

Full Length Research Paper

Complexation of BaCl₂ with glutathione (GSH) in blood components

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Barium is a divalent alkaline earth metal and can exist only in combination forms. Studies have proved that barium can cause different toxic effects. The human body can be exposed to barium in drinking water, food and air. In medical field, barium is used in diagnostic procedure. Among its various combinations, barium chloride is considered most important in its related toxicities, because of its water solubility. In the human body, glutathione is present in almost all type of cells and acts as an antioxidant and also arrests the toxic xenobiotics like metals and drugs through conjugate formation. This study was designed to investigate the possible interaction of barium with extracellular (separated-plasma) glutathione (GSH) and intracellular (separated-cytosolic fraction) GSH. The renowned Ellman's method of thiol quantification was used to determine the effect of barium chloride on the levels of extracellular and intracellular GSH. The results showed that a decrease in the concentration of GSH was affected by barium chloride. This decrease was enhanced by increasing the concentrations of barium chloride and also by the time elapsed. Hypothetically, this decrease in the levels of GSH may be attributed to the conjugation formation of GSH with barium metal.

Key words: 5,5-Dithiobis-(2-Nitrobenzoic acid) (DTNB), glutathione (GSH), barium chloride (BaCl₂), absorbance (ABS).

INTRODUCTION

Barium (Ba) (Weast et al., 1987), atomic number 56, atomic weight 137.3 g/mol and oxidation state 2⁺ is the 5th element in group II of the periodic table (Robert et al., 2007). It is a soft silvery white metal, decolorizes to silver yellow on exposure to air and cannot exist freely in nature and thus occurs in combination forms (Weast et al., 1987). These combinations have different solubilities in different solvents (WHO, 2001). Among its halides, barium

chloride (BaCl₂) is water soluble and thus it is considered as the main cause of barium related toxicities (Patnaik and Pradyot, 2002). Barium is used as a contrast medium to enhance the X-rays imaging and improve the visibility of different structures like the gastrointestinal tract (GIT), blood vessels and body fluids (Thomson and Varma, 2010). Thus, barium in its sulfate form is employed in diagnosis of different conditions (Shroy and Robert, 1995; Bushberg et al., 2001), like Crohn's disease (Baumgart and Sandborn, 2007), achalasia (Spiess and Kahrilas, 1998), gastro esophageal reflux disease (Numans et al., 2004), esophageal cancer (Enzinger and Mayer, 2003) and Schatzki ring (Pezzullo et al., 2003). Animal studies

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and human case reports suggest that barium can cause different toxic effects depending on whether it enters the human body through the gastrointestinal or respiratory tract. It may cause bronchoconstriction (Hicks et al., 1986), cardiac arrhythmias, tachycardia, irregular blood pressure (Wetherill et al., 1981), GIT hemorrhage; bowel obstruction and stomach rupture (Boyd and Abel, 1966). Apart from other defense mechanisms of the human body, glutathione (GSH) present in all the cells of the human body (Verjee and Behal, 1976) also takes part in the detoxification and metabolism of xenobiotics like metals and many drugs (Hayes and Pulford, 1995). The level of glutathione is between 0.1 and 10 mM in human tissues (Verjee and Behal, 1976). Glutathione is a tripeptide (γ -glutamyl-cysteinyl-glycine), having molecular weight 307 (Meister and Anderson, 1984) and containing an unusual peptide bond between the amine group of cysteine and the carboxyl group of the glutamate side chain (Pompella et al., 2003). GSH exists in two forms in the human body; reduced GSH and disulfide oxidized glutathione (GSSG) (Kaplowitz et al., 1985). Biosynthesis of reduced glutathione takes place in a two steps in its enzymatic controlled process, from its three constituent amino acids in cytosol of human cells (Meister, 1988a). The transport of glutathione, out of the cell, takes place through a carrier-dependent mechanism (Townsend et al., 2003). GSH acts as antioxidant and thus protect the tissues from injury due to these toxic oxygen radicals (Garcia-Ruiz and Fernández-Checa, 2006). GSH conjugates with xenobiotics and metals like silver, aluminum, mercury, zinc, lithium, vanadium and arsenic, thus detoxifies them (Meister, 1988b). GSH reverses the adverse effects of cigarette smoke on the human defense system (Townsend, 2007). GSH assists the human body in the activation of lymphocytes and leukocytes and also in the production of cytokines (Townsend, 2007). GSH also maintains the essential thiol status in human body (Lu, 1999). It regulates the activity of many enzymes through protein glutathionylation (Sies, 1999) and regulates the nitric oxide cycle (Fang et al., 2002).

