HPLC-Determination of Serum Concentrations of Homocysteine for Women with Recurrent Miscarriage in Diyala Governorate

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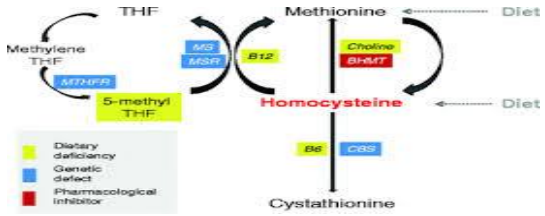
Abstract
This research determined the amount of homocysteine in the blood serum, by using the High-Performance Liquid Chromatography Technique (HPLC) and the model SYKAMN German[1]. High levels of homocysteine in the serum, are a medical condition called hyperhomocysteinemia. Hyper homocysteinemia produces a significant increase in the chance of miscarriage in early pregnancy loss. The results showed an increase in homocysteine in pregnant women with repeated trimester miscarriage with high homocysteine values. Homocysteine concentrations for three groups, the following values (31.5 µmol / L - 36.5 µmol / L) for women with Recurrent Miscarriage and homocysteine concentrations for pregnancy between the following values (17.5 µ mol / L - 20.6µmol / L) while healthy women were (14.5µmol / L - 11.6 µmol / L.
Keywords: Homocysteine, HPLC, Stage, Recurrent pregnancy loss, miscarriage.

1. Introduction

Two or more consecutive pregnancy losses are characterized as recurrent pregnancy loss (RPL)[2].
Because recurrence rates and risk factors are comparable after two successive pregnancy losses, some doctors believe that two subsequent pregnancy losses would be enough to diagnose Recurrent Miscarriage (RM)[3]. Repeated loss may happen at any stage of gestation, whether those are early or late. If an embryo is lost within the first trimester of pregnancy, it is referred to as early pregnancy loss.
Frequent miscarriages might happen at any pregnancy stage, whether early or late. If an embryo is lost within the first trimester of pregnancy, it is referred to as early pregnancy loss. Late pregnancy loss occurs when a fetus is lost after the first trimester of pregnancy. Late pregnancy losses are less common than early pregnancy losses, accounting for approximately 1% of all pregnancies [4]. A miscarriage is a major problem that women suffer from all over the world. A World Health Organization survey showed that approximately 25 million unsafe miscarriages occurred between 2010 and 2014, equivalent to 45% of the total miscarriages in the world[5]. The exact pathophysiological mechanism of miscarriage remains unrecognized in around 50% of remaining recurrent pregnancy loss RPL cases [6]. Some of the causes of recurrent miscarriage: Are chromosomal abnormalities, Endocrine Etiologies, Thrombophilia, karyotype abnormalities, and other causes.
Through the conversion of S-adenosyl methionine from dietary protein rich in sulfur amino acids, it originates in humans [7]. Homocysteine is an amino acid not given by the food that may be transformed into cysteine or recycled into methionine, a necessary amino acid, with the assistance of particular B vitamins. Men and women have different homocysteine levels, with a normal range often ranging between 5 and 15 micromol/L. When values reach 15 micromol/L, hyper homo cysteinemia results [8].
Homocysteine as presented by Dayal and Lentz in (fig .1) is a non-essential amino acid that, with the help of a certain vitamin B, may be transformed into cysteine or recycled into methionine. [9].



