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USE OF HPLC/ED TO MONITORING SOME CLINICALLY IMPORTANT SULPHUR COMPOUNDS

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Supervisor: **prof. Ing. Karel Ventura, CSc.**

References

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University of Pardubice, Faculty of Chemical Technology, Department of Analytical Chemistry. Supervisor: prof. Ing. Karel Ventura, CSc.

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. The aim was to verify the levels of free LA in the plasma of 40 individuals (non-supplemented with LA). We measured the levels of LA using a high-performance liquid chromatography (HPLC) method with coulometric detection. Our limit detection (LOD) was 1.85 nmol/L and was better than the LODs in other studies. The levels of free LA in plasma of voluntary blood donors were not detectable in all cases, the presented results show that endogenous concentrations of LA are less than 1.85 nmol/L. This method is suitable for further studies in order to determine LA levels in patients after supplementation.

The effect of LA on the viability of erythrocytes was monitored. For the determination of the cell viability the Trypan blue exclusion test was used. The high concentrations of LA had a statistically significant effect on the number of erythrocytes already after the 24-hour incubation time.

The next aim was to verify the importance of homocysteine as a biomarker in clinical practice. Moreover, the aim is to obtain a general overview about the whole methionine metabolism for patients, suffering from coronary artery disease at different degrees of severity, as well as to verify the immediate effect of a percutaneous coronary intervention on the methionine level and its metabolites. We measured the total plasma level of homocysteine, cysteine, methionine and cysteinyl-glycine using HPLC with coulometric detection.

Abstrakt

Kyselina lipoová (LA) hraje významnou úlohu při léčbě mnoha onemocnění. Existuje mnoho studií, které se zabývaly stanovením exogenních hladin LA po suplementaci, ale informace o endogenních hladinách jsou stále rozporuplné. Cílem této práce bylo vyvinout vhodnou metodu pro stanovení hladin volné LA. Jedná se o vysokoúčinnou kapalinovou chromatografii s elektrochemickou detekcí (HPLC). Vzorky krve byly získány od 40 nesuplementovaných dobrovolných dárců. Limit detekce této metody byl 1,85 nmol/L a byl nižší ve srovnání s ostatními studiemi. Nicméně i přesto nebyly

hladiny LA v plasmě u všech 40 dobrovolných dárců detekovány. Výsledky naší studie ukazují, že endogenní koncentrace volné LA v plasmě jsou pravděpodobně nižší než 1,85 nmol/L. Tato metoda je vhodná pro studie zabývající se stanovením hladin LA u suplementovaných pacientů.

Dále byl sledován vliv LA na životaschopnost (viabilitu) erytrocytů. Ke stanovení viability buněk byla použita metoda vylučování trypanové modři. Na počet erytrocytů měly vysoké koncentrace statisticky významný vliv již po 24 hodinové inkubaci.

Dalším cílem bylo ověřit význam homocysteinu jako biomarkeru v klinické praxi, získat obecný přehled o celkovém metabolismu methioninu u pacientů s onemocněním koronárních artérií a ověřit okamžitý vliv perkutánní koronární intervence na hladiny methioninu a jeho metabolitů. Hladiny vybraných aminothiolů v plasmě byly stanoveny metodou HPLC s coulometrickou detekcí.

Keywords: cytotoxicity; electrochemical detection; erythrocytes; homocysteine; lipoic acid; coronary artery disease; percutaneous coronary intervention; plasma; high-performance liquid chromatography

Klíčová slova: cytotoxicita; elektrochemická detekce; erytrocyty; homocystein; kyselina lipoová; onemocnění koronárních artérií; perkutánní koronární intervence; plasma; vysokoúčinná kapalinová chromatografie

CONTENTS

LIST OF ABBREVIATIONS

AI	Index of atherogenity
BMI	Body Mass Index
CAD	coronary artery disease
Cys	cysteine
DES	drug-eluting stent
EDTA	ethylenediamine tetra-acetic acid
HCl	hydrochloric acid
Hcy	homocysteine
HDL-C	high-density lipoprotein cholesterol
HPLC	high-performance liquid chromatography
LA	lipoic acid
LDL-C	low-density lipoprotein cholesterol
LOD	limit of detection
LOQ	limit of quantification
Met	methionine
NS	not significant
PCI	percutaneous coronary intervention
RV	re-vasculization
SPE	solid-phase extraction
SSA	5-sulfosalicylic acid
TC	Total cholesterol
TG	triglycerides

INTRODUCTION

Atherosclerosis is a narrowing of the arteries caused by the buildup of plaque. This disease silently and slowly blocks arteries, putting blood flow at risk. It is the usual cause of heart attack, strokes, and peripheral vascular disease. Antioxidant stress plays an important role in the pathogenesis of cardiovascular diseases. Lipoic acid is a powerful antioxidant found naturally in diets. This antioxidant retains its properties in both forms and takes an important position in the antioxidant network. In addition, lipoic acid is known as a scavenger of free radicals such as hydroxyl radical, singlet oxygen or peroxynitrite. Lipoic acid may also possess a lipid lowering effect indicated with low plasma total cholesterol and low-density lipoprotein cholesterol levels and reduced athero-lesion formation. By current knowledge it seems to be perspective to use to drug-eluting stent as an antioxidant in patients with the coronary artery disease. On the other hand, lipoic acid has also oxidant activity and can induce apoptosis in a variety of cells including erythrocytes. Owing due to its proapoptotic potency lipoic acid has been suggested for the cancer therapy. Therefore, this is the question of the safety and toxicity of the lipoic acid to human organism.

