DETERMINATION OF GLUTATHIONE CONCENTRATION AFTER ITS INTERACTION WITH CADMIUM NITRATE TETRAHYDRATE BY USING ELLMAN'S MODIFIED METHOD

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ABSTRACT

Industrial waste is main source of environmental pollution especially of cadmium which is toxic to all organisms including humans because it interact with the thiol containing cellular components like Glutathione which is a tripeptide present in human body inside as well as outside the cells. So we have studied the interaction of Glutathione (GSH) with cadmium Nitrate tetrahydrate in aqueous medium by using modified Ellman's method. For this purpose we studied the concentration and time dependent effect of cadmium nitrate tetrahydrate(CNT) on Glutathione in aqueous medium by using UV-visible spectrophotometer. Different concentrations of cadmium nitrate tetrahydrate were introduced to constant concentration of Glutathione to determine the affect of cadmium on concentration of GSH. It was found that higher concentration of Cd cause greater decrease in GSH concentration than lower concentration of cadmium nitrate tetrahydrate with the passage of time, at different time of intervals, there was a gradual depletion in the concentration of GSH that was indicating either binding of cadmium with GSH or conversion of GSH (reduced form of GSH) into GSSG (oxidized form of GSH).

Key words: Cadmium Nitrate Tetrahydrate, Concentration, Time dependent, Reduced Glutathione, Ellman's modified method.

INTRODUCTION

Glutathione consists of a linkage called as unusual peptide linkage that is between carboxyl group of glutamate side chain and amine group of cysteine. (kretzschmar M., 1990).Oxidation of reduced glutathione converts it into its oxidized form. (Irwin M., et al 1975).Atherosclerosis appears linked to oxidative damage to the vessel wall. Increased lipid peroxide, decreased GSH peroxides levels. And lower levels of the protective eicosanoid prostacy link (PGI2) have been documented in human and animal atherosclerotic arteries (Stamler JS et al 1996). Oxidative stress within atherosclerotic arteries depletes GSH and other antioxidants and results in a shift in the so-called "prostaglandin" (more correctly, eicosanoid) balance from anti-inflammatory towards pro-inflammatory (Miura K, 1992).

GSH can produce coronary vasodilatation when added to isolated, perfused rodent heart; very likely due to its normalizing effect on prostaglandin synthesis (Ochi H et al., 1992).When 70% to 80% GSH is depleted , it is called GSH depletion threshold and at this level cell will die. (Kidd PM, 1997; Gul M et al., 2000).Failure of Mitochondria is especially implicated in degeneration of retina (Cai J, 2000) and in Parkinson's disease (Kidd PM, 2000).

Cadmium is the cause of various diseases including cancer, respiratory disease and cerebrovascular diseases, especially the rates of popular mortality due to these diseases are higher in cadmium contaminated areas than those areas which are not cadmium contaminated. Breast cancer and endometrial cancer are also associated with chronic exposure to cadmium (J. A McElroy et l., 2006;A Agenta et al.,2008).

MATERIALS AND METHODS

CHAMICALS AND REAGENTS

Potassium dihydrogen phosphate (Merck), Glutathione (GSH) (Fluka), Sodium Hydroxide (Fluka), Ellman's Reagent, (Sigma), HCl 35% (Kolchlight), Cadmium nitrate tetrahydrate (Aldrech),Distilled water Double refined distilled water, UV-Visible spectrophotometer (UV-1601) (Schimadzu, Japan), pH meter: Nov:2010 model (Scientific Nova Company Ltd Korea), Balance Analytical model AX200 (Schimadzu, Japan), Magnetic Stirrer, Memmert oven model U-30854 (Schwabach,Germany), 50ml beakers (Pyrex Iwaki Glass, Japan) 200 µl, 500 µl, 1000 µl micro- pipette digital (Scorex Swiss Finland) were used in this research work.

