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Research Article

REVERSED PHASE HPLC DETERMINATION OF TOTAL HOMOCYSTEINE, CYSTEINE, CYSTEINYL GLYCINE, GLUTATHIONE IN PLASMA OF EPILEPTIC PATIENTS

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ABSTRACT

The fast and accurate reversed-phase HPLC method was predicated on qualitative and quantitative estimation of the total concentrations of the essential thiols in the plasma of adults volunteers compared with epileptic patients, which is l-cysteine (Cys) total homocysteine (tHys), cysteinyl glycine (Cys-Gly), glutathione (GSH) and cysteamine (Cstm), respectively. The plasma samples of (50) epileptic patients were classified into three groups; the non-anti-epileptic drug consisted of 20 patients (12 male, eight female) who were mainly diagnosed with idiopathic generalized epilepsy. The mean age was 34 ± 13 (range 19–48) years. The second group consisted of 30 patients receiving two types of anti-epileptic drugs, 18 patients (12 male and six female) receiving Carbamazepine, and 12 patients (7 males and five female) receiving phenytoin. The mean age of the patients was 32 ± 11 Compared with 31 healthy volunteers (20 male and 11 female). The mean age of the controls was 31 ± 9 (range 26–49 years). The mean duration of treatment of patients receiving the anti-epileptic drug was six months). All measurement was done using pre-column derivatization with bromobimane, and the derivative of each standard mixture were baseline separated on C18-DB (50 x 4.6 mm ID) column, 3µm particle size. The regression coefficients for the separated and standard deviation SD within-run ranged from 0.09 to 8.40 µmol/L, and between-run ranged from 0.15to 9.16 µmol/L; the analytical procedure gave good linearity in the range between 1.25 to 20 µmole/ l, the detection limit was 0.1 µmole /L for all the thiol groups. Analytical

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recovery was 96.9–107.4 %; the mean concentration of plasma cysteine and total homocysteine was slightly higher in males than females; it was 221± 75 for adult males and 190± 44 µmol/L for adult females, while t-homocysteine was (10.55 ±2.45 vs. 9.79 ± 1.88 µmol/L, the results observed that the value of cysteine and homocysteine were significantly higher in epileptic patients using Carbamazepine and phenytoin than in healthy volunteers. Mean values for glutathione were lower, while cysteinyl glycine showed no significant difference in healthy and epileptic patients and no sex- and age-dependent.

KEYWORDS

HPLC; Homocysteine; Epilepsy; Anti-epileptic drugs.

INTRODUCTION

Homocysteine, an amino acid containing sulfur, is produced because of methionine metabolism. Prolonged administration of anti-epileptic drugs frequently leads to the development of hyperhomocysteinemia in patients. Previous investigations have explored the effects of valproic acid, lamotrigine, and levetiracetam on plasma concentrations of homocysteine, aminothiols, and glutathione.(1). Homocysteine can undergo catabolism to cysteine or remethylation to methionine (1,2). Precursors of glutathione include cysteine and γ-glutamyl cysteine. When glutathione breaks down through the cystathionine beta-synthase enzyme, it results in homocysteinemia and significantly increased levels of homocysteine in the bloodstream. Patients with homocystinuria may experience up to a 20% increase in seizures (2). Mildly elevated levels of homocysteine can arise as a result of deficiencies in certain vitamins. This form of hyperhomocysteinemia has been widely recognized as a significant risk factor for vascular disease, owing to its ability to induce damage to vascular endothelial cells and expedite the progression of atherosclerosis. Furthermore, it exerts an influence on the blood coagulation system, giving rise to a prothrombotic state (2, 3).

Cysteine metabolism disorders encompass conditions such as cystinosis, which is an autosomal recessive disease resulting from defects in lysosomal transport (3), and cystinuria, a prevalent hereditary disorder that impacts the transport of amino acids (4). Cysteamine is employed to deplete cystine from cells affected by nephropathic cystinosis in order to treat children with this condition (5). In cases of cystinuria, sulfhydryl-containing compounds like 2-mercaptopropionylglycine (MPG) can reduce cystine excretion (6).

