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Determination of reduced glutathione by High Performance Liquid Chromatography in patients with renal stones and Type 2 Diabetes Mellitus

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Abstract : High- Performance Liquid Chromatography Technique of Fluorescence Detector (HPLC-FLD) was used for the detection of sulfur glutathione disulfide (GSSG) based on a derivatization glutathione with ortho-phthalaldehyde (OPA) at pH 12. This technique is characterized by high sensitivity, high specificity , and high selectivity for many of the compounds and in particular the glutathione in serum samples of patients with renal stones. Standard glutathione (20µl, 10mg/dL) was injected and measured at reference conditions to set and fix the real retention time (RT), then 20µl of patent serum samples were injected and measured at the same conditions. Conditions of separation were; acetonitrile: H2O (60:40 ml) as isocratic mobile phase, column C18-ODS (25cm x 4.6 mm x 5µm), 20 µl injection volume of sample, and flow rate 1.0 ml/min at 35ºC through Ex= 350 nm Em = 450 nm Florescence detector Spectrophotometer.
The study showed that significant differences in glutathione levels among control samples, samples of patients with renal stone alone and samples of patients with renal stone and type 2 diabetes mellitus. The result was explain the role of glutathione as antioxidant is present in the pathogenesis of nephrolithiasis.
Keywords: Nephrolithiasis, HPLC-FLD, Glutathione, Type 2 DM.

Introduction

Nephrolithisis is common clinical disorder affecting up to 5% of the general population in the USA[1]. The prevalence of renal stone disease has been rising in both sexes, being estimated that about 5% of American women and 12% of men will develop a kidney stone at some time in their life[2]. Nevertheless, in certain areas of the world, as in the Middle East, the lifetime risk appears to be even higher[3]. There has been heightened awareness of renal stone disease in children as well[4]. Recurrence rates of 50% after 10 years and 75% after 20 years have been reported[5,6]. Renal stones are aggregates of crystals that are formed in supersaturated urine (urine is usually supersaturated in terms of its salt components).

Renal stones come in different types, and the formation of a specific stone-type depends upon the presence of particular risk factors. 1) The most common renal stone, and a main component in stones of mixed composition, is calcium oxalate. This type may occur as multiple stones or may recur, can induce pain with both passage and obstruction, and is commonly caused by treatable metabolic disorders of hypercalciuria. 2) Similar to calcium oxalate stones, uric acid stones induce the same adverse effects but differ with their rarer occurrence (only 5% of renal stones). Uric acid stones are also translucent and, unlike the other stones, cannot be distinguished by radiographs. 3) Struvite stones are generated by infections of urease-containing microorganisms that are capable of hydrolyzing the urea in urine to carbon dioxide and ammonia. When urine

pH exceeds 7.2, struvite stones may form, and the resulting obstruction can fill the renal collection system and erode into the renal tissue[7,8].

Clinical manifestations are characterized by lumbar pain of sudden onset (the location of pain depends on the location of stone in the urinary tract) that may be accompanied by nausea and vomiting, gross or microscopic hematuria Diagnosis of renal stone in the acute setting is beyond the scope of the present update but in brief, is represented by urinalysis and imaging. Urinalysis often reveals hematuria but the latter is absent in approximately 9% of cases[9]. Crystalluria is occasional and the presence of leucocyturia may suggest associated urinary tract infection. Unenhanced helical computed tomography (CT) scan, the most sensitive and specific radiographic test [10,11] , is becoming the diagnostic procedure of choice to confirm the presence of kidney and especially of ureteral stones [12] . of radiation and elevated costs must be considered [13] . Since renal ultrasound (US) provides information about obstruction [14] but may miss ureteral stones, the association of US with conventional abdominal X-ray may help [15]. Renal colic must be differentiated from musculoskeletal pain, herpes zoster, pyelonephritis, appendicitis, diverticulitis, acute cholecystitis, gynecologic disease, ureteral stricture or obstruction due to blood clot, polycystic kidney disease.