PREPARATION OF STOCK SOLUTIONS

0.2M Phosphate buffer was prepared by taking 42.4 ml of 0.2M sodium hydroxide solution into a 250 ml volume metric flask, to this 50 ml of 0.2M Potassium dihydrogen phosphate solution was mixed and total volume was made 200 ml with quantity sufficient of distil water. 1mM of Glutathione (GSH) solution was prepared by taking/dissolving 15.375 milligram of L. Glutathione (GSH) in 50ml of 0.1 N hydrochloric acid solution. DTNB or 5,5-dithobes-2-nitrbenzoic acid 1mM solution was prepared by dissolving 19.8 mg into 50 ml of phosphate buffer pH 7.6. 2mM stock solution of cadmium nitrate tetrahydrate was prepared by dissolving 61.8 mg in 50 ml of distil water.

STANDARD CURVE

After preparing 1mM stock solution of GSH (Glutathione), its different concentrations (table no.1) were prepared and from each of the GSH dilution 200µl was taken and added into 2300µl of 0.2 M phosphate buffer pH 7.6 then 500µl of 1mM 5,5-Dithiobis,2-nitro benzoic acid (DTNB) was added, these five mixtures were well shaken and incubated for five minutes. After incubations period, absorbance of each mixture was recorded at fixed wavelength λ max: 412 nm. DTNB blank was prepared by adding 500µl DTNB (5,5-dithiobis-2-nitrobenzoic acid) to 2500µl phosphate buffer pH 7.6. Absorbance of DTNB was also taken at same fixed wavelength λ max: 412nm. By subtracting absorbance of DTBN blank from absorbance of each of the mixture, a real absorbance of each mixture was obtained as shown in table 1. According to table 1 (having different absorbances of different GSH dilutions) standard curve was drawn as shown in figure 1.

Table #1

Different concentrations of Glutathione plus 5, 5-dithiobis-2 -nitrobenzoic acid (DNTB) Mixture							
Absorbance of 5,5-dithiobis- 2-nitrobenzoic acid (DTNB) blank solution was 0.057							
S No	**Used Conc. of GSH**	**Conc.(Final)) Of GSH in Mixture**	**ABS 1st**	**ABS 2nd**	**ABS 3rd**	**3 readings average**	**absorbance* (Real)**
1	**.2mM**	13.33 µM	.215	.213	.210	.213	.156
2	**.4mM**	26.66 µM	.365	.362	.360	.362	.305
3	**.6mM**	40 µM	.525	.523	.519	.522	.465
4	**.8mM**	53.33 µM	.688	.685	.680	.684	.627
5	**1mM**	66.66 µM	.845	.841	.838	.841	.784
***Absorbance(Real)= ABS of Mixture- ABS of DTNB blank solution.**							

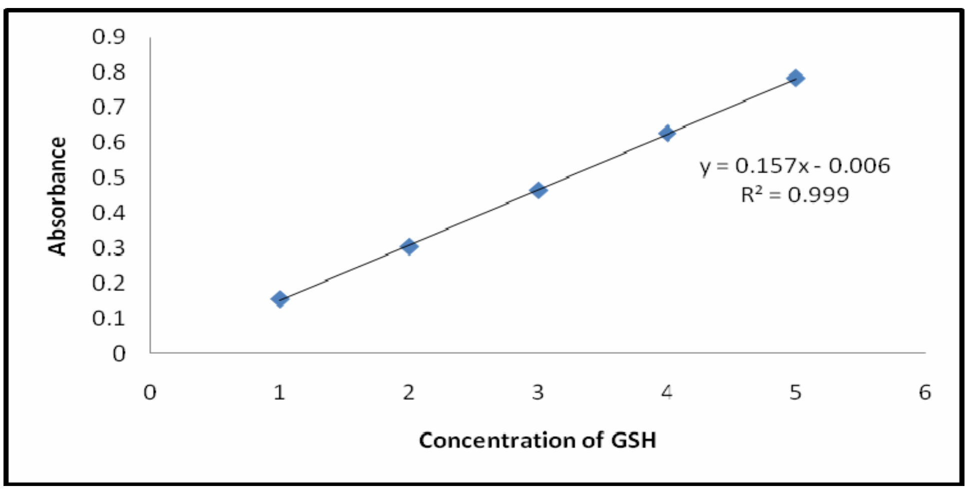

Fig 1: Standard Curve for Glutathione (GSH)

