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Quantitation and characterization of glutathionyl haemoglobin as an oxidative stress marker in chronic renal failure by mass spectrometry

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Abstract

Objectives: Glutathionyl haemoglobin (GS-Hb) belonging to the class of glutathionylated proteins has been investigated as a possible marker of oxidative stress in different chronic diseases. The purpose of this study was to examine whether glutathionyl haemoglobin can serve as an oxidative stress marker in non-diabetic chronic renal failure patients on different renal replacement therapies (RRT) through its quantitation, and characterization of the specific binding site of glutathione in haemoglobin molecule by mass spectrometric analysis.

Design and methods: The study group consisted of non-diabetic chronic renal failure patients on renal replacement therapy (RRT): hemodialysis (HD), continuous ambulatory peritoneal dialysis (CAPD) and renal allograft transplant (Txp) patients. Haemoglobin samples of these subjects were analyzed by liquid chromatography electrospray ionization mass spectrometry for GS-Hb quantitation. Characterization of GS-Hb was done by tandem mass spectrometry. Levels of erythrocyte glutathione (GSH) and lipid peroxidation (as thiobarbituric acid reacting substances) were measured spectrophotometrically, while glycated haemoglobin (HbA1c) was measured by HPLC.

Results: GS-Hb levels were markedly elevated in the dialysis group and marginally in the transplant group as compared to the controls. GS-Hb levels correlated positively with lipid peroxidation and negatively with the erythrocyte glutathione levels in RRT groups indicating enhanced oxidative stress. *De novo* sequencing of the chymotryptic fragment of GS-Hb established that glutathione is attached to Cys-93 of the beta globin chain. Mass spectrometric quantitation of total glycated haemoglobin showed good agreement with HbA1c estimation by conventional HPLC method.

Conclusions: Glutathionyl haemoglobin can serve as a clinical marker of oxidative stress in chronic debilitating therapies like RRT. Mass spectrometry provides a reliable analytical tool for quantitation and residue level characterization of different post-translational modifications of haemoglobin.

Keywords: Oxidative stress; Chronic renal failure; Dialysis; Transplantation; Glutathionylation

Introduction

Oxidative stress has been defined as a loss of counterbalance between free radicals and/or reactive oxygen species (ROS) production and antioxidant defence mechanisms. Chronic renal failure is accompanied by very complex long-term manifestations such as accelerated aging, atherosclerosis, heart disease, polyneuropathies and amyloidosis [1–3], which may be related to the hyper-production of free radicals.

End-stage renal disease (ESRD) patients on renal replacement therapy (RRT–dialysis/transplantation) are subjected to enhanced oxidative stress as a result of reduced anti-oxidant systems (vitamin C and selenium deficiency, reduced intracellular levels of vitamin E, reduced activity of the glutathione

Abbreviations: CRF, chronic renal failure; ESRD, end-stage renal disease; HD, hemodialysis; CAPD, continuous ambulatory peritoneal dialysis; Txp, transplantation; RRT, renal replacement therapy; GS-Hb, glutathionylated haemoglobin; Gly-Hb, glycated haemoglobin.

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antioxidant system) and increased pro-oxidant activity (advanced age, high frequency of diabetes, chronic inflammatory state, uremic syndrome, bio-incompatibility of dialysis membranes and solutions) [4]. There is growing evidence, from experimental and clinical studies, that oxidative stress may be implicated in the pathogenesis of atherosclerosis and other complications of ESRD [5]. This hypothesis is based on studies that have conclusively demonstrated an increased oxidative burden in uremic patients, before and particularly after renal replacement therapies, as evidenced by increased or decreased concentrations of multiple enzymatic/non-enzymatic biomarkers of oxidative stress [6–8].