Stone formation usually results from an imbalance between factors that promote urinary crystallization, and those that inhibit crystal formation and growth[16]. The main determinants of calcium oxalate (CaOx) supersaturation are oxalate and calcium concentration, while the latter associated to urinary pH determines calcium phosphate supersaturation. Urinary pH itself is the main determinant of uric acid supersaturation [17].

Type 2 diabetes is the result of failure to produce sufficient insulin and insulin resistance. Elevated blood glucose levels are managed with reduced food intake, increased physical activity, and eventually oral medications or insulin. Type 2 diabetes is believed to affect more than 15 million adult Americans, 50% of whom are undiagnosed. It is typically diagnosed during adulthood. However with the increasing incidence of childhood obesity and concurrent insulin resistance, the number of children diagnosed with type 2 diabetes has also increased worldwide[18].

Glutathione (GSH) is an important tripeptide thiol (g-glutamyl cysteinyl glycine) antioxidant (Figure 1) and widely distributed in biological fluids and tissues [19]. It is involved in many physiological functions, such as the detoxification of xenobiotics, the transport of amino acids, the stabilization of cell membranes, and the synthesis of proteins and DNA. It plays an essential role in protecting cells from the toxic effects of oxidizing agents, ionizing agents, and free radicals. Altered glutathione concentrations may play an important role in various diseases and/or pathological conditions, such as inflammation[20], autoimmune disorders [21], hepatic damage[22], sepsis, apoptosis[23], cancer, and chronic diseases[24]. GSH is an important biomarker of oxidative/nitrosative stress, and it has been widely used in clinics[25].

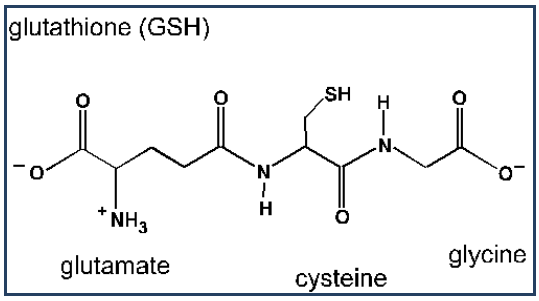


Figure 1 : The chemical structure of glutathione

Materials and methods

A. Patients

Patient groups consist of (80) samples from (30) women and (50) men, divided into two groups. First group (A) consist of (n=40) samples from (15) women and (25) men with renal stone while second group (B) were (n=40) samples from (15) women and (25) men with renal stone and type 2 DM. The age of patients were ranged from (30 - 65) years.

All samples were collected from Urology Center in Al Hilla Teaching Hospital in Hilla City, Based on the history: patients with positive history of renal stone, patients with radio-opaque stone or radio-lucent stone ultra sonic, KUB and/or CT scan examination. The patients were free from chronic diseases except type 2 DM. Pregnancy women and patient under renal medical treatments were excluded. All patients underwent full history and physical examination.

Control groups (C) consist of (n= 40) samples of healthy volunteers from (15)women and (25)men . They were collected from medical staff who were free from signs and symptoms of renal stones, age ranged from (30 - 65) years, all of them were non-smokers , free from DM, hypertension and no family history of renal stones .

B. Samples Collection

Fasting blood samples were collected, and used with plain tubes were subjected to centrifugation at 3,000 rpm for 10 min at 4°C to obtain serum. Serum were stored at (-20°C) immediately after separation in multiple eppendorf till analysis.

C. **Chemicals:**

Chemicals that have been used in the present study obtained from thoughtful international companies were high purified as follows; acetonitrile, methanol, ethanol, and distilled water for HPLC. Another chemicals were used in this study as follows; standard glutathione and Ortho-Phthalaldehyde (OPA).

D. **Instrument:-**

Instrument that have been used in the present study were modern and sophisticated analysis instruments as follows; Preparative and Revers Phase-High Performance Liquid Chromatography (Pre-HPLC,RP-HPLC) with fluorescence detector, Lovibond pH meter 200, Magnetic Stirrer with Hot plate, Sensitive Balance, Centrifuge Hettich EBA 20, and Water Bath Grant.