MATERIALS AND METHODS

Materials used are L-glutathione (Fluka), 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) (sigma), barium chloride (BaCl_2) (Peking, China), potassium dihydrogen phosphate (Merck), disodium edetate (Riedle Dehean Ag sleeze Hannover), pharmaceutical grade sodium chloride (NaCl) (Merck), sodium hydroxide (NaOH) (Fluka AG), and hydrochloric acid 35% (HCl) (Merck). These reagents were utilized as purchased without any further purification. Also, chloroform (Merck), distilled water (double refined) and ethanol (Merck), ultraviolet (UV)-Visible (automatic double beam) spectrophotometer: UV. Dec 610 (Jasco), UV-1600 (Schimadzu), Oven: Memmert Model U-30, 854 Schwabach (Germany), pH Meter: Model NOV-210 (Nova Scientific company ltd. Korea), centrifuge (H-200, Kokusan Ensink company Japan), hot plate 400 (England), graduated micropipette (Scorex Swiss Finland), Sartorius balance, magnetic stirrer, Eppendorf tubes (Plastic 101), siliconized glass test tubes, sterile pyrogen free disposable syringes

(B.D), and disposable rubber gloves were used.

Glassware, including graduated cylinders, pipettes, beakers, test tubes, and flasks of different volumes, funnels, and glassware were properly washed with distilled water, detergent powder and organic solvents and were dried in an oven for 2 h at 110°C.

Ellman's method (1960)

Preparation of required solutions

BaCl_2 of 244.28 mg of was dissolved in 1000 ml of normal saline to obtain 1 mM isotonic solution of BaCl_2 . GSH of 30.74 mg (molecular weight 307.4) was dissolved in 100 ml of HCl (0.1 N) solution for preparing 1 mM GSH solution. To prepare 0.2 M phosphate buffer (pH 7.6), 50 ml of 0.2 M monobasic phosphate solution was mixed with 42.4 ml of 0.2 M NaOH solution. We prepared 100 ml of 1 mM DTNB (Ellman's reagent) by dissolving 39.6 mg of DTNB (molecular weight 396.35) in 0.2 M phosphate buffer. 1.861 g of disodium edetate (EDTA-2Na, molecular weight 372.2) was dissolved in 10 ml of distilled water for preparing 0.5 M EDTA-2Na solution. A 5 mM EDTA-2Na solution was obtained by diluting 1 ml of the 0.5 M EDTA-2Na to 100 ml with distilled water. 0.9 g of pharmaceutical grade NaCl was dissolved in water and diluted to 100 ml to obtain a 0.9% NaCl solution (Normal Saline). 30 ml of chloroform was mixed with 50 ml of ethanol for preparation of the 3:5 chloroform:ethanol mixture.

Isolation of separated-plasma

A syringe was first rinsed in 0.5 M EDTA-2Na solution and then blood was taken in it from the vein of a volunteer. 1.8 ml of the blood was centrifuged for 5 min at 10,000 rpm, in a 2 ml Eppendorf's tube. Then, 0.5 ml of the supernatant plasma was collected, mixed with 50 μ l of the 5 mM EDTA-2Na, and was stored in refrigerator for further use. This process was repeated until the required amount of the separated plasma was obtained.