In recent years, the importance of aminothiols in the cardiovascular disease has been discussed. Homocysteine is a sulphur amino acid derived from essential amino acid methionine. Cysteine, structurally like to homocysteine, seems to be a risk factor for coronary artery disease according to some authors, but other studies provided contrary results. A lot of studies about homocysteine have been published recently. It has been shown, that homocysteine is an independent risk factor for cardiovascular disease. However, information concerning homocysteine as a biomarker remains controversial. Although the majority of available literature shows statistically significant differences in the total plasma homocysteine between coronarographic negative or positive subjects, some authors published no or very closed significance. The relation between the degree of severity proved coronary artery disease and total plasma homocysteine also remains unambiguous. Firstly, the severity of this disease may be investigate by different methods. Additionally, a lot of information was published about homocysteine, but this association was discussed only in several studies during last ten year.

AIMS

The aims of this thesis are:

- the development of a suitable method for the determination of the endogenous levels of lipoic acid
- the verification of a possibility to use the method for determining the levels of lipoic acid after supplementation
- the determination of the cytotoxicity of lipoic acid to erythrocytes
- to verify the importance of homocysteine as a biomarker in clinical practice
- to obtain a general overview about the whole methionine metabolism for patients suffering from coronary artery disease at different degrees of severity
- to verify the immediate effect of a percutaneous coronary intervention on the methionine level and its metabolites

Acknowledgement

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EXPERIMENTALPART

1 HPLC determination of endogenous lipoic acid in human plasma

We measured the levels of free LA using an HPLC method with very sensitive coulometric detection after previous sample preparation including deproteination and solid phase extraction.

1.1 Blood collection

Three milliliters of venous blood were 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.

Non-supplemented blood donors:
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 kilograms and more) and a good state of health. Exclusion criteria are stated in Table 1. Informed consent in writing was obtained from all individuals.

Supplemented blood donors:
Plasma samples were collected from 3 voluntary blood donors (2 women, 1 man). The average age was 35±8 years. This group was supplemented with LA (500 mg per tablet; 1 hour after consumption).

1.2 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.

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.

1.3 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.

1.4 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.

2 The determination the cytotoxicity of lipoic acid to erythrocytes

The effect of LA on the viability of erythrocytes was monitored**.** Plasma samples were collected from voluntary blood donors, non-supplemented with LA. The venous blood was centrifuged at 750 *x* g; the separated erythrocytes were washed three times with phosphate buffer, diluted with Ringer's solution, and injected into 12-well culture plates. Lipoic acid solutions, which had previously been diluted in different rations, were added to the erythrocyte suspension. The incubation time was 24 and 48 hours at 37°C. For the determination of the cell viability the Trypan blue exclusion test was used.

3 HPLC determination of total plasma homocysteine, cysteine, methionine and cysteinyl-glycine

We measured the total plasma level of homocysteine (Hcy), cysteine (Cys), methionine (Met) and cysteinyl-glycine using the HPLC method with very sensitive coulometric detection.

3.1 Subjects

Sixty-eight patients (41 men and 27 women) with proved coronary artery disease (CAD) were included in study A. These patients had acute CAD (acute myocardial infarction with ST elevation and 6 without ST elevation, unstable angina pectoris), chronic CAD (stable angina pectoris) with progressive course or were hospitalized for re-vasculization (RV) completion after acute CAD survived recently. A lot of patients with acute or chronic CAD were forced to undergo percutaneous coronary intervention (PCI) immediately after coronary angiography. Twenty-nine patients had acute form of CAD. There were 22 patients in the group with chronic CAD and 17 patients in the group with RV

completion. The study B includes 44 patients (35 men and 9 women) who were appropriated from study A. These patients underwent a coronary angiography with a subsequent PCI. The samples were collected before PCI, control samples were collected 24 and 48 hours after PCI.

Patients in all groups were receiving aspirin, calcium antagonists, beta-blockers, angiotensin converting enzyme inhibitors, nitrates, per-oral antidiabetics. The study protocol was approved by the Human Ethics Committee of the Regional Hospital of Pardubice (13th December 2013). Informed consent was obtained from all subjects in writing.

3.2 Blood collection

Venous blood was collected into a Vacutainer Tube containing EDTA as anticoagulant. Blood samples were centrifuged at 1 600 *x* g for 10 min at 4 °C and the aliquots were stored at –80 °C until preparation for analysis. The time between blood sampling and freezing of the plasma was no more than 60 minutes.

3.3 HPLC conditions

We have measured a total plasma level of Hcy, Cys, Met and cysteinyl-glycine using HPLC method with coulometric detection. Briefly, measurement of plasma total thiols involves reduction and detection of generated thiols. The system consisted of a pump (LC-10AD vp, Shimadzu, Kyoto, Japan), a Rheodyne injector (Model 9725i, Rheodyne, L.P., Rohnert Pack, CA, USA) with a 20 μL loop and a 80 *x* 4.6 mm RP-C18 column (HR-80, particle size, 3 μm; ESA, Inc). The mobile phase consisted of sodium dihydrogen phosphate, octane sulfonate and acetonitrile. We used Coulochem III electrochemical detector (ESA) including Model 5010A analytical cell for monitoring thiols in eluate. The software program Clarity (DataApex, Prague, Czech Republic) was used for data integration. Total cholesterol, triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), albumin and glucose were analysed using routine methods in laboratories of Department of Clinical Biochemistry of Hospital Pardubice region, Pardubice Hospital.

RESULT AND DISCUSSION

Determination of endogenous lipoic acid in human plasma

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.* gave 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).