Methods:-

A. Detection,Qualitative and Quantitative Determination of Glutathione Using HPLC-FLD

Standard material and Samples were analyzed by High Performance Liquid Chromatography (HPLC) system, SHIMADZU model 10AV-LC equipped with binary delivery pump model LC-10AV, the eluted peaks were monitored by fluorescence detector FLD-20A. The condition of separation are listed in table (1)**.**

B. Preparation and analysis of standard solution

1. A standard solution of GSH was prepared by weighing out the desired mass of 10 mg dissolved in the methanol grade for HPLC up to 100ml .
2. A weight of 0.50 mg of OPA was dissolved in absolute methanol, and adjusted by NH4OH (5N) to pH=12 and brought to a final volume of 5 mL.
3. A volume of 50 µL of standard was added to (20µL) of OPA and vortex for 1 minute.
4. 20 µl of mixture was injected into HPLC.
5. Obtaining results that contain the peak top, retention time, and area under the peak for standard as it shown in Figure (2).
6. Make the necessary calculations.

C. Preparation and analysis of control and patient samples :

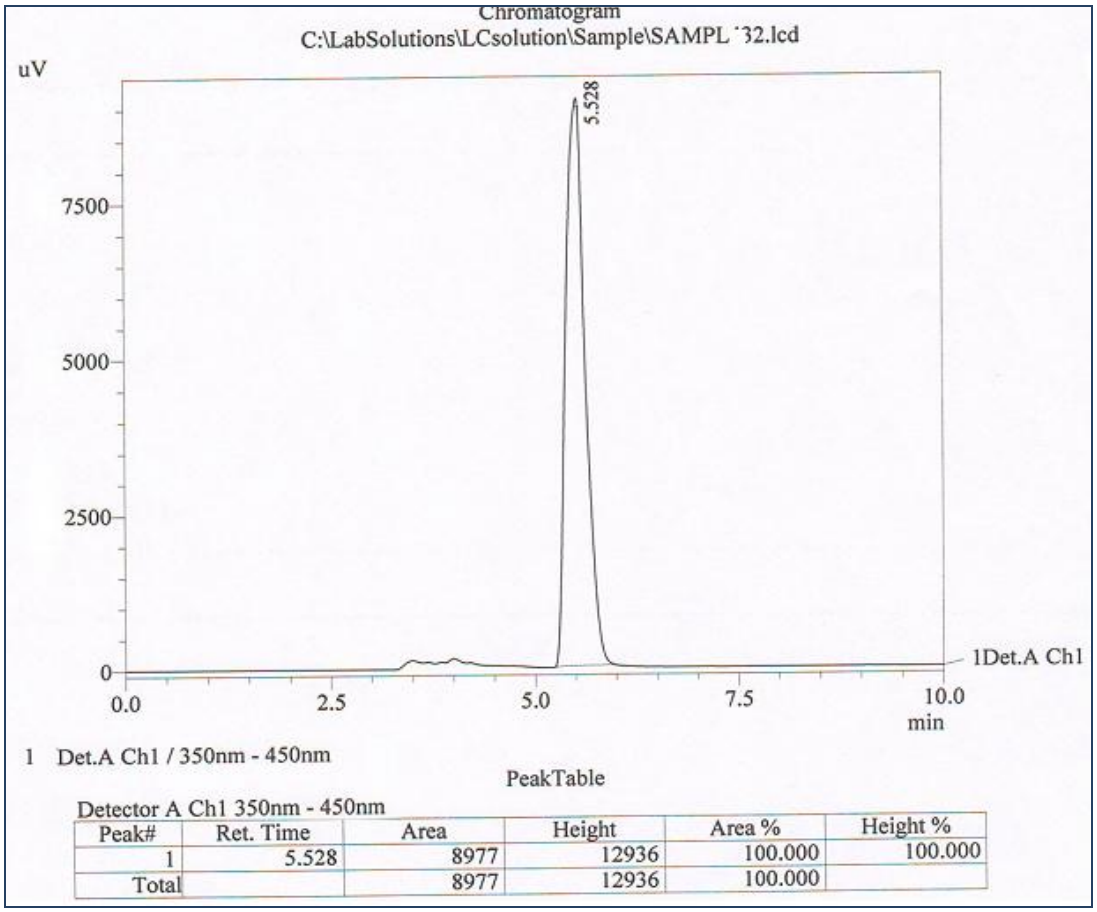
1. A volume of 50 µL of serum was added to (20µL) of OPA
2. After 1 min vortex , 20 µL from sample was taken and injected into the HPLC .
3. Obtaining results that contain the peak top, retention time, and area under the peak for standard as it shown in (Figures 3, 4 and 5).
4. Make the necessary calculations.