Isolation of cytosolic fraction

The red blood cells fraction was obtained from Eppendorf's tube after removal of plasma. This fraction was washed 3 times with normal saline. For the washing procedure, normal saline was introduced to the tube and after centrifugation for 5 min, the supernatant was discarded. Subsequently, 0.5 ml of the 5 mM EDTA-2Na solution was mixed with an equal volume of the washed red cells fraction and was kept in refrigerator for 1 h.

After 1 h, 0.6 ml of cold chloroform:ethanol mixture was added and mixed to precipitate the hemoglobin from the red cells. A drop of distilled water was added, mixed and centrifuged for 10 min at 12,000 rpm. After centrifugation, the supernatant pale yellow cytosolic fraction was collected and stored in refrigerator for further use. By repeating this process the required volume of separated-cytosolic fraction was obtained.

Ethical considerations

This study was performed in the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Gomal University, Dera Ismail Khan, Pakistan in well equipped laboratories. All the steps of the study were approved by institutional committee in accordance to the guidelines of the National Institution of Health (NIH), Pakistan. For this research, three healthy male volunteers (25 to 35 years) donated their venous blood. They were given a volunteer protocol,

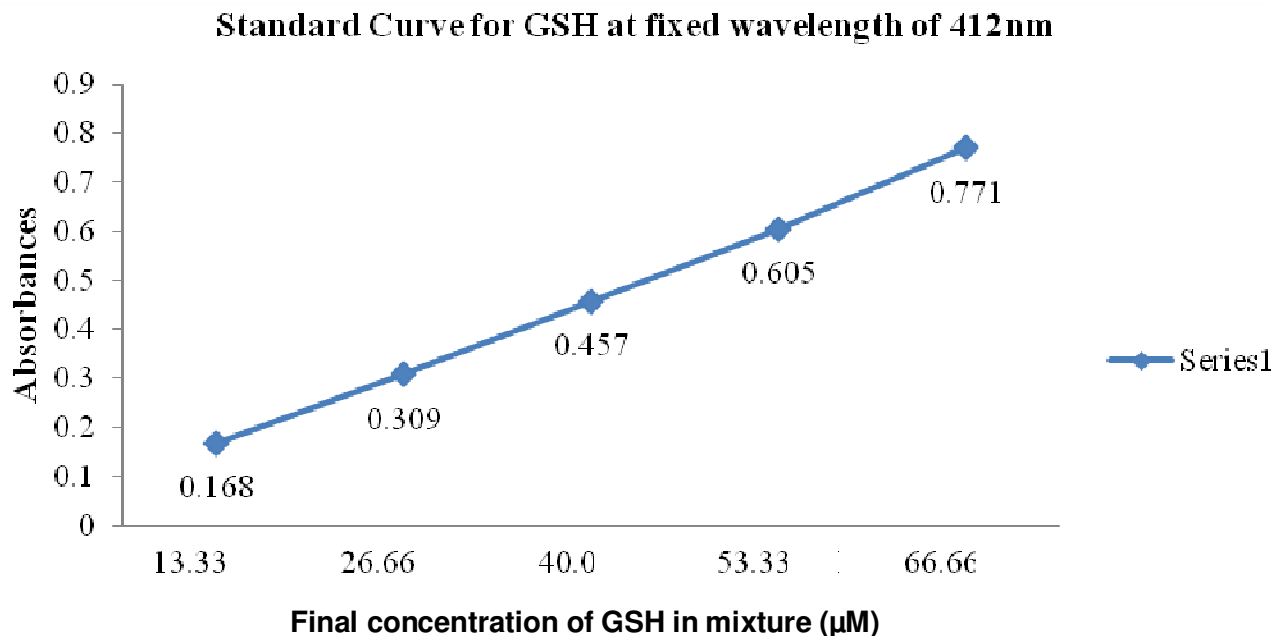


Figure 1. Standard calibration curve for GSH at 412 nm.

stating the terms and conditions of the testing. The protocols were signed by each of them individually. They were properly examined by a physician and it was ensured that they were fit to donate the required blood samples.

