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ORIGINAL ARTICLE

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

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Abstract This paper describes the development and validation of an HPLC method for the determination of protein bound and total lipoic acid in human plasma. The essential steps in the total lipoic acid assay include reduction of disulfide bridge with tris(2-carboxyethyl)phosphine, derivatization via thiol group with 1-benzyl-2-chloropyridinium bromide and HPLC analysis of S-pyridinium derivative. Protein-bound lipoic acid is first separated from free lipoic acid with the use of liquid extraction, converted to its reduced counterpart then processed as total lipoic acid. The method is reproducible, precise and accurate. The inter- and intraday related standard deviation varied from 1.5% to 11.5% and from 1.8% to 19.6%, respectively, while recovery is in the range of 80.0–106.0% and 80.4–110.8%, respectively. The mean concentration of total lipoic acid in healthy donors after supplementation with 600 mg and 1200 mg was 0.67 ± 0.40 μmol L^{-1} (137.6 ± 82.1 μg L^{-1}) and 1.57 ± 0.34 μmol L^{-1} (323.34 ± 70.07 μg L^{-1}), respectively.

1. Introduction

1,2-Dithiolane-3-pentanoic acid ($C_8H_{14}O_2S_2$), commonly known as lipoic acid (LA), is a direct and an indirect antioxidant used both in the prevention and treatment of diseases related to oxidative stress. During the dynamic transformations taking place in living organisms, LA is metabolized to some catabolites e.g. dihydrolipoic acid (DHLA), lipoamide, methyl lipoate and other (Kataoka, 1998). Moreover, it has been proven that LA forms mixed disulfides not only with GSH, but also with the cysteinyl sulfhydryl in peptides (Ishii et al., 2010). To the best of our knowledge humans are supplemented by LA but not by DHLA. LA is administered for the treat-

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ment of various diseases, such as cardiovascular, diabetes, neurodegenerative and liver disorders (alcohol-induced damage, heavy metal intoxication and mushroom poisoning) (Bilska and Włodek, 2005; Bustamante et al., 1998; Gorąca et al., 2011; Rochette et al., 2013). Furthermore, LA is used as a nutritional supplement in many countries. The use of dietary supplements without proper control raises controversy as they are not drugs, and so do not apply to the rules of drug control. To clarify the function of LA in biochemical and clinical practice, its identification and determination especially in plasma and urine is essential (Borowczyk et al., 2015). However, biological samples contain a large number of individual compounds, what leads to difficulties in determination of the analytes. Therefore, reliable analysis of these samples is a fundamental challenge for researchers. In an ideal case, no sample preparation would be necessary for the proper analysis of a sample, as every manipulation can lead to loss of analyte and hence loss of accuracy. Plasma is recognized as the most commonly analyzed biological fluid. In human plasma, albumin (HSA) is the most abundant protein, that exhibits a set of quite diverse bio-functions, such as: regulation of plasma osmotic pressure, the source of amino acids during starvation time, acting as an antioxidant, as well as binding and transport of endogenous and exogenous compounds (Kawakami et al., 2006). HSA exists in both reduced and oxidized forms. Reduced form of HSA contains free thiol group at Cys^{34}, which is able to form mixed disulfides with low-molecular-mass thiols. Thiols bounding to HSA via disulfide bond are reversible; thus, HSA can act as specific reservoir of plasma thiols (Oettl and Stauber, 2007). Since DHLA possesses thiol groups it can be also efficiently bounded to HSA. For this reason we decided to elaborate the procedure enabling determination of both free and protein bounded LA (ProtS-LA).

High separation capacity of the HPLC makes it the preferred technique for analysis of biological samples. Several HPLC methods which exploit fluorescence (Haj-Yehia et al., 2000; Niebch et al., 1997; Satoh et al., 2007; Witt and Rüstow, 1998), electrochemical (Khan et al., 2011a, 2010; Khan et al., 2011b; Teichert and Preiss, 2002, 1995) and mass spectrometry (Chen et al., 2005; Chng et al., 2010; Ishii et al., 2010; Montero et al., 2012; Trivedi et al., 2004) detection are available for quantification of LA and DHLA in plasma. All mentioned above detection modes exhibit some advantages and have some limitations. Mass spectrometry detector is considered as an universal, highly sensitive and provides additional information concerning the structure of analytes. On the other hand, high purchase and maintenance costs as well as the necessity of handling by highly qualified staff results in low popularity of this kind of detection in clinical laboratories. Moreover, for these reasons renewed interest in the classical HPLC with fluorescence or UV detection as a tool for biological thiols quantification is observed. Fluorescence detectors are well known for their high sensitivity and selectivity. Nevertheless, LA does not exhibit a natural fluorescence; thus, for signal enhancement a derivatization reaction is usually employed. Electrochemical detectors enable direct LA determination but suffer from troublesome service of detection. UV detector is known for its stability, universality and low demand in terms of maintenance. Since molar absorptivity coefficient of LA appears to be low, the derivatization reaction is needed when UV detection is used. To the best of our knowledge, only one paper offers HPLC-UV method for LA determination in plasma (Ezhilarasi et al., 2014). In this report, we describe our efforts aimed at the elaboration of new procedures for the determination of total and protein bounded in plasma by HPLC with ultraviolet detection.