IN AQUEOUS MEDIUM, THE AFFECT OF DIFFERENT CONCENTRATIONS OF CADMIUM NITRATE TETRAHYDRATE ON GSH (GLUTATHIONE)

Six test tubes were numbered in sequence from test tube No1 to test tube No6. In each of the test tube 2ml of 1mM GSH (Glutathione) solution was added, to each of the test tube (containing 2ml of 1mM GSH) 2ml of each cadmium nitrate tetrahydrate dilution was mixed starting from higher concentrations of metal salt to the lower concentration of metal salt. All these mixtures are called mixture -1. The concentration of GSH in these mixtures was 0.5mM while metal concentrations were 0.00005mM to 1mMand respectively starting from higher concentration to lower concentration of cadmium nitrate tetrahydrate. All the mixture -1 were well shaken and left for

10 minutes (incubation time). After 10 minutes (incubation time) 0.2ml (200 µl) from each mixture -1 was mixed with 2.3ml (2300 µl) of phosphate buffer pH 7.6, to this mixture 0.5ml (500 µl) of 1mM DTNB solution was mixed. These are mixture- 2 (containing 200 µl of 1mM Glutathione plus different concentrations of cadmium nitrate tetrahydrate plus 2300 µl phosphate buffer pH 7.6 plus 500 µl DTNB). The mixture -2 were well shaken and left for five minute (incubation time). After five minutes absorbance of each mixture -2 was recorded at fixed wavelength λ max: 412nm by using UV-visible spectrophotometer. The absorbances of all the mixture -2 were converted into GSH concentration by using Ellman's modified method (as described in standard curve). Affect of different concentrations of cadmium nitrate tetrahydrate on GSH (Glutathione) is shown in table 2, figure 2.

Effect of different concentrations of cadmium nitrate tetrahydrate (CNT)i.e. from 0.0001mM to 2.0mM on the chemical status of glutathione in aqueous medium

Concentration of Glutathione (GSH) in final mixture was 33.33µM
Absorbance of 5,5-dithiobis- 2-nitrobenzoic acid (DTNB) blank solution was 0.061

S No	Used conc: of CNT	Conc: of CNT(Final)	ABS 1st	ABS 2nd	ABS 3rd	Mean of 3 reading	abs(Real) * / conc: of GSH after reaction with CNT		abs(Real) */ conc: of GSH Blank	
							ABS	Conc. Of GSH (µM)	ABS	Conc. Of GSH (µM)
1	.0001mM	.003 µM	.621	.614	.610	.615	.554	3.57	.785	5.04
2	.001mM	.03 µM	.565	.560	.555	.560	.499	3.22	.785	5.04
3	.01mM	.33 µM	.490	.486	.485	.487	.426	2.75	.785	5.04
4	.1mM	3.33 µM	.421	.415	.412	.416	.355	2.30	.785	5.04
5	1mM	33.33 µM	.307	.303	.297	.302	.241	1.57	.785	5.04
6	2mM	66.66 µM	.230	.224	.223	.226	.165	1.09	.785	5.04

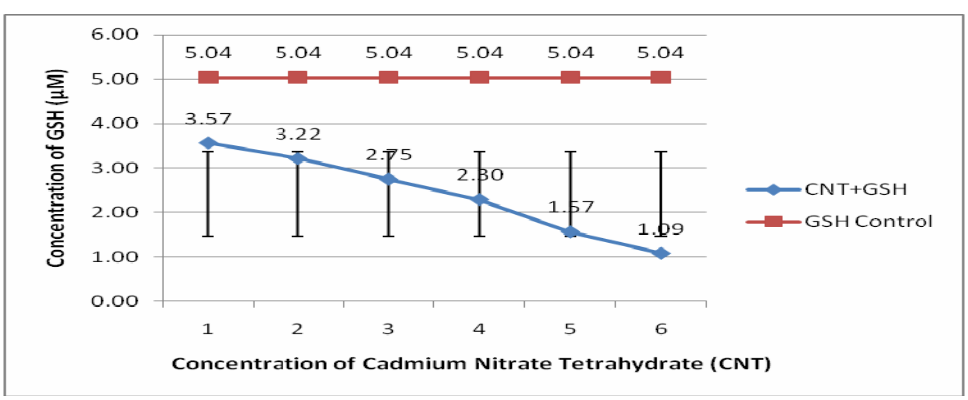

Figure 2.◆Effect of different concentrations (0.0001mM, 0.001mM, 0.01mM, 0.1mM, 1.0mM & 2.0 mM) of CNT on the chemical status of glutathione (GSH) in aqueous medium. GSH■ontrol. Results are the mean ±SE of 3 experiments.