Preparation of biological samples and determination of biochemical inorganic parameters

We used the Ellman's modified method for the quantification of extracellular (separated-plasma) thiol content and intracellular (separated-cytosolic fraction) thiol content (Ellman, 1959).

Extracellular (separated-plasma) GSH content

According to the prescribed procedure, 1 ml of separated-plasma was added to each of 5 test tubes. Then 1 ml of 0.2, 0.4, 0.6, 0.8 and 1 mM of BaCl_2 solutions were added to these test tubes, respectively. Reading sample from each of these test tubes were prepared by taking 0.2 ml of the separated-plasma- BaCl_2 mixture, and was mixed with 2.3 ml of 0.2 mM phosphate buffer and 0.5 ml of the 1 mM DTNB solution. A separated-plasma GSH control/blank sample was also prepared by diluting with phosphate buffer and adding DTNB solution, without introducing the BaCl_2 solution. Similarly, a blank DTNB sample was also prepared by diluting 0.5 ml the sample to 3 ml with phosphate buffer. Then, all these prepared samples were analyzed by UV-Visible spectrophotometer at fixed wavelength of 412 nm, while the reference cell contains phosphate buffer solution. Two of the samples contain the lowest and the highest concentration (0.2 and 1 mM) of BaCl_2 , respectively, and the blank sample, was also analyzed for determination of the time-dependent effect at different time intervals from 0 to 150 min. The real absorbances of each sample were calculated by subtracting the absorbance of blank DTNB solution and the remaining concentration of GSH in each was determined from the standard curve of GSH at 412 nm, as per prescribed procedure of Ellman's method (Ellman, 1959).

Intracellular (separated-cytosolic fraction) GSH content

Another 5 test tubes were taken for separated-cytosolic fraction and 1 ml of separated-cytosolic fraction was added to each one. To these 5 test tubes, 1 ml of 0.2, 0.4, 0.6, 0.8 and 1 mM of the BaCl_2 were added, respectively. Then 0.2 ml of each of these test tubes was diluted with 2.3 ml of 0.2 mM phosphate buffer and 0.5 ml of 1 mM DTNB solution. A sample of DTNB blank was prepared by mixing 0.5 ml of the 1 mM DTNB solution with 2.5 ml of the 0.2 mM phosphate buffer solution. Similarly, a separated-cytosolic fraction blank sample was also prepared according to the standard procedure of Ellman's method (Ellman, 1959). All these samples were analyzed by UV-Visible spectrophotometer at a fixed wavelength of 412 nm, against the phosphate buffer solution containing the reference cell. Similarly, the time-dependent effect of the lowest and the highest concentrations of BaCl_2 were also observed at different intervals from 0 to 150 min. The remaining concentrations of GSH in the samples were calculated from their real absorbances, from the GSH standard curve.

RESULTS

Standard curve

The standard curve (Figure 1) of the increasing concentrations (13.33 to 66.66 μM) of GSH was prepared by following Ellman's method (Ellman, 1959).

Effect of BaCl_2 on the level of extracellular (separated-plasma) GSH

It was observed that the BaCl_2 caused a decrease in the extracellular (separated - plasma) GSH content. This