2. Materials and methods

2.1. Chemicals and reagents

1-Benzyl-2-chloropyridinium bromide (BCPB), a derivatization reagent, was synthesized in our laboratory as described earlier (Bald et al., 1996). Chloroform, hydrochloric acid, perchloric acid (PCA), sodium hydroxide and tris(hydroxymethyl) aminomethane (TRIS) were purchased from J.T. Baker (Deventer, the Netherlands). Acetic acid was purchased from Chempur (Piekary Śląskie, Poland). HPLC-grade acetonitrile (MeCN) and methanol (MeOH) were obtained from Labscan (Dublin, Ireland). Lipoic acid (LA), dihydrolipoic acid (DHLA), human serum albumin (HSA), 1-octanol and tris (2-carboxyethyl)phosphine hydrochloride (TCEP) were purchased from Sigma–Aldrich (St. Louis, USA). Stock standard solutions were prepared by dissolving an appropriate amount of LA (final concentration 0.1 mol L^{-1}) in 0.2 mol L^{-1} NaOH, BCPB (0.1 mol L^{-1}) and HSA (50 g L^{-1}) in water, and TCEP (0.25 mol L^{-1}) in TRIS buffer (pH 9). Stock solutions of LA and BCPB were kept at 4 °C for several days without a noticeable change of content. TCEP solution was prepared daily. The working solutions were prepared by dilution with water as needed.

2.2. Sample collection

Blood samples were donated by 17 apparently healthy, ethnically homogenous volunteers dosed with commercially available LA capsules (600 or 1200 mg of LA) for 30 days. During the study, no additional medications were allowed except LA. Blood (2 mL) was drawn, with a tourniquet applied, into a tube containing EDTA in the morning 1 day before the first LA dose and 1 day after last LA dose. Blood was centrifuged under standard conditions (10 min, 1000 g, within < 20 min after collection) and plasma was used for the determination of LA. Informed consent was obtained from all volunteers, and this study was approved by the Bioethics Committee of Regional Chamber of Physicians in Krakow (127/KBL/OIL) and University of Łódź (22/KBBN-UŁ/II/2015). Plasma samples were stored at −78 °C until analysis. On the day of analysis, samples were thawed and processed as described below.

2.3. Preparation of Albumin-Cys^{34}-S-LA

HSA (50 g L^{-1}) dissolved in 0.1 mol L^{-1} phosphate buffer pH 7.4 was converted to albumin-Cys^{34}-S-LA (HSA-LA) by incubation with 2-fold molar excess of DHLA or LA at 36.6 °C for 24 h. Excess LA was removed from HSA-LA by a quintuple extraction with chloroform as described in Section 2.5.2.

2.4. Separation of protein and LA

Portions of 50 μL of water or 50 g L^{-1} HSA were each placed in eppendorf tube and spiked with 10 μL of standard solutions with the increasing amount of LA to provide a concentration in the range 0.1–20 μmol L^{-1}. Next, the mixtures were acidified with 7.5 μL of 3 mol L^{-1} PCA, vortex-mixed and centrifuged at 12,000*g* for 5 min. The supernatant was transferred to new tubes and alkalized to pH 7 by the use of 2.5 mol L^{-1} NaOH, 25 μL of 0.5 mol L^{-1} TRIS buffer (pH 9) and 5 μL of 0.25 mol L^{-1} TCEP were added and vortex-mixed. After 15 min, 10 μL of 0.1 mol L^{-1} BCPB was added and the mixture was vigorously shaken by hand for 10 s. Next,

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the sample was kept at room temperature for 15 min and was acidified with 15 μL of 3 mol L^{-1} PCA. The mixture was then transferred to an autosampler vial and injected (5 μL) into the HPLC system.

A 50 μL of water or 50 g L^{-1} HSA was spiked with standard solution of LA (final concentration 10 μmol L^{-1}). Next, 25 μL of 0.5 mol L^{-1} TRIS buffer (pH 9) and 5 μL of 0.25 mol L^{-1} TCEP were added, vortex-mixed and allowed to stand for 15 min at room temperature. Next, 10 μL of 0.1 mol L^{-1} BCPB was added, vigorously mixed, held on 15 min and acidified with 15 μL of 3 mol L^{-1} PCA. At the end, different volume of MeCN (0–90 μL) or MeOH (0–80 μL) were added, vortex-mixed and put aside for 15 min, followed by centrifugation (5 min, 12,000*g*). Supernatant was transferred to an autosampler vial and injected (5 μL) into the HPLC system.