Results and Discussion

1. Using area under the peak to calculate the aqueous extract concentration by the following equation: -

Sample con.(mg/dl) = (standard con. X area of sample) / area of standard

2. The results of standard was calculated by using the area under curve(Figure 2).

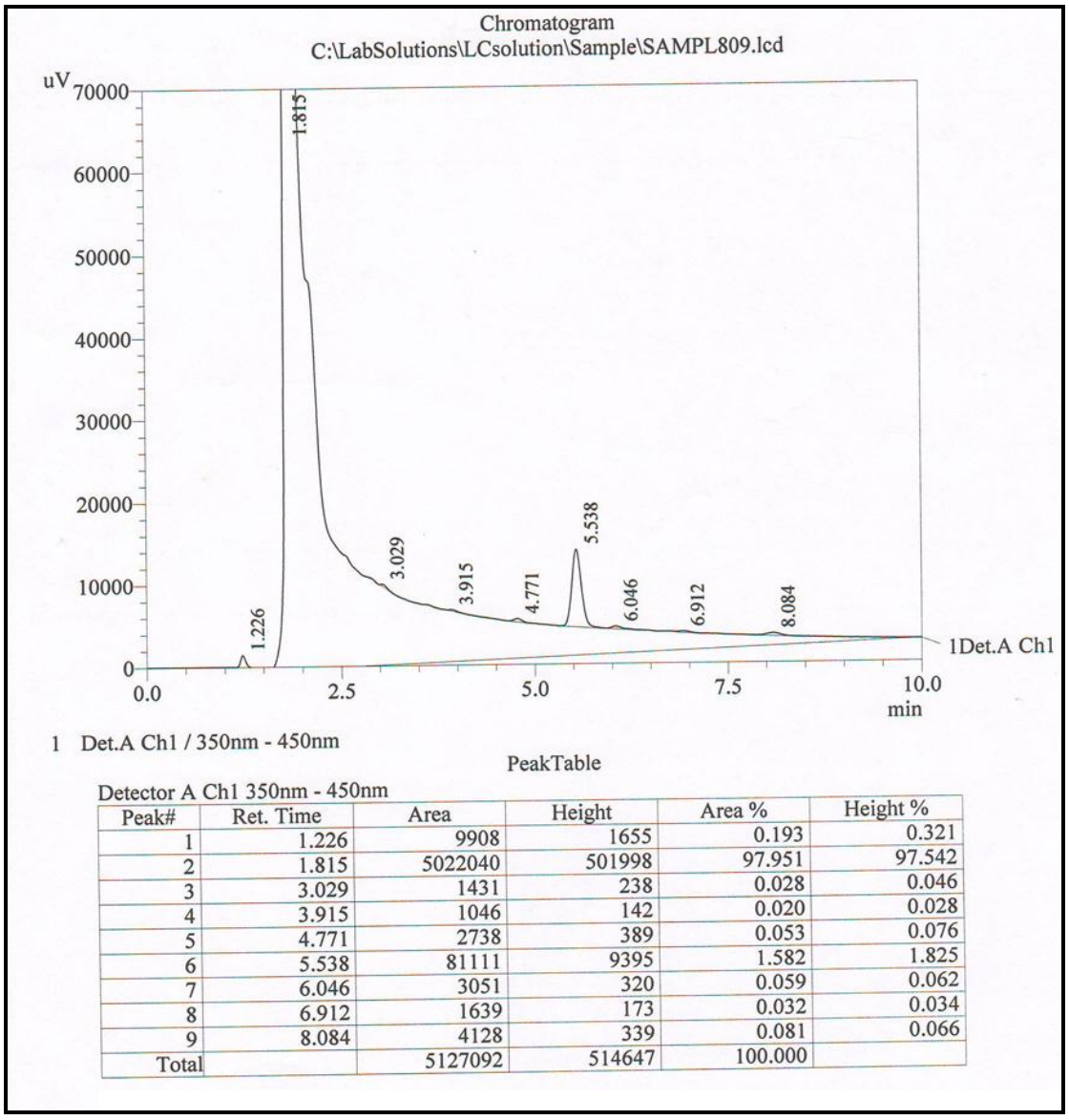


PeakTable

Detector A Ch1 350nm - 450nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	5.528	8977	12936	100.000	100.000
Total		8977	12936	100.000	