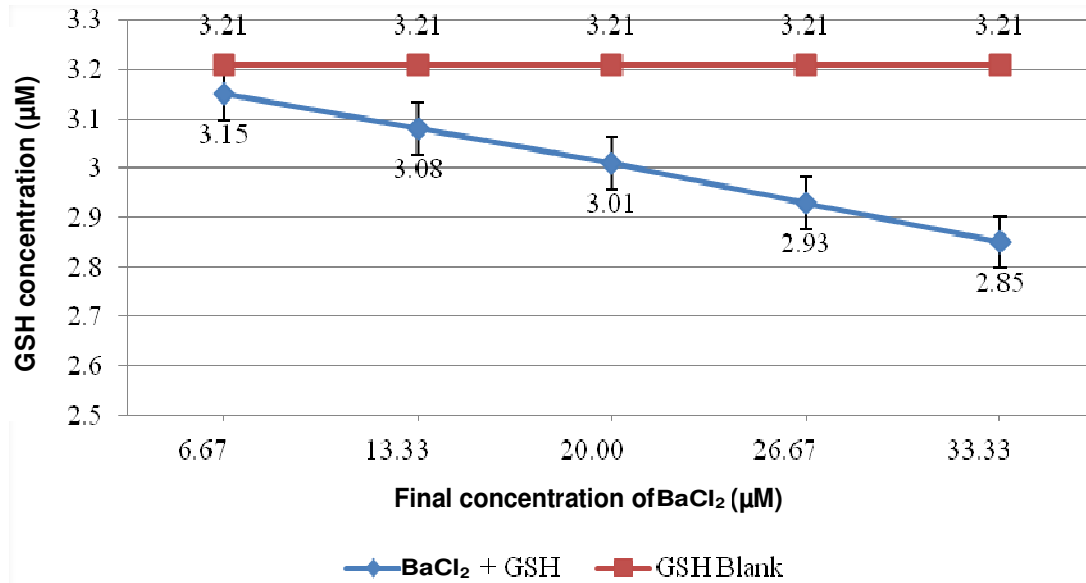


Figure 2. Effect of BaCl₂ on extracellular plasma GSH content. ■Control (1 ml 0.9% NaCl/1 ml of separated-plasma). ♦BaCl₂ (6.67 to 33.33 µM) + separated-plasma. Results are the mean ± SEM of 3 experiments of plasma GSH.

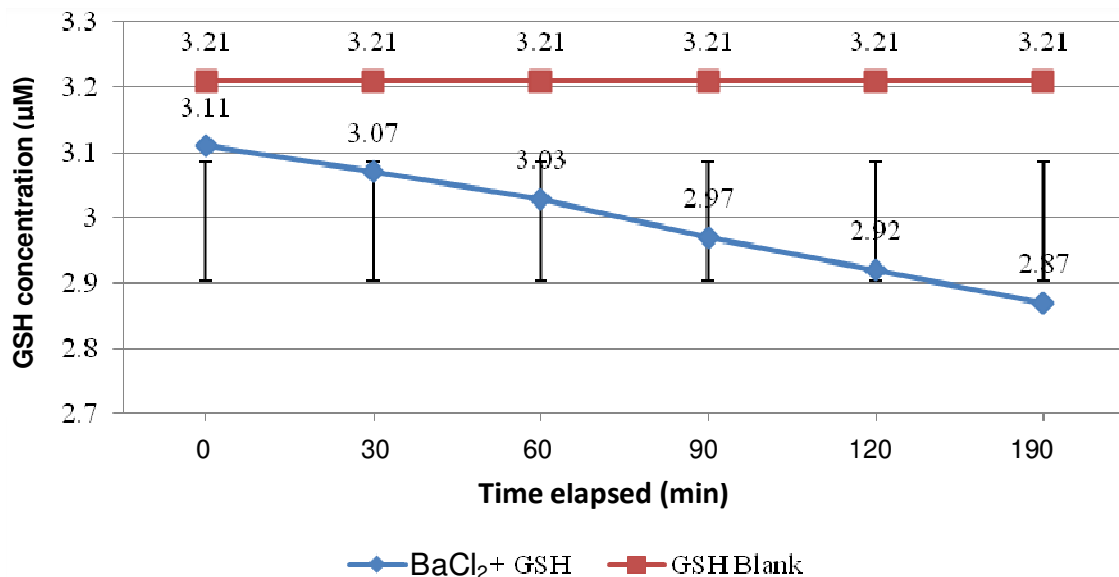


Figure 3. Effect of 0.2 mM BaCl₂ on the extracellular plasma. GSH content with time incubation period (0 to 150 min). ■ Control (1 ml 0.9% NaCl/1 ml of separated-plasma). ♦ BaCl₂ (0.2 mM)+separated-plasma. Results are the mean ± SEM of 3 experiments of plasma GSH.

decrease was enhanced by increasing concentrations of BaCl₂ as shown in the Figure 2.