The aim of this work was to verify the levels of free LA in the plasma of 40 individuals (non-supplemented with LA). We measured the levels of LA

using an HPLC method with coulometric detection. The mobile phase was optimized in order to obtain the best separation of LA in the shortest time. Standard solution of LA and plasma were used for studying the mobile phase composition. Several mobile phases (different buffers containing acetonitrile or methanol) were assessed. The separation was optimized after studying the effect of the concentration of NaH_2PO_4 (from 5 to 100 mmol/L) and acetonitrile concentration. The retention behavior was studied with respect to the pH value of the mobile phase in the range of 2.0-5.0. The optimal pH 2.8 was chosen for the best separation and detection of LA. The criteria used were the resolution, electrode sensitivity, baseline stability and the analysis speed. The hydrodynamic voltammogram analysis was performed to optimize conditions for the accurate determination of LA. It was carried out by injection of 10 μL solution of LA (1 μmol/L) and measuring the current produced by LA at the electrodes; the voltammogram indicated that the highest response for LA was observed at 590 mV. The flow rate of mobile phase was tested in the range of 1-2 mL/min. The optimum flow rate was 1 mL/min. Due to the expected low endogenous levels of LA, we had to maximize the sensitivity of the electrochemical detector. The optimal sensitivity for which we were able to stabilize the baseline was 20 nA. A higher sensitivity caused the signal to be affected by the mobile phase.

After about 50 injections, the electrode potentials were set to -1000 mV at each electrode for 15 min with the mobile phase at a flow rate of 1 mL/min, followed by 1-24-hours acetonitrile/water (1:1, v/v) rinse and by 30-min 2-propanol/methanol (1:4, v/v) rinse at a flow rate of 1 mL/min (electrodes off). This procedure was used to remove impurities from electrodes to achieve electrode sensitivity and baseline stability.

Many protein precipitants are acids. Commonly used acids are metaphosphoric acid, trichloroacetic acid, perchloric acid and 5-sulfosalicylic acid (SSA) and hydrochloric acid (HCl). Organic solvents have been used instead of acids. The following different precipitant reagents were added to the blood plasma and standard solution: SSA (10%), HCl (2 mol/L), methanol and acetonitrile. All acids used as protein precipitants interfered with the HPLC analysis. Only acetonitrile as a protein precipitant led to the satisfactory recoveries and did not interfere with the HPLC analysis.

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. SPE of the samples was studied using Discovery DSC-Ph sorbent. The volume, flow rate, and composition of

solutions were studied with the aim of removing the interferences with a minimal loss of LA. The volume assayed was 1 mL at 1-3 mL/min. The optimum flow rate was 1 mL/min. Methanol (100%) provided the best elution of the LA.

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: $y = 139.21x + 250.78$, $r = 0.9997$. Calibration curve of plasma samples spiked with LA is shown in Fig.1.

The LOD and the limit of quantification (LOQ) values were 1.85 and 5.18 nmol/L. The LOD was better in comparison to the 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).

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 confirming that the levels of LA are less than 4.9 nmol/L (Teichert and Preiss, 2002). This method was used in order to determine LA plasma levels of supplemented voluntary blood donors. This method is suitable for further studies in order to determine LA levels in patients after supplementation.

The HPLC chromatograms of plasma samples with standard additions of LA are shown in Fig. 2.

