SHORT COMMUNICATION

The issue of HPLC determination of endogenous lipoic acid in human plasma

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Abstract:

Lipoic acid (LA) is used extensively as a therapeutic agent for the treatment of various diseases. Many methods have been reported for the determination of LA plasma levels and its metabolites after its supplementation, but available information concerning endogenous plasma levels is still rare. Studies, which directly focused on determining the endogenous plasma levels, provided highly controversial results, less than 4.9 nmol/L or 143.7 – 197.0 nmol/L.

The main aim of this study was to verify the levels of free LA in the plasma of 40 individuals (17 women, 23 men). This group was non-supplemented with LA and met the conditions for incorporation into the blood donors register. We measured the levels of LA using an HPLC method with very sensitive coulometric detection after previous sample preparation including deproteination and solid phase extraction with phenyl cartridge.

Our limit of detection was 1.85 nmol/L and was better than the LODs in studies, which directly focused on determining the endogenous plasma levels of LA, 2.4 nmo/L and 4.9 nmol/L respectively. However, the levels of free LA in the plasma of non-supplemented voluntary blood donors were not detectable in all cases. The presented results of our study show that endogenous concentrations of LA are less than 1.85 nmol/L.

KEYWORDS: Lipoic acid, endogenous levels, human plasma, HPLC, coulometric detection

Abbreviations used:

HPLC, High performance chromatography;

LA, Lipoic acid;

LOD, Limit of detection;

SPE, Solid-phase extraction.

1.INTRODUCTION

Lipoic acid (LA) is used extensively as a therapeutic agent for the treatment of various diseases, and many clinical studies showed that supplementation with LA has beneficial effects for patients suffering from diabetes mellitus, cataract, cardiovascular diseases, etc. Many methods have been reported for the determination of exogenous plasma LA and its metabolites (Rochette et al., 2013; Borowczyk et al., 2015).

The available information concerning endogenous plasma levels is still rare and controversial. In 1992, Teichert and Preiss published values in the range of 4.8 – 121.0 nmol/L (Teichert and Preiss, 1992). However, in 2002 these same authors assumed that levels are 4.9 nmol/L or less (Teichert and Preiss, 2002). Montero et al. give not detectable contents for LA in human plasma (Montero et al., 2012). On the other hand, Khan et al. published that the range is 143.7 – 197.0 nmol/L (Khan et al., 2011).

2.EXPERIMENTAL

2.1Chemicals and reagents

Phosphoric acid, sodium hydroxide, hydrochloric acid, dichloromethane, sodium phosphate monobasic and LA were obtained from Sigma-Aldrich (Steinheim, Germany). Nitrogen was in purity 4.6 (Linde Gas, Czech Republic). Methanol, acetonitrile and 2-propanol were purchased from Merck (Darmstadt, Germany).

2.2 Blood collection

Three millilitres of venous blood was collected into tube containing ethylene diamintetraacetic acid as an anticoagulant (Greiner Bio-One, Kremsmünster, Austria). The plasma aliquots were stored in duplicates at -80 °C.

Plasma samples were collected from 40 voluntary blood donors (17 women, 23 men). The average age was 43.9 ± 9.4 years. This group was non-supplemented with LA and met the conditions for incorporation into the blood donors register in the Department of Transfusion of the Regional Hospital of Pardubice. Inclusion criteria were: age (18-65 years), body weight (50 kilogrammes and more) and a good state of health. Exclusion criteria are stated in Table 1. Informed consent in writing was obtained from all individuals.

2.3 Standard solution and plasma sample preparation

Lipoic acid was dissolved in a solution of 0.5 M sodium hydroxide to obtain a stock solution of 50 mmol/L. For linearity verification, calibration standards were prepared in the concentration range 1-80 nmol/L. The same examples of chromatograms are shown on the Figure 1.

