Original Article

Evaluation of serum concentrations of homocysteine using high-performance liquid chromatography for patients with Preeclampsia

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Abstract

Preeclampsia is a condition that causes persistently high blood pressure during pregnancy or the postpartum period. It's frequently linked to excessive protein levels in the urine, new-onset blood platelet depletion, or indicators of brain difficulty including seizures and/or visual difficulties. It's detected by an increase in the expecting mother's blood pressure after the 20th week of pregnancy, and it's only seen in humans. Preeclampsia raises the risk of maternal and perinatal morbidity and death, whether it is isolated or combined with preexisting hypertension. Preventing maternal and fetal morbidity/mortality requires early detection of high-risk variables and biomarkers for preeclampsia. Preeclampsia causes pathophysiological changes both locally (defective placentation and decreased placental perfusion) and systemically (hypertension) (angiogenic imbalance and altered vascular endothelium function). Preeclampsia is characterized by an angiogenic imbalance, and angiogenic indicators might be useful tools for detecting and diagnosing afflicted pregnancies early on.

Keywords: Preeclampsia, serum concentrations, liquid chromatography.

1. Introduction

Preeclampsia is a condition that causes persistently high blood pressure during pregnancy or the postpartum period. It's frequently linked to excessive protein levels in the urine, new-onset blood platelet depletion, or indicators of brain difficulty including seizures and/or visual difficulties. It's detected by an increase in the expecting mother's blood pressure after the 20th week of pregnancy, and it's only seen in humans. Preeclampsia raises the risk of maternal and perinatal morbidity and death, whether it is isolated or combined with preexisting hypertension. Preventing maternal and fetal morbidity/mortality requires early detection of high-risk variables and biomarkers for preeclampsia. Preeclampsia causes pathophysiological changes both locally (defective placentation and decreased placental perfusion) and systemically (hypertension) (angiogenic imbalance and altered vascular endothelium function). Preeclampsia is characterized by an angiogenic imbalance, and angiogenic indicators might be useful tools for detecting and diagnosing afflicted pregnancies early on. (1)

Endothelial dysfunction and cardiovascular disease are connected to homocysteine, and excessive homocysteine levels have been associated to preeclampsia. (2) The exact etiology of preeclampsia (PE) is yet unclear. The missing link in the pre-eclampsia etiology might be serum homocysteine. There was a clear reduction in antioxidant activity and an increase in lipid peroxides in preeclampsia.

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Homocysteine levels in the blood decrease throughout normal pregnancy, paralleling physiologic albumin reduction and folic acid supplementation, but spike in pre-eclampsia. In a study on early pregnancy losses, hyperhomocysteinemia was observed to diminish total vascular surface and therefore early pregnancy loss. (3)

There are some conflicting findings of maternal circulating levels of homocysteine in women. Large bodies of studies report hyperhomocysteinemia in preeclamptic women compared with normal pregnant women 4-13. However, some studies have shown that there is no difference in circulating concentrations of homocysteine between mild preeclampsia and healthy pregnant women 4,11, and between mil and severe preeclampsia4,14. Therefore, a cross sectional study was designed to examine maternal serum levels of homocysteine in preeclampsia(4)

To establish illness pathophysiological pathways, homocysteine levels must be calculated. The study of these items is one of the most important practical undertakings in the world of biological and pharmaceutical sciences.. (Turnell D, Cooper J.1989)(5)

Chemical derivatives in the form of pre- or post-column columns have been used in conjunction with high-performance liquid chromatography to solve these problems (HPLC). One of the most commonly used methods is precolumn OPA derivation. Because of its fast interaction with the amino group of amino acids, this chemical is a crucial amino acid derivative agent, resulting in extremely light materials. Sulfhydryl agents have an effect on the stability of these compounds, and some research suggests that 3-mercaptopropionic acid (3-MPA) is more stable than 2-mercaptoethanolamine (2-ME). (Kaspar H, Dettmer K, et al.2009)(6)

