A Simple and Specific Method for Estimation of Lipoic Acid in Human Plasma by High Performance Liquid Chromatography

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Abstract

A rapid high performance liquid chromatographic method for determination of lipoic acid in human plasma was developed and validated. The method involved extraction of lipoic acid in ethanol consisting of 50 mM disodium hydrogen phosphate: acetonitrile: methanol in the ratio of 50:30:20. The separation was done using a C18 column (150 mm) and detection was carried out using UV detection at 201 nm. The assay was found to be linear in the range of 0.78-50 µg/ml with the correlation coefficient of 0.9998. Intra and inter-day variations were determined by processing each standard concentration in duplicate for five consecutive days. The average recovery of lipoic acid from plasma was 113%. The developed method demonstrates better sensitivity, precision, accuracy, stability and linearity when compared with the methods previously used. The method is simple and can be used for the determination of lipoic acid in basic research studies as well as in standard clinical laboratories.

Keywords: HPLC; Lipoic acid; Antioxidant; Free radicals; Co-factor; Therapeutic agent; Thiocetic acid; Vitamin E; Vitamin C

Introduction

α-Lipoic acid (LA) (1, 2-dithiolane-3-valeric acid, 1, 2-dithiolane-3-pentanoic acid, 6, 8-dithiooctanoyc acids, thiotic acid) (Figure 1) is a crucial cofactor in the pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase multienzyme complexes which are responsible for the production of acetyl-CoA in metabolic pathways [1]. It is also known as a metabolic antioxidant synthesized in human and animal cells and affords a protection to the cellular membrane by interacting with vitamin C and glutathione, which in turn recycles other antioxidants such as vitamin E to their reduced state and both oxidized and reduced forms of LA (Figure 2) reacts with reactive oxygen species and reactive nitrogen species, preventing damage due to oxidative stress in cells [2]. Many studies have proved its potential therapy for chronic diseases associated with oxidative stress [3]. Because of its potent antioxidant properties, LA has been tested for its potential therapeutic effects in a variety of pathological conditions and used as an effective therapeutic agent in various diseases, including diabetes, mitochondrial cytopathies, cardiovascular diseases, hepatitis, cataract, radiation damage, HIV infections, also it plays a role in neurodegenerative disorders and neurovascular abnormalities associated with diabetic neuropathy and in delaying or inhibiting the development of neuropathy in diabetes [4-7].

Because of its extensive and multidisciplinary therapeutic use, a simple and accurate method for analysis of LA would be useful. Different analytical methods have been developed so far to determine the LA content in biological samples (tissue and plasma) various foodstuffs, both from animal and plant sources, in dietary supplements and in pharmaceutical preparations. Only few studies have been reported for the estimation of LA in the human body fluids in the minimum number of samples. New and rapid method should be developed to assay the LA in a large number of samples in various clinical stages to know the significance which should be economical and reproducible.

The methods so far reported by various studies include the use of the different extraction procedure to improve the recovery and of different

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analytical techniques to improve the sensitivity of measurements. However, most of these methods faced limitations either in terms of recoveries or sensitivities. This required the development of a rapid and reliable method to determine LA with better sensitivity in human plasma. Among the different available forms, free alpha-LA is of more interest due to its more pharmacological and therapeutic function.

The microbial assay, which was the first one to be reported for the determination of LA in human serum suffered from reproducibility [8,9]. Methods using liquid chromatography–mass spectrometry (LC–MS) [10,11] and gas chromatography–mass spectrometry (GC–MS) have been reported; however, these methods are quite expensive and unaffordable for many laboratories [12-14].

High performance liquid chromatographic (HPLC) is one of the most widely used analytical methods with different detection modes including ultraviolet [15], fluorescence [16,17] and electrochemical detection (ECD) [18-20]. Although HPLC with fluorescence detection was reliable for the determination of LA in biological fluids, the sample pre-treatment and intensive extraction involves derivatization which is tedious and cumbersome [12,16]. The HPLC method using an ECD with a dual gold–mercury electrode was found to be highly sensitive, but the electrodes lost sensitivity after 30–50 injections and had to be reconditioned [14,21].

However, there is still a need to improve the chromatographic conditions for better sensitivity, simple sample preparation and shorter run time. UV detector is a very common and primarily used detector for HPLC analysis. Since LA contains a carboxyl group it can be detected by using UV detector. Hence the study is aimed to develop and validate a simple, specific and reproducible method for quantitation of LA in human plasma by HPLC.

Experimental

Reagents and standards

Alpha-LA pure powder was purchased from Sigma Chemical Company, India; methanol, acetonitrile, chloroform, ethanol (HPLC grade), and disodium hydrogen phosphate (Na2HPO4) were purchased from Qualigens India. Deionized water was processed through a Milli-Q water purification system (Millipore, USA). Pooled human plasma was obtained from a Blood Bank in Chennai, India.

Instrumentation

Chromatographic analysis was performed using HPLC system (Shimadzu Corporation, Kyoto, Japan) consisted of two pumps (LC-10ATvp) and an auto sampler (SIL-HTA) with built in system controller (SCL-10Avp). Class VP-LC workstation was used for data collection and acquisition. The analytical column was a C18, 250×4.6 mm ID, 5µ particle size protected by a compatible guard column (Lichrospher 100 RP-18e, Merck, Germany). The mobile phase comprised of 50mM Na2HPO4 (pH adjusted to 2.7): acetonitrile: methanol (pH adjusted to 2.7): acetonitrile: methanol in the ratio of 50:30:20. Prior to the preparation of mobile phase, the solvents were degased separately using a Millipore vacuum pump with 0.45 µm filter paper. Sonication was done for 10 min to remove dissolved gas. The UV detector was set at 201 nm. The chromatogram was run for 5 minutes at a flow rate of 1 ml/min, with column oven temperature maintained at 40°C. Unknown concentrations were derived from linear regression analysis of the peak height of the analyte vs. concentration curve. The linearity was verified using estimates of correlation coefficient (r).