Similarly, the decrease in intracellular GSH level was more pronounced under the effect of the 0.2 mM (Figure 3) and 1mM (Figure 4) of BaCl₂ with time elapse. Figures 3 and 4 shows the time dependent decrease in intracellular GSH.

Effect of BaCl₂ on the level of intracellular (separated-cytosolic fraction) glutathione

A decrease was observed in the level of extracellular GSH which was enhanced with increasing concentrations of BaCl₂, as shown in the Figure 5.

Decrease was found in the intracellular GSH level,

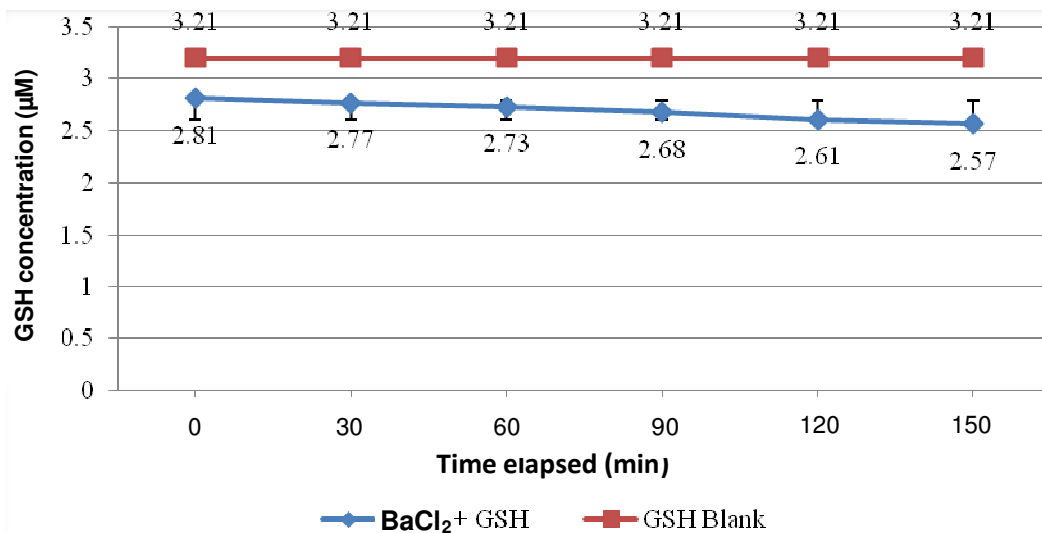


Figure 4. Effect of 1 mM BaCl₂ on the extracellular plasma. GSH content with time incubation period (0 to 150 min). ■ Control (1 ml 0.9% NaCl/1 ml of separated-plasma). ♦ BaCl₂ (1 mM)+separated-plasma. Results are the mean ± SEM of 3 experiments of plasma GSH.