Glutathione, in its reduced form (GSH), is an important intracellular antioxidant compound. It prevents oxidation of essential protein thiol groups induced by oxidative stress by scavenging free radicals and detoxifying electrophiles [9]. Glutathione deficiency contributes to oxidative stress, which plays a key role in aging and pathogenesis of many diseases [10]. GSH is oxidized to its dimeric form GSSG in response to an oxidative perturbation. However, GSSG is rapidly reduced by the action of glutathione reductase. If GSSG accumulates within the cell, it can form protein–glutathione adducts via reversible thiol exchange reactions referred to as *S*-glutathionylation of protein [11,12]. Protein *S*-glutathionylation has been implicated in the buffering of oxidative stress, stabilization of extracellular proteins, protection of proteins against irreversible oxidation of critical cysteine residues, and regulation of enzyme activity. Protein *S*-glutathionylation may be induced by changes in the intracellular redox potential as well as by the formation of ROS [13,14]. Glutathionylated proteins (GSSPs) are more stable than GSSG and less prone to enzymatic reduction by glutathione reductase [15]. Thus, in addition to the ratio of GSH to GSSG, the content of glutathionylated proteins (GSSPs) can serve as indicators of oxidative stress. Within the RBCs, GSSG is found to react with haemoglobin forming glutathionyl haemoglobin (GS-Hb), which may serve as a useful clinical marker of oxidative stress.

In recent years, a number of non-enzymatic post-translationally modified proteins like haemoglobin adducts have been investigated in quest of new markers for disease, for diagnostic and/or prognostic purposes. A classic example is glycated haemoglobin (HbA1c), which is used as an indicator of time averaged glucose concentration in diabetic patients (6–8 weeks). Regular monitoring of glycated haemoglobin in diabetics is recommended to prevent long-term diabetic complications. Recently GS-Hb, another non-enzymatic post-translational modification, has been recognized as a probable marker in relation to oxidative stress in certain chronic diseases. Significant increase in glutathionyl Hb concentration, compared to its low value in healthy individuals has been found in diabetes mellitus, hyperlipidemia, Friedreich ataxia, chronic renal failure–conditions where oxidative stress has been implicated [16–19]. Characterization of glutathionyl haemoglobin at the residue level could be important to assess the functional alteration as possible adaptive mechanism in response to oxidative stress.

In this study, we have evaluated the degree of oxidative stress by quantitation of GS-Hb in different RRT groups using liquid chromatography–electrospray ionization mass spectrometry (LC/ESI-MS) in conjunction with other traditional markers of oxidative stress. The site of glutathionylation has been established using tandem mass spectrometry. The degree of haemoglobin glycation measured by mass spectrometry is in good agreement with established methodology.

Materials and methods

Patients

The study group consisted of patients in the age group of 30 to 68 years, who were recruited after obtaining written consent. The study was approved by the hospital (Manipal Hospital, Bangalore) ethics committee. Hemodialysis (HD) group consisted of 15 patients (male = 8, female = 7) undergoing regular dialysis of 12 h/week with 4 h in duration, using bicarbonate dialysate and polysulfone membrane dialyzer. The peritoneal dialysis (CAPD) group consisted of 12 continuous ambulatory peritoneal dialysis patients (male = 5, female = 7) under dialysis regimen for at least 6 months with a minimum of three exchanges per day with dextrose dialysate (2% to 4.5%). The transplant (Txp) group consisted of 15 stable renal allograft patients (male = 9, female = 6) with minimum 1 year post transplant period. Exclusion criteria were patients with diabetes, intercurrent infections, chronic inflammatory conditions and smokers. Control group consisted of 15 non-diabetic subjects (male = 8, female = 7) with no history of renal failure or other organ diseases.

Biochemical and clinical parameters

Fasting serum samples (blood collected in plain tubes without anticoagulant) was used for routine biochemical analysis on an automated chemistry analyzer (Dade Behring, USA) employing standard methodology using the kits supplied by the manufacturer. HbA1c was estimated by high-performance liquid chromatography (HPLC) using an automated Variant HPLC system (Bio-Rad, USA).

Glutathione estimation

Reduced erythrocyte glutathione (GSH) was estimated by the modified method of Beutler et al. [20] with minor modifications and GSH concentration was expressed in μmol/L whole blood.

Lipid peroxidation

Erythrocyte lipid peroxidation was measured as thiobarbituric acid reacting substances (TBARS) by the method of Jain et al. [21]. Molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ of 1,1′,3,3′-tetramethoxypropane (MDA) was used to calculate the TBARS concentration and expressed in nmol/g Hb.