2.5. Recommended analytical procedure

2.5.1. Determination of total LA

Plasma samples (50 μL) were mixed with 25 μL of 0.5 mol L^{-1} TRIS buffer (pH 9) and 5 μL of 0.25 mol L^{-1} TCEP. After 15 min of reduction, 10 μL of 0.1 mol L^{-1} BCPB was added, then sample was vortex-mixed and kept at room temperature for 15 min (derivatization reaction). Next, to attain a separation of LA derivative from protein, 15 μL of 3 mol L^{-1} PCA and 70 μL of MeCN were added, vortex mixed and put aside for 15 min, followed by centrifugation (5 min, 12,000*g*). Supernatant was transferred to an autosampler vial and injected (5 μL) into the HPLC system.

2.5.2. Determination of protein bound LA

Plasma samples (50 μL) were treated with 5 μL of 3 mol L^{-1} PCA and 100 μL of chloroform and vigorously vortex-mixed for 10 min. After extraction, the mixture was centrifuged (5 min, 12,000*g*) and organic as well as water layers was decanted. The proteins were resuspended with 50 μL of 0.5 mol L^{-1} TRIS buffer (pH 9) and processed according to the procedure described in Section 2.5.1.

2.6. Chromatographic analysis

The analyses were performed using a 1220 Infinity LC Agilent system consisted of a binary pump integrated with degasser, autosampler, column oven and diode-array detector. The whole HPLC system was controlled by OpenLAB CDS ChemStation Edition software. All separations were achieved through a Zorbax C-18 (150 × 4.6 mm, 5 μm) analytical column (Agilent Technologies). The mobile phase consisted of 2% acetic acid solution, pH 2.36 (component A) and MeCN (component B). For the determination of LA in plasma, gradient elution was used: 0–5 min, 10–40% B; 5–6 min, 40–10% B; 6–8 min, 10% B. Flow rate of mobile phase was 1 mL min^{-1} at 25 °C. The peak of 2-S-pyridinium derivative of LA was monitored at 321 nm. LA-BCPB peak was identified by comparison of its retention time as well as diode-array spectra, taken at real time of analysis, with that of the authentic standard.

2.7. Validation study

2.7.1. Selectivity

Six different LA-free plasma samples, obtained from apparently healthy donors were used to evaluate the selectivity of the method. This was done by investigating the potential interferences at the chromatographic peak region for LA derivative using the recommended procedure and chromatographic conditions described in Sections 2.5.1 and 2.6, respectively. Moreover, the test of peak purity by diode-array detector was performed.

2.7.2. Intra-day and inter-day variation

The intra-day accuracy and precision were estimated by analyzing five plasma samples spiked with LA at three different levels 0.1, 1 and 20 μmol L^{-1}. The inter-day precision and accuracy were evaluated on five consecutive days in a week at the same LA levels in plasma. The spiked plasma samples were prepared according to a recommended procedure described in Section 2.5.1. The results were expressed as the reproducibility of the recovered amount and determined concentration as the mean ± SD and the RSD% calculated from the data obtained.

2.7.3. Calibration

Total LA: To prepare the calibration standards used to determine LA in human plasma, portions of 50 μL of blank plasma were each placed in eppendorf tube and spiked with 10 μL of standard solutions with the increasing amount of LA to provide a concentration of 0.1, 0.5, 1, 2, 5, 10 and 20 μmol L^{-1} in plasma. The calibration standards were prepared in triplicate according to recommended procedure described in Section 2.5.1.

Protein bound LA: To prepare the calibration standards used to determine ProtS-LA in human plasma, portions of 50 μL of blank plasma were each placed in eppendorf tube and spiked with the increasing amount of HSA-LA to provide ProtS-LA concentration of 0.5, 1, 1.5, 2, 2.5 and 3 μmol L^{-1} plasma. The calibration standards were prepared in triplicate according to recommended procedure described in Section 2.5.2.

The calibration curves were obtained by least-square linear regression analysis of relationship between peak areas of the LA derivative and LA concentrations. The slope of the calibration curve was used to calculate the LA concentrations in plasma samples.

2.7.4. Limit of detection and quantification

To investigate the lower limits of detection (LOD) and quantification (LOQ) LA-free blank plasma samples were spiked with a decreasing concentration of the standard solution of LA and subsequently subjected to all steps of the analytical procedure. The study was repeated until the signal-to-noise ratio reached 3:1 and 6:1 for LOD and LOQ, respectively.

2.7.5. Stability experiment

Short-term stability of 2-S-pyridinium derivative of LA in the final analytical solution was tested. Human plasma was spiked

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with LA (final concentration 20 μmol L^{-1}) and immediately processed according to the procedure described in Section 2.5.1. Acidified final analytical solution containing MeCN was stored in the autosampler at room temperature and injected into the HPLC system without delay and consecutively after 1.5, 3, 4, 7, 10, 13, 16 and 19 h.

Short- and long-term stability of LA in plasma was also evaluated. Human plasma was spiked with LA (final concentration 20 μmol L^{-1}), split into four portions and kept at 24, 4, −20 and −78 °C. 50 μL of plasma was assayed according to procedure described in Section 2.5.1 at time zero and in successive intervals.

3. Results and discussion

Utility of BCPB has been proven during determination of low molecular aminothiols in biological samples (Bald et al., 1996; Chwatko, 2013; Chwatko et al., 2014; Kuśmierek and Bald, 2009a, 2009b; Sypniewski and Bald, 1996). It has been also used as an effective derivatization reagent for LA determination in human urine (Chwatko et al., 2014). This method is based on the reduction of LA to DHLA, conversion of DHLA to UV-absorbing BCPB derivative and quantification by HPLC. The aim of present work was to extend the above derivatization scheme for the determination of total and protein-bound LA (ProtS-LA) in human plasma. Importantly, dedicated to urine analytical scheme cannot be directly applied for the determination of LA in plasma samples. The main problem has concerned physicochemical properties of LA and the presence of proteins in the sample. Therefore, individual conditions for each step of the analytical procedure were optimized.

3.1. Sample preparation

Sample preparation influences nearly all of the later assay steps and for this reason it is critical for unequivocal identification and quantification of the analytes. DHLA reacts with BCPB (Fig. 1) within 15 min in water solution (pH 9) to form a stable thioether, 2-S-pyridinium derivative (LA-BCPB). This stable derivative has a well-defined maximum at 321 nm. To measure the total LA (the sum of DHLA, LA and ProtS-LA) it is necessary to cleave disulfide bonds prior to derivatization. Such approach makes free sulfhydryl groups accessible to BCPB. For this reason, we used TCEP in TRIS buffer at pH 9, at room temperature for 15 min. Our results show that both reduction and derivatization require longer times for plasma samples than for urine (Chwatko et al., 2014). A crucial step in plasma sample preparation is LA and DHLA separation from protein. LA and DHLA are the amphiphilic molecules having both hydrophilic and hydrophobic fragments and can specifically interact with the surface of proteins. Several methods, such as liquid-liquid extraction (LLE) (Chng et al., 2010; Ezhilarasi et al., 2014; Haj-Yehia et al., 2000; Khan et al., 2011a, 2010, 2011b; Montero et al., 2012; Niebch et al., 1997; Satoh et al., 2007; Trivedi et al., 2004; Witt and Rüstow, 1998), solid phase extraction (Khan et al., 2010; Teichert and Preiss, 2002, 1995) and deproteinization (Chen et al., 2005; Chng et al., 2010; Khan et al., 2011b) can be utilized for removal of LA from protein. In our study, we tested deproteinization procedure and LLE for determination of total LA and ProtS-LA, respectively.

During acidic deproteinization, plasma proteins markedly adsorb LA; consequently, the concentration of LA in the solution is lower than expected. In our study, HSA solutions containing LA in the range 0.5–30 μmol L^{-1} were deproteinized with PCA and assayed for LA content. We observed a lower LA concentration in final analytical solutions for HSA samples compared to standard solution (Fig. 2A). Recovery of free LA was calculated with the use of the following formula:

$$\text{Recovery } (\%) = \text{LA amount in HSA sample} / \text{LA amount in standard solution} \times 100\%.$$

The recovery was found to be between 32.4% and 38.9%, meaning that more than 60% of free LA adsorbed on HSA. Similar problem concerned separation of proteins and BCPB derivative of LA. Our experiments have proven that addition of MeCN or MeOH to PCA significantly increases LA-BCPB recovery (Fig. 2B). Moreover, better results were obtained when the mixture of PCA and MeCN was used. In this case, the recovery reaches 100% while application of MeOH and PCA mixture afforded only 76%. Our results are comparable with those obtained by other investigators for the mixture of MeCN and metaphosphoric acid (recovery, 98%) (Khan et al., 2011b). Finally, for deproteinization of plasma samples for determination of total LA the mixture of PCA and MeCN was used.

Since LA shares structural similarity to the medium chain fatty acids e.g. octanoic acid it is preferably bounded by sit II in albumin (Atukeren et al., 2010; Kawakami et al., 2006). An accurate separation of free LA from proteins is essential for determination of ProtS-LA in plasma samples because incomplete removal of free LA causes overestimation of ProtS-LA. Thus, LLE with chloroform, dichloromethane, diethyl ether, ethyl acetate or 1-octanol was considered. As described earlier, dichloromethane, diethyl ether and ethyl acetate were widely used for LA extraction from plasma. Unfortunately, one can interpret provided results as inconsistent due to the extraction recoveries in case of dichloromethane, diethyl ether and ethyl acetate being in the range of 87.0–98.4%, 75.0–80.3% and 58.1–90.3%, respectively (Haj-Yehia et al., 2000; Montero et al., 2012; Khan et al., 2011b, 2011a, 2010; Trivedi et al., 2004; Witt and Rüstow, 1998). Additionally, when chloroform/ethanol mixture was used for LA extraction from plasma samples better recovery of the analyte was obtained as compared to the other solvents used (Montero et al., 2012). Then, we have decided to test chloroform and 1-octanol as the extraction solvents. Our results show that both solvents completely extracted free LA from acidified HSA and plasma samples. Additionally, after extraction of HSA containing free LA (10 μmol L^{-1}) the LA-BCBP peak in protein fraction was not observed. Due to the better stability of proteins as well as better separation from the liquid phases for further experiments chloroform was used.

3.2. Chromatography

Because of unique separation parameters of HPLC technique, it has frequently been used in the analysis of biological samples. In order to provide a good performance of the assay

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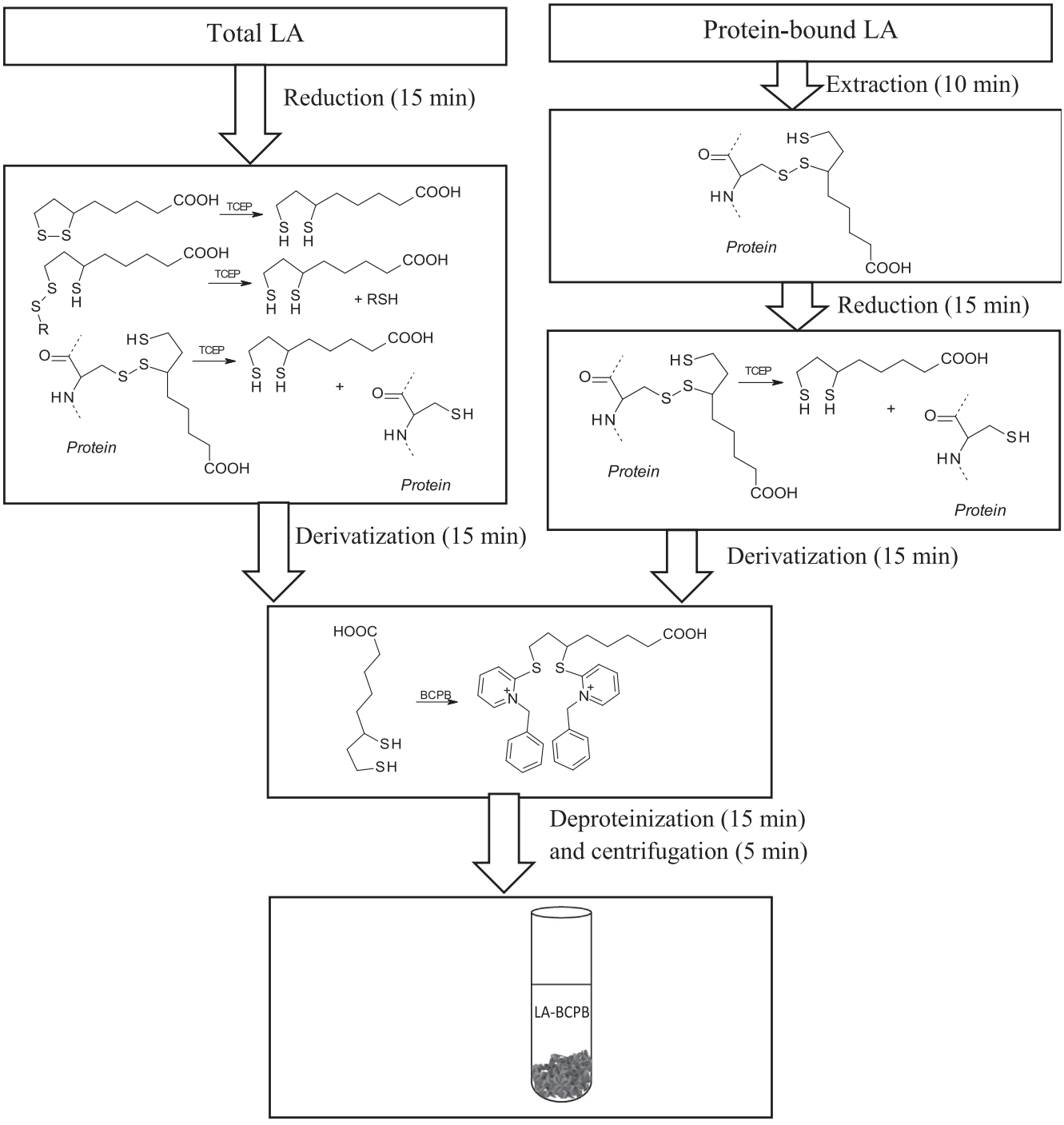

Figure 1 Determination strategy for estimation of total and protein-bound LA in plasma.

the chromatographic conditions were optimized in terms of mobile phase MeCN and acetic acid concentration as well as column temperature. As expected, an increase in MeCN content in the mobile phase from 20 to 30% resulted in decrease of retention factor from 8 to 1 and simultaneous increase in peak height from 6 to 32 mAU. The effect of acetic acid concentration was tested in a range of 0.5–3%. The retention factor of LA-BCBP slightly decreased (1.4–1) while peak height increased (16–40 mAU) with increase of acetic acid concentration. Temperature of the column in the range of 15–40 °C slightly influenced retention parameters. Based on the results described above, we have chosen chromatographic conditions specified in Section 2.6. Chromatographic profiles obtained for the standard, blank and spiked protein fraction as well as total LA after supplementation are depicted in Fig. 3. As can be seen endogenous components do not significantly interfere with LA-BCBP peak. Under these conditions LA-BCPB is eluted after 5.49 ± 0.01 min (RSD = 0.18%, n = 10).

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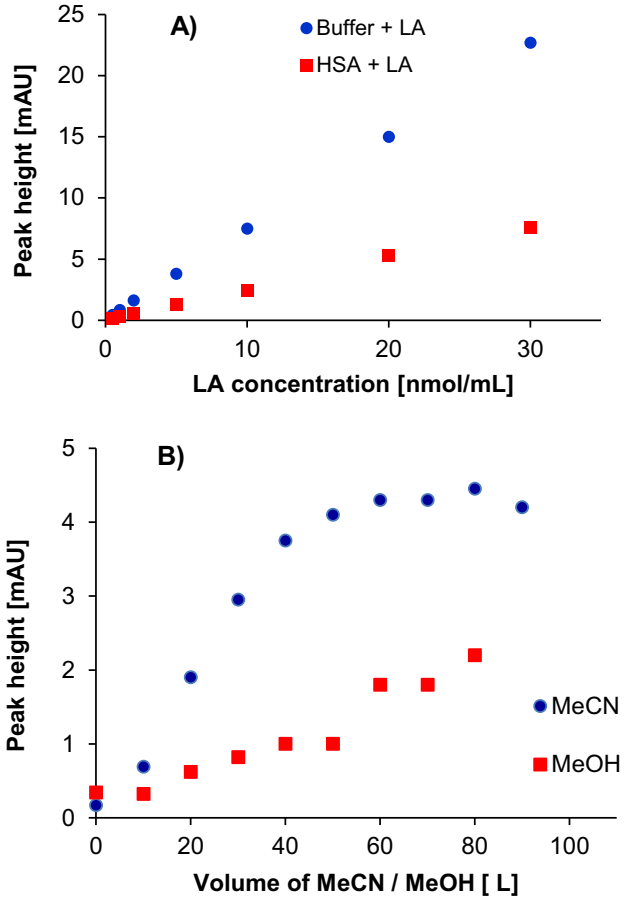

Figure 2 Influence of different conditions to separation of LA and LA-BCPB from protein. (A) Acid deproteinization of HSA containing LA in the range 0.5–20 μmol L^{-1}; (B) The relationship between MeCN/MeOH volume and separation of LA-BCPB from protein.

3.3. Validation study

The method was validated for selectivity, precision, recovery, linearity, limit of detection and quantitation, and stability according to the guidelines for analytical methods (Ravichandran et al., 2010; Wille et al., 2011).

The selectivity of our method was demonstrated by providing the absence of interfering peaks with a signal obtained for the analyte. For this purpose, six various blank plasma samples and plasma spiked with LA were analyzed and the chromatograms showed sufficient separation of LA-BCPB from the matrix components. Furthermore, examination of peak purity showed that LA-BCBP peak was not attributable to more than one component.

The accuracy of an analytical method may be defined as the closeness of the measured value obtained by the method to the true value, whereas the precision as the degree of agreement among individual test results obtained by replicate measurements (Ravichandran et al., 2010; Wille et al., 2011). The precision is expressed as the relative standard deviation (RSD), while the accuracy as the percentage of analyte recovery and calculated by expressing the mean measured amount as a percentage of the added amount. In our study three concentra-