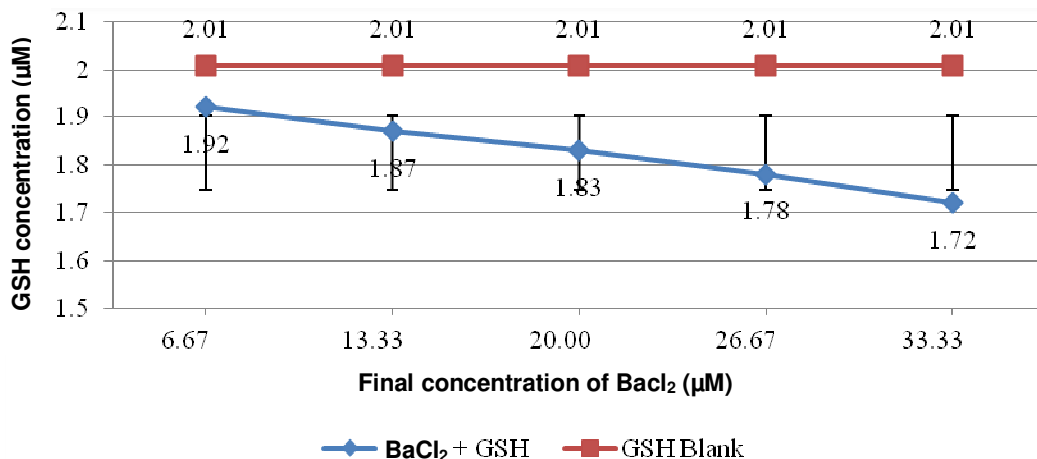


Figure 5. Effect of BaCl₂ on intracellular cytosolic fraction GSH content. ■ Control (1 ml 0.9% NaCl/1 ml of separated-cytosolic fraction). ♦ BaCl₂ (6.67 to 33.33 µM)+separated-cytosolic fraction. Results are the mean ± SEM of 3 experiments of cytosolic fraction GSH.

under the effect of 0.2 mM (Figure 6) and 1 mM (Figure 7) solutions of BaCl₂ was more pronounced when we increased the incubation period. Figures 6 and 7 shows the time dependent effect of BaCl₂ on the level of intracellular GSH.

DISCUSSION

Different case reports and animal studies have shown that barium has various toxic effects (Tarasenko et al., 1977; Shankle and Keane, 1998). Barium is generally used as a medical contrast agent in computed

tomography (Buckwalter and Kenneth, 2010) and in fluoroscopy (Shroy and Robert, 1955). During this diagnostic procedure and through food, drinking water and air, human may be exposed to different amount of barium (Schroeder et al., 1972).

GSH, found in human tissues (Verjee and Behal, 1976), takes part in the detoxification of xenobiotics, like drugs and metals (Hayes and Pulford, 1995). During detoxification, the GSH forms conjugates with xenobiotics and thus the levels of GSH are depleted (Hayes and Pulford, 1995). It was thus felt necessary to study the interaction of extracellular and intracellular glutathione with barium metal. The chloride salt of barium (BaCl₂) was

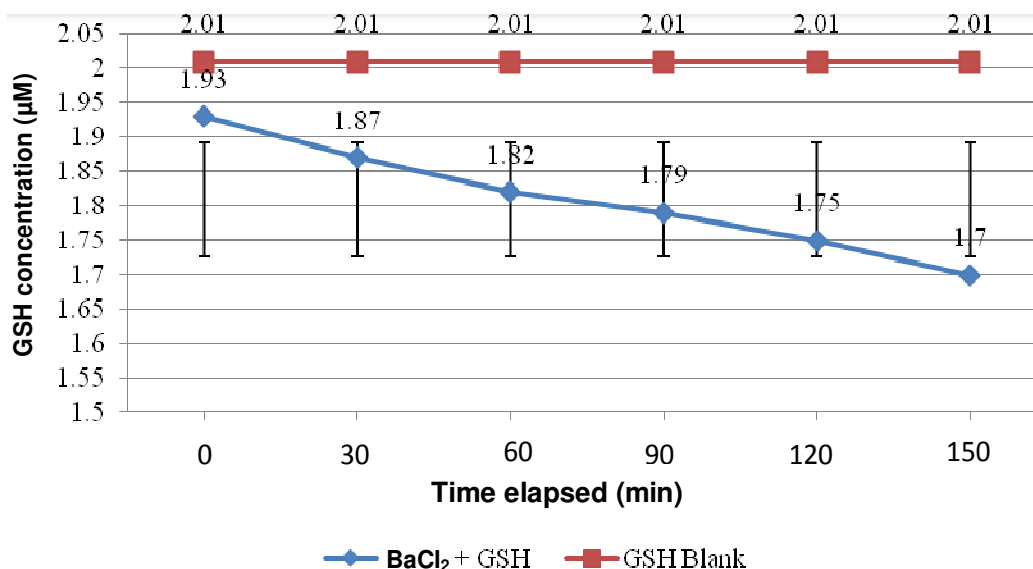


Figure 6. Effect of 0.2 mM BaCl₂ on the intracellular cytosolic fraction. GSH content with time incubation period (0 to 150 min). ■ Control (1 ml 0.9% NaCl/1 ml of separated-cytosolic fraction). ♦ BaCl₂ (0.2 mM)+separated-cytosolic fraction. Results are the mean ± SEM of 3 experiments of plasma GSH.

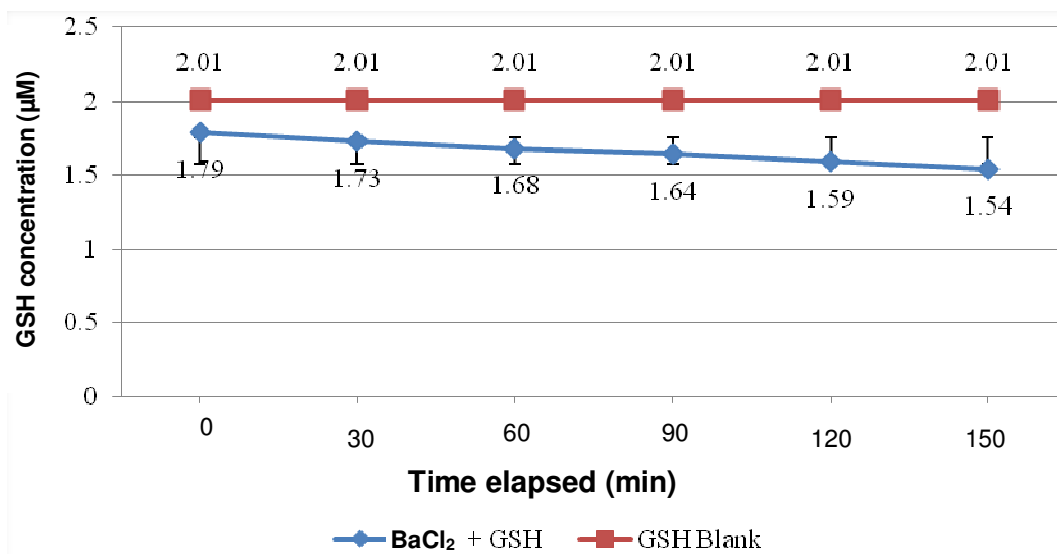
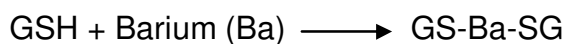


Figure 7. Effect of 1 mM BaCl₂ on the intracellular cytosolic fraction. GSH content with time incubation period (0 to 150 min). ■ Control (1 ml 0.9% NaCl/1 ml of separated-cytosolic fraction). ♦ BaCl₂ (1 mM)+separated-cytosolic fraction. Results are the mean ± SEM of 3 experiments of plasma GSH.

was chosen, which is water soluble and is thus considered the most important of all combinations in barium related toxicities (Patnaik and Pradyot, 2002).

BaCl₂ was found to cause a decrease in the level of extracellular GSH (separated-plasma GSH) as well as in the level of intracellular GSH (separated-cytosolic fraction GSH). It was also found that this effect is more pronounced with increasing concentration of barium and with the prolongation of the incubation period.

If we consider the conjugating and reducing power of GSH (Kidd, 1997) and also the 2⁺ oxidation state of barium, we presume that the level of GSH decreases due to its conjugation with barium. A hypothetical pattern of the reaction in which GSH forms conjugate with barium is given as follows:



Though, this study was not designed to know about the exact mechanism of the reaction between GSH and barium in the separated human blood components; however, the findings can be utilized to further explore the role of GSH in the metabolism and detoxification of an important diagnostic agent, barium.

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