



### PALLADIUM (II) CHLORIDE ( $\text{PdCl}_2$ ) SPECTROPHOTOMETRY TO DETERMINE LIPOIC ACID CONCENTRATION IN PLASMA AND LEUKOCYTES

NA Yudhaswara<sup>1</sup>, AR Prijanti<sup>2,3</sup>, M Sadikin<sup>2,3</sup>

<sup>1</sup>*Master Program of Biomedical Sciences, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia*

<sup>2</sup>*Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia*

<sup>3</sup>*Center of Hypoxia and Oxidative Stress Studies, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia*

\*Corresponding author : [aniretno@gmail.com](mailto:aniretno@gmail.com)

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#### ABSTRACT

**Background:** Lipoic acid is a substance contained in intra- and extracellular that act as a coenzyme of pyruvate dehydrogenase, as well as an antidote, chelating agent, and antioxidant. Measurement of lipoic acid is needed to determine the amount of lipoic acid that performs its functions either as a coenzyme or an antioxidant. Besides, this measurement requires a special tool such as High Performance Liquid Chromatography (HPLC) and a process that is available in rural or simple laboratories.

**Objective:** A common and easy tool such as a spectrophotometer was conducted and could expected to be a tool of lipoic acid determination in body fluid such as plasma.

**Methods:** Measurement of lipoic acid using spectrophotometry with UV methanol and visible  $\text{PdCl}_2$  has been tested and compared to HPLC measurement that was valid and reliable in drug measurement or pharmaceutical preparations.

**Results:** Determination of lipoic acid in plasma and leukocytes using  $\text{PdCl}_2$  produced replicable, reliability and valid result, with high accuracy, precision and was not different from lipoic acid measurement using HPLC,  $p=0.99$ . While UV methanol was different compare to HPLC  $p=0.0001$  or was not valid.

**Conclusion:** The measurement of lipoic acid using  $\text{PdCl}_2$  visible spectrophotometry can be applied to determine the levels of lipoic acid and DHLA in plasma and equal to HPLC result.

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**Keywords :** HPLC, lipoic acid,  $\text{PdCl}_2$ , spectrophotometry, UV-methanol

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## INTRODUCTION

Alpha-lipoic acid (LA) or thioctic acid or 1,2-dithiolane-3-pentanoic acid,  $C_8H_{14}O_2S_2$  is a disulfide compound commonly found in all prokaryotic and eukaryotic cells, particularly in liver cells. The free form of alpha-lipoic acid is water- and fat-soluble. The end-carboxylate group determines its solubility in water.

The lipoic acid carbon framework is a hydrophobic group; therefore, its water solubility becomes limited and affects structure stability in the cell system. In the body, lipoic acid is synthesized by every cell, especially in liver cells. Lipoic acid has known as a coenzyme of several important reactions, including pyruvate dehydrogenase and ketoglutarate dehydrogenase. Clinically, lipoic acid is widely used in various treatments such as heavy metal poisoning, mushroom poisoning, degenerative atherosclerosis, and polyneuropathy diabetes.[1]

In the body, lipoic acid is present in two forms: oxidized state and the reduced state as dehydrolipoic acid (DHLA) (Figure 1). Those forms can react with reactive oxygen species (ROS) such as superoxide radicals, hydroxyl radicals, and hypochlorite acids; therefore, lipoic acid is widely used as an antioxidant. Lipoic acid has a unique ability as an antioxidant that is soluble in water and fat. Both the oxidized and reduced forms of lipoic acid can be well absorbed in oral preparations.[2,3]

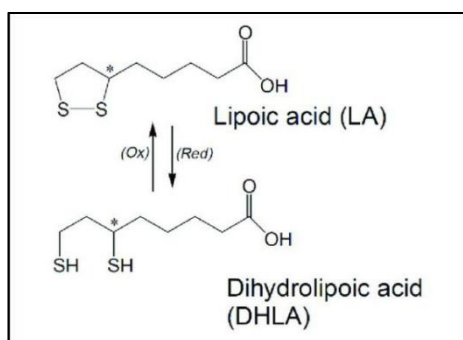


Figure 1. Lipoic acid and dihydrolipoic acid structure

The potential of lipoic acid and DHLA as an antioxidant in blocking and preventing oxidative stress states has not been studied, either as an endogenous or exogenous antioxidant. Various degenerative diseases are related to stress oxidative that is determined by the availability of antioxidants. Lipoic acid deficiency leads to disruption of carbohydrate and fat metabolism due to dysregulation of pyruvate dehydrogenase and PPAR alpha-gamma. In addition, the deficiency of lipoic acid lowers the body's resistance to heavy metals such as mercury, zinc, cadmium, and copper. In contrast, lipoic acid overdose causes nausea and kidney disorders. The use of lipoic acid in the treatment of diabetic neuropathy has been reported to have a significant effect.[4]

Colorimetry[5], gas chromatography[6], gas chromatography-mass spectrometry, [7,8] high-performance liquid chromatography (HPLC) [9-12], stripping voltametry[13], and capillary electrophoresis[14] have been used to determine the amount and levels of lipoic acid in tablet and drug preparations. However, the determination of lipoic acid in biological fluids is still limited and uses only HPLC. Therefore, it is necessary to develop a method that can easily measure the lipoic acid.

Kinetic spectrophotometry[15,16] has been applied to determine the lipoic acid in the tablet based on the effect of this compound's catalyst in the reaction with iodine-azide. The spectrophotometric method relies on the presence of sulfur groups in lipoic acid. This sulfur group will complex with palladium (II) chloride ( $PdCl_2$ ) in the Britton-Robinson buffer solution, producing a yellow that can be absorbed in specific wavelengths.[14] The sulfide ring in alpha-lipoic acid can also provide a maximum absorbance value at ultra-violet wavelengths[17] so that the amount and levels of lipoic acid in tablets can be measured.[16] This may be an

approach to measuring lipoic acid in various biological fluids. Therefore, looking at the potential and benefits of lipoic acid, developing a valid, reliable, and practical method to measure lipoic acid in plasma is needed.

## **MATERIAL AND METHODS**

### **Study design**

This research was an exploratory study to determine plasma lipoic acid levels in several methods and validate lipoic acid determination using visible-PdCl<sub>2</sub> and UV-methanol spectrophotometry compared to HPLC.[17] This research was carried out in Biochemistry and Molecular Biology Laboratory, Faculty of Medicine, Universitas Indonesia, and the Regional Health Laboratory of Jakarta. This research started with validation lipoic acid measurement with palladium (II) chloride and UV-methanol spectrophotometry compared to HPLC. Lipoic acid was measured in blood plasma and leukocytes (white blood cells) from 30 volunteers. This study had received ethical approval from the Medical Research Ethics Committee of Faculty of Medicine, University of Indonesia with number: 1038/UN2.F1/ETIK/2016.

### **Sample optimization**

The optimization samples include treating samples using methanol and TCA 15% to precipitate protein and treating 1% H<sub>2</sub>O<sub>2</sub> as an oxidizing agent to form lipoic acid in an oxidized form. After the sample was added, the mixture was centrifuged and then discharged the clear solution. After that, the sample was tested with three methods. Protein concentrations were measured using Christian-Ward Burg at 280 nm wavelength. Absorbance value at

the smallest wavelength 280 nm is the optimum optimization.

### **UV-methanol spectrophotometer method**

A fresh stock solution of 1000 ng/mL lipoic acid in methanol and a series of lipoic acid solutions with concentrations of 100, 200, 400, 600, 800 ng/mL series were made. The maximum UV wavelength was determined by using 200 ng/mL lipoic acid. Then the absorbance values of standard solutions, sample solutions were determined using maximum UV 210 nm [18] or 225 nm after derivative.[15]

### **Visible-PdCl<sub>2</sub> spectrophotometer method**

Principally, the lipoic acid level can be determined by visible PdCl<sub>2</sub> spectrophotometric because the sulfide group in lipoic acid forms a complex lipoic acid-PdCl<sub>2</sub> when extracted in pH 2.2 and KCl. A fresh standard solution of lipoic acid (1000 ng/mL) was prepared. PdCl<sub>2</sub> solution is available as 0.24 mL concentrated HCl per 100 mL of solution. The buffer solution of Britton-Robinson pH 2.2 was prepared by mixing phosphate, boric acid, and acetic acid 0.4 M, then titrated with 0.2 M NaOH to pH 2.2. Determination of absorbance was performed by visible spectrophotometer by finding a certain wavelength using 500 ng/mL solution. The determination of lipoic acid concentration in plasma, 1 mL of optimized plasma was added 0.2 mL PdCl<sub>2</sub> 5 mM, 5 mL buffer of Brighton-Robinson pH 2.2, added 1mL KCl and aquadest until volume reach 10 mL. The mixture was then centrifuged for 10 min to produce a homogeneous solution, and the absorbance was measured at the wavelength 350-800 nm.[19] Genesys 10S UV-VIS spectrophotometer with xenon

lamp dual beam was used in this experiment.

### HPLC method

The determination of lipoic acid levels with HPLC (Shimadzu MS.18) was performed at the Regional Health Laboratory of Jakarta (Labkesda), Rawasari, East Jakarta, using lipoic acid standard 100, 300, 500, and 700 ng/mL. Acetonitrile and 0.1 % acetic acid (1: 1) was used as mobile phase in a column diameter of 2.1x50 mm. The experiment was carried out by adding 300 µL of the sample and 300 µL of acetonitrile; then it was centrifugated at 4°C for 5 min. Then, the acetonitrile, the standard, and the test solution were injected. Then the data were read by computerized with MassLynk V.40.[7]

### Preparation of standard curves

Preparation of standard graphs and curves was performed from each method. The concentration used was according to the Lambert Law. Linear equation was generated by  $y = mx + c$ , which  $y$  = absorbance,  $x$  = concentration of solution.

### Measurement of lipoic acid in the cells

Lipoic acid in white blood cells was determined, which sample was collected by centrifugation of whole blood at 12000 rpm for 10 minutes. Three layers of blood were separated to obtain white blood cells. The white blood cells were added with H<sub>2</sub>O (1:1 volume), then stirred for 5 min. Cells were lysis and then optimized by adding 15% TCA and methanol (cell volume : TCA : methanol = 5:1:4). Then, it was centrifuged to obtain supernatant and measured the level of lipoic acid with three methods.[20]

### Statistical analysis

The data were tested for normality with the Shapiro-Wilk method. If the data distribution is normal and homogeneous, the analysis is continued with the Anova test and continued T-test calculation by SPSS 20.  $p < 0,05$  was considered as significant.

## RESULTS

### Measurement of lipoic acid using UV-methanol spectrophotometry, PdCl<sub>2</sub> spectrophotometry, and HPLC method

First, we compared the lipoic acid level determined by using UV spectrophotometry, PdCl<sub>2</sub> spectrophotometry, and the HPLC method. The result shows that there was a significant difference between the three methods (Anova test,  $p < 0.05$ ) (Figure 2). Interestingly, there was no significant difference between lipoic acid levels using the PdCl<sub>2</sub> visible method and HPLC as the gold standard, while there was a very significant difference between lipoic acid levels determined by UV-methanol spectrophotometry and HPLC. This significant difference can be proven by Anova statistical analysis, using Post Hoc (LSD) analysis ( $p = 0.001$ ).

