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Determination of lipoic acid in human plasma by HPLC-ECD using liquid–liquid and solid-phase extraction: Method development, validation and optimization of experimental parameters

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ABSTRACT

A rapid, inexpensive, sensitive and specific HPLC-ECD method for the determination of lipoic acid in human plasma was developed and validated over the linearity range of 0.001–10 μg/ml using naproxen sodium as an internal standard (IS). Extraction of lipoic acid and IS from plasma (250 μl) was carried out with a simple one step liquid–liquid extraction using dichloromethane. Similarly solid-phase extraction was carried out using dichloromethane as extraction solvent. The separated organic layer was dried under the stream of nitrogen at 40 °C and the residue was reconstituted with the mobile phase. Complete separation of both lipoic acid and IS at 30 °C on Discovery HS C18 RP column (250 mm × 4.6 mm, 5 μm) was achieved in 6 min using 0.05 M phosphate buffer (pH 2.5 adjusted with phosphoric acid):acetonitrile (50:50, v/v) as a mobile phase pumped at the rate of 1.5 ml/min using electrochemical detector in DC mode at the detector potential of 1.0 V. The limit of detection and limit of quantification of lipoic acid were 200 pg/ml and 1 ng/ml, respectively. While on column limit of detection and limit of quantification of lipoic acid were 10 and 50 pg/ml, respectively. The absolute recoveries of lipoic acid with liquid–liquid and solid-phase extraction were 98.43, 95.65, 101.45, and 97.36, 102.73, 100.17% at 0.5, 1 and 5 μg/ml levels, respectively. Coefficient of variations for both intra-day and inter-day were between 0.28 and 4.97%. The method is validated and will be quite suitable for the analysis of lipoic acid in the plasma of human volunteers as well as patients with diabetes and cardiovascular diseases.

1. Introduction

Alpha-lipoic acid (ALA), usually referred as thioctic acid is chemically known as 1,2-dithiolane-3-valeric acid or 6,8-dithiolane octanoic acid, or 6,8-thioctic acid is a naturally occurring antioxidant compound that is found in plants, animals and various microorganisms [1,2]. The chemical structure of α-lipoic acid is shown in Fig. 1. Alpha-lipoic acid (LA) and its reduced form dihydrolipoic acid (DHLA) together constitute the universal antioxidant redox couple of many biological systems [2]. It is a cofactor for mitochondrial alphaketo-acid dehydrogenase and a free radical scavenger that scavenges hydroxyl radicals, hypochlorous acid, peroxyradicals, superoxide radicals, and singlet oxygen [2–5]. It is also used as a chelating agent that is used as an antidote in iron, copper, mercury, arsenic, and cadmium poisoning [2,6]. Lipoic acid recycles others antioxidants such as glutathione, vitamins C and E to their reduced state and maintains body antioxidant capacity [4,7]. It is also used as a therapeutic agent in various diseases and was found to be useful in patients with diabetes, mitochondrial cytopathies, cardiovascular diseases, hepatitis, cataract, radiation damage, HIV infections, heavy metal poisoning, neurodegenerative disorders, and neurovascular abnormalities associated with diabetic neuropathy [1,2,8]. Because of its extensive and multidisciplinary therapeutic use, a specific, fast and reliable method of analysis is required that can be easily applied by standard clinical laboratories. Such type quantification of lipoic acid is crucial in biochemical, nutritional and pharmacokinetic studies particularly related to the role of LA in the homeostasis of body redox status, as well as its beneficial antioxidant role in various diseases [2]. Numerous chromatographic methods have been reported for the determination of lipoic acid and its metabolites in pharmaceutical preparations [6,9], biological fluids and food samples [2,10].

Various analytical methods are currently reported for the analysis of lipoic acid in the body fluids including thin layer chromatography (TLC), high performance liquid chromatography (HPLC), gas chromatography (GC), polarography, colorimetric and microbiological assay, liquid chromatography–mass spectrometry (LC–MS) [1,11,12], gas chromatography–mass spectrometry

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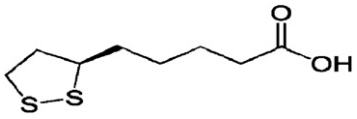

Fig. 1. Chemical structure of α-lipoic acid.