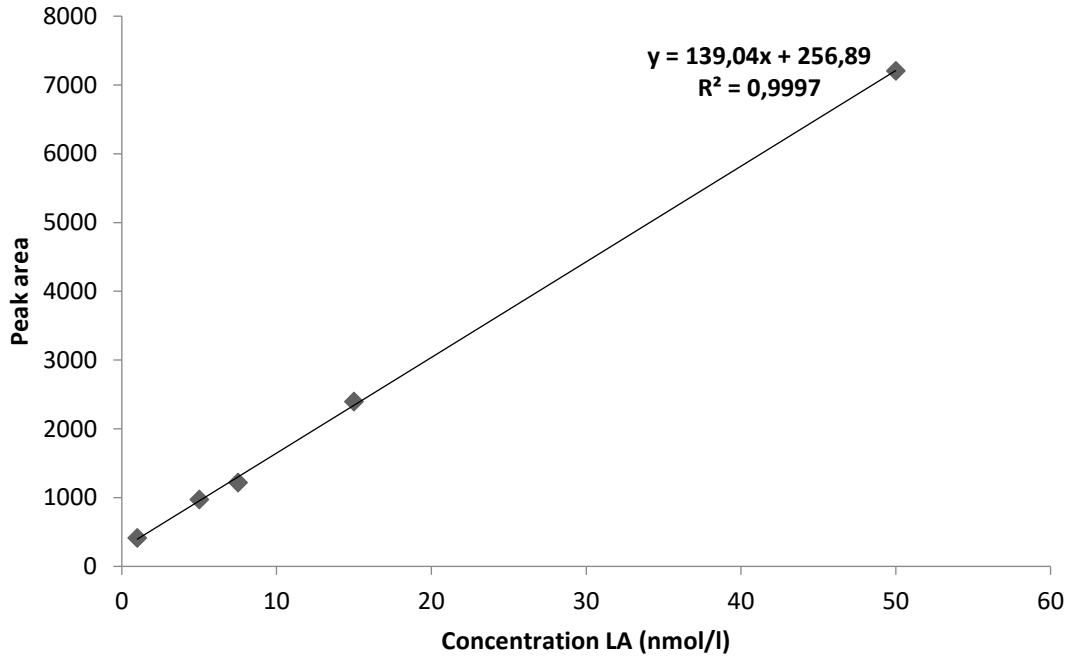

Fig. 1: *Calibration curve of plasma samples spiked with LA.*

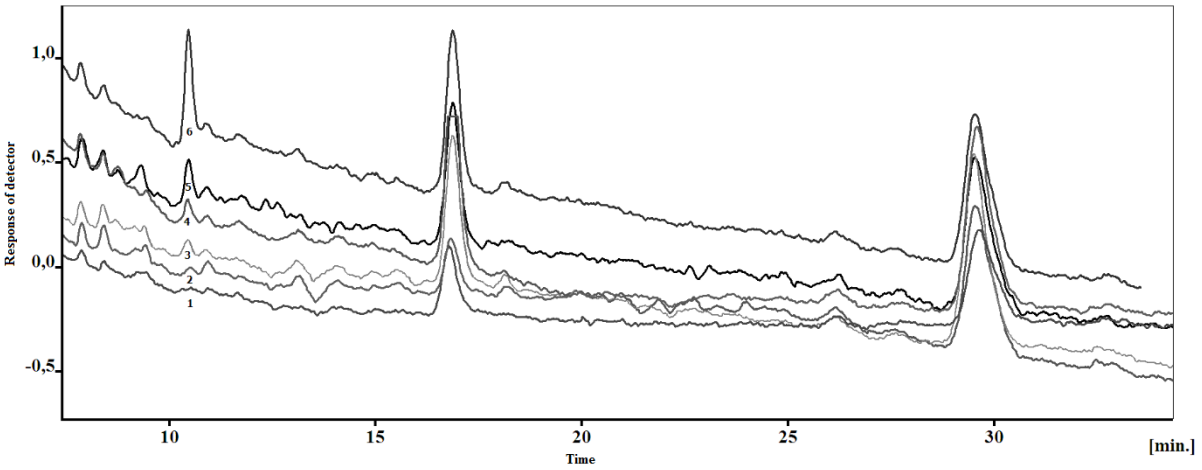

Fig. 2: *The HPLC chromatograms of plasma samples with standard additions of LA. HPLC conditions: isocratic elution (mobile phase: 20 mmol/L NaH_2PO_4 and acetonitrile, 62:38, v/v, pH 2.8); stationary phase was an analytical column LiChroCart 250 x 4 mm, Purospher Star RP-18e, 5 µm, fitted with a guard column LiChroCart 4 x 4 mm, Purospher Star RP-18e, 5 µm. The flow rate was kept constantly at 1 mL/min, separation run at 37°C. The detection was electrochemical (E_G = +750 mV, E_1 = +450 mV, E_2 = +590 mV). The sensitivity was 20 nA.*

The next aim was the verification of a possibility to use the method for the determination of exogenous LA after supplementation. Rochette *et al*. published an optimal therapeutic dose of LA 200–1800 mg LA/ day; plasma levels after supplementation were 15-20 µmol/L (Rochette *et al*., 2015). Chen *et al*. supplemented healthy volunteers (n=10) with a single dose of 600 mg LA leading to the plasma levels of 16±4.2 µmol/L (Chen *et al*., 2005). Moini *et al*. measured the levels of LA after a single dose of 200 and 600 mg LA; plasma levels were 3.1±1.5 µmol/L and 13.7±8.2 µmol/L, respectively (Moini *et al*., 2002).

This method was used to verify plasma levels of LA in three voluntary blood donors. The donors were supplemented with a single dose of 500 mg LA, blood collection was carried out 1 hour after consumption of the tablet. The plasma levels of LA were 1.42, 1.92 and 4.73 µmol/L. The analytical parameters of LA analysis were satisfactory. 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 0.5-20 µmol/L. The regression equation and correlation co-efficient retrieved from the calibration curve of LA were: $y = 1081.734x - 49.181$, $r = 0.999$. The LOD and LOQ were 0.17 and 0.34 µmol/L, respectively. The mean of recovery was 105.9±8.9%. The HPLC conditions: isocratic elution (mobile phase: 20 mmol/L NaH_2PO_4 and acetonitrile, 62:38, v/v, pH 2.8). The flow rate was kept constantly at 1 mL/min, separation run at 37°C. The detection was electrochemical (E_G = +750 mV, E_1 = +450 mV, E_2 = +590 mV). The sensitivity was 2 µA.

Determination of the cytotoxicity of lipoic acid to erythrocytes

Lipoic acid, a nutrient with both antioxidant and oxidant activity, can induce apoptosis in a variety of cells including erythrocytes (Bhavsar *et al.*, 2010). Owing due its proapoptotic potency LA has been suggested for the cancer therapy (Dorsam *et al*., 2016; Zhang *et al*., 2010). On the other hand, according to the recent studies it seems to be perspective to use stents with an LA coating as an antioxidant in patients with CAD (Lim *et al*., 2016; Lim *et al*., 2014).

To the best of our knowledge, there is no available information in the literature concerning the concentration of LA which can be released from the drug-eluting stent (DES). The high concentrations could damage the blood components, especially erythrocytes.

For this reason, the effect of LA on the viability of erythrocytes was monitored. For the determination of the cell viability the Trypan blue exclusion test was used. The effect of low concentrations of LA on viability and the number of erythrocytes after 24 and 48-hours incubation was first tested. The concentrations of LA were 1, 5 and 10 μmol/L. The effect of high concentrations of LA (50, 100 and 250 μmol/L) on the viability and the number of erythrocytes after 24 and 48-hours incubation was also tested.

The concentrations of LA (1, 5 and 10 μmol/L) did not cause a statistically significant change regarding the viability or number of erythrocytes after the 24 and 48-hour incubation time. The high concentrations of LA (50, 100, 250 μmol/L) did neither cause a statistically significant change regarding the viability of erythrocytes after the 24 and 48-hour incubation time. On the other hand, the high concentrations of LA had a statistically significant effect on the number of erythrocytes already after the 24-hour incubation time.

Our results agree with the study published by Bhavsar *et al*. (Bhavsar *et al*., 2010). The high concentrations of LA released from DES could have a negative effect on erythrocytes and may cause an anemia. It will be necessary to consider the application of DES with LA in patients with hematological disease, which significantly reduces the levels of erythrocytes. The results are shown in Fig. 3 – 4.