IN AQUEOUS MEDIUM, THE AFFECT OF CADMIUM NITRATE TETRAHYDRATE ON GLUTATHIONE (GSH) WITH TIME

Similar procedure as described in affect of different concentration of cadmium nitrate tetrahydrate on glutathione was repeated for determination of affect of cadmium nitrate tetrahydrate on the chemical status of glutathione at different time intervals (incubation time). Readings / absorbances were taken at 0min:, 20min:, 40min:, 60min:, 90min:, 120min: and the results are shown in table 2, figure 3. The figure 3 shows a comparison between the affect of lowest used concentration of cadmium nitrate tetrahydrate and the highest used cadmium nitrate tetrahydrate concentration on GSH from 0min: to 120min(incubation time).

Table #3

Effect of different concentrations of cadmium nitrate tetrahydrate (CNT) on chemical status of Glutathione (GSH) at different time intervals (incubation time)

Concentration of Glutathione (GSH) in final mixture was 33.33µM
ABS(Absorbance) of 5,5-Dithiobis, 2-nitrobenzoic acid (DTNB) blank solution was 0.060

S.#	Used CNT con: (µ M)	Conce: (Final) of CNT (µM)	0 mint		20 mint		40 Mint		60 Mint		90 Mint		120 Mint	
			ABS	Conc	ABS	Conc	ABS	Conc	ABS	Conc	ABS	Conc	ABS	Conc
1	0.0001mM	.003 µ M	.554	3.57	.521	3.36	.487	3.14	.453	2.92	.421	2.72	.398	2.57
2	0.001mM	.03 µ M	.499	3.22	.465	3.00	.436	2.82	.411	2.66	.383	2.48	.365	2.36
3	0.01mM	.33 µ M	.426	2.75	.385	2.49	.357	2.31	.331	2.15	.299	1.94	.256	1.67
4	0.1mM	3.33 µ M	.355	2.30	.302	1.96	.255	1.66	.213	1.39	.185	1.22	.155	1.03
5	1.0mM	33.33 µ M	.241	1.57	.207	1.36	.167	1.10	.142	.94	.117	0.78	.083	0.57
6	2.0mM	66.66 µ M	.165	1.09	.142	0.94	.122	0.82	.101	.68	.086	0.59	.061	0.43
Real abs*/conc: of GSH Blank			.785	5.04	.785	5.04	.785	5.04	.785	5.04	.785	5.04	.785	5.04