(Hcy) grades vary between men and women when (Hcy) levels are high, which reveals that there is an issue with homocysteine metabolism [10]. Abnormally high levels of homocysteine in the serum, are a medical condition called hyperhomocysteinemia. Early pregnancy loss is three times more likely when you have hyperhomocysteinemia.[11]. impairment of chorionic villous vascularization is linked to hyperhomocysteinemia[12]. The exchange between fetus and mother is essential for average fetal growth, but defects of chorionic villous vascularization provoke pregnancy loss[13].To evaluate the pathophysiological processes underlying illnesses, the content of amino acids (homocysteine in our study) must be known[14]. One of the most crucial practical projects in the area of biopharmaceutical sciences is the analysis of these components[15]. There has been a lot of interest in measuring amino acids in many materials, including plasma, urine, and cerebrospinal fluid[16]. Ion exchange chromatography with precolumn

doi.org/10.31838/hiv22.02.383 Received: 17.04.22, Revised: 24.04.22, Accepted: 08.08.22

derivatization using ninhydrin or ophthalaldehyde (OPA) has been used to analyze amino acids throughout the past fifteen years[17]. This approach, however, is a little challenging, expensive, and time-consuming [18]. To solve these issues high-performance ance liquid chromatography (HPLC) has recently been adopted in collaboration with chemical derivatives in the form of pre-or post-columns. One of the most widely used techniques is the Precolumn OPA-derivatization [18, 19]. Due to its fast interaction with the first amine group of amino acids, which results in the creation of products with strong fluorescence characteristics, this chemical is an efficient derivative factor for amino acids. However unstable they may be, the derivatives of indole can still be used to get precise findings[20]. The addition of sulfhydryl agents to these derivatives is necessary for their stability. According to certain investigations, 3-mercaptopropionic acid (3-MPA) creates a product that is more stable than the typical 2-mercaptoethanol amine (2-MEA). This study used pre-column derivatization with OPA coupled with 3-MPA to assess homocysteine of plasma for women with recurrent miscarriage[21].

2. Material and Methods

This study was conducted at Baqubah teaching Hospital, Diyala Governorate, in the Analytical Chemistry Laboratory in the College of Education Pure Science) and at the Ministry of Science and Technology's Environmental and Water Laboratories in the period between (October 2021) to march,2022). The study included (120) women, 40 control subjects and 80 patients Their ages were between 18-42 years., were diagnosed clinically by specialized doctors. To deter determine the amount of homocysteine in the blood serum, about (5ml) of blood was collected from women by venous. Placed in a gel tube to separate the serum. The serum was separated from the blood after it had been allowed to clot at room temp by centrifugation (10 min. at 3000 rpm). Serum specimens were taken and kept at (-20oC) for future homocysteine measurements.

3. Sample preparation

By adding 10% trichloroacetic acid (TCA) the plasma proteins were precipitated and then the solution was centrcentrifuged0000 rpm for 5 minutes, the supernatant was drained, and filtration by using a syringe filter with a pore size of 0.45 m. the highest purity components were used. The samples were made by mixing 250 µl of sample the e with 500 µl of methanol. The borate solution was made by dissolving 5.4 gm. of powdered tetra borate in 100 ml of water. 250 µl methanol, 250 µl 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 the solution was collected and combined with 100 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 Labor at ories The method given by Mohammad Abadi and Arezoo Mirfazeli (2016)[1] 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 × 4.6mm) as the column separation. Florescence Ex = 330nm.

Em = 445 nm detector. After both the sample solution to berate 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. Then the mobile phase is passed on the separation column for a period, notless thanr half an hour. 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 assign 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 High of more than 100 bars can be used to achieve 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 80, patients and 40 samples, for the control group. The homocysteine concentration in the samples was calculated using the following formula:

$$Csam = \frac{\text{Cst} \times \text{Asam}}{\text{Asat}}$$

Csam = Concentration of samples, Cst= Concentration of standard

Where, Cst = concentration of a standard ard substance, Asam = apex area of the model

Determination of serum concentrations of

homocysteine using HPLC for women with Recurrent Miscarriage, Ast = apex area of standard material

3. 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 women with Recurrent Miscarriage were compared with the groups of healthy women, as the total number of samples studied with abortion group was 40 samples, the group of pregnancy was (40) and healthy people (40) samples from Diyala Governorate and the biochemical variables were recorded to ensure that they had with Recurrent Miscarriage, as well as healthy pregnancy to make sure. They were free from Recurrent Miscarriage and the results were as follows: (Table 1) showed homocysteine concentrations for three group groups following values between (31.5 µmol / L - 36.5 µmol / L) women with Recurrent Miscarriage and homocysteine concentrations for pregnancy between the following values

(17.5 µ mol / L - 20.6µmol / L) while healthy women were (14.5µmol / L - 11.6 µmol / L. 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 seethe lection, the 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.