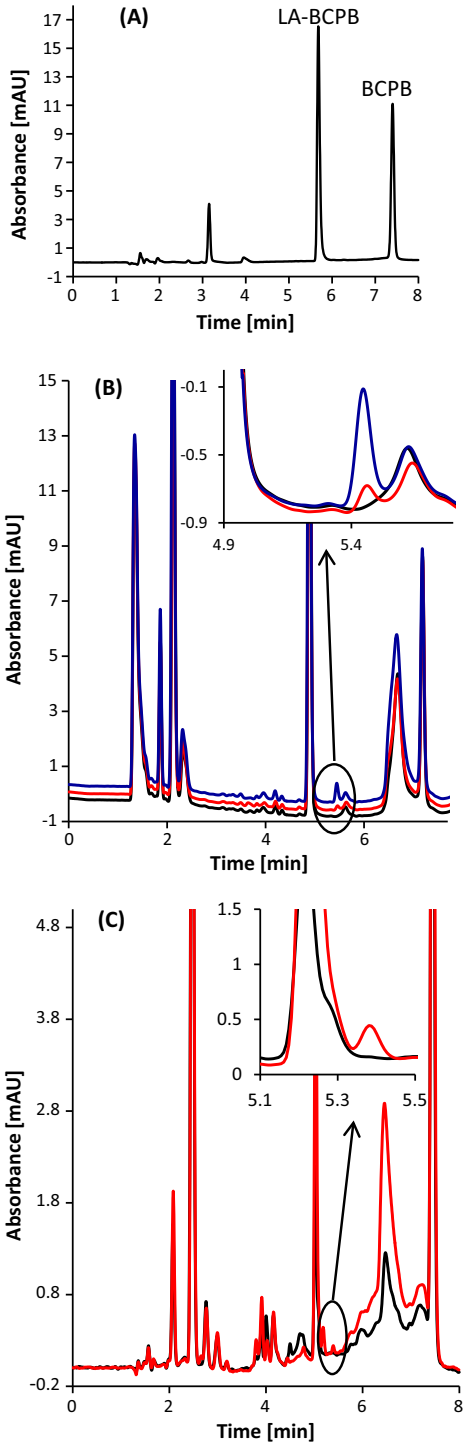

Figure 3 Representative chromatograms of water standard solution and plasma samples. (A) Standard water solution of LA, concentration 15 μmol L^{-1}. (B) Profile of plasma obtained from volunteer before (black line) and after oral administration of 600 mg (red line) and 1200 mg (blue line) of LA with total LA amounting 0.0 μmol L^{-1}, 0.53 μmol L^{-1} (108.3 μg L^{-1}) and 2.08 μmol L^{-1} (430.0 μg L^{-1}), respectively. (C) Profile of blank (black line) and spiked (red line) with 3 μmol L^{-1} ProtS-LA plasma samples. Chromatographic conditions are as described in the text.

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tions of LA were tested: one at the LOQ, one near the expected amount of analyte, and one near the upper boundary of the standard curve. The intra- and inter-day recovery and precision values were calculated and are shown in Table 1. The criteria for acceptability of the data included accuracy within ±15% from the nominal values and a precision within ±15% RSD, except for LOQ, where it should not exceed ±20% for accuracy as well as precision (Wille et al., 2011). Obtained in our study values fulfill these requirements.

The relationship between detector response and LA and ProtS-LA concentrations was continuous and repeatable, and was demonstrated using a seven-point and six-point calibration curves, respectively. The calibration curves were linear in the tested range from 0.1 to 20 μmol L^{-1} and from 0.5 to 3 μmol L^{-1} for total LA and ProtS-LA, respectively. The equations for the linear regression line were $y = 1.295x + 0.0726$ for total LA and $y = 0.330x - 0.018$ for ProtS-LA; outliers were not excluded. The coefficients of correlation for the calibration regression were 0.9998 and 0.9901 for total LA and ProtS-LA, respectively.

The LOD and LOQ for total LA and ProtS-LA were experimentally established to be 0.05 and 0.1 μmol L^{-1} for total LA and 0.2 and 0.5 μmol L^{-1} for ProtS-LA, respectively. The analyte response at LOQ level fulfills the criteria for precision and accuracy (Wille et al., 2011), i.e. precision of 20% and accuracy of 80–120%. The LOQ and/or LOD for total LA determination by this method compare favorably with others' recently reported values which were obtained by the HPLC method with UV detection (Ezhilarasi et al., 2014) and match with the HPLC method with fluorescence detection (Niebch et al., 1997). Other HPLC methods for LA determination in plasma based on fluorescence (Haj-Yehia et al., 2000; Satoh et al., 2007; Witt and Rüstow, 1998), mass spectrometry (Chen et al., 2005; Trivedi et al., 2004) and electrochemical (Khan et al., 2011a, 2010, 2011b; Teichert and Preiss, 2002, 1995) detection have better LOQ and/or LOD than our method.

Table 1 Evaluation of the intra-day and inter-day precision and accuracy for total LA and ProtS-LA in plasma obtained by the proposed method, $n = 5$.