Sample preparation

Venous blood anticoagulated with EDTA was the sample of choice. Plasma and buffy coat were aspirated off after centrifugation at 805×*g* for 10 min. The packed cells were washed with 0.9% NaCl thrice before lysis with eight volumes

of ice-cold distilled water. The haemolysates were centrifuged at 12,880×*g* for 10 min to remove the erythrocyte membranes. The clear supernatant was diluted 100 times with distilled water prior to mass spectrometric analysis.

Quantitation of glutathionyl haemoglobin

Electrospray Ionization mass spectra were acquired in positive ion mode using a single quadrupole mass spectrometer (Hewlett Packard HP 1100 MSD series). 12 μL of sample was injected through a C18 reverse phase analytical column (Zorbax, 4.6 mm × 150 mm, 5 μm), kept at ambient temperature (25 °C). Hb was eluted using a linear gradient of acetonitrile from 20% to 90% in 35 min with distilled water and acetonitrile containing 0.1% acetic acid as the mobile phase. The flow rate was maintained at 0.2 mL/min. Retention time for alpha and beta chains of haemoglobin were 20.2 min and 20.9 min, respectively. Data acquisition was set to full scan mode and the spectra were acquired over the mass range 200–3000 *m/z* with a scan time of 2.12 cycles/s. Mass scale calibration was done using ES tuning mix supplied by the manufacturer. Deconvolution of the multiply charged species was performed using the Chemstation software.

Since charge distribution of molecular ions of globin chains and their adducts are identical in mass spectra, it can be approximated that signal intensities can be correlated with their abundance. The most intense signals for molecular ions corresponding to α and β subunits and their corresponding adducts were distributed predominantly in the range of 17 to 21 protonated species. GS-Hb and Gly-Hb were quantitated from the signal intensities of their respective molecular ions according to the following equation [22]:

$$\text{GS}-\text{Hb}_\beta\ \% = \frac{\text{GS}-\text{Hb}_\beta \times 100}{(\beta + \text{GS}-\text{Hb}_\beta + \text{Gly}-\text{Hb}_\beta)}$$

$$\text{Gly}-\text{Hb}_\beta\% = \frac{\text{Gly}-\text{Hb}_\beta \times 100}{(\beta + \text{GS}-\text{Hb}_\beta + \text{Gly}-\text{Hb}_\beta)}$$

$$\text{Gly}-\text{Hb}_\alpha\ \% = \frac{\text{Gly}-\text{Hb}_\alpha \times 100}{(\alpha + \text{Gly}-\text{Hb}_\alpha)}$$

Characterization of glutathionyl haemoglobin

GS-Hb was prepared, *in vitro,* by incubating human haemoglobin (3.5 g/dL) obtained from the RBC lysates, with a large excess of GSSG (67 mmol/L) in 0.5 mol/L NH_4HCO_3 buffer (pH 8.0) for 12 h at 37 °C.

Enzyme digestion of GS-Hb was performed using chymotrypsin. 5 μL of GS-Hb sample was diluted with 40 μL of 0.05 mol/L NH_4HCO_3 buffer (pH 8.0). 5 μL of enzyme stock solution (0.5 mg/mL) was added and kept for 12 h of incubation at 37 °C.

MS analysis

Samples were analyzed using an Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Germany) in the reflectron mode using a 90-ns time delay, and a 25-kV accelerating voltage in the positive ion mode. The system utilizes a 50 Hz pulsed nitrogen laser, emitting at 337 nm. α-Cyano-4-hydroxy cinnamic acid was used as a matrix.

Tandem mass (MS/MS) spectra were acquired by selecting the precursor mass with a 10-Da window, and fragments were generated in post source decay mode (PSD). A single acquisition run was the sum of at least 50 series with 600 total added shots to generate the MS/MS spectra. Mass spectra were analyzed using Flex-analysis software, provided by the manufacturer.

Statistical analysis

Quantitative data are expressed as mean ± SD. Group comparison was done by Student's *t*-test and $p<0.05$ was considered significant. Univariate regression analysis was done using online statistical analysis programme.