Elevated levels of homocysteine in the plasma present a substantial risk for a range of diseases, including arterial and venous thrombosis, myocardial infarction, and chronic renal insufficiency. Hyperhomocysteinemia has been linked to cardiovascular diseases as well as specific anti-epileptic drugs (7). Prolonged administration of these medications can also result in hyperhomocysteinemia and reduced serum concentrations of folate, vitamin B12, and vitamin B6, all of which play crucial roles in homocysteine metabolism.

Conversely, specific anti-epileptic drugs including carbamazepine, phenytoin, phenobarbital, and primidone have the potential to induce hepatic

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enzymes (9) and activate hepatic cytochrome P450 and glucuronyl transferase (10).

The measurement of reduced (GSH) and oxidized glutathione in plasma is imperative for assessing the cellular and tissue redox and detoxification status. This assessment assists in predicting the protective function of glutathione against oxidative damage induced by free radicals (11).

Determining the concentration of major thiols in plasma using an amino acid analyzer is time-consuming. However, a highly accurate and sensitive high-performance liquid chromatographic (HPLC) method has been developed to simultaneously measure thiol groups in serum, plasma, and urine (12,13,14).

In this paper, we present a rapid HPLC method that utilizes 3µm particles in a highly efficient short (5 cm) column chromatography for quantitatively determining total homocysteine levels as well as cysteine, cysteinyl glycine, and glutathione levels in plasma. This method is particularly useful for monitoring drug therapy involving cysteamine. Additionally, we investigated the impact of two commonly used anti-epileptic drugs (phenytoin and carbamazepine) on homocysteine metabolism.

Materials and Methods

Chemicals:

Chemical standards including l-Homocysteine, l-cystine, cysteinyl glycine (reduced form), glutathione (reduced form), and cysteamine were procured from Sigma. NaBH4, EDTA, 1-octanol, hydrochloric acid, formic acid, acetic acid, acetonitrile of HPLC grade, bromobimane, ammonium nitrate, ammonium formate, and NaOH were also sourced from Sigma Chemical. Dimethyl sulfoxide was obtained from Aldrich Co. The Phenomenex ODS column with dimensions of 50 × 4.6 mm ID and a particle size of 3 µm was acquired from Super.

Sample collection:

Following an overnight fasting period, blood samples were obtained from both healthy volunteers and epileptic patients between the hours of 8 and 10 a.m., prior to their morning administration of the anti-epileptic drug. Venous blood was drawn from the antecubital vein while the subjects were in a seated position. One milliliter of blood was collected in EDTA-containing tubes. These tubes were promptly cooled on ice and centrifuged within 15 minutes at 2000g for a duration of 10 minutes. To minimize the release of Homocysteine from blood cells, plasma was rapidly separated and subsequently frozen at -20 °C until HPLC analysis, which took place within two weeks.

Pre-column derivatization The derivatization procedure was conducted as follows:

In a centrifuge vial, the following components were added: 10 µL of 4 mol/L NaBH4 (dissolved in a solution comprising 333 mL/L dimethyl sulfoxide and 66 mmol/L NaOH), five µL of a solution containing two mmol/L EDTA and two mmol/L DTT (dithiothreitol), five µL of 1-octanol, and five µL of 2 mol/L HCl.

Subsequently, this mixture was transferred to a derivatization vial that contained ten µL of plasma. After allowing for an incubation period of 1 minute at room temperature, an additional 25 µL of N-ethyl morpholine buffer (2 mol/L, pH 8.0) and 10 µL of 25

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mmol/L bromobimane (in a ratio of 1:1 acetonitrile/H2O by volume) were introduced to the derivatization vial.

Following another minute of incubation, a portion consisting of 20 µL from this mixture was loaded into the column.

Chromatography:

The separation process was carried out using a Shimadzu 2010A liquid chromatogram equipped with a binary delivery pump model 2010. The eluted peaks were monitored by a fluorescence detector 2010A, which operated at an excitation wavelength of 390 nm and an emission wavelength of 478 nm. The data obtained were analyzed using the Shimadzu Chromatopack-C-R8A software.

For the separation of the derivatized sample, a reversed-phase (50 × 4.6 mm ID Phenomenex ODS) column was employed. The column was initially equilibrated with a buffer consisting of 30 mmol/L ammonium nitrate and 40 mmol/L ammonium formate at pH 3.6 (buffer A), along with acetonitrile (B). Thiols were eluted from the column using a linear gradient ranging from 10% to 100% buffer B over eight minutes, at a flow rate of 1.5 mL/min. The column was equilibrated for five minutes before each run and maintained at a temperature of 30°C. Retention times for each analyte were determined using external calibrators at three different concentrations.

To assess recovery and precision, various concentrations of cysteine, cysteinyl glycine, glutathione, homocysteine, and cysteamine were spiked into plasma samples. Concentrations in the biological samples supplemented with calibrators were determined in three replicates, and analytical recoveries were calculated (Table 3). Intra-assay precision was evaluated by analyzing three replicates of the same sample on the same day, while inter-assay precision was determined by analyzing the same samples on five consecutive days (Table 2). Results indicate that intra-assay precision exhibited relative standard deviations ranging from 0.08 to 5.2 µmol/L, while inter-assay precision ranged from 0.12 to 8.54 µmol/L.

Linearity and the limit of detection were assessed using a serial dilution method of the stock standard for each sample. The assays were conducted within specified ranges: 1.5–300 µmol/L for cysteine, and 0.625–100 µmol/L for cysteinyl glycine, glutathione, homocysteine, and cysteamine. Correlation coefficients ranging from 0.9945 to 0.9999 were obtained, indicating good linearity. The limit of detection for calibrator samples, defined as the concentration producing a signal-to-noise ratio greater than 5, was approximately 0.1 µmol/L for all analytes.

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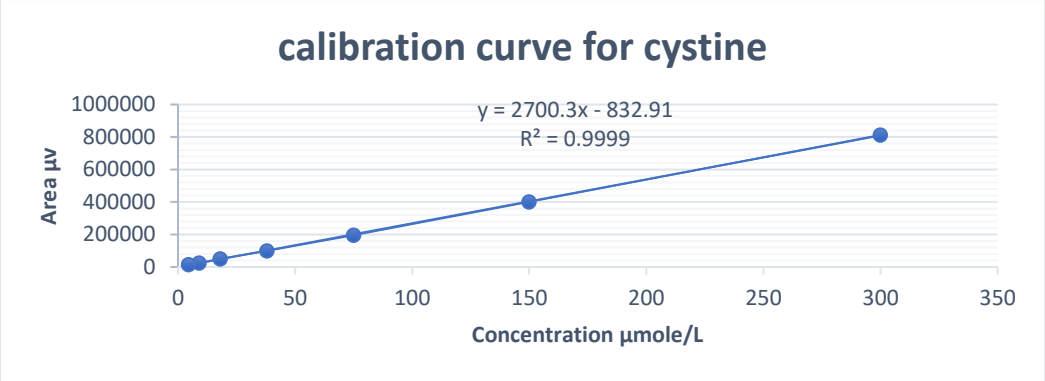