Four hundred and fifty microliters of the plasma were added to 50 µl of calibration standards of LA. After having added 250 µl of acetonitrile, the mixture was vortexed for 2

min and then centrifuged at 25 000 x g for 10 min at 4 °C. Twenty microliters of 2 M hydrochloric acid were mixed with 450 µl of the upper layer for solid-phase extraction (SPE). Fifty microliters of water were added to 450 µl of plasma sample. The next procedure was identical with the standard preparation.

2.4 Extraction procedure

An SPE Phenyl cartridge (1 ml; DSC-Ph; Supelco, Bellefonte, USA) with the Visiprep Vacuum Manifold (Supelco, Bellefonte, USA) was used for the extraction. Conditioning was carried out by passing 3 ml of methanol and 3 ml of water. Four hundred and fifty microliters of deproteinized plasma was loaded and passed through the cartridge with a flow rate of 3 ml/min, then washed with 5 ml of water and dried under full vacuum for 5 min. The samples were eluted with 1 ml of methanol and collected into a plastic Eppendorf tube. The eluate was evaporated to dryness using a gentle nitrogen stream at 40 °C. The residue was re-dissolved in 100 µl of acetonitrile/water (1:1) and 10 µl were injected into the HPLC system.

2.5 HPLC conditions

The HPLC system consisted of a pump (LC-10ADvp, Shimadzu, Kyoto, Japan), a Rheodyne injector (Model 9725i, Rheodyne, L.P., Rohnert Park, CA, USA), a LiChroCart 254-4, Purospher®Star RP-C18, 5 µm analytical column (Merck, Darmstadt, Germany), fitted LiChroCart 4-4, Purospher®Star RP-C18, 5 µm guard column, and the electrochemical detector was a Coulochem III (ESA, Chelmsford, MA, USA) including a Model 5010 Analytical cell (electrode 1 was set to 450 mV, electrode 2 to 590 mV) and Model 5020 Guard cell (750 mV). The gain range was 20 nA. Data were collected and analysed with the software package Clarity (DataApex, Prague, Czech Republic).

The mobile phase consisted of 20 mM NaH_2PO_4 (pH 2.8, adjusted by H_3PO_4) and acetonitrile (62:38, v/v). The flow rate was 1 mL/min at a temperature of 37 °C.

3.RESULTS AND DISCUSSION

We found a limitation in a very important part of the sample preparation, in the extraction. Dichloromethane of high purity (for HPLC, ≥ 99.9%) contains the essential stabilizer amylene (1-pentene). In addition, we tested a next high purity dichloromethane (>99.5%) with the lowest concentration of stabilizer available, but it had similar chromatographic properties to LA and their separation was difficult. Moreover, gradient elution cannot be used with this electrochemical detection.

Taking into account the problematic liquid-liquid extraction, we chose a SPE. We combined and modified previous methods (Teichert and Preiss, 1992; Teichert and Preiss, 1995; Khan et al., 2010; Teichert and Preiss, 1997; Khan et al., 2011; Teichert and Preiss, 2002). The resulting chromatograms indicated that the Phenyl cartridges and the C8 cartridges are more suitable for LA extraction than the others. Moreover, the CN cartridges are inappropriate due to their very low recovery.

The calibration curve was obtained by plotting the peak responses areas against the concentrations of LA using a linear least squares regression and showed good linearity in the range of 7.5-50 nmol/L. The regression equation and correlation co-efficient retrieved from the calibration curve of LA were: $y = 139.21x + 250.78$, $r = 0.9997$.

The limit of detection (LOD) and the limit of quantification values were 1.85 and 5.18 nmol/L. The LOD was better than previous studies, 2.4 nmo/L and 4.9 nmol/L respectively (Khan et al., 2011; Teichert and Preiss, 2002). However, the levels of LA in plasma of 40 non-supplemented voluntary blood donors were not detectable in all cases. Similarly, plasma level of LA was not detectable in study Montero et al. (Montero et al. 2012).