To overcome these issues, several standard medical analysis devices, such as spectrophotometers like the ELISA system, can be used to do a laboratory study of amino acids (homocysteine). This instrument, however, is not available owing to a paucity of chemicals, high prices, and difficulties extracting them from the original source. To improve the precision of the task and to withstand any exceptional circumstances that may happen during laboratory work, high-performance liquid chromatography (HPLC) technology was employed to analyze the amino acids and use the reference compounds for use in the study. (2012, Zhang X, Zhao T, et al.)(7) (D. A. Skoog, D. West, et al., 2013)(8)

2. Material and methods

Blood samples (5 ml) were collected from (15) fasting patients women for a period of approximately (12) hours, and their ages ranged between (20-42) years, and were diagnosed clinically by specialized doctors; and (15) from healthy people as a control group, between 1/8/2021 and 15/9/2021 in Baqubah Teaching Hospital / Diyala Governorate. The samples (serum) were stored at -20 °C before the investigation to guarantee that the level of homocysteine in the blood serum could be assessed..

3. Sample preparation

Plasma proteins were precipitated using a 10% trichloroacetic acid solution (TCA). The supernatant was drained after centrifugation at 10000 rpm for 5 minutes, and filtering was done with a syringe filter with a pore size of 0.45 m. The components employed were of the purest possible quality. 250 liters of material were mixed with 500 liters of methanol to create the samples. The protein in the plasma is precipitated by adding 10% trichloroacetic acid (TCA), after which the solution is agitated out in a centrifuge for 5 minutes at 10,000 cycles, the clear solution is discarded, and the sediment is kept. All of the materials utilized are extremely pure. 5.4 gram powdered tetraborate was dissolved in 100 ml water to make the borate solution. To create the derivative solution, 2250 l methanol, 250 l buffer borate solution, and 25 l 3-MPA were added to 0.025 gm. OPA.

The samples were made by combining (250 l) of the sample with (500 l) of methanol in an incubator at laboratory temperature for 5 minutes, centrifuged with 5000 cycles for 5 minutes, and then combining (250 l) of the transparent solution with 100 l l of borate buffer solution. The amino acid homocysteine is determined by adding (50 l) of (OPA / MPA-3) solution to the sample, which is then kept at room temperature for two minutes before analysis.

4. Hplc condition

The tests were carried out at the Environmental and Water Laboratories of the Ministry of Science and Technology. Using the High-Performance Liquid Chromatography Technique (HPLC) and the model SYKAMN German, (Fahime Mohammad Abadi, Arezoo Mirfazeli, et al.2016) created a technique. At a flow rate of 1 ml/min, the mobile process comprised of acetonitrile, buffer, and DW (60: 10: 30), with C18-NH2 (25 cm * 4.6 mm) as the column separation. Florescence detector (Ex = 330 nm, Em = 445 nm).

After both of the sample solution to be separated and the mobile phase solution have been prepared and placed in the device's designated locations, the required separation column has been installed in the device's designated locations according to the type of separation and the material to be separated, and the mobile phase has been passed through the separation column for at least half an hour, the device injects a small amount of the sample solution to be separated, such that the detector sees chromatograms and the outcome is a top and a sign for each It's made up of the sample's components, and the area under the top of the separated material is computed and compared to the area under the top of a standard substance of the same concentration to determine the

separated material's concentration. If the separation is successful, each peak indicates a component of the mixture to be separated. To produce exceptional results, a high pressure of above 100 bars might be applied.

The results were collected in the form of chromatogram peaks (peaks) graphs 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, and this is an illustrative review of the results

Accounts

The homocysteine concentration in the samples was

calculated using the following formula :

$$C_{sam} = \frac{Cst\ xAsam}{Asat}$$

Where,

Csam = sample concentration (sample)

Cst = concentration of standard substance

Asam = apex area of the model

Ast = apex area of standard material

5. Results

Amino acids may be evaluated and separated utilizing a 1 ml/min flow rate with an excitation wavelength of 330 nm and an emission wavelength of 445 nm. The statistical values of women with Preeclampsia were compared to groups of healthy people, because the total number of samples studied with Preeclampsia 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 Preeclampsia, as well as healthy people to ensure that they were free of Preeclampsia, and the results were as follows: Table 1 shows homocysteine concentrations in Preeclampsia patients ranging from (µ g / ml 53.7 to µg / ml 58.65) and healthy women ranging from (µg / ml 1.13 to µg / m l 1.35)

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.

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

No	Control	Patient
1	1.14	56.48
2	1.13	57.89
3	1.33	56.44
4	1.14	58.41
5	1.25	56.55
6	1.14	57.48
7	1.13	56.44
8	1.35	55.78
9	1.14	54.69
10	1.32	58.65
11	1.30	54.47
12	1.21	57.46
13	1.25	54.69
14	1.24	53.56
15	1.33	54.68

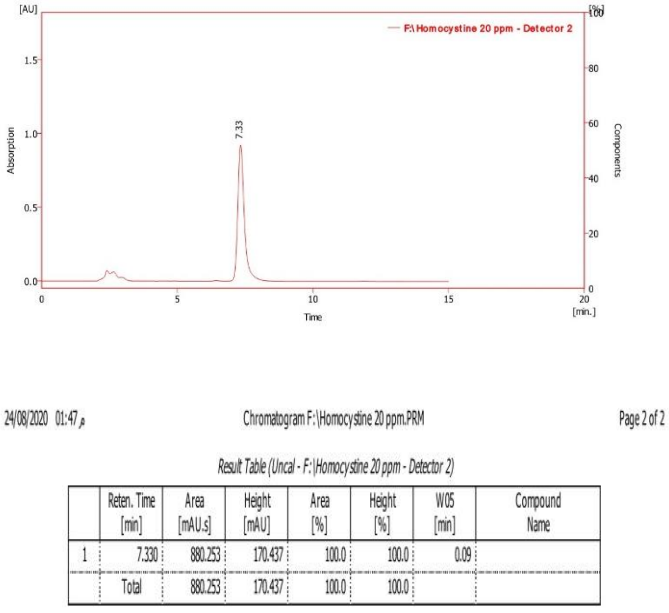
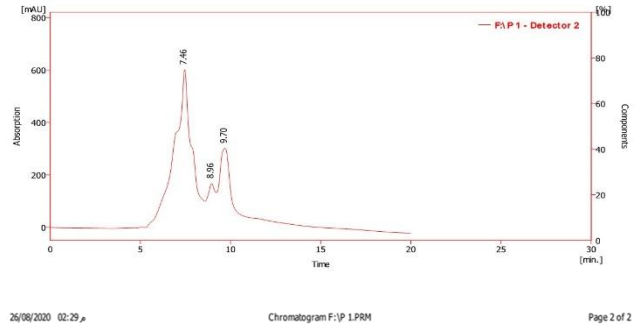


Fig. 1: A chromatogram of the homocysteine standard material



Result Table (Uncal - F:\P 1 - Detector 2)

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	7.460	16194.174	420.509	81.7	71.8	0.46	
2	8.957	498.954	37.188	2.5	6.4	0.22	
3	9.697	3117.370	127.601	15.7	21.8	0.41	
	Total	19810.498	585.298	100.0	100.0		

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

Because the value of their concentrations in the standard and in the selection, solution was given as an equal end value, all values of 100 and their units were treated as a percentage p percent while introducing the quantity of homocysteine into the standard solution of the computer. This allows you to easily compute the percentage obtained with the indicated quantity in the preparation by comparing the area of the standard solution's tops to the area of the test solution's tops. It's what he's used to working with.