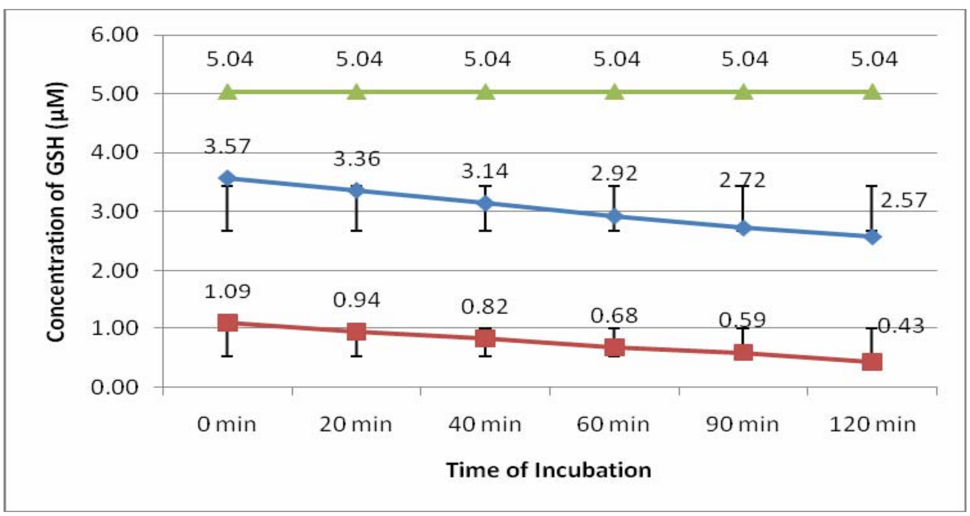

Figure 3. Effect of different concentration of CNT on glutathione (GSH) in aqueous medium with time (i.e. 0 min: ,20 min:,40 min:,60 min:,90 min:,120 min:) GSH Control Effect of lowest used CNT concentration (0.0001mM)) Effect of highest used CNT (2.0mM). Results are the mean ± SE of 3 experiments.

RESULTS

IN AQUEOUS MEDIUM, THE AFFECT OF CADMIUM NITRATE TETRAHYDRATE ON GSH (GLUTATHIONE)

Different concentrations of CNT (cadmium nitrate tetra hydrate) have different affect on the chemical status /modulation of glutathione (GSH) at 0 min: (i-e absorbance of mixture -2 after five minutes incubation time) depending upon different concentrations of cadmium nitrate tetrahydrate (CNT),the affect on glutathione(GSH) is shown in table 2 and figure 2, Which clearly indicates that greater the used metal concentration greater is the decrease in the GSH (Glutathione) concentration in aqueous medium and vice versa.

IN AQUEOUS MEDIUM, AFFECT OF DIFFERENT CONCENTRATIONS OF CADMIUM NITRATE TETRAHYDRATE ON GSH (GLUTATHIONE) AT DIFFERENT TIME OF INCUBATION

Table 3 and figure 3 shows that as the time passes different concentrations of cadmium nitrate tetrahydrate have a gradual decreasing affect on the concentration of Glutathione (Reduced form of GSH). In figure 3 the comparison of lowest used and highest used concentration of cadmium nitrate tetrahydrate is clear that cadmium nitrate tetrahydrate is depleting GSH gradually and continuously from 0 min: to 120 min.

DISCUSSION

As cadmium in the form of different salts is an environmental pollutant, it is also contaminating the soils due to industrial wastes, so in both form - as environmental pollutant and as well as soils contaminants has made us to investigate its possible effect on the chemistry of reduced glutathione(GSH) in aqueous medium. The depletion of GSH due to its interaction with cadmium nitrate tetrahydrate is a clear indication of formation of some intermediates like Cd – S - G etc. or the conversion of GSH into GSSG. Results suggest that either due to the interaction of cadmium with reduced form of Glutathione (GSH), there is formation of $GS^{\bullet}$ (Thiyl) and thus two $2GS^{\bullet}$can easily interact with each other forming GSSG (oxidized form of Glutathione) or as cadmium has binding capacity with thiols including glutathione so there is formation of cadmium glutathione complex.

However exact mechanism of action of cadmium nitrate tetrahydrate on the chemical status of reduced form of glutathione (GSH) was not possible to determine under the conditions in which this research work was conducted. The results indicate that cadmium nitrate tetrahydrate with the passage of time shows that affect of different concentrations of cadmium nitrate tetrahydrate is continuous and gradual.

POSSIBLE REACTIONS

$$Cd(NO_3)_2 \longrightarrow Cd^{+2} + 2NO_3^{-1}$$

$$2GSH \longrightarrow 2GS^{-1} + 2H^{+}$$
$$Cd^{+2} + 2GS^{-1} \longrightarrow Cd(GS)_2$$
$$2H^{+} + 2NO_3^{-1} \longrightarrow 2HNO_3$$
$$2GS^{-1} \longrightarrow GSSG$$

CONCLUSION

Decreased in concentration of reduced form of glutathione by introducing different concentrations of cadmium nitrate tetrahydrate clear cut indicates the interaction of cadmium with GSH (reduced form of glutathione) and the possible reactions may be as shown above. Thus increasing quantity of cadmium as polluted of environment as well as soils is globally a serious threat to human health.

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