4.CONCLUSIONS

The results of our study show that endogenous concentrations of LA are less than 1.85 nmol/L. These results are in agreement with the conclusion of a previous study that levels of LA are less than 4.9 nmol/L (Teichert and Preiss, 2002).

CONFLICT OF INTEREST

The authors declare no potential conflicts of interest.

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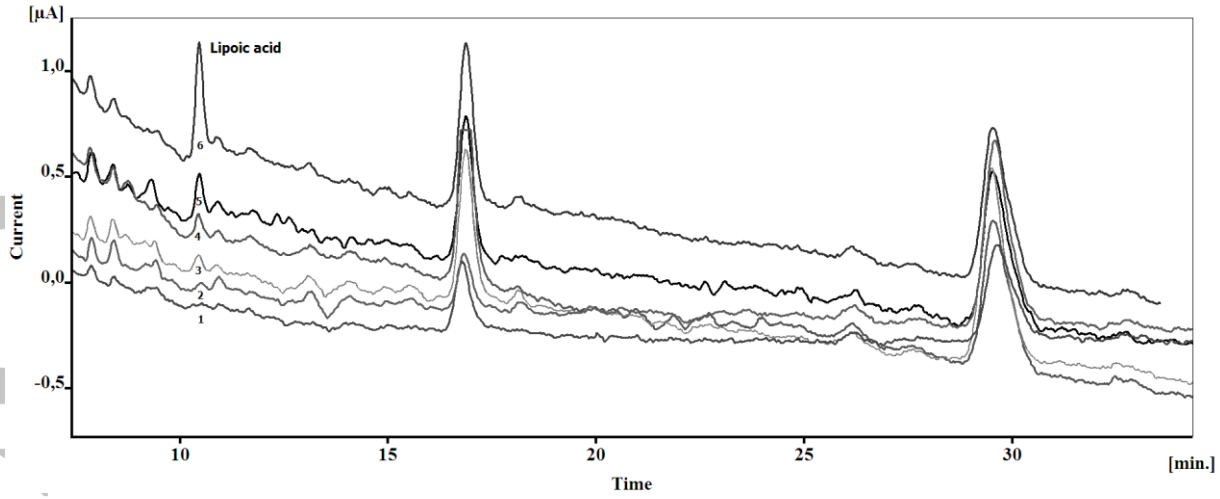

Figure 1. Chromatograms of calibration standards, 1- plasma without addition of LA, 2- plasma with addition of 1 nmol/L LA, 3- plasma with addition of 5 nmol/L LA, 4-plasma with addition of 7.5 nmol/L LA, 5- plasma with addition of 15 nmol/L LA, 6- plasma with addition of 50 nmol/L LA.

Table 1 Exclusion criteria for voluntary blood donors

The permanent exclusion	The provisional exclusion (6 months)	The provisional exclusion (1 month)	The provisional exclusion (1-2 weeks)	The provisional exclusion (Various time)
Infection hepatitis B or C in the past	Surgery and injury	Influenza	Pooling out a tooth	Surgery with disposable stitching (into healing)
Syphilis or tuberculosis in the past	Gynaecological intervention	Angina	Minor surgical intervention in mouth	Infection hepatitis A (1-2 years)
Some tropical disease in the past	Invasive examination	Antibiotic treatment	Cold	Infection mononucleosis (1-2 years)
Severe allergy	Tattoo	Vaccination (Influenza, hepatitis B)	Herpes	Borreliosis (1-2 years)
Haemophilia	Piercing and earring	Tick attaching		Non-infection diseases of heart, lung ,kidney and liver (time is various according to severity of disease)
Epilepsy	Acupuncture	Sojourn outside Europe		
HIV positivity	Sojourn in the tropics	Breast-feeding (after ending)		
Cancer (in the past too)	Gravidity (childbirth or termination)			
Severe psychiatric diseases				
Severe chronic diseases				