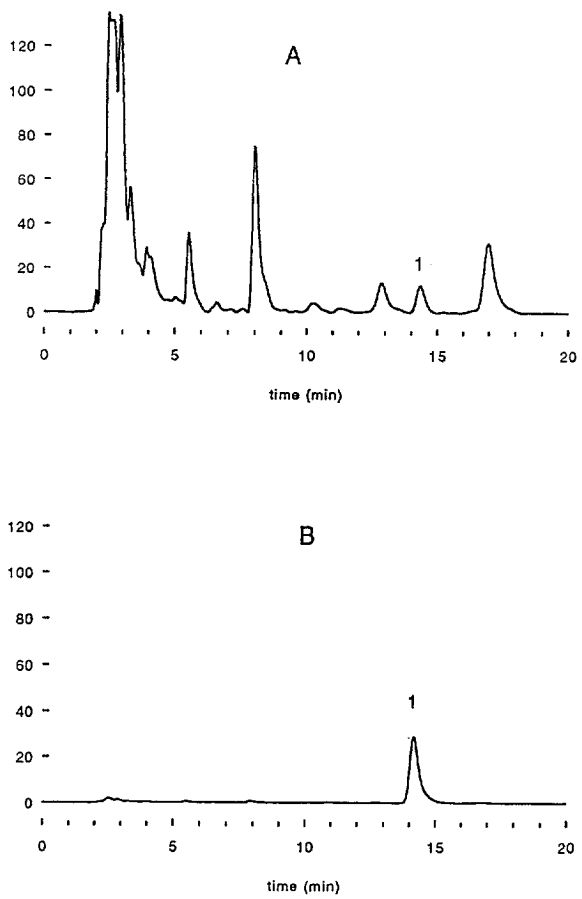

Fig. 2. Chromatograms of plasma extracts. (A) Blank plasma (1 nA full scale). (B) Plasma 10 min after drug administration (200 mg, single oral dose) (50 nA full scale). Peak 1 = lipoic acid.

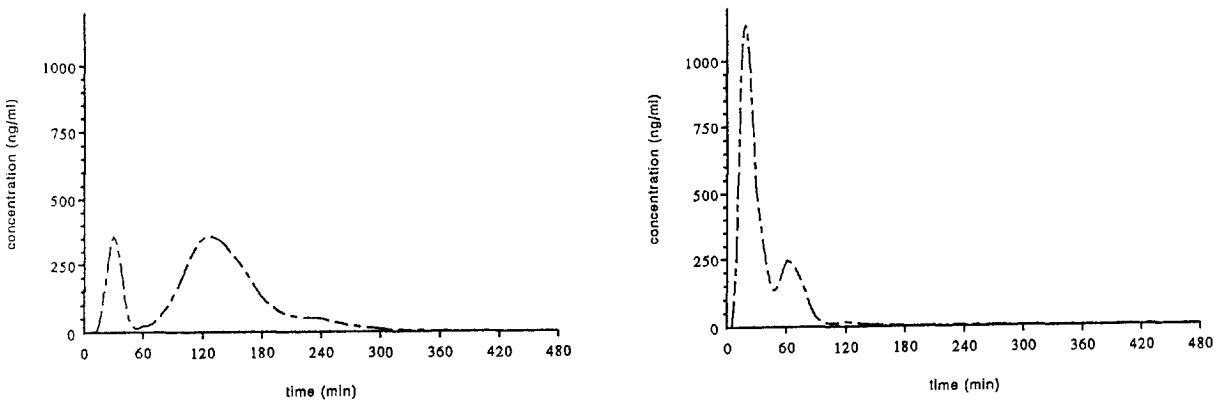

Fig. 3. Plasma lipoic acid concentration–time profiles following oral administration (200 mg) to two volunteers.

ng/ml level and 97.7% at the 1000 ng/ml level. Before calibration the intersection value of the linear curve over the range 10–450 ng/ml was subtracted from all measured y-values to eliminate the physiological lipoic acid level of blank plasma.

The detection limit was 1 ng/ml at a signal-to-noise ratio of 3:1.

The assay method was applied to determine plasma levels of lipoic acid in humans. Fig. 2 illustrates representative chromatograms of plasma extracts before and after oral administration of 200 mg of lipoic acid to a human volunteer. The plasma concentrations–time profiles in two volunteers are shown in Fig. 3. Plasma concentrations were measured for 10 h following oral administration of 200 mg of the drug and the decline in plasma concentration appeared to be biphasic. Fig. 3 also demonstrates that the sensitivity of this assay is adequate to define the elimination profile of lipoic acid in humans. Using the present assay a metabolite was detected at a retention time of 11 min, but could not be identified up to now.

4. Conclusions

A convenient, reliable and highly sensitive method for the analysis of lipoic acid in plasma has been established. The method consists of enzymatic hydrolysis of the sample to release the bound lipoic acid, solid-phase extraction of lipoic acid and electrochemical detection. A variety of methods have been published for the quantitative analysis of lipoic acid. Many of them, however, lack the necessary specificity or sensitivity needed for single-dose lipoic acid disposition studies in man. Therefore, up to now, information on pharmacokinetic data in humans was scarcely available, although lipoic acid is widely used in drug therapy.

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