LA added (μmol L^{-1})	Intra-day			Inter-day		
	LA found ± SD (μmol L^{-1})	RSD (%)	Recovery (%)	LA found ± SD (μmol L^{-1})	RSD (%)	Recovery (%)
Total LA						
0.1	0.11 ± 0.02	19.6	108.9	0.09 ± 0.01	10.5	91.7
1.0	1.02 ± 0.04	3.4	102.3	0.93 ± 0.06	6.7	92.9
20	22.2 ± 0.40	1.8	110.8	20.8 ± 0.31	1.5	103.9
ProtS-LA						
0.5	0.40 ± 0.05	12.9	80.4	0.40 ± 0.05	11.5	80.0
1.5	1.58 ± 0.25	15.6	105.3	1.64 ± 0.25	15.3	109.4
3.0	3.13 ± 0.38	12.1	104.3	3.18 ± 0.23	7.4	106.0

Table 2 Concentrations of total LA in human plasma before and after supplementation with LA.

Gender	Age (year)	LA concentration in μmol L^{-1} (μg L^{-1})		
		Before supplementation	After supplementation	
			600 mg	1200 mg[b]
F	39	n.d.	0.16 (33.9)	1.36 (281.0)
M	41	0.08[a]	0.66 (135.4)	1.26 (260.7)
F	43	n.d.	0.53 (108.3)	2.08 (430.0)
F	40	n.d.	1.08 (223.5)	1.28 (264.1)
F	28	n.d.	1.35 (277.6)	1.54 (318.3)
F	55	n.d.	1.39 (287.8)	1.87 (386.0)
F	43	n.d.	0.18 (36.9)	
M	47	0.015[a]	0.27 (56.5)	
F	39	0.015[a]	0.26 (54.5)	
F	28	n.d.	0.57 (118.5)	
F	39	n.d.	0.32 (65.0)	
M	63	n.d.	0.39 (81.3)	
M	57	n.d.	0.51 (105.0)	
M	30	n.d.	0.82 (169.3)	
M	53	n.d.	0.82 (169.3)	
M	50	n.d.	0.89 (182.8)	
F	62	n.d.	1.13 (233.6)	

n.d., not detected.
[a] Concentration below LOQ.
[b] Supplementation with 1200 mg of LA processed one year later.

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The stability of LA *ex vivo* at 24, 4, −20 and −78 °C in plasma was tested. Under these temperatures LA in plasma samples was found to be stable for at least 2, 10 days, 26 weeks and 6 months, respectively. The residual percentages of LA after mentioned periods of time were 96.5%, 102.5%, 98.7% and 103.0%, respectively and indicating no stability problems. Our stability results correspond to those obtained earlier at 24 (Khan et al., 2011b), −20 (Khan et al., 2011a; Chen et al., 2005) and −80 °C (Trivedi et al., 2004) and are inconsistent with the study provided by Khan et al. (2010) which demonstrated that spiked plasma samples were stable for only 1 week at −80 °C. Other examinations have shown that exogenous LA in plasma samples is stable at −70 °C for 6 months (Teichert and Preiss, 1995). LA-BCPB in final analytical solution containing MeCN was found to be stable at room temperature for 4 h as the concentration of analyte was found to be 103.6% of the initial concentration.

3.4. Application to authentic plasma samples

The optimized procedure for the determination of total LA and ProtS-LA was applied to the analysis of plasma samples of apparently healthy volunteers, 28–63 years old (7 men and 10 women), before and after supplementation. In 14 of the 17 plasma samples collected before taking the drug the LA levels were below the LOD and in 3 samples below the LOQ. After oral administration of 600 and 1200 mg of LA, its concentration in plasma was in the range 33.9–287.8 and 260.7–430.0 $\mu g\ L^{-1}$ plasma, respectively (Table 2). Furthermore, in all plasma samples ProtS-LA was not observed.

4. Conclusion

We reported the new HPLC based methodology for determination of total LA and ProtS-LA in plasma. Since absorption of UV radiation by LA is low, it was derivatized with BCPB following the reductive cleavage of the —S—S— bond with TCEP. Use of BCPB as derivatization reagent enhances the detectability because formed S-pyridinium derivative exhibits relatively high molar absorptivity coefficient ($\varepsilon = 1 \times 10^4\ L \times mol^{-1}\ cm^{-1}$). Analytical figures of merit, demonstrated during the method validation procedure, justify a conclusion that present procedure is reliable and robust. It should be emphasized that established sample preparation procedure allows effective differentiation of ProtS-LA from free LA. Importantly, this solution can be utilized in other HPLC assays including those coupled with mass spectrometry or fluorescence detection. Developed method has also several other advantages over previously published assays. To the best of our knowledge there is only one other HPLC UV method allowing LA determination in human plasma (Ezhilarasi et al., 2014) while our method has better LOQ and LOD. An additional advantage is the short chromatographic run time and employment of small amount of reagents (eco-friendly method). In conclusion, the method is suitable for clinical studies and controlling the use of LA supplements.

Author contribution statement

G.C. supervised all experiments and prepared the manuscript, M.K. did sample preparation and HPLC analysis, M.I. did statistical analysis of the data, A.K. involved preparation of standards and HPLC analysis, A.B-W. did collection of samples, B.M. did setting up of the clinical experiment, and R. G. gave contribution in the manuscript preparation.

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