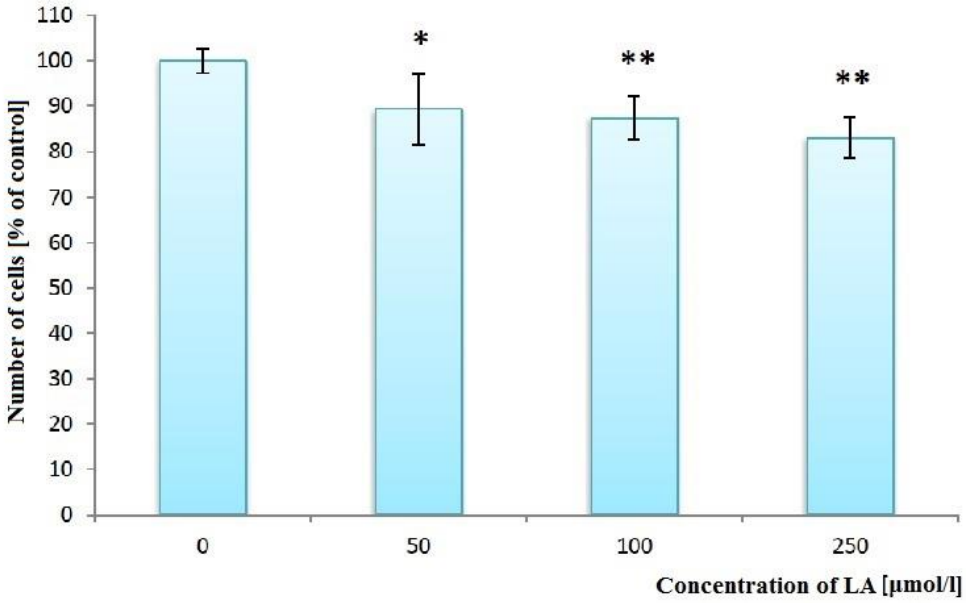

***Fig. 3**: The effect of high concentrations of LA on the number of erythrocytes after **24-hours** incubation. The figure shows a mean value with a standard deviation. The symbol * indicates results at the limit of statistical significance; the symbol ** indicates a statistically significant difference over the control.*
**p ≤ 0,05; ** p ≤ 0,01*

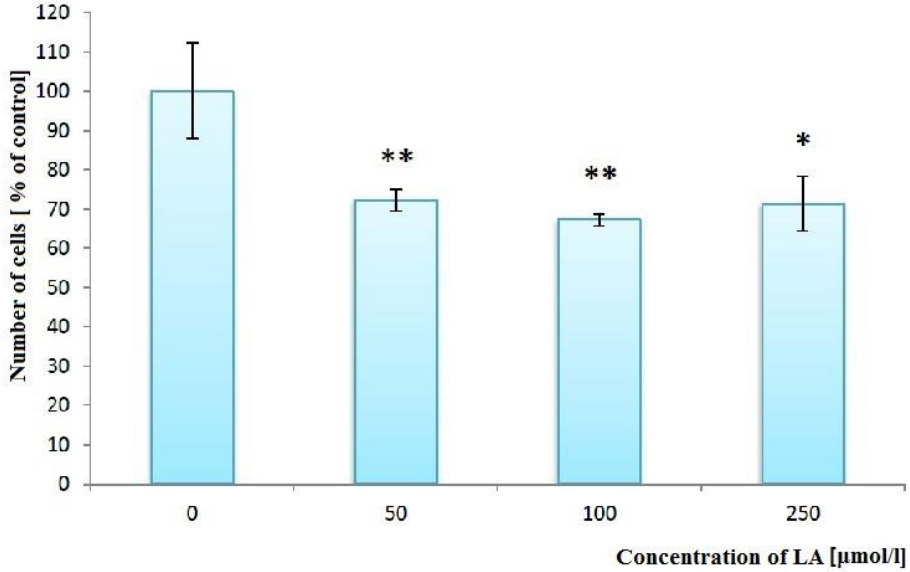

***Fig. 4:** The effect of high concentrations of LA on the number of erythrocytes after **48-hours** incubation. The figure shows a mean value with a standard deviation. The symbol * indicates results at the limit of statistical significance; the symbol ** indicates a statistically significant difference over the control.*
**p ≤ 0,05; ** p ≤ 0,01*

HPLC determination of total plasma homocysteine, cysteine, methionine and cysteinyl-glycine

Homocysteine is a sulphur amino acid derived from essential amino acid Met. It is metabolized by the remethylation pathway to Met again, in case of Met deficiency. The second possibility is the trans-sulphuration pathway via cystathionine to Cys, which is important for synthesis of other thiol-taurine or glutathione. Glutathione is the main antioxidant, which is degraded to cysteinyl-glycine – highly reactive thiol that generates reactive oxygen species (Ganguli *et al.*, 2015; Schalinske *et al.*, 2012). A lot of studies about Hcy have been published recently. It has been shown, that Hcy is an independent risk factor for cardiovascular disease. However, information concerning Hcy as a biomarker remains controversial (Ganguli *et al.*, 2015; Schalinske *et al.*, 2012). The next aim of this study was to verify its relevance as a biomarker in a clinical practice and the next aim was to obtain general overview about the whole methionine metabolism in CAD patients. We measured the total plasma level of Hcy, Cys, Met and cysteinyl-glycine using HPLC with coulometric detection. The HPLC chromatogram of Hcy, Cys, Met and Cysteinyl-glycine is shown in Fig. 5.