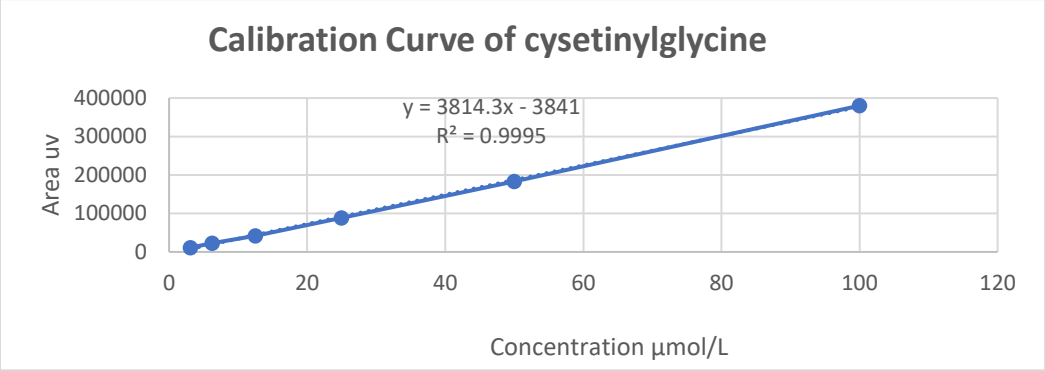

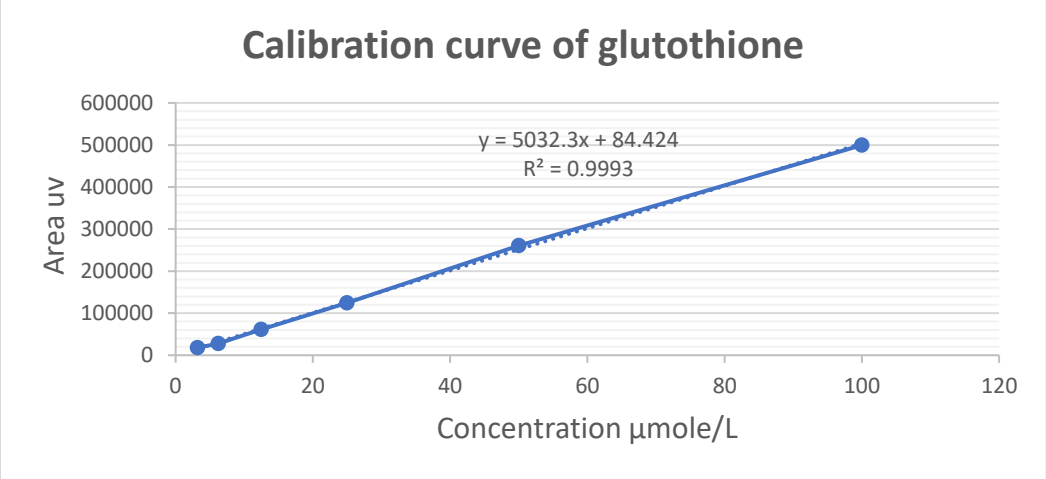

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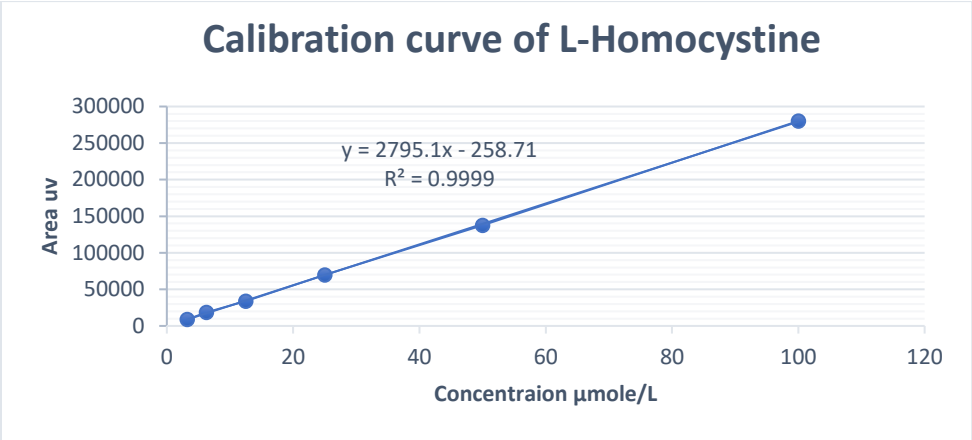