Figure 2 : The curve of standard glutathione reduced



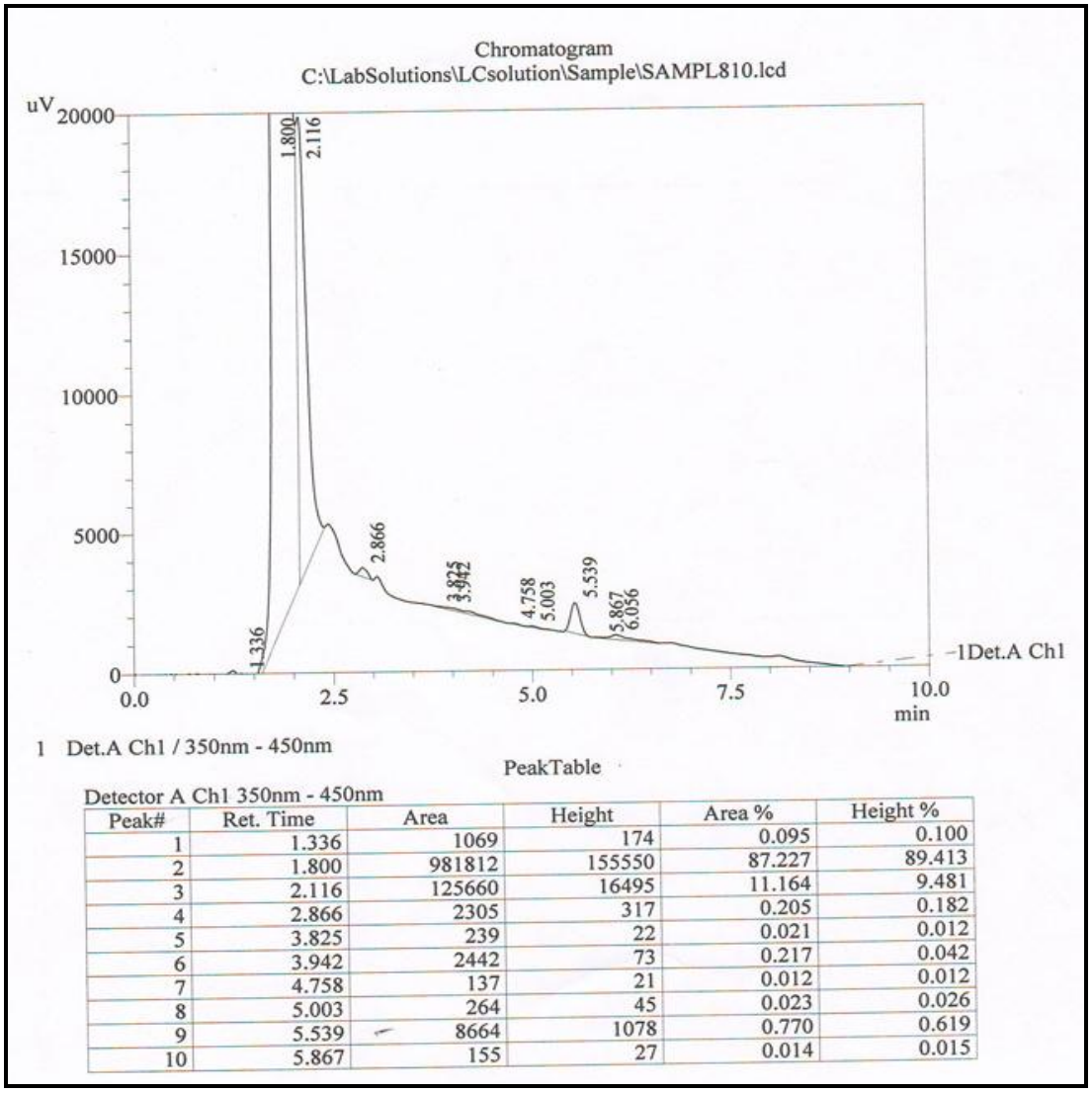
1 Det.A Ch1 / 350nm - 450nm

PeakTable

Detector A Ch1 350nm - 450nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	1.226	9908	1655	0.193	0.321
2	1.815	5022040	501998	97.951	97.542
3	3.029	1431	238	0.028	0.046
4	3.915	1046	142	0.020	0.028
5	4.771	2738	389	0.053	0.076
6	5.538	81111	9395	1.582	1.825
7	6.046	3051	320	0.059	0.062
8	6.912	1639	173	0.032	0.034
9	8.084	4128	339	0.081	0.066
Total		5127092	514647	100.000	

Figure 3 : the area under curve of glutathione (Control sample)