Preparation of standard solution

Stock standard solution of (1mg/ml) of LA was prepared by dissolving the pure powder in methanol. The working standards of LA were prepared in pooled human plasma. The concentrations of LA in the working standards were 0.78, 1.56, 3.125, 6.25, 12.5, 25 and 50 µg/ml.

Sample preparation

Three hundred microlitres of the blank/standard was mixed with 300 µl of ethanol and 1.8 ml of chloroform, vortexed for 2 min and centrifuged for 5 min at 2500 rpm. Six hundred microlitres of the supernatant was separated and evaporated in nitrogen evaporator for 10 min. The dried residue was reconstituted with 100 µl of the mobile phase and 50 µl of the sample was directly injected into the HPLC column.

Validation procedure

The accuracy and linearity of alpha-LA standards were evaluated by analyzing a set of standards ranging from 0.78 to 50 µg/ml. Unknown concentrations were derived from linear regression analysis of the peak height of the analyte vs. concentration curve. The linearity was verified using estimates of correlation coefficient (r). Inter day and between intra day variations were determined by processing each standard concentration in duplicate for five consecutive days.

Precision studies were carried out on the basis of injection repeatability of spiked plasma samples. In order to evaluate the precision of the method, three different concentrations of alpha-LA were prepared in pooled plasma and analyzed in duplicate on three consecutive days.

For the recovery experiment, known concentrations of LA were prepared in pooled human plasma samples spiked with 0.78 and 3.125 µg/ml of LA and assayed. The percentage of recovery was calculated by dividing sample differences with the added concentrations. Recovery experiments were carried out on three different occasions.

The sensitivity of the method was evaluated by quantifying the limits of quantification (LOQ) and detection (LOD) for alpha-LA. These values were estimated mathematically from the standard curve equations. The LOQ and LOD were obtained by multiplying the standard deviation (S.D.) of the Y-axis intercepts by 10 and 5 respectively.

Results and Discussion

Several HPLC methods have been described to measure LA levels in human plasma. Some of them are quite complex and lengthy because of the extraction procedures involved in the sample preparation. Although a spectrophotometric method has been reported very recently, the sensitivity of that method is quite low and requires a large volume of sample [22].

This method was optimized under different chromatographic conditions in order to validate various parameters. Several methods, such as liquid-liquid extraction, deproteinization using solvents like chloroform, ethanol and acetonitrile for sample preparation have been used [6,11,23,24]. Among the various solvents used as mobile phase, 50 mM Na2HPO4 (pH adjusted to 2.7): acetonitrile: methanol in the ratio of 50:30:20 produced a good resolution peak. While developing the method, the run time was kept at 8 min (Figure 3). For the selection of optimal flow rate, mobile phase was pumped at different flow rates in the range of 1–2 ml/min. Better separation of the target compound was achieved at 1 ml/min flow rate.
In the present study, simple extraction procedure with ethanol and chloroform was standardized which resulted in better recovery of the analyte obtained as compared in the other solvents used. However the solvent used in the study provide satisfactory extraction efficiency. Under these chromatographic conditions, sufficient resolution was obtained with a good resolution peak at a retention time of 3.5 min. Temperature is an important parameter that greatly affects the chromatographic analysis. To study its effect on the analysis of LA, column oven temperature variations were evaluated in the range of 25–45°C. The sensitivity and retention time of analyte were greatly influenced by column oven temperature. The chromatographic peak LA was found to be sharp at 40°C and resolution of the peak decreased with increasing temperature. The UV detector was used in the range of 201–401 nm. The detector at which the analyte showed optimal response was selected as the optimized range for the determination of LA. The UV detector at 201 nm showed an optimal response and it was used for measurement of LA content. This was performed using photodiode array (PDA), though LA can be detected using UV detector, as the detection wavelength falls within the range of UV detector. The method was validated by evaluating the linearity, precision, recovery, LOD and LOQ, specificity and sensitivity. Complete separation of the target peak was achieved in 5min. Plasma LA was separated as a discrete peak.
The calibration curve of LA standard solutions constructed using seven concentrations shows good linearity in the range 0.78 to 50.0 µg/ml as shown in Figure 5. A linear relationship was observed between peak heights and the concentrations over this range with a correlation coefficient (r) of 0.9998.

The peak for LA was observed linear at different concentration. The calibration curve of LA standard solutions constructed using seven concentrations shows good linearity in the range 0.78 to 50.0 µg/ml as shown in Figure 5. A linear relationship was observed between peak heights and the concentrations over this range with a correlation coefficient (r) of 0.9998.

The linearity, reproducibility (inter-day and intra-day) of plasma LA data is presented in Table 1. The inter-day and intra-day co-efficient variation (CV %) for standards containing 0.78-50 µg/ml ranged from 3.8 to 17.9% (Table 3). The LOD and LOQ values were 0.39 and 0.78 µg/ml respectively.

Conclusions

In summary, the HPLC-UV method described for the estimation of LA in human plasma is simple, sensitive, reproducible and precise with a recovery of 113%. The assay is less laborious, economical and less time consuming than other reported methods previously. Plasma concentration of LA was determined in healthy volunteers (n=10), the mean plasma LA concentration with standard deviation was 0.4570 ± 0.26 µg /ml. The validated HPLC-UV method may be applied to the measurement of LA levels in clinical practice. Further the LA concentrations can be confirmed by LC-MS quantification; thereby this method may be applied in future studies with shorter run time thereby large number of samples can be quantitated.

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