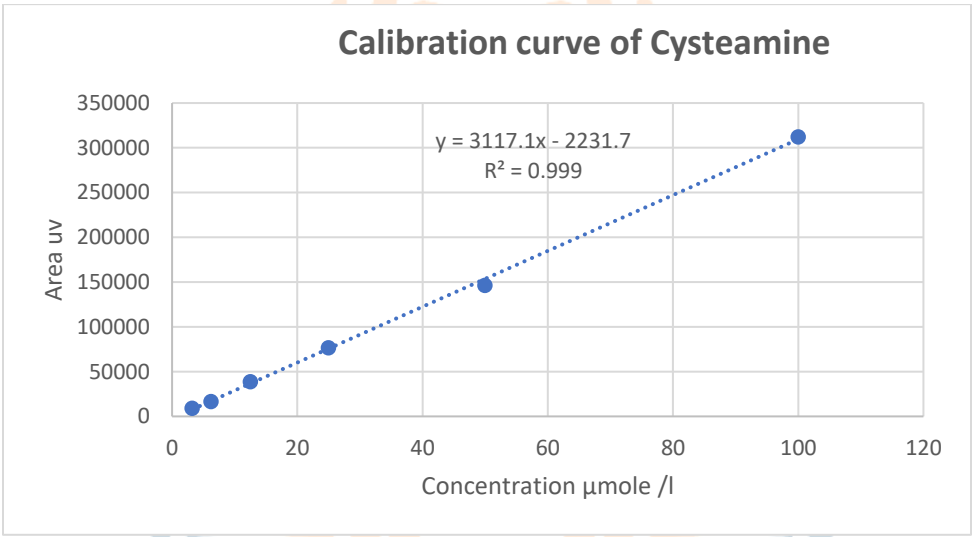

Fig 1: Calibration curve of each thiol at different concentrations with regression equation used for calculating each concentration in the sample.

Table 1: Levels of homocysteine and thiols groups for control and epileptic patient's.

groups	tHcy (µmol/L)	cys (µmol/L)	Cys-gly (µmol/L)	GSH (µmol/L)
Non-anti-epileptic drug group n=20	11.32±3.87 n=18 M	243±29	63.5 ±7.4	11.87 ±5.65
	9.8± 2.97	193 ±39	57.98 ±5.82	11.73±4.65

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	n=12 F			
Phenytoin (n =12)	18.32±3.21 n=7 M	264 ±48	59 ±16	11.6 ±6.41
	16.89±3.22 n=5 F	201 ±55	60.97 ±23	11.08 ±6.01
Carbamazepine (n = 18)	20.08± 6.7 n= 12M	275 ±33	54.67 ±5.94	10.87±4.88
	18.43±5.74 n=6 F	211 ±47	54.77 ±4.97	9.89.50 ±4.13
Controls (n = 31)	10.55±2.45 n=20 M	221 ±75	57.7 ±6.87	12.96 ±5.13
	9.78±1.88 n=11 F	190 ±44	56.3 ±6.44	12.32 ±3.77

Table 2: Regression analysis data of standard calibration from 1.5 to 300 µg/g for cystine and 0.625-100 for other thiol .from regression program we applied to get linearity and LOD.

Subjects	Linearity range µg/ml	Regression equation	R^2	S/N	LOD µg/g
Cystine	1.5-300	Y=2700.3 X - 823.9	0.9999	0.0166	0.10
Cysteinyl-glycine	0.625-100	Y=3814.3 X - 3841	0.9990	0.0166	0.10
glutathione	0.625-100	Y=5032.3 X -84.4	0.9993	0.0166	0.10
Homocysteine	0.625-100	Y= 2795.1 X - 258.7	0.9945	0.0166	0.10
cysteamine	0.625-100	Y=3117.1 X - 2331.7	0.9999	0.0166	0.10

Table 3: Precision assay of homocysteine and thiols in plasma of healthy volunteers.

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subject	Intra-assay n=3		Inter-assay n=5	
	Mean µmole/l	SD Mean µmole/l	Mean µmole/l	SD Mean µmole/l
cysteine	256	5.2	254.2	8.54
Cysteinyl-glycine	59.7	1.10	56.6	2.02
glutathione	12.32	1.38	12.02	0.54
Homocysteine	10.12	0.32	10.43	0.72
cysteamine	3.06	0.08	2.98	0.12

The Recovery studies of spiked the blank with different concentration and recalculated the found concentration from the chromatograms, using the fallowing equation:

Recovery %= found concentration from the chromatogram / added concentration x 100

Table 4: Recovery of homocysteine and thiol group in plasma of healthy volunteers.

subject	µmole/l			
	Origin sample	added	measured	Mean % recovered
cysteine	101	300	399	98.01
cysteinyl-glycine	66	150	217	101.15
glutathione	12.1	83	96.0	107.40
Homocysteine	9.8	83	92.5	96.9
cysteamine	0.0	83	81.5	98.19