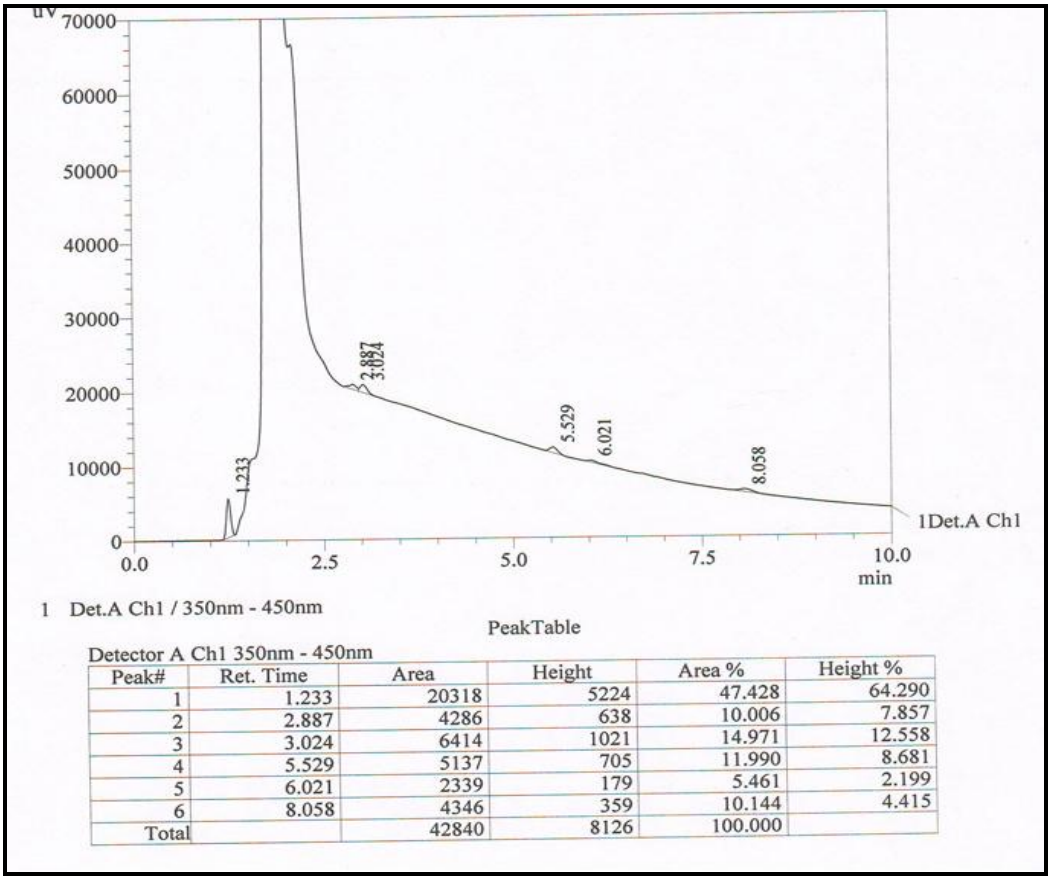


PeakTable

Detector A Ch1 350nm - 450nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	1.336	1069	174	0.095	0.100
2	1.800	981812	155550	87.227	89.413
3	2.116	125660	16495	11.164	9.481
4	2.866	2305	317	0.205	0.182
5	3.825	239	22	0.021	0.012
6	3.942	2442	73	0.217	0.042
7	4.758	137	21	0.012	0.012
8	5.003	264	45	0.023	0.026
9	5.539	8664	1078	0.770	0.619
10	5.867	155	27	0.014	0.015

Figure 4 : the area under curve of glutathione (Renal stone only Sample)



PeakTable

Detector A Ch1 350nm - 450nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	1.233	20318	5224	47.428	64.290
2	2.887	4286	638	10.006	7.857
3	3.024	6414	1021	14.971	12.558
4	5.529	5137	705	11.990	8.681
5	6.021	2339	179	5.461	2.199
6	8.058	4346	359	10.144	4.415
Total		42840	8126	100.000	

Figure 5 : the area under curve of glutathione (Renal stone with DM sample)

3. The results were calculated for all samples (control and patients) by using area under curve (Figures 3, 4 and 5) and was found that : the average level of glutathione in control group was (54.35 – 50.48) mg/dL which was for (male – female) , the average level of glutathione in patient with renal stone only group was (13.79 – 13. 08) mg/dL which was (male – female) and the level for group of renal stone and type 2 DM patients was (5.88 – 5.35) mg/dL for (male – female).

Discussion

Results shows that conditions of separation and analysis methods were applied successfully, very efficient, and accurate for glutathione determination. Quantitative results and mathematical calculations obtained from HPLC shows that glutathione was detected and measured by applying the special equations of this technique by using the concentration, area of standard material and area of sample.

High-Performance Liquid Chromatography is one of the most important comparing techniques between samples and standard material. Qualitative analysis shows the appearance chromatogram peak for standard material, control group samples, renal stone patient group sample and renal stone with DM patient sample at very closely retention time of (5.528, 5.538, 5.539, 5.529 min) respectively; indicate that these analyst compounds have the same molecular weight, physical, and chemical properties. Thus these analyst compounds are same.