The patients were divided into three groups according to the severity of their disease. The characteristics of patients with a different severity of proved CAD are shown in the Table 2. Table 3 shows plasma aminothiols levels in patients with the different severity of CAD. The dynamic of aminothiols after PCI is shown in Table 4.

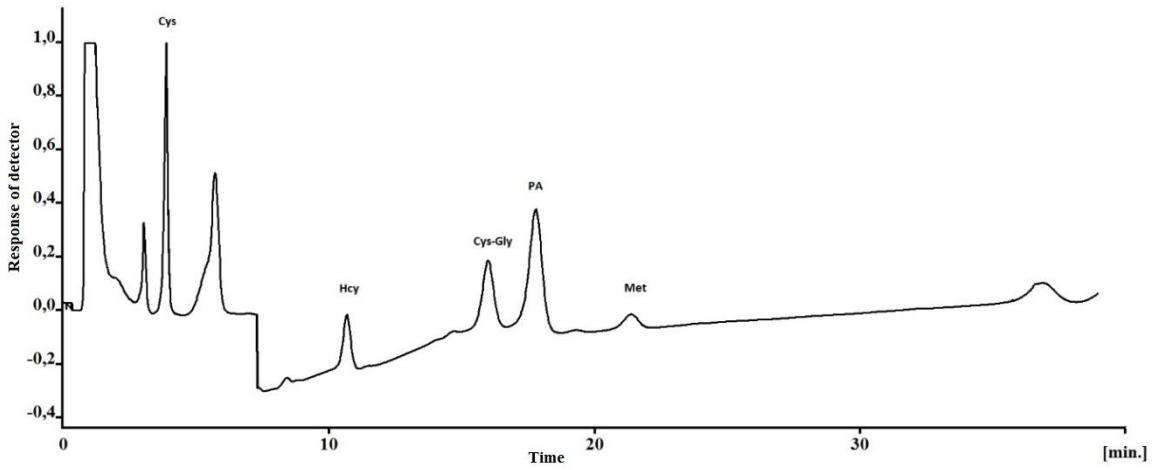

Fig. 5: *The HPLC chromatogram of Hcy, Cys, Met and Cysteinyl-glycine. HPLC conditions: isocratic elution (mobile phase: 20 mmol/L* NaH_2PO_4 *and 10 mmol/L* $C_8H_{17}NaO_3S$; *acetonitrile, 95:5, v/v, pH 2.82); the flow rate was kept constantly at 1 mL/min, separation run at 25°C. The sensitivity was 100 µA (0.1-7min);5µA (7.1-40min).*
Abbreviations: penicillamine; internal standard (PA)

Table 2 Characteristics of patients with a different severity of proved CAD

	Acute CAD (n = 29) 71.3 years (39–90)	Chronic CAD (n = 22) 72.9 years (49-90)	Completion RV (n = 17) 67.3 years (43-84)	*p* value
Male (n)	15 (51.7 %)	12 (54.5 %)	14 (82.4 %)	-
	67.0 years (39-83)	68.7 years (49-84)	66.3 years (43-83)	
Female (n)	14 (48.3 %)	10 (45.5 %)	3 (17.7 %)	-
	75.4 years (52-90)	77.9 years (57-90)	72 years (60-84)	
Smokers	4 (13.8 %)	0 (0 %)	4 (23.5 %)	<0,05*°
Ex-smokers	6 (20.7 %)	8 (36.4 %)	6 (35.3 %)	NS
Hypertension	20 (68.9 %)	20 (90.9 %)	14 (82.4 %)	<0,05*
Diabetes	6 (20.7 %)	10 (45.5 %)	3 (17.7 %)	NS
Prediabetes	2 (6.9 %)	1 (4.6 %)	1 (5.9 %)	NS
Kidney disease	1 (3.5 %)	0 (0 %)	1 (5.9 %)	NS
BMI	27.9 ± 4.3	28.3 ± 3.4	29.8 ± 4.5	NS
Glucose (mmol/L)	6.6 ± 2.7	7.4 ± 2.5	6.2 ± 2.1	NS
Albumin (g/L)	40.6 ± 8,8	38.4 ± 5,4	35.5 ± 2.6	<0,05§
TC (mmol/L)	4.3 ± 1.1	4.1 ± 0.8	3.5 ± 0.9	<0,05§
LDL-C (mmol/L)	2.5 ± 0.7	2.5 ± 0.7	2.0 ± 0.8	<0,05§
HDL-C (mmol/L)	1.0 ± 0.3	1.0 ± 0.3	1.0 ± 0.2	NS
TG (mmol/L)	1.4 ± 0.6	1.5 ± 0.6	1.2 ± 0.7	NS
AI	3.4 ± 1.0	3.1 ± 1.4	2.3 ± 0.9	<0,01§; <0,05°
Statins	9 (31.1 %)	9 (40.9 %)	10 (58.8 %)	NS
Fibrates	0 (0 %)	2 (9.1 %)	0 (0 %)	NS

** The comparison of patients with acute CAD and chronic CAD*
† The comparison of patients with acute CAD and completion of RV
° The comparison of patients with chronic CAD and completion of RV
Abbreviations: Body Mass Index (BMI), Total cholesterol (TC), Index of atherogenity (AI), not significant (NS), coronary artery disease (CAD), re-vasculization (RV)

Table 3 Plasma aminothiols levels in patients with the different severity of CAD

	Acute CAD	Chronic CAD	Completion RV	*p* value
Homocysteine (μmol/L)	26.7 ± 9.6	17.9 ± 6.1	21.6 ±5.2	<0.001*
Cysteine (μmol/L)	391.8 ± 115.7	355.2 ± 114.7	271.9 ± 101.1	<0.01§; <0.05°
Cysteinyl-glycine (μmol/L)	36.1 ± 11.6	29.7 ± 11.3	28.5 ± 11.9	<0.05§
Methionine (μmol/L)	28.9 ± 8.12	26.1 ± 6.7	19.2 ± 5.9	<0.001§; <0.01°