A- Standard **B- epileptic Patient Sample**

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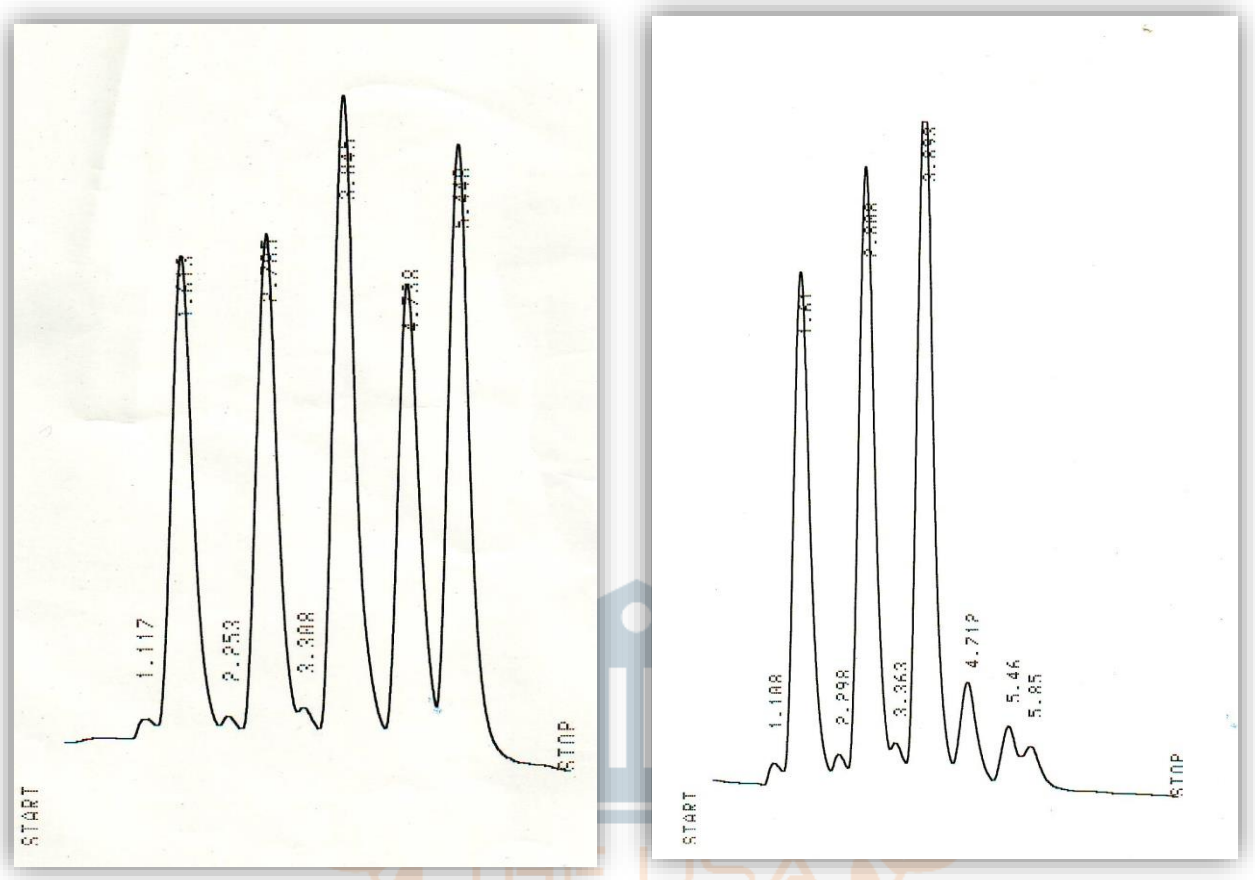

1- Rt= 1.63 min cystine.

2- Rt= 2.785 min cysteinyl glycine.

3- Rt= 3.845 min glutathione.

4- Rt= 4.738 min Homocysteine.

5- Rt= 5.448 min cysteamine.