(Table 1) showed homocysteine concentrations for three groups:

No	Control (µmol / L)	Pregnancy (µmol / L)	Abortion (µmol / L)
1	5 – 15	18.6	30.1
2	8.5	17.5	31.5
3	9.9	18.9	33.6
4	11.2	18.9	31.5
5	10.5	18.7	31.5
6	10.8	19.6	32.6
7	9.8	18.9	32.6
8	9.8	19.5	35.6
9	10.5	19.8	32.5
10	11.4	20.1	34.6
11	10.6	18.9	35.6
12	10.5	19.6	32.5
13	10.8	19.8	34.0
14	9.8	20.4	35.6
15	11.2	20.6	34.5
126	11.2	20.1	35.6
17	11.5	20.3	34.5
18	11.5	18.9	36.6
19	10.5	18.9	32.6
20	9.8	19.6	34.5
21	9.5	19.5	32.6
22	9.7	20.5	34.5
23	9.5	20.4	34.5
24	9.6	20.3	33.6
25	10.5	19.5	33.5
26	10.7	19.6	32.6
27	10.6	19.8	34.5
28	9.8	20.5	36.5
29	9.7	18.9	32.6
30	9.6	19.6	35.6
31	9.4	18.7	34.5
32	9.8	18.6	35.6
33	10.5	19.3	32.6
34	10.5	19.6	32.6
35	10.4	19.8	32.6
36	10.9	20.6	32.6
37	9.8	20.6	32.6
38	9.4	19.6	34.5
39	9.9	18.9	35.6
40	10.2	19.8	35.6

Fig. 2 shows a plot of the homocysteine chromatogram standard curve, with a description of

the peaks and some statistical values for retention period, apex area and percentage, height, quantity, and unit form.

Chromatography Laboratory

HPLC

30/12/2021 11:45 ص	Chromatogram F:\ \st homocysteine 10 ppm PRM		Page 1 of 1
Sample Info:			
Sample ID	: st homocysteine 10 ppm	Amount	0
Sample	: st homocysteine 10 ppm	ISTD Amount	0
Inj. Volume [mL]	: 0.1	Dilution	1
Autostop	: 20.00 min	External Start	: Start - Restart, down
Detector 1	: Detector 2	Range 1	: Bipolar, 2000 mAU, 10 Samp. per Sec.
Subtraction Chromatogram: (None)		Matching	: No Change