Results

Table 1 shows clinical parameters of patient groups compared to the control group. Decrease in GSH was more pronounced in the dialysis group with no significant changes in the transplant group. Similarly, an increase in the lipid peroxidation of erythrocytes as indicated by increased TBARS levels was significantly higher in the dialysis group compared to the transplant group. Decreased GSH, observed in the dialysis group, correlated negatively with the erythrocyte TBARS ($r=-0.63$; $p<0.001$). A positive correlation was observed between the duration of RRT and GS-Hb ($r=0.744$, $p=0.001$) in the dialysis group.

Fig. 1 shows the ESI mass spectrum of haemoglobin from a control subject. Panel A and B shows mass spectra of alpha and beta globin chains with their adducts, respectively. Deconvolution of the mass spectral signals corresponding to different charged states yielded almost precise masses (±2 Da) of Hbα, Hbβ and their adducts. The alpha and beta set consisted of the following masses: α (15,125 Da; $M_{\text{calculated,}}$ 15,126 Da), glycated α (15,287 Da; $M_{\text{calculated}}$ 15,288 Da), β (15,866 Da; $M_{\text{calculated,}}$ 15,867 Da), glycated β (16,028 Da; $M_{\text{calculated,}}$ 16,029 Da) and glutathionylated β (16,171 Da; $M_{\text{calculated,}}$ 16,172 Da). Fig. 2 shows the ESI-MS of alpha globin chain and its adduct for RRT groups. Panels A, B, and C correspond to transplant, CAPD and HD, respectively, with their deconvoluted mass spectra shown as insets. Fig. 3 shows the ESI-MS of beta globin chain and its adducts for the patient category in the same order. We observed augmented intensities for glutathionyl haemoglobin in HD and CAPD compared to the transplant group and relatively higher peak intensities for glycated adducts in both the globin chains in CAPD group compared to the other two groups.

In all patient groups, we were able to quantify the amount of GS-Hb in terms of percentile values (see Materials and methods), and these values were significantly higher in HD and CAPD followed by Txp with respect to control group as shown in Table 2.

Table 1
Clinical and biochemical parameters of study subjects

	Control (n=15)	Transplant (n=15)	CAPD (n=12)	HD (n=15)
Age (years)	49±7.6	49.1±6.8	54.2±7.1	50.1±8.5
RRT duration (months)		47.2±32.3	24.9±12.1	28±17.3
Hemoglobin (g%)	14.27±1.51	13.10±1.49	10.93±1.76[a]	9.74±1.56[a]
Creatinine (mg/dL)	0.9±0.14	1.26±0.26	8.92±2.31	8.60±2.84
Cholesterol (mg/dL)	155.47±80.8	181.8±38.81[a]	181.4±36.01[a]	146.07±41.13[ns]
Triglycerides (mg/dL)	100.13±26.79	169±55.41[c]	170.27±60.85[c]	167.07±63.68[b]
HDL cholesterol (mg/dL)	44.93±7.50	42.93±11.0[ns]	38.2±8.94[a]	34.6±7.01[b]
LDL cholesterol (mg/dL)	90.50±19.36	104±18.40	109.15±30.29	78.05±41.27
Albumin (g/dL)	4.26±0.46	3.75±0.55	3.52±0.41	3.45±0.39
CRP (mg/L)	3.10±2.1	9.5±7.85[a]	15.74±12.1[c]	14.62±10.63[c]
HbA1c (%)	5.4±0.36	5.85±0.51	6.26±0.77[c]	5.62±0.67
Oxidative stress markers in erythrocytes				
GSH (mmol/L)	2.26±0.31	2.50±0.55[ns]	1.72±0.49[b]	1.65±0.29[c]
TBARS (nmol/g Hb)	4.15±0.79	5.19±1.07[a]	8.11±1.99[c]	9.78±3.07[c]

Data are the mean±SD. n=no of subjects.
[a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$ – compared to control group; ns – not significant.

GSH and GS-Hb showed negative correlation ($r=-0.66$, $p<0.001$) whereas lipid peroxidation and the GS-Hb levels showed positive correlation ($r=0.6$, $p<0.001$). The average glycated haemoglobin (Hbα and Hbβ) