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DETERMINATION OF SERUM CONCENTRATIONS OF HOMOCYSTEINE USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR PATIENTS WITH DIABETES TYPE 2

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ABSTRACT : Introduction and Goals: Homocysteine measurements are useful for metabolic tests and assessing a patient's health situation. The aim of this study was to use reverse phase high performance liquid chromatography (RP-HPLC) techniques with o-phthalaldehyde (OPA) pre-column derivatization and 3-mercaptopropionic acid to test plasma amino acids (3-MPA). Concentrations of homocysteine in serum from 15 selected sick men aged 27–62 years were determined by method of HPLC. In conclusion, certain clinical samples, because of the ease of preparation and accurate calculation, determining homocysteine using OPA/3-MPA derivatives and RP-HPLC is a successful method. The aim of this study is to see how homocysteine affected diabetes in middle-aged and elderly Iraqi adults. Use OPA with 3-MPA as a pre-column derivation.

Key words : HPLC, OPA/3MPA, homocysteine, diabetes type 2.

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INTRODUCTION

Amino acids are needed for a number of processes. These substances are the building blocks of proteins and can also act as precursors in the biosynthesis of a number of important biological and physiological compounds. Energy metabolism, neurotransmission and fat transport are all dependent on them (Go *et al*, 2008). In order to assess the pathophysiological pathways of diseases, homocysteine must be calculated. The study of these products is one of the most important practical programs in the field of biomedical and pharmaceutical sciences. (Turnell and Cooper, 1989). To address these issues, the effective high-performance liquid chromatography (HPLC) approach has been used in conjunction with chemical derivatives in the form of pre- or post-column columns. One of the most commonly used techniques is precolumn OPA derivation. Because of its fast interaction with the amino group of amino acids, this compound is an important amino acid derivative agent, resulting in the development of highly fluorescent materials. The addition of sulfhydryl agents to these compounds affects their stability and some experiments suggest that 3-mercaptopropionic acid (3-MPA) provides a more stable substance than 2-mercaptoethanolamine (2-ME) (Kaspar *et al*, 2009). The amino acid homocysteine play a major role in various metabolic processes in the human body and their dangerous effects on their health and their relationship to diabetes and their complications such as atherosclerosis, heart attacks, blood clots and possibly Alzheimer's disease, as researchers and doctors are no longer interested in the past is important for homocysteine, but in recent years there has been an increase in interest in this topic, especially for patients with type 2 diabetes (Serra *et al*, 2005).

Homocysteine is an indicator of fitness; there is a remarkable association between homocysteine levels and patients' overall wellbeing, and homocysteine levels are a feature of both positive and unhealthy influences (Ajith *et al*, 2005). To prevent these issues, the laboratory study of amino acids (homocysteine) and can be performed conveniently using certain popular medical analysis instruments, such as spectrophotometers like the ELISA system, but it is not possible to use this tool due to a shortage of chemicals, high costs and difficulties

extracting them from the original originator. To increase the accuracy of the work and to tolerate any specific conditions that may occur during laboratory work, the high-performance liquid chromatography (HPLC) technique was used to analyze the amino acids and use the standard compounds for use in the study (Zhang *et al*, 2012; Skoog *et al*, 2013).

MATERIALS AND METHODS

This study was conducted between 1/8/2020 to 1/9/2020 were collected in Baqubah teaching Hospital, Diyala Governorate (5 ml) blood samples were obtained from 15 sick men whom fasting for a period of approximately (12) hours and their ages ranged between (27-62) years, were diagnosed clinically by specialized doctors and (15) from healthy people as a control group. To ensure that the amount of homocysteine in the blood serum is determined, the samples (serum) were kept at -20°C before the time of study.

Sample preparation

A 10% trichloroacetic acid solution was used to precipitate plasma proteins (TCA). After centrifugation at 10000 rpm for 5 minutes, the supernatant was drained, and filtration was performed using a syringe filter with a pore size of 0.45 m. The components used were of the highest purity possible. The samples were made by mixing 250 l of sample with 500 l of methanol.

The protein in the plasma is precipitated by adding 10% of trichloroacetic acid (TCA), then the solution is shaken out in a centrifuge, with 10,000 cycles for a period of 5 minutes, the clear solution is removed and the sediment is retained. All materials used are of a high degree of purity. The borate solution was made by dissolving 5.4 gm. of powdered tetraborate in 100 ml of water. 2250 µl methanol, 250 µl l buffer borate solution, and 25 µl 3-MPA were added to 0.025 gm. OPA to make the derivative solution.

The samples were prepared by taking (250 µl) of the sample and mixing it with 500 µl of methanol in an incubator at laboratory temperature for 5 minutes, then centrifuged with 5000 cycles for 5 minutes and (250 µl) of the transparent solution was taken and mixed with 100 µl l of borate buffer solution. The amino acid homocysteine is deduced by adding (50 µl) of (OPA / MPA-3) solution to the sample, which is then held in the incubator at room temperature for two minutes before the examination.

HPLC condition

The tests were conducted at the Ministry of Science and Technology's Environmental and Water Laboratories. The method given by Fahime Mohammad Abadi and Arezoo Mirfazeli (2016) was developed using the High-Performance Liquid Chromatography Technique (HPLC) and the model SYKAMN German. The mobile process consisted of acetonitrile, buffer and DW (60 : 10 : 30) at a flow rate of 1 ml/min, with C18-NH2 (25 cm × 4.6 mm) as the column separation. Florescence Ex = 330 nm, Em = 445 nm Detector.

After both the sample solution to be separated and the mobile phase solution are prepared and placed in the place designated for them in the device and the required separation column is installed according to the type of separation and the material to be separated in the place designated for it inside the device and then the mobile phase is passed on the separation column for a period not less For half an hour, then the device injects a small amount of the sample solution with the microliter so that the sample is transferred to the column and passes through the moving phase through the separation column in which the material is separated, which then comes out, so that the result appears in the form of chromatograms to the detector and the result appears in the form of a top and a sign for each. It is composed of the components of the sample and the area under the top of the separated material is calculated and compared to the area under the top of a standard substance with the same concentration, so that the concentration of the separated material can be known. Each peak represents a part of the mixture to be separated if the separation is successful. A high pressure of more than 100 bars can be used to achieve an outstanding performance.

Through the work that was conducted, the results were collected in the form of graphs of chromatogram peaks (peaks) and tables for each sample separately, as well as the standard material to show some statistical values, detention time, peak area and percentage, height, quantity and type of unit used, and this is an illustrative review of the results that emerged for the standard subject, homocysteine, the patient group, which numbered 15, patients and 15 samples, for the control group

Accounts

The homocysteine concentration in the samples was calculated using the following formula :

$$C_{sam} = \frac{Cst \times Asam}{Asta}$$

Where,

Csam = sample concentration (sample)

Cst = concentration of standard substance

Asam = apex area of the model

Ast = apex area of standard material

RESULTS

With an excitation wavelength of 330 nm and an emission wavelength of 445 nm, amino acids can be analyzed and separated, using a 1 ml/min flow rate. The statistical values of men with diabetes were compared with the groups of healthy people, as the total number of samples studied with diabetes was 15 samples and the group of healthy people (15) samples from Diyala Governorate and the biochemical variables were recorded to ensure that they had diabetes, as well as healthy people to make sure. They were free from diabetes and the results were as follows :

Table 1 showed homocysteine concentrations for type 2 diabetes patients the following values between (µg/ml 22.3-µg/ml 28.9) and homocysteine concentrations for healthy people between the following values (µg/ml 0.86 -µ g/ml 1.23).

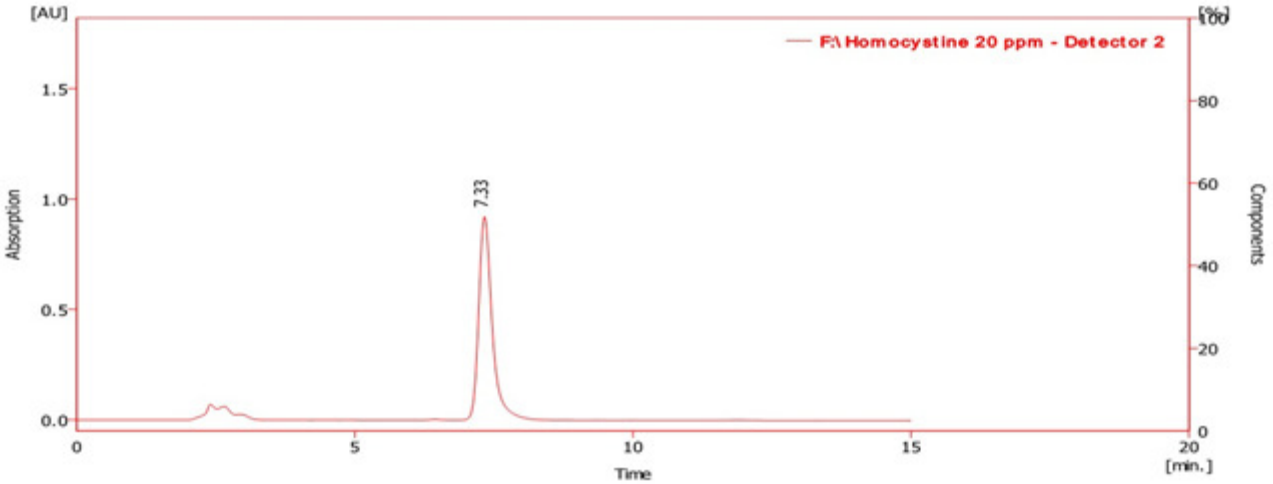
While introducing the amount of homocysteine into the standard solution of the computer, all values of 100 and their units were considered as a percentage p% because the value of their concentrations in the standard and in the selection, solution was presented as an equal end value. This helps to calculate the percentage achieved with the declared amount in the preparation directly by comparing the area of the tops of the standard solution with the area of the tops of the test solution. It is what he usually works with while analyzing some chemicals, such as medicines.

Fig. 1 shows a plot of the homocysteine norm's chromatogram standard curve, with a description table of the peaks and some statistical values for retention period, apex area and percentage, height, quantity and unit form.

Fig. 2 shows chromatograms plotting of one of the homocysteine patients' standard curves versus the standard solution, with a description table of the peaks and some statistical values such as (peaks), retention time, apex area, percentage, height, quantity and unit form.

DISCUSSION

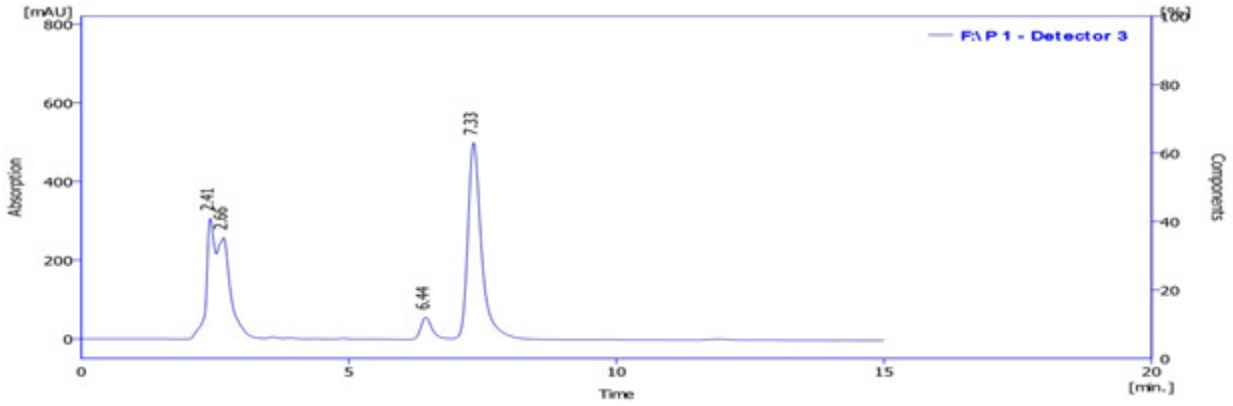
The aim of this study was to see whether reverse-phase chromatography (RP-HPLC) could be used to extract homocysteine from a mixture of OPA-3MPA derivatives. The findings demonstrate that this approach can separate amino acids chromatographically. The use of polar solvents and thiol-containing compounds including 3-MPA in the) Simons and Johnson (1978) analysis to establish the structure of the OPA derivation showed that the presence of these compounds has a significant impact on the fluorescence property of isoindole derivatives. These compounds have an important effect on fluorescence intensity when combined with borate buffer. (Simons and Johnson, 1978). Tornell *et al*. demonstrate that in the sample preparation process, using methanol alone as an organic solvent increases the chromatogram and amino acid separation solution precision, which is close to the current research (Turnell and Cooper, 1989).



Result Table (Uncal - F:\Homocystine 20 ppm - Detector 2)

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	7.330	880.253	170.437	100.0	100.0	0.09	
	Total	880.253	170.437	100.0	100.0		

Fig. 1 : A chromatogram of the homocysteine standard material.



Result Table (Uncal - F:\P 1 - Detector 3)

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	2.410	929.554	153.070	14.3	24.0	0.13	
2	2.660	968.548	99.031	14.9	15.5	0.19	
3	6.437	431.520	41.993	6.6	6.6	0.18	
4	7.333	4185.933	342.999	64.2	53.8	0.20	
	Total	6515.556	637.092	100.0	100.0		

Fig. 2 : The chromatogram of the first test sample for homocysteine.

The proteins in this study were denatured with TCA. Frank et al. used HPLC technologies with pre-column derivation using OPA / 3-MPA to test a fast and effective solution for amino acid analysis. They discovered that this easy and optimized technique requires a small sample size and the use of an OPA/3-MPA mixture, which is close to the current study (Frank and Powers, 2007). Which leads to a high rate of customer retention. Qureshi *et al* (1989) on the other hand, discovered that HPLC using the OPA / 2-ME combination was a good method for amino acid analysis in both patients and healthy people, with high specificity and reproducibility. There was no significant difference in the accuracy of amino acid chromatograms when SSA 5- sulfosalicylic acid was compared to TCA (Qureshi *et al*, 1989). The aim of the study by Schwarz et al. was to compare ion exchange chromatography with RP-HPLC in combination with a pre-column derivation using OPA-3MPA for amino acid analysis (IEC). They demonstrated that HPLC has a positive experience with IEC technology and suggest it as a tool for clinically relevant amino acid separation (Schwarz *et al*, 2005). We discovered through our work and research in this area that there is a very significant increase in homocysteine concentration from the normal level in men with chronic diabetes compared to healthy people in good health, as shown by some studies and research in this area, which we will mention through this research. The findings, as seen in Table 1, revealed substantial variations in the average concentration of total homocysteine in the blood serum of type 2 diabetes patients relative to the healthy population (Rudy *et al*, 2005). The relationship between the level of homocysteine in the blood serum and fat peroxidation in patients with angina pectoris was studied by Abduljabbar *et al* (2019) with the level of homocysteine measured by the color enzyme method and the level of malondaldehyde measured by the lipid peroxidation index by optical spectroscopy and the results showed a significant increase. Malondaldehyde serum levels are considerably higher in patients with angina pectoris than in the general population, and blood serum levels are significantly higher in patients with angina pectoris than in the general population (Abduljabbar *et al*, 2019). Researchers Meltem et al (2017) tested the relationship between homocysteine and psoriasis since the study included 50 psoriasis patients and homocysteine levels were measured using the HPLC method of high-performance liquid chromatography.

When they used the HPLC instrument to assess homocysteine and vitamin B12, AL-Jumaily *et al* (2000) discovered a connection between homocysteine concentration and hepatitis. They found that all patients with cirrhosis or chronic hepatitis had high homocysteine concentrations, as well as an increase in the vitamin, as compared to healthy subjects. B12 is an essential vitamin. Using high-performance liquid chromatography (HPLC) and a UV detector, researcher AL-Baldawi (2006) analyzed homocysteine concentrations in patients with high blood pressure, as well as homocysteine concentrations in blood plasma. The levels of homocysteine plasma were much higher in patients than

Table 1 : shows homocysteine concentrations micrograms per milliliter (µg/ml) for patient and healthy groups.

No	Control	Patient
1	1.23	22.3
2	0.98	25.9
3	0.87	28.7
4	1.01	25.6
5	0.97	24.7
6	0.87	25.9
7	1.14	26.7
8	1.13	27.8
9	0.98	25.8
10	0.86	28.9
11	0.87	28.6
12	0.96	27.5
13	1.00	25.6
14	1.08	27.8
15	0.89	25.8

in the healthy group and there were no significant differences between patients (Male and female) (AL-Baldawi, 2006). The cause for the noticeable rise in homocysteine in the blood serum was confirmed by the evidence obtained from the patients, when it was discovered that this increase was due to other disorders that follow type 2 diabetes, such as cardiovascular disease (stroke and angina) and high blood pressure, and these findings arrived. According to a survey by a consortium of researchers, they claimed that this rise is due to vascular disease caused by the destruction of homocysteine in the artery lining, which is supported by medical evidence (Donner *et al*, 1998; Christen *et al*, 2000). High levels of homocysteine, on the other hand, are linked to the progression of cardiovascular disease. A rise of 5 mol/L in homocysteine concentration was shown to double the likelihood of developing cardiovascular disease in a related analysis by the researcher (Framingham) (Selhub, 2006).

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