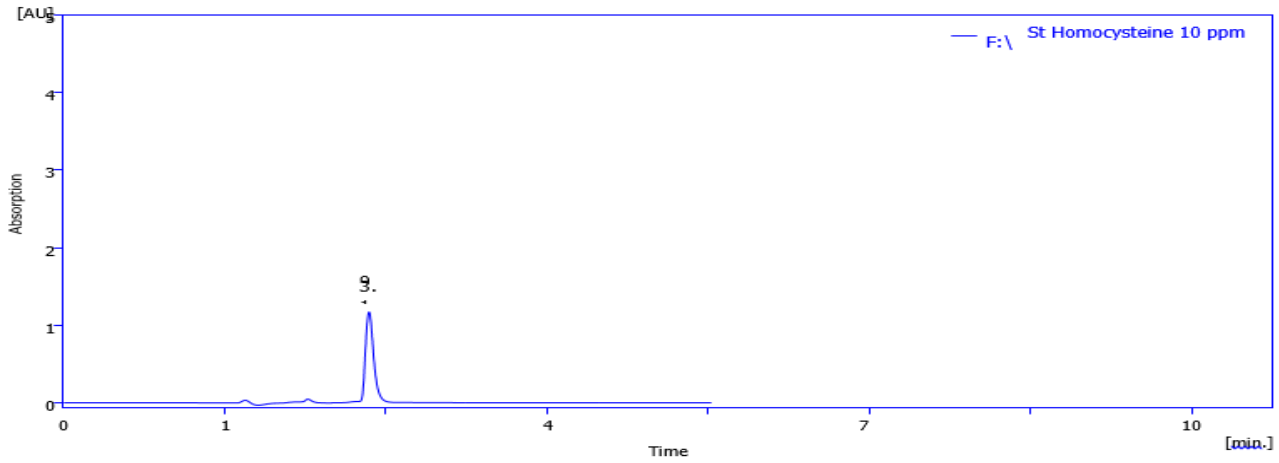


Table (Uncal - F:\ st homocysteine 10 ppm)							
	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W 05 [min]	Compound Name
1	3.19	803.255	957.223	100.0	100.0	0.10	
	Total	803.255	957.223	100.0	100.0		

4. Discussion

This investigation's purpose was to determine 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 (homocysteine) chromatographically. The usage of polar organic solvents and substances containing thiols, such as 3-MPA in the) Simons and Johnson (1978) [22, 23]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[24] Tornell et al. demonstrate[25] 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[26, 27] on the other hand, discovered that HPLC using the OPA / 2-ME combination was a good method for amino acid (homocysteine in this research) analysis in both patients and healthy people, with high specificity and reproducibility.

We discovered through our work and research in this area that compared to the RPL non-pregnant and control groups, the concentration of serum Hcy in the RPL pregnant group increased significantly (Table1). There was also a substantial rise in serum Hcy levels in the RPL nonpregnant group as compared to the control group. There were also previous studies reporting an increase in the homocysteine concentration in women who suffer from recurrent miscarriage compared with the control group[28]

The results showed an increase in homocysteine in pregnant women with recurrent pregnancy loss high levels of homocysteine are associated with vitamin B12 deficiency [29]. Then it refers to the assessment of food impacts. Genetic factors such as mutation in the methylene tetrahydrofolate reductase (MTHFR) enzyme gene, malnutrition, and vitamin B12 or folic acid malabsorption, The MTHFR gene mutation reduces enzyme activity and elevates blood homocysteine levels.[30]. Impairment of chorionic villous vascularization is linked to hyperhomocysteinemia [13]. Higher levels of homocysteine have been linked to more pregnancy problems and undesirable pregnancy outcomes, including abnormalities, particularly neural tube defects. [31]. The danger that comes from hyperhomocysteinemia and prenatal poisoning might be the cause [32] or homocysteine can potentially interact with hemostatic genetic determinants leading to increasing the thrombogenic potential [33]. Attributing to cellular methylation that affects gene expression or because

of the incorrect incorporation of uracil into the DNA, which causes damage, or because of the enhanced capacity of homocysteine to activate factor V and inactivate protein C, thrombomodulin, and heparin sulfate. DNA [32]. An endothelial cell surface glycoprotein called thrombomodulin encourages the activation of the anticoagulant protein C and prevents thrombin from acting as a procoagulant [34]. As a result, there will be less protein C activation, which might lead to thrombosis and disseminated intravascular coagulation.[35] homocysteine does not decrease thrombomodulin synthesis but directly inhibits the thrombomodulin cofactor activity and then the inhibition of thrombomodulin contributes to decreased protein C activation[36].

Source of Funding: The study's design, analysis, production of the article, or scholarly publishing were all done independently; financing or other outside influences were not involved.

Ethical Clearance: The regional ethics committee authorized the project (College of Education Pure Science.

Conflict of Interest - (nil).

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