Standard UV-methanol curve was done using concentrations of 0, 100, 200, 400, 600 and 800 ng/mL. Then measured the absorbance obtained by the equation of line  $y = 0.004x - 0.082$  with  $R = 0.99$ . The determination of plasma lipoic acid level using UV methanol method in sample of healthy blood plasma using volume ratio of 1: 1.

On the measurement of lipoic acid concentration in plasma, 5 mL of deproteinized plasma add with 5 mL methanol and then measured the

absorbance at 225 nm and obtained the mean value was  $309 \pm 64$  ng/mL, the lowest was 240 ng/mL and the highest was 505 ng/mL.

The measurement of lipoic acid by methanol spectrophotometric method has been tested on the drug, but has not been tested in plasma and therefore the test is compared to standard lipoic acid testing with HPLC. Standard lipoic acid

measurement using HPLC MS with combination of methanol and acetonitrile 1:1 phase and columns with 2.1 x 50 mm diameter obtained peak at 0.8 min. The result of standard curve chromatogram the of lipoic acid with the ratio between lipoic acid (ng/mL) axis x and the y peak area of  $y = 17.587x - 698.75$ , with  $R = 0.9993$ . There was an average value of 30 plasma lipoic acid of  $197.11 \pm 58.11$ .

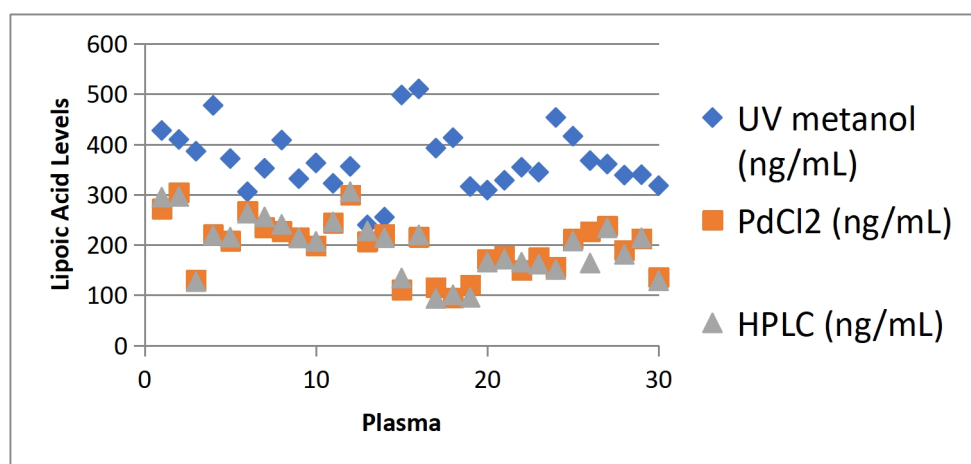


Figure 2. Levels of lipoic acid from each method, Anova test showed  $p < 0.05$