(GC–MS), and capillary electrophoresis [13,14]. Although GC–MS is a powerful technique for the quantification of LA in biological fluids but it is expensive and beyond the reach of many laboratories [2,9]. HPLC is the more suitable and appropriate method for the evaluation of lipoic acid due to its high sensitivity, specificity, inexpensive sample preparation and ease of samples handling procedures in clinical practice [15]. HPLC with different detection modes including ultraviolet [16], fluorescence [2,17] and electrochemical [10,15,18,19] have been reported for the quantification of lipoic acid in dietary supplements and biological fluids. HPLC-UV method has limitations due to the lack of strong chromophore in lipoic acid and its high detection limits [9]. HPLC with fluorescence detection, proved to offer a good method for the determination of α-lipoic acid in biological fluids but require lengthy derivatization and laborious sample preparation steps [2,17]. HPLC with electrochemical detection seems to be a method of choice in terms of sensitivity, short run time and low cost for most of the standard laboratories to quantify α-lipoic acid in biological fluids as compared with other reported analytical methods [1,2,9,11–14,16,17].

The goal of our suggested work was to develop a rapid, sensitive and reliable HPLC-ECD method for the quantification of α-lipoic acid in biological fluids with commercially easily available IS. The method is fast and cost effective as compared with the other reported HPLC-ECD methods for the quantification of lipoic acid in biological fluids [10,15]. The method was successfully applied for the determination of lipoic acid in human plasma using liquid–liquid and solid-phase extraction. This method can also be applied for the analysis of lipoic acid in pharmaceutical preparations and food supplements with slight modifications in extraction protocol.

2. Experimental

2.1. Chemicals and reagents

Alpha-lipoic acid and retinyl palmitate were obtained from Sigma–Aldrich (Oslo, Norway). Naproxen sodium (purity 99.3%) was kindly provided by Saydon Pharma Pvt. Ltd. (Peshawar, Pakistan). HPLC grade acetonitrile, methanol, and analytical grade potassium dihydrogen phosphate, phosphoric acid, dichloromethane, ethyl acetate, diethylether and hydrochloric acid were also purchased from Sigma–Aldrich (Oslo, Norway). Purified water was prepared with Millipore (Milford, USA) distillation apparatus and used for buffer preparation. The plasma was obtained from volunteers in the pharmaceutical laboratory at Department of Pharmacy, University of Peshawar (Pakistan).

2.2. Equipments

The study was carried out using a PerkinElmer HPLC system (Norwalk, USA) consisted of a pump (series 200), on-line vacuum degasser (series 200), autosampler (series 200), column oven (series 200), linked by a Pe Nelson network chromatography interface (NCI) 900 with a DECADE II electrochemical detector (Antec Leyden, Netherlands), with Flexcell flow cell has an effective volume of 0.5 μl and consists of three electrode configuration with a working glassy carbon electrode, a HyREF reference electrode (REF) and the auxiliary electrode (AUX), KCl Ag/AgCl. The whole HPLC system was controlled by PerkinElmer Total Chrom Workstation Software (version 6.3.1). The data was acquired and quantified using this software. The separation was performed using Discovery HS C_{18} RP chromatographic column (250 mm × 4.6 mm, 5 μm; Bellefonte, USA), protected by a PerkinElmer C_{18} (30 mm × 4.6 mm, 10 μm; Norwalk, USA) pre-column guard cartridge. Supelclean LC-18 SPE cartridges (1 ml; particle size 56.2 μm; Bellefonte, USA) with the Visiprep™ Vacuum Manifold were used for solid-phase extraction.

2.3. Chromatographic conditions

Chromatographic analysis of lipoic acid and naproxen sodium was performed using electrochemical detector in DC mode applying the detector potential of 1.0 V. The mobile phase composed 50% each of acetonitrile and 0.05 M potassium dihydrogenphosphate (pH 2.5, adjusted with 85% phosphoric acid) was pumped at the rate of 1.5 ml/min keeping the column oven and detector temperature at 30 °C. The sample (20 μl) was injected into HPLC system by autosampler.

2.4. Preparation of standard solutions

The stock solutions of quality control standards for lipoic acid and internal standard naproxen sodium each 1 mg/ml were prepared in the mobile phase. The stock solution of lipoic acid was further diluted with the same solvent to obtain various dilutions in the range of 1–10,000 ng/ml and the concentration of the IS was kept constant (1.0 μg/ml).

2.5. Blood collection and plasma sample preparation

Blood was collected in the ethylene diamintetraacetic acid (EDTA) tube from the healthy volunteers (Pharmacy Graduates in Department of Pharmacy, University of Peshawar, aged 20–25 years) who had given their informed consent. The study protocol was approved by the ethical committee of Department of Pharmacy, University of Peshawar. The collected blood was centrifuged at 1252 × *g* for 15 min at 4 °C to separate the plasma. The plasma was stored at −80 °C until analysis. At the time of analysis the plasma was thawed at room temperature and spiked with appropriate concentrations of lipoic acid to prepare 1, 500, 1000, 2500, 5000, and 10,000 ng/ml dilutions of lipoic acid. An equal volume (10 μl/ml) of internal standard (1 mg/ml) was added to each lipoic acid dilution (10 ml tube) to make IS concentration 1 μg/ml in each sample. Following extraction the samples were analysed and calibration curve was constructed in the range of 1.0–10,000.0 ng/ml.

2.6. Liquid–liquid extraction

Plasma (250 μl) was transferred to glass vial (≈5 ml) then internal standard and LA were added. The acetonitrile (1 ml) was added, vortexed for 1 min and extracted with 2 ml dichloromethane. The resulting mixture was then vortex and centrifuged at 959 × *g* at 0 °C for 10 min, the organic layer was separated and dried under nitrogen at 40 °C. The residue was reconstituted with 1.0 ml mobile phase vortexed and centrifuged again. The clear solution was separated and transferred to autosampler vial and injected into HPLC equipped with loop size of 20 μl.

2.7. Solid-phase extraction

Solid-phase extraction [10], with a little modification was applied for the recovery of lipoic acid from plasma. Supelclean LC-18 SPE cartridges (1 ml; particle size 56.2 μm; Bellefonte, USA) with the Visiprep™ Vacuum Manifold were used for solid-phase

extraction. Conditioning was performed according to the manufacturer guidelines, simply by passing methanol (1 ml) through the cartridge followed by equilibration with water (1 ml), pH 3.0 adjusted with phosphoric acid. The plasma (250 μl), water (3 ml) and concentrated HCl (10 μl) were loaded and passed through the cartridge with a flow rate of 3 ml/min, then washed with 2 ml of water (adjusted to pH 3.0 with phosphoric acid), column was then dried by air and analytes were eluted with 1 ml of dichloromethane (1 ml) at a flow rate of 3 ml/min. The eluate was collected in glass tubes, and dried under a gentle stream of nitrogen at 40 °C. The residue was reconstituted with mobile phase (1 ml) and 20 μl was injected by autosampler into the HPLC system.

2.8. Chromatographic conditions optimizations

Various chromatographic conditions such as mobile phase composition, detector potential, flow rate, extraction solvent, and column oven temperature were optimized for the analysis of lipoic acid using RP-HPLC-ECD system in isocratic mode.

2.8.1. Detector potential optimization

The applied ECD potential was varied in the range of 500–1000 mV. The voltammogram was constructed between applied potential and detector response. The optimum detector potential at which both analytes showed good peak response was selected for the evaluation of lipoic acid and naproxen sodium.

2.8.2. Mobile phase optimization

Various organic solvents like methanol, acetonitrile, tetrahydrofurane (0.05%) and phosphate buffer (0.025–0.05 M) in different combinations were applied and finally acetonitrile: 0.05 M phosphate buffer pH 2.5, adjusted with phosphoric acid (50:50, v/v), was selected for the analysis of both analytes. The peak response of lipoic acid and naproxen sodium was recorded against all combination of acetonitrile: 0.05 M phosphate buffer (20:80; 30:70; 40:60; 50:50; 60:40). The mobile phase composition that analyzed the target peaks in a shorter run time with greater sensitivity and peak resolution was selected.

2.8.3. Flow rate optimization

Flow rate of the mobile phase was evaluated in the range of 1–2 ml/min, however good resolution was obtained at the flow rate of 1.5 ml/min. The flow rate 1.5 ml/min was selected and optimized under various chromatographic conditions.

2.8.4. Temperature optimization

The temperature of column oven was varied in the range of 25–45 °C to study its effects on the chromatogram of analyte and IS. The effects of temperature on the sensitivity, retention time, and peak resolution of both the compounds were studied from the chromatograms of these analytes. The changes observed in the chromatograms with respect to temperature were recorded.

2.8.5. Extraction solvent/procedure optimization

Lipoic acid and naproxen sodium were extracted from plasma using both liquid–liquid and solid-phase extraction procedures. In liquid–liquid extraction various organic solvents including ethyl acetate (EA), dichloromethane (DCM), diethyl ether (DEE), acetonitrile, and a 50:50 mixture of DCM:DEE were used to obtain the maximum recovery of both lipoic acid and IS. The plasma (250 μl) was spiked with 0.5, 1.0, and 5.0 μg/ml lipoic acid and 1 μg/ml IS. Then samples vortexed for 10 min, extracted and analysed. The recovery was determined at three concentration levels of lipoic acid. Similarly, solid-phase extraction was applied at three concentration levels of lipoic acid keeping the internal standard concentration 1 μg/ml and recoveries were determined.

2.8.6. Selection of internal standard

Different compounds were tried to be used as internal standard. Among all these compounds Vitamin A palmitate, retinyl acetate, tocopherol acetate and naproxen sodium were assessed to be used as an internal standard. Among these compounds the one that showed good resolution and instrumental response with the lipoic acid was selected as internal standard. Then naproxen sodium was used as an internal standard in the present studies.

2.9. Method validation

The specificity, accuracy, precision, sensitivity, linearity, recovery, limits of detection (LOD) and limit of quantitation (LOQ), robustness, stability of samples and system suitability parameters were evaluated in order to validate the developed method.

The specificity of the chromatographic method was evaluated by analysis of the analytes in mobile phase, blank plasma, and 1:1 mixture containing 1 μg/ml each of lipoic acid and naproxen sodium and plasma samples spiked with 1 μg/ml each of lipoic acid and naproxen sodium.

The accuracy of the method was determined by % recovery method. The % recovery of the analyte was determined at three concentrations by spiking the plasma (250 μl) with 0.5, 1.0 and 5.0 μg/ml of lipoic acid and 1 μg/ml IS. Each sample was injected in triplicate and recovery was determined according to the following equation:

$$\text{Recovery} = \frac{[C] \times 100}{[A] + [B]} \quad (1)$$

where A = response ratio of the analyte with reference to IS in the mobile phase; B = response ratio of analyte with reference to IS in the control plasma; C = response ratio of the analyte with reference to IS in spiked plasma.

The linearity of the method was assessed from the calibration curves constructed at six concentrations points of lipoic acid in the mobile phase and spiked plasma. Calibration curves were constructed by plotting the response ratios (ratios of peak areas of analyte to internal standard) versus concentration of lipoic acid using a linear least squares regression. The resulting plot slope (m), intercept (b), correlation coefficient (r) and standard error (E_s) were calculated from the regression equation using Microsoft Excel 2007.

Precision study was carried out on the basis of injection repeatability and analysis repeatability of spiked plasma samples. Injection repeatability was determined by repeated injection (n = 10) of plasma sample spiked with 1 μg/ml each of lipoic acid and internal standard into HPLC. The retention time and peak area repeatability data obtained as mean, standard deviation (±SD) and covariance (% RSD), were expressed as a measure of precision of the method. Analysis repeatability was determined by analyzing plasma samples (n = 5) spiked with 1 μg/ml each of analyte and internal standard, prepared individually from same human plasma and the results were obtained as repeatability of recovered amount, expressed by mean, standard deviation (±SD), and covariance (% RSD).

The intra-day and inter-day variations were studied by analyzing plasma samples spiked with lipoic acid and IS (1 μg/ml), at 8:00, 16:00, and 24:00 h, for 1 week at alternate days. The results were expressed as mean, standard deviation (±SD), and covariance (% RSD). The recovered amounts were calculated in the form of concentration by the following equation:

$$C = \left(\frac{X}{Y}\right) \times \left(\frac{A}{B}\right) \times C_s \times F_D \quad (2)$$

where X and Y are peak areas of the analyte in plasma samples and 1:1 mixture, respectively; A and B are peak areas of the internal standard in 1:1 mixture and plasma samples, respectively; C_s is the concentration of analyte in the 1:1 mixture; and F_D is the dilution factor.

The sensitivity of the method was evaluated by quantifying the limit of detection (LOD) and limit of quantification (LOQ) for lipoic acid. The limit of detection (LOD) of the analyte is the concentration at which signal-to-noise ratio (S/N) is three and limit of quantification (LOQ) is the minimum concentration of analyte that can be determined at an acceptable precision and accuracy under rated conditions of analysis by a given method. For LOD and LOQ quantification dilutions of lipoic acid were prepared in the range of 0.1–5 ng/ml. The LOD was then determined from the peaks by the software at signal-to-noise ratio (S/N) of three, while LOQ was determined by measuring the analyte response with precision and accuracy of <20%, respectively.

The robustness/ruggedness of the reported method was assessed by bringing small deliberate changes in the various chromatographic conditions, like mobile phase composition (±2%), column oven temperature (±5 °C), and flow rate of mobile phase (0.2 ml/min).

Stability studies of standard stock solutions stored at, 25 °C for 24 h, 4, −20, and −80 °C for 1 month were carried out, respectively. Similarly, the stability studies of spiked plasma samples stored at −80 °C were carried out for 1 week. Each sample was injected in triplicate and the % stability was calculated by the following equation:

$$\%\,\text{stability} = \frac{S_t}{S_0} \times 100 \tag{3}$$

where S_t is stability of analyte at time t, and S_0 is stability at initial time.

Various parameters such as the retention factor (k), separation factor (α), tailing factor (T), resolution (R_s), and efficiency (N), were calculated as suitability tests for evaluation of chromatographic system using the following equations:

$$k = \frac{t - tM}{tM} \tag{4}$$

$$\alpha = \frac{k2}{k1} \tag{5}$$

$$T = \frac{A_{5\%h} + B_{5\%h}}{2 \times A_{5\%h}} \tag{6}$$

$$R_s = \frac{1.18 \times (t_2 - t_1)}{w_{h2} - w_{h1}} \tag{7}$$

$$N = 5.54 \times \left(\frac{t}{w_h}\right) 2 \tag{8}$$

where t_M and t is the retention time of un-retained samples (mobile phase) and analyte, respectively; $A_{5\%h}$ and $B_{5\%h}$ is the bandwidth of the front half and tail half of the peak at 5% of the peak height, respectively. w_h is the bandwidth at half-height (w_{h1} and w_{h2} are bandwidths at half-height of peaks 1 and 2, respectively). 1 and 2 are the adjacent components ($t_1 < t_2$).

3. Results and discussion

The present method developed for determination of lipoic acid in human plasma using naproxen as an internal standard is rapid, robust and easy to automate. Complete separation of lipoic acid and naproxen sodium was achieved within 6 min with good instrumental response. Some endogenous compounds have been co-extracted with the applied extraction procedure but none of these interfere with the lipoic acid analysis. The various chromatographic conditions and experimental parameters were optimized and the method was validated in accordance with standard guidelines [20].

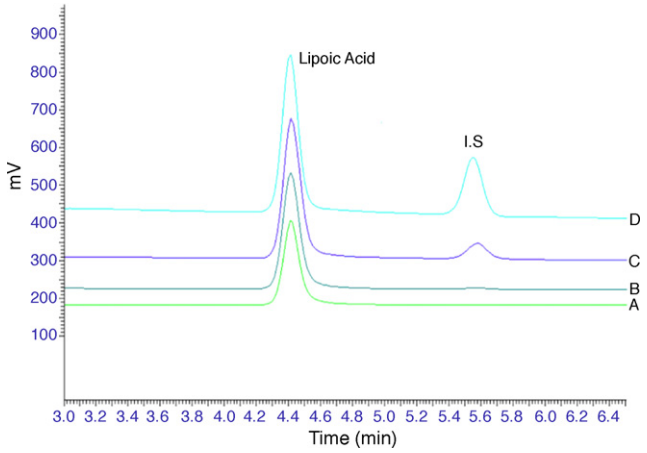

Fig. 2. Hydrodynamic voltammogram showing response of lipoic acid (LA) and naproxen sodium (IS) at different detector voltage. *Chromatogram*: (A) detector response at 700 mV; (B) detector response at 800 mV; (C) detector response at 900 mV; (D) detector response at 1000 mV.

3.1. Optimization of chromatographic conditions and experimental parameters

The various experimental parameters and chromatographic conditions were optimized and the method was found suitable for the analysis of lipoic acid in human plasma. Among the various organic solvents used as mobile phase acetonitrile and 0.05 M phosphate buffer (pH 2.5 adjusted using phosphoric acid) with 50:50 ratio offered good resolution of the peaks, best sensitivity and better retention times of the analytes were observed. Similarly, flow rate of the mobile phase was adjusted 1.5 ml/min as better resolution of peaks of analyte and IS and better peak shapes were observed at this flow rate. At higher flow rate although retention times of both analytes decreased but their area response decreased. The voltammogram indicates that highest response for lipoic acid and naproxen sodium was observed at 1.0 V. The response of naproxen sodium decreased significantly by decreasing the detector voltage and below 0.7 V it was not detected at all as shown in Fig. 2. Selection of column oven temperature was based on better sensitivity, good resolution peaks and shorter retention times of both analytes (lipoic acid and naproxen sodium). The sensitivity and retention times of both analyte and internal standard were affected by column oven temperature considerably. Peak response increased and retention time decreased of both analyte and internal standard upto 30 °C, while above that temperature sensitivity of both analytes decreased although retention time decreased. The peaks of both the compounds were sharp at 30 °C and became broad above that temperature. Internal standard was selected on the basis of its sensitivity, specificity, stability and compatibility with lipoic acid and extraction procedure. Retinyl acetate, retinyl palmitate, tocopherol acetate, and naproxen sodium were evaluated as internal standard with lipoic acid. Among all these compounds naproxen sodium showed the better results in term of sensitivity, good recovery and retention time compared with the other internal standards under study. Lipoic acid and naproxen sodium were dissolved in methanol to prepare stock (1 mg/ml) solutions of both analytes. The stock solutions were further diluted with the mobile phase to prepare the appropriate dilutions. Proteins were precipitated with acetonitrile and both liquid–liquid and solid-phase extractions were applied for the recovery of analytes. Dichloromethane was used for the extraction of lipoic acid from

plasma samples as it has shown best recoveries for lipoic acid and IS. Solid-phase extraction was better in the sense that better recoveries were achieved in a short time with a small amount of extraction solvent.

3.2. Method validation

The method was validated by evaluating the linearity, precision, specificity, sensitivity, recovery, limits of detection and limits of quantitation, robustness, stability of solutions and system suitability parameters. Our laboratory results showed that the method is fully validated and accurate for the determination of lipoic acid in plasma using electrochemical detector. Complete separation of the target peaks was achieved in 6 min, by analyzing the standard solutions, blank plasma and spiked plasma samples. Representative chromatograms of mobile phase, blank plasma, and 1:1 mixture (1 μg/ml of lipoic acid and naproxen sodium each) are shown in Fig. 3.

The calibration curves of lipoic acid standard solutions, spiked plasma samples, and spiked plasma samples corrected for blank plasma constructed at six concentration levels show good linearity in the range of 0.001–10 μg/ml, as shown in Fig. 4. Regression equation and correlation coefficient (r) calculated from the calibration curves of standard solutions, spiked plasma samples, and spiked plasma samples corrected for blank plasma for lipoic acid are shown in Table 1. Similarly, the standard error (E_s), calculated for standard solutions, spiked plasma, and spiked plasma samples corrected for blank plasma, of lipoic acid was 0.115, 0.278, and 0.278, respectively as shown in Table 1.

Accuracy of the method determined on the basis of percent recovery at 0.5, 1 and 5 μg/ml concentration, for lipoic acid was 104.50, 95.43, and 103.06%, respectively, as shown in Table 1. The repeatability (injection repeatability, analysis repeatability), and intermediate precision (intra-day, inter-day studies) are presented in Tables 1 and 2, respectively.

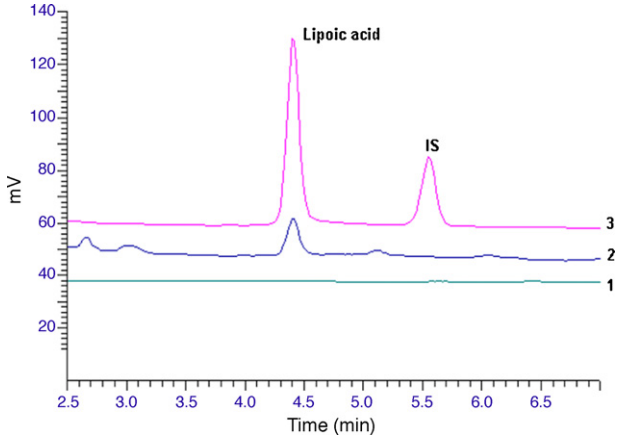

Fig. 3. Representative RP-HPLC-ECD chromatograms of various samples analyzed under specified conditions. *Chromatograms*: (1) the solvent blank (mobile phase); (2) the blank plasma sample; (3) 1:1 mixture containing 1 μg/ml lipoic acid and internal standard (IS) each.

Recovery of lipoic acid was measured at three concentration using liquid–liquid and solid-phase extraction. The % recoveries with liquid–liquid and solid-phase extraction were in the range of 95.40–99.84% and 97.36–102.73% at all three concentrations studies, respectively. The recoveries obtained with liquid–liquid and solid-phase extraction are shown in Tables 1 and 3, respectively. The absolute recovery of the internal standard at a single concentration of 1 μg/ml was recorded 95.53%.

Sensitivity of the method was determined on the basis of quantification of LOD and LOQ values of lipoic acid. The LOD and LOQ

Table 1
Concentration range, linearity, accuracy, repeatability, recovery, and sensitivity of the developed method.

S/no.	Parameters	Lipoic acid
1	Concentration range (μg/ml)	0.001–10
2	*Linearity*	
(a)	Standard solutions	
	Regression equation	$y = 0.0035x + 0.177$
	Correlation coefficient, r	0.9999
	Standard error, E_s	0.115
(b)	Spiked plasma samples	
	Regression equation	$y = 0.0036x + 0.462$
	Correlation coefficient, r	0.9998
	Standard error, E_s	0.278
(c)	Corrected plasma samples	
	Regression equation	$y = 0.0036x + 0.114$
	Correlation coefficient, r	0.9998
	Standard error, E_s	0.278
3	*Accuracy* (*% recovery ± SD; % RSD*)	
	0.5 μg/ml	104.50 ± 2.59; 2.47
	1.0 μg/ml	95.43 ± 0.56; 0.59
	5.0 μg/ml	103.06 ± 0.80; 0.78
4	*Precision*	
	Repeatability	
(a)	Injection repeatability	
	Retention time (min) ± SD; % RSD)	4.38 ± 0.01; 0.12
	Peak area, 1 μg/ml ± SD; % RSD)	73,242 ± 5664; 7.71
(b)	Analysis repeatability (amount recovered, 1 μg/ml ± SD; % RSD)	0.8824 ± 0.0092; 1.043
5	*Recovery* (*amount recovered, μg/ml ± SD; % RSD; % recovery*)	
	0.5 μg/ml	0.494 ± 7.74; 1.51; (98.80)
	1.0 μg/ml	0.954 ± 5.59; 0.58; (95.40)
	5.0 μg/ml	4.992 ± 4.55; 0.09; (99.84)
6	*Sensitivity*	
(a)	Limit of detection (ng/ml)	0.200
(b)	Limit of quantification (ng/ml)	1.000

Table 2
Intra-day and inter-day studies performed for lipoic acid.

S/no.	Parameters	Alpha-lipoic acid Mean ± SD; % CV
1	*Intra-day repeatability*	
	Lipoic acid: 0.5 μg/ml (amount recovered, μg/ml)	0.4619 ± 0.0096; 2.0782
	Lipoic acid: 1 μg/ml (amount recovered, μg/ml)	0.9730 ± 0.0028; 0.2885
	Lipoic acid: 5 μg/ml (amount recovered, μg/ml)	4.5340 ± 0.0143; 0.3148
2	*Inter-day repeatability*	
	Lipoic acid: 0.5 μg/ml (amount recovered, μg/ml)	0.4308 ± 0.0138; 3.1411
	Lipoic acid: 1 μg/ml (amount recovered, μg/ml)	0.8895 ± 0.0182; 2.0421
	Lipoic acid: 5 μg/ml (amount recovered, μg/ml)	4.4822 ± 0.1967; 4.388

Table 3
Comparison of liquid–liquid and solid-phase extraction procedures for lipoic acid.

Extraction solvents	% Recovery Lipoic acid
Liquid–liquid extraction	
Dichloromethane	98.42
Diethyl ether	80.26
Ethyl acetate	75.27
Dichloromethane and diethyl ether (50:50)	88.45
Acetonitrile	92.34
Concentration (μg/ml)	% Recovery
Solid-phase extraction	
0.5 μg/ml	97.36
1 μg/ml	102.73
5 μg/ml	100.17

values were 0.25 and 1.00 ng/ ml, respectively as presented in Table 1 and Fig. 5.

The stability studies showed that the standard solutions of lipoic acid were stable for at least 1 month when stored at −80 °C, however, spiked plasma samples were stable for only 1 week when stored at −80 °C. The degradation rate of both standard solutions and spiked plasma sample stored at 25 °C was higher than the samples stored at 4, −20, and −80 °C. Standard solutions of lipoic acid were more unstable at room (25 °C) (5.03% loss), refrigerator (4 °C) (2.48% loss), freezer (−20 °C) (1.16% loss), as well as deep freezer temperature (−80 °C) (0.70% loss) as compared with naproxen sodium, the % loss of which was 4.52, 2.07, 0.46 and 0.39% at room, refrigerator, freezer, and deep freezer temperature, respectively. These values are within the assay variability limits [1].

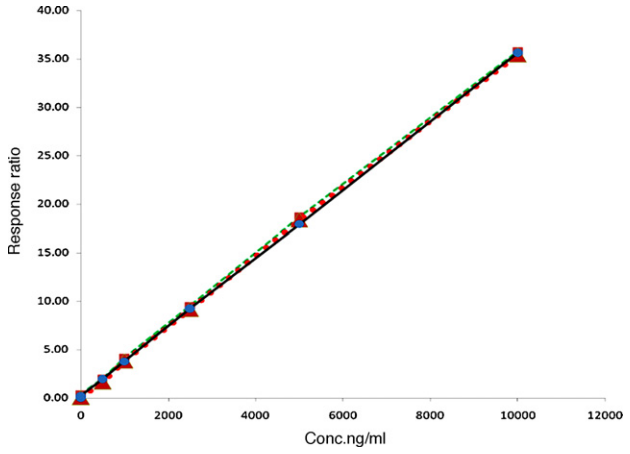

Fig. 4. Calibration curves for α-*lipoic acid*. Solid black line (—) represents standard samples ($y = 0.0035x + 0.177$, $r = 0.9999$; Dashed green line (- - - - - - - -) represents spiked plasma samples ($y = 0.0036x + 0.462$, $r = 0.9998$; and dotted red line (. . . . . .) represents spiked plasma samples corrected for the blank plasma ($y = 0.0036x + 0.114$, $r = 0.9998$). *Note:* Each point is a mean of triplicate injections. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

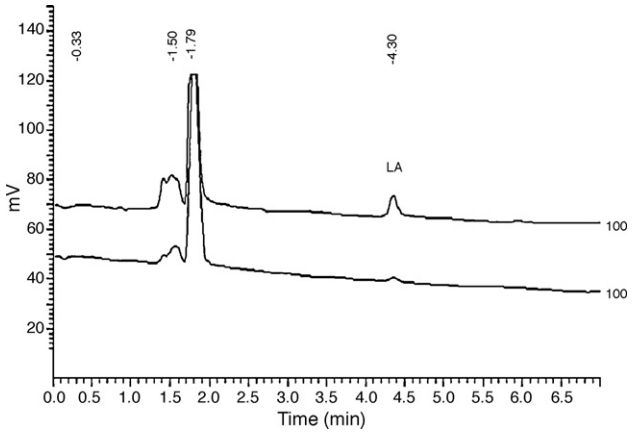

Fig. 5. Chromatograms showing limit of detection (LOD) and limit of quantification (LOQ) of lipoic acid (LA).

Table 4
System suitability specifications and tests.

	Suitability tests and specifications	
Column length (mm)	250	
Internal diameter (mm)	4.6	
Particle size (μm)	5	
Back pressure (psi)	1675–1820	
	A[a]	IS[b]
Retention factor, *k*	1.93	2.67
Separation factor, *α*	9.67	1.38
Tailing factor, *T*	1.01	0.93
Resolution, R_s	9.59	3.71
Asymmetric factor, A_f	0.95	1.11
Efficiency or number of theoretical plates, *N*	3711	5172

[a] Alpha-lipoic acid.
[b] Naproxen sodium.

Method robustness evaluated by bringing minor changes in different chromatographic conditions such as mobile phase composition, column oven temperature, flow rate and detector voltage, resulted negligible changes in the peak area and retention time of the analytes.

Results of system suitability parameters presented in Table 4, were in the permissible range [20].

4. Application of the method

Our developed and validated HPLC-ECD method was applied for the assessment of oxidative stress through measuring plasma concentration of lipoic acid in healthy volunteers and patients with diabetes and cardiovascular diseases. This method is a part of bio-

chemical analysis of blood samples collected from healthy human volunteers and patients with diabetes and cardiovascular diseases. This method will be used for the determination of lipoic acid in the clinical practice. Our data obtained from healthy volunteers showed best peaks resolution, and the plasma concentration of lipoic acid was found within the normal range. This method can also be applied for the investigation of lipoic acid in foods, food supplements, pharmaceutical preparations and others complex biological matrices if suitable modifications are made in the extraction procedure used for these compounds.

5. Conclusion

The reported optimized and validated HPLC-ECD method for the determination of lipoic acid in human plasma was rapid, simple, economical, accurate, sensitive, precise, selective and reproducible. The method was optimized using various chromatographic parameters and validated according to standard guidelines [20]. Various parameters such as mobile phase composition, stationary phase, flow rate, detector voltage, column oven temperature and internal standard were evaluated and selected on the basis of repeated trials. The selected method was also validated on the basis of specificity, sensitivity, linearity, stability, precision, recovery, robustness and system suitability. In comparison with other reported methods [10,15], the present method is rapid (with the short analysis time), precise, accurate and economical. In addition, the method is based on single step liquid–liquid or solid-phase extraction procedures. Plasma concentration of lipoic acid was determined in human volunteers (data not shown). This method can also be used for the investigation of lipoic acid in biological matrices and in pharmacokinetic studies.

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