Fig 2: Separation of total Homocysteine and thiol groups on reversed-phase column (50 x 4.6 mm ID) mobile phase 0.01M potassium Hydrogen Phosphate: acetonitrile (92:8, v/v), the eluted compound monitored by fluorescence, excitation 273 nm and 428 nm emission flow rate 1.5 ml/ min.

RESULTS AND DISCUSSION

In our study, a fast liquid chromatographic column (FLC) was utilized with a particle size of 3 µm and a length of 50 mm. This method allowed for baseline separation of all thiol groups in just 5 minutes, as demonstrated in Figure 2.

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The concentrations of total plasma homocysteine, glutathione, cysteinyl glycine, and cysteine were determined in 31 healthy volunteers and 50 epileptic patients. The patient groups were classified as follows: non-anti-epileptic drug users (n=20), phenytoin users (n=12), and Carbamazepine users (n=18). It was observed that the total plasma homocysteine concentrations in the control group (n=31) were slightly higher in males than females.

Plasma cysteinyl glycine did not show significant changes among the patient groups. However, glutathione concentrations were slightly decreased in epileptic patients.

The mean levels of homocysteine and cysteine were significantly higher in the antiepileptic drug group compared to the non-anti-epileptic drug and control groups. In the Carbamazepine group, the mean value of homocysteine ranged from 20.08 to 18.43 µmol/L for males and females respectively, while the mean value of cysteine ranged from 264 to 211 µmol/L for both sexes. Similarly, in the phenytoin group, the concentration range for homocysteine was 18.32 to 16.89 µmol/L for males and females respectively, while for cysteine it was 264-211 µmol/L for both sexes. These values were significantly higher compared to the non-anti-epileptic drug and control groups, as shown in Table 1. There was a significant difference in homocysteine and cysteine levels between the non-anti-epileptic drug group and the control group for both males and females.

Previous research has suggested a possible link between increased homocysteine levels and epileptogenesis. Patients with high plasma concentrations of homocysteine (50–200 µmol/L) have been reported to experience frequent seizures. It has also been suggested that seizures cannot be optimally controlled when homocysteine levels exceed 20 µmol/L. Our study aligns with previous reports, as we found high homocysteine concentrations in patients receiving monotherapy.

Furthermore, our data reveals a significant correlation between elevated homocysteine levels and the use of antiepileptic drugs in patients with epilepsy. The study encompassed two groups, namely epileptic patients who were not receiving antiepileptic drugs and a control group consisting of healthy individuals. The findings showed slightly higher levels of homocysteine in epileptic patients not undergoing drug treatment compared to the control group. This implies that antiepileptic drugs have a more substantial impact on the development of hyperhomocysteinemia in epileptic patients than the underlying diseases.

The primary objective of this research is to elucidate the influence of phenytoin and Carbamazepine on homocysteine metabolism. These medications, known as enzyme inducers, are proposed to directly modulate liver enzyme activity. This modulation potentially results in the depletion of cofactors involved in homocysteine metabolism, such as folic acid, pyridoxal 5′-phosphate, and Vitamin B12. Consequently, these observed changes play a crucial role in altering homocysteine levels.It is well-known that patients on phenytoin often have low serum folic acid levels. Previous reports have also indicated an association between carbamazepine use and low folic acid status.

Previous studies involving patients with hyperhomocysteinemia who received antiepileptic drugs along with supplementation of pyridoxine, riboflavin, and folic acid for one month showed a decrease in all redox products. This suggests that common antiepileptic drugs have unfavorable effects on homocysteine levels. However, there was only a slight change in homocysteine concentrations among

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epileptic patients who were not receiving antiepileptic drugs.

CONCLUSION

In conclusion, the use of antiepileptic drugs in patients is associated with an anticipated occurrence of hyperhomocysteinemia. Previous studies have indicated that elevated levels of homocysteine can be effectively reduced through supplementation with its cofactors, namely folic acid, Vitamin B12, and Vitamin B6. It is theorized that by lowering plasma homocysteine concentrations to below ten µmol/L, it may be possible to prevent up to 25% of cardiovascular diseases. Given the prevalence of patients receiving antiepileptic drug therapy, routine screening and treatment for elevated homocysteine levels are recommended.

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Disclosure:

The authors stated that they do not have any conflicting interests.

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