** The comparison of patients with acute CAD and chronic CAD*
† The comparison of patients with acute CAD and completion of RV
° The comparison of patients with chronic CAD and completion of RV
Abbreviations: coronary artery disease (CAD), re-vasculization (RV)

Table 4 The dynamic of aminothiols after PCI

Dynamics (μmol/L)	Before PCI	24h after PCI	48h after PCI	*p* value
Homocysteine	22.0 ± 7.9	22.4 ± 7.3	22.9 ± 7.1	NS
Cysteine	285.5 ± 108.0	282.2 ± 109.9	286.5 ± 110.7	NS
Cysteinyl-glycine	24.8 ± 6.6	28.7 ± 8.1	28.3 ± 8.2	< 0.05*§
Methionine	23.7 ± 7.5	27.5 ± 8.5	27.5 ± 8.6	< 0.05*§

** The comparison of levels before PCI and 24h after PCI*
† The comparison of levels before PCI a 48h after PCI
Abbreviations: no significant (NS), percutaneous coronary intervention (PCI)

We did not find any significant differences in the prevalence of diabetes, pre-diabetes and kidney diseases between the selected groups. We also did not find any significant differences between the number of smokers and hypertonic patients. We found significant differences between the levels of albumin, total cholesterol, LDL-C and the index of atherogenity in the selected groups. The levels of Hcy at those patients with acute CAD were significantly higher compared to the chronically ill patients. Our results support the hypothesis that total plasma of Hcy seems to be a good biomarker of the severity of the proved

disease. Homocysteine increases with an instability of the disease and a need for the treatment. Those patients who returned for the completion of the RV had significantly decreased levels of the total amount of plasma Cys, cysteinyl-glycine and Met before the second intervention, compared to those patients with an acute or chronic form of the disease. The condition before completion of the RV is probably associated with a higher oxidative stress, when the transsulfuration pathway is preferred, because it increases Cys consumption. The Cys seems to act more as an antioxidant than a risk factor in patients with CAD. 44 patients underwent a coronary angiography and subsequently PCI. The sample collections took place before the intervention and control samples were taken 24 and 48-hours after the intervention. We have not found any statistical differences between the levels of Hcy and Cys before and 24 and 48-hours after the intervention. The levels of Met and cysteinyl-glycine were significantly higher 24 hours and 48 hours after PCI compared to concentrations before the intervention.

Our findings agree with the results from Basati *et al*. who found the highest level of the total plasma Hcy in unstable angina patients, followed by stable angina patients and controls (Basati *et al*., 2014). Similarly, Liu *et al*. have showed an increasing prevalence of hyperhomocysteinemia from the controlled non-coronary heart disease group via stable and unstable angina pectoris patients to the group with acute myocardial infarction in their study (Liu *et al*., 2015). On the other hand, Vizzardi *et al*. concluded that Hcy level is poorly correlated with the severity of CAD in the group of unstable angina patients (Vizzardi *et al*., 2007).

The study published by Chai *et al*. supports the hypothesis that the total Hcy level is an independent predictor of multiple-vessel disease in patients with chest pain undergoing coronary angiographic study (Chai *et al*., 2011). Similarly, Oudi *et al*. and Shenoy *et al*. concluded that elevated levels of the total Hcy appear to be associated with a greater number of diseased arteries (Oudi *et al*., 2010; Shenoy *et al*., 2014). On the other hand, Kwon *et al*. concluded that no correlation was found between the Hcy level and the extent of CAD on the basis of the number of stenotic coronary arteries (Kwon *et al*., 2016).

Yurtdas *et al*. showed in their research that there is an important pathophysiological link between increased levels of the plasma Hcy, the degree of the ischemic findings, and the severity of the slow flow in slow coronary flow patients (Yurtdas *et al.*, 2013).

Our results support the hypothesis that the total plasma Hcy is probably not applicable in early diagnostic of stenosis in coronary arteries, but seems to be a good biomarker of the severity of the proved CAD. In all probability, the plasma cysteine employs as an antioxidant in CAD patients.

CONCLUSIONS

High-performance liquid chromatography with very sensitive coulometric detection for the determination of LA in human plasma was optimized. Due to the expected low endogenous levels of LA, we had to maximize the sensitivity of the electrochemical detector. In particular, we chose a high volume of plasma samples, we tried to minimize the dilution of samples during the deproteination and we modified the SPE procedure. Our limit of detection was 1.85 nmol/L which is 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). 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. In conclusion, a simple, sensitive and accurate assay has been developed for the determination of LA in human plasma after supplementation.

In the next part of this study, the effect of LA on the viability of erythrocytes was monitored. For the determination of the cell viability the Trypan blue exclusion test was used. The effect of a low concentration of LA (1, 5, 10 μmol/L) and a high concentration of LA (50, 100, 250 μmol/L) on the viability and the number of erythrocytes after 24 and 48-hours incubation was studied. The whole range of the concentrations did not cause statistically significant changes in the viability of erythrocytes even after 48-hour incubation. Low concentrations of LA also did not cause statistically significant changes in the number of erythrocytes after 48-hours of incubation. However, the high concentration of LA had a statistically significant effect with respect to the number of erythrocytes after 24-hours incubation.

In the last part of this study, we measured the total plasma level of Hcy, Cys, Met and cysteinyl-glycine using HPLC with coulometric detection. The patients were divided into three groups according to the severity of their disease. We did not find any significant differences in the prevalence of diabetes, pre-diabetes and kidney diseases between the selected groups. We did not find any significant differences between the number of smokers and hypertonic patients. We found significant differences between the levels of albumin, total cholesterol, LDL-C and the index of AI in the selected groups. The levels of Hcy at those patients with acute coronary artery disease were significantly higher compared to the chronically ill patients. Those patients who returned for the completion of RV had significantly decreased levels of the total amount of plasma Cys, cysteinyl-glycine and Met before the second intervention, compared to those patients with an acute or chronic form of the disease. 44 patients underwent a coronary angiography and subsequently PCI. The sample collections took place before the intervention, 24 and 48-hours after PCI. We have not found any statistical differences between the levels of Hcy and Cys before and 24 and 48-hours after the intervention. The levels of Met and cysteinyl-glycine were significantly higher 24 and 48-hours after PCI compared to concentrations before the intervention. Our results support the hypothesis that the total plasma Hcy is probably not applicable in the early diagnosys of the stenosis in the coronary arteries, but seems to be a good biomarker of the severity of the proved CAD. In all probability, the plasma cysteine employs as an antioxidant in CAD patients.

List of papers published by the author

Sechovcová S., Královcová P., Ventura K., Kanďár R., Analytické metody pro stanovení kyseliny lipoové. *Chemické Listy* 111, 11-16, 2017

Sechovcová S., Královcová P., Kanďár R., Ventura K., The issue of HPLC determination of endogenous lipoic acid in human plasma. *Biomedical Chromatography* 32, 4172, 2017

Kanďár R., Žáková P., Frohlichová M., Štramová X., Bencová S., Determination of coproporphyrine I and III in human urine using HPLC with fluorescence detection. *Scientific Papers of the University of Pardubice*, Series A, Faculty of Chemical Technology 16, 87-98, 2010

Drábková P., Bencová S., Štramová X., Žáková P., Kanďár R., Determination of epinephrine, norepinephrine and dopamine in human urine using high-performance liquid chromatography with coulometric detection. *Scientific Papers of the University of Pardubice*, Series A, Faculty of Chemical Technology 19, 25-38, 2013

Results of the thesis have been presented in 2 posters at international and 3 posters at national conferences by the author since 2010.

REFERENCES

Basati G., Ravazi A.E., Abdi A., Sarrafzedegan B. *Miomark Med 8.* 2014, p. 405-412.

Bhavsar S.K., Bobbala D., Xuan N.T., Foller M., Lang F. *Cell Physiol Biochem 26.* 2010, p. 859-868.

Dorsam B., Fahrer J. *Cancer Lett 37.* 2016, p. 12-19.

Ganguly P., Alam S.F. *Nut J 6.* 2015, p. 14.

Chai H.T., Chen Y.L., Chung S.Y., Tsai T.H., Yang Ch.H.,Chen H. Ch.,Sung P.H., Sun Ch.K., Chang L.T., Fan Ch.Q., Yip H.K. *Int Heart J 52.* 2011, p. 280-285.

Chen J., Jiang W., Cai J., Tao W., Gao X., Jiang X. *J Chromatogr B 824.* 2005, p. 249.

Khan M.I., Iqbal Z., Ahmad L., Shah Y., Watson D.G. *J Chromatogr B 878.* 2010, p. 2782.

Khan M.I., Iqbal Z., Watson D.G.,Khan A., Khan I. *J Chromatogr B 879.* 2011, p. 1725.

Kwon S.W., Kim J.Y., Suh Y.J., Lee D.H., Yoon Y.W., Lee B.K., Yung Y.H., Choi E.Y., Hong B.K., Rim S.J., Kwon H.M. *Korean Circ J 46.* 2016, p. 154-160.

Lim K.S., Park J.K., Jeong M.H., Bae I.H., Nah J.W., Park D.S., Kim J.M., Kim J.H. et al. *J Mater Sci: Mater Med 66.* 2016.

Lim K.S., Park J.K., Jeong M.H., Bae I.H., Nah J.W., Park D.S., Kim J.M., Kim J.H. et al. *Macromol Res 24.* 2016.

Lim K.S., Jeong M.H., Bae I.H., Park J.K., Park D.S., Kim J.M., Kim J.H. et al. *J Cardiol 64.* 2014, p. 409.

Liu Ch., Yang Y., Peng D., Chen L., Luo J. *Saudi Med J 36.* 2015, p. 839-846.

Schalinske K.L., Smazal A.L. *Adv Nutr 3.* 2012, p. 755-762.

Moini H., Packer L., Saris N.E.L. *Toxikol Appl Pharmacol 182.* 2002, p. 84.

Montero O., Ramírez M., Sánchez G.A., Gonzáles C. *Biomed Chromatogr 26.* 2012, p. 1228-1233.

Oudi M.E., Aouni Z., Mazigh C., Khochkar R., Gazoueni E., Haouela H., Machghoul S. *Exp Clin Cardiol 15.* 2015, p. 25-28.

Shenoy V., Mehendale V., Prabhu K., Shetty R., Rao P. *Ind J Clin Biochem 29.* 2014, p. 339-344.

Teichert J., Preiss R. *J Chromatogr B 672.* 1995, p. 277.

Teichert J., Preiss R. *Methods Enzymol 279.* 1997, p. 159.

Teichert J., Preiss R. *J Chromatogr B 769.* 2002, p. 269.

Vizzardi E., Nodari S., Fiorina C., Metra M., Dei Cas L. *Cardiol 107.* 2007, p. 354-359.

Yurtdas M.. Ozcan I.T., Seyis A.S., Camsari A., Cicek D. *J Cardiol 61.* 2013, p. 138-143.

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	Gynecological intervention	Angina	Minor surgical intervention in mouth	Infection hepatitis A (1-2 years)
Some tropical disease in the	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				