The results shows that a significant differences among the average level of glutathione when comparing its levels between control and patient group which was lower by more than ten times from control group. The type 2 DM with renal stone group showed lower glutathione level about two times from renal stone patients.

The significant decrease in level of glutathione has been reported that the conditions which enhance peroxidation and depletion of thiol content increase the oxalate binding activity, which in turn promotes nucleation and aggregation property of stone matrix protein fractions. This behavior is also associated with

peroxidized mitochondria and nuclei, suggesting that the peroxidation can be a causative factor for the initial stage of stone formation. Reduction in serum level of Glutathione here can be explained due to the consumption of the antioxidant enzyme by increasing the lipid peroxidation.

Conclusions

Glutathione (GSH) is an important antioxidant in the human. GSH is capable of preventing damage to important cellular components caused by reactive oxygen species such as free radicals, peroxides, lipid peroxides and heavy metals. It is a tripeptide with a gamma peptide linkage between the carboxyl group of the glutamate side chain and the amine group of cysteine, and the carboxyl group of cysteine is attached by normal peptide linkage to a glycine. Thiol groups are reducing agents in glutathione. Glutathione reduces disulfide bonds formed within cytoplasmic proteins to cysteines by serving as an electron donor. In the process, glutathione is converted to its oxidized form, glutathione disulfide (GSSG).

The HPLC-fluorescence method for the determination of GSH in serum was developed and validated. This method is more economical and has a lower limit of detection and the derivatization procedure is finished under mild conditions without high temperatures and long derivative times, which influence the stability of GSH. In addition, only a small amount of the samples (50 μL) were needed in the analysis procedure, which indicates that it is more suitable to analyze biological and clinical samples.

The significant differences in glutathione level was explained by the role of lipid peroxidation and anti oxidant enzyme in pathogenesis of nephrolithiasis.

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