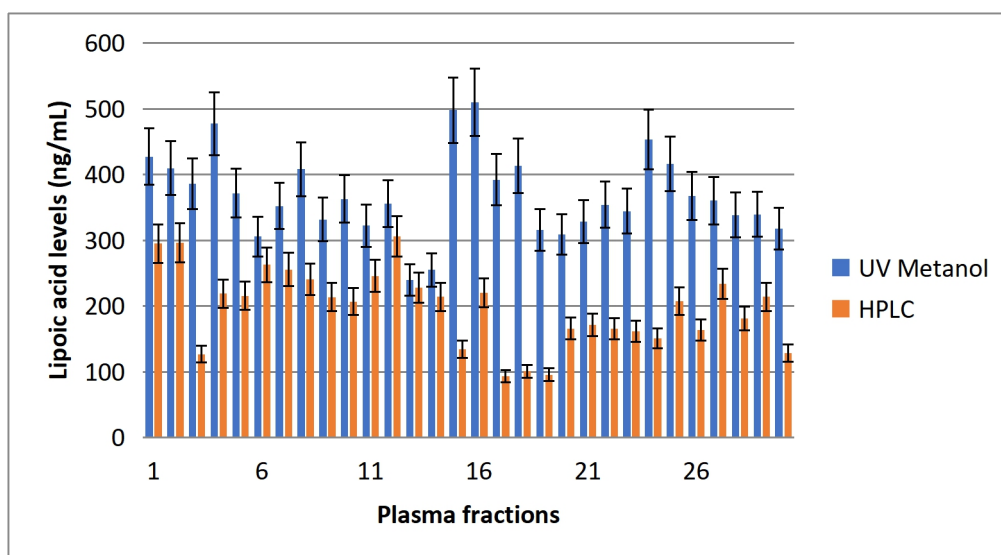


Figure 3. Lipoic acid levels determined by methanol UV spectrophotometry and HPLC standard

#### Analysis of lipoic acid using visible $\text{PdCl}_2$ method

Specific wavelength was searched using lipoic acid solution 500 ng/mL, characterized by the appearance of a peak

at 480 nm wavelength. The color of the lipoic acid- $\text{PdCl}_2$  complex was rather orange. The standard curve of visible  $\text{PdCl}_2$  was made using the concentration of 0, 100, 200, 300, 400, and 500. Then the

absorbance was measured and obtained equation line  $y = 0.0005x + 0.0492$  with  $R^2 = 0.99$ . After that, the absorbance was measured, and the mean value calculated was  $196.32 \pm 54.543$  ng/mL, with the minimum value 94.6 ng/mL and maximum value 304.8 ng/mL (Table 1)

Determination of plasma lipoic acid by the standard method was performed with HPLC. We had a standard

curve of lipoic acid from the result of the chromatogram, with the formula obtained as  $y = 17,587x - 698.75$ , with  $R = 0.99$ . The average value of 30 plasma lipoic acid was  $197.11 \pm 58.11$  ng/mL (Table 1). Figure 4 shows that there was no significant differences between lipoic acid levels using  $\text{PdCl}_2$  visible method and HPLC standard ((Post Hoc (LSD) analysis,  $p=0.959$ ). Concentration of lipoic acid in three methods are showed in Table 1.

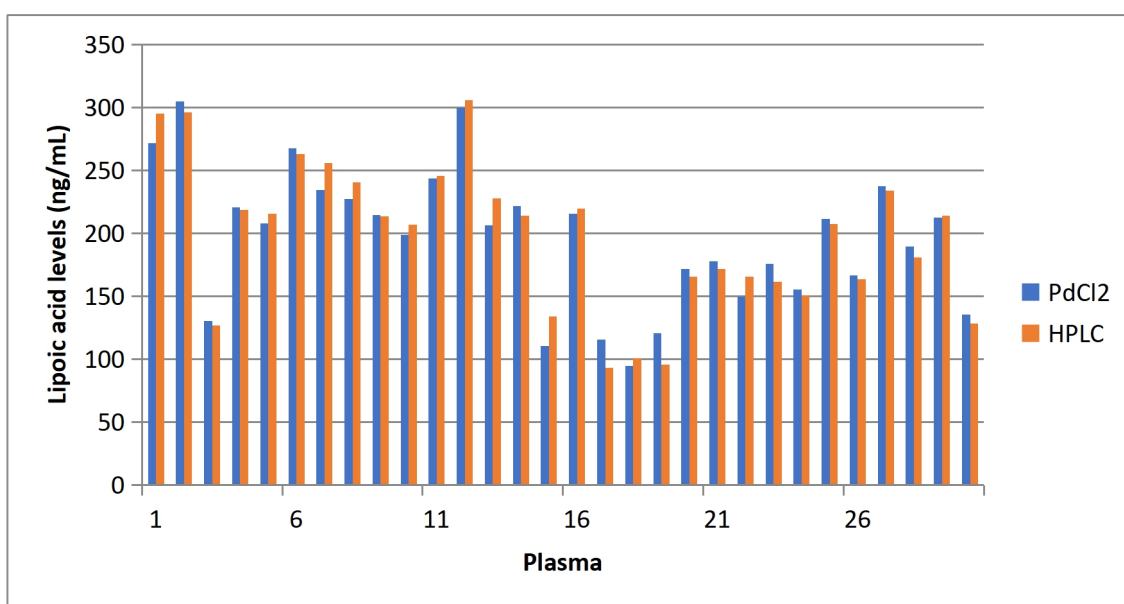


Figure 4. Lipoic acid levels determined by using visible  $\text{PdCl}_2$  method and HPLC standard

### Measurement of lipoic acid in leukocytes

White blood cells were chosen as a sample for measuring lipoic acid level because it is easy to collect, and leukocyte represents many types of cells. Lipoic acid present in the cytoplasm or mitochondria that act as the coenzyme of pyruvate dehydrogenase. The result shows that lipoic acid level was almost constant and homogeneous as  $33.39 \pm 9.2$  ng/million cells (Table 2).

Tabel 1. Concentration of lipoic acid in several methods

Samples	UV methanol (ng/mL)	$\text{PdCl}_2$ (ng/mL)	HPLC (ng/mL)
Mean	368,86	196,32	197,11
SD	63.85	54.54	58.52

Tabel 2. Concentration of Lipoic acid in Leukocytes

Samples n = 30	Leukocytes million/mL	Lipoic acid concentration ng/million cells
Mean	7.28	33.39
SD	1.75	9.2



## **DISCUSSION**

Lipoic acid has two form, as Lipoic acid (LA), and Dihydrolipoic acid (DHLA). Determination of lipoic acid concentration by UV-Methanol spectrophotometric method is based on the existence of sulphide bonding group on lipoic acid that gives specific absorbance value at certain wavelength.[20,21] Methanol determination of maximum wavelength used lipoic acid solution 200 µg / mL, it showed that the maximum wavelength was 225 nm after derivative.[15]

Accuracy refers to the notion of accuracy (similarity). The test results show that the chosen method has a 103.3% recovery range that states the accuracy rate fulfills the acceptability requirement. A recovery value close to 100% indicates that the method has good accuracy in showing the degree of conformity of the average of a measurement proportional to the actual value.

Precision (repeatability) testing is performed to see the proximity between test results performed repeatedly on the sample. Testing is performed using repeatability to obtain the system's accuracy in responding to the detected analyte. As a condition of acceptance is used Horwitz coefficient of variation equation, according to AOAC (Association of Official Analytical Chemist) as a reference.[21] A method's precision is said to qualify acceptability if the value is less than a 5% coefficient of variations. The precision test is performed by measuring the sample solution three times. Based on the research results obtained in percent for a precision test of 0.93%. The value fulfills the requirements of AOAC. It informs that the tool's operational system and the analyst have a good precision value against the method with a relatively constant response, although the measurement

results have a precision value that matches the requirements.

Limit of detection (LOD) and limit of quantitation (LOQ) have strong interconnection that the discussion cannot be separated. Practically, the way of evaluation of both measurements can be said no significant difference. The difference between the two is only on the quantitative nature of the data obtained. If the smallest concentration that can be detected at the detection limit is defined but not necessarily quantitatively, whereas, in the definition of quantitative limit, the smallest concentration of the analyte can be measured quantitatively. Using the linear regression from the standard curve of calibration, LOD can be measured as three times the deviation standard than divided by the line's slope. Besides, LOQ is equal to 10 times the deviation standard and then divided by the line's slope. Results obtained that LOD's value was 90 ng/mL, and LOQ was 30 ng/mL.

## **CONCLUSION**

The visible-PdCl<sub>2</sub> method can be applied to determine lipoic acid levels (LA) and DHLA in plasma and equal to the HPLC. Therefore, it can be applied to measure lipoic acid in the absence of HPLC facility with high accuracy and precision.

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