6. Discussion

The goal of this work was to examine if homocysteine could be extracted from a combination of OPA-3MPA derivatives using reverse-phase chromatography (RP-HPLC). The data show that this method can chromatographically separate amino acids. The presence of polar solvents and thiol-containing chemicals such as 3-MPA in the) Simons et al (study to determine the structure of the OPA derivation indicated that these compounds have a considerable influence on the fluorescence property of isoindole derivatives. When coupled with borate buffer, these chemicals have a significant impact on fluorescence intensity. (SS Simons, DF Johnson.1978)(9)

Tornell et al. show that using methanol alone as an organic solvent boosts the chromatogram and amino acid separation solution precision in the sample preparation procedure, which is similar to the current research. Turnnell, D., and Cooper, J. (1982)(5).

TCA was used to denature the proteins in this work. To evaluate a rapid and effective method for amino acid analysis, Frank et al. employed HPLC technologies with pre-column derivation employing OPA / 3-MPA. They determined that this simple and optimized approach necessitates a small sample size and the use of an OPA / 3-MPA combination, which is similar to what is used in the current work. (Powers RW.2007, Frank MP)(10)

Using HPLC technology, researchers G. Minniti and A. Piana evaluated free and protein bound homocysteine in blood plasma. Initially, the sample was reduced with tri-n-butylphosphine, the proteins were precipitated with trichloroacetic acid (10%), and the derivatives were separated using an HPLC apparatus with the same inverted phase and fluorometric detection. A Hewlett-Packard system (HP 1090 LC) and an HP 1046-A fluorescence detector fitted with a column of 12534 mm, 5 mm column were used for separation and quantification. Excitation at 385 nm and emission at 515 nm were used to determine the fluorescence intensity.(11)

The researcher Ubbink JB (12) measured total homocysteine (tHcy) concentrations using HPLC and fluorometric detection, which is the most commonly used method for determining tHcy concentrations, using monobromobimane and ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F) is highly reactive and fluorescent towards thiols and thus requires complex chromatography Because thiol is photosensitive, it must be protected from light in order to produce valid findings. Electrochemical detection frequently use HPLC.

Andersson et al. (13) developed an HPLC method for measuring total homocysteine using di-ion chromatography followed by post-column derivatization and spectroscopic detection with good accuracy (1.5% within the intra-batch; 2.5% between the inter-batch), and sensitivity Less than 50 nm, dual-ion reverse phase chromatography was used at PH = 2.4, post-column derivatization with -dithiopyridine 4,4, and chromatography at 324 nm. With a smaller sample size, shorter duration, and little dithiothreitol (DTT), Powers HJ, Moat SJ (14) and Tuschl K.et al (15) improved the tandem MS approach. The feedstock, however, was only 49% at 8.9 M, and the LOQ limit of quantification was around 4 M. Nexo et al and colleagues (38) discovered two immunological methods: fluorescent polarization immunoassay (FPIA) and enzyme-linked immunosorbent assay (EIA)) in their novel homocysteine measurements for the European Demonstration Project, which involved six sites in four countries.They were compared with HPLC and gas chromatography-mass spectrometry (GC-MS), they considered linearity and accuracy in five plasma samples, established a correlation using patient samples and evaluated long-term performance, the results obtained by GC-MS were set as a standard for comparison. . In general, all the linear methods showed in a wide range at 5-45 μm and FPIA method showed low inaccuracy in all ranges while the EIA method was characterized by high throughput and small sample size, and more accurate if manual EIA is used then EIA is more suitable for screening purposes.

This analytical technique has chosen a pH value for the mobile phase and a flow rate for the liquid phase of 1 ml/min via our research. Through the vandimeter relationship, and by presenting the standard chromatogram of homocysteine and comparing it to the chromatograms of the examined samples,

it is a known flow rate in HPLC, with its precision and speed.We see a large congruence in the times of detention as well as a small standard deviation, which confirms that the measurement accuracy is high in this type of analysis, and we see a significant difference in the results when we compare homocysteine concentrations in standard solutions to sample solutions. The levels of homocysteine concentrations in the first table, as well as the other tables, were higher than the values coming from the conventional comparison approach for healthy adults. As seen in Table (1)

We can conclude from the foregoing that the HPLC method is preferred over other methods in the analysis of samples, such as homocysteine, for a variety of reasons, including: it does not require a long analysis time, it has a low economic cost when compared to non-HPLC methods using a syringe containing 20 microliters of the studied solution, and it can be repeated many times with great confidence and accuracy, whereas when using non-HPLC methods, the analyzes can In addition to the interactions that occur while assessing an acid in a mixture of acids, these approaches are unfavorable due to the length of time it takes to separate and analyze each acid separately. The analyst must remember that interferences that can arise when employing non-HPLC techniques in the analysis, which obstruct the measurement process, can occasionally result in the entire analysis method failing.

Now we'll go over some of the benefits of working through this work and some of the researchers' work using HPLC technology and other techniques, as well as the differences and similarities between the pathological condition and the causes of preeclampsia and its diseases, which are still of interest to researchers. Using biophysical, biochemical, and spectroscopic approaches, several attempts have been made to find biomarkers of pre- and post-eclampsia. Preeclampsia blood serum was analyzed using high-performance liquid chromatography (HPLC) and a fluorescence detection technique, which revealed low and high amounts of free amino acids, particularly homocysteine levels, which were considerably raised in preeclampsia patients.

The determination of homocysteine shortage or excess in plasma or serum has become a critical diagnostic process that necessitates the use of precise, quick, and low-cost detection technologies. Researchers Birte Vester and K. (16) Rasmussen worked in this field with groups of researchers and scientists in order to arrive at the approaches we indicated previously, and the outcomes were positive. Researchers G. Minnitia and his colleagues worked on the same field seven years later, and good findings were obtained despite certain technical challenges in the effort (11). In terms of health, homocysteine has a significant impact on the health of the pregnant mother and her child, as evidenced by some studies. For example, Hoque et al. (17) found that homocysteine levels increased significantly in preeclampsia, but that this increase was the most dominant in preeclampsia, which is consistent with our findings.

according to Singh U. et al .did yet another research (18) Endothelial dysfunction of the arteries involved in placental infarction and placental transit abnormalities, which leads to abruption and recurrent miscarriage, fetal development limitation, and anxiety, were shown to be the cause of hyperhomocysteinemia,.

While some studies have indicated that homocysteine levels are lower in the early third trimester than during pregnancy, the differences between primary subjects and controls in our investigation are likely to be underestimated. Because prior research has indicated that hyperhomocysteinemia early in pregnancy might raise the risk of preeclampsia later in pregnancy, measuring homocysteine levels is suggested. Because of the lack of vitamins associated with hyperhomocysteinemia, such as folic acid deficiency and vitamins B6 and B12, it is recommended to take the above-mentioned supplements in the blood during pregnancy and even before pregnancy, and these findings may be useful for further research into the causes and consequences of high homocysteine in preeclampsia, as well as its potential intervention. (19)

All of these studies, research, and ongoing efforts have proven the validity of hypotheses based on the direct influence on the mother's and child's health caused by pre-eclampsia, which affects mostly pregnant and post-pregnancy moms. Severe preeclampsia causes multiple deaths and morbidity in both mother and child, especially when it occurs before the due date of natural childbirth, which is between 24 and 34 weeks of pregnancy (end of the sixth month and beginning of the second half of the ninth month), and treatment is difficult to come by. This disease's only known symptom is delivery. Some obstetricians urge for early labor to avoid major consequences for the mother, including as eclampsia (fits) and renal failure, while others favor a more predictable strategy to labor deferral to lower the child's mortality and morbidity associated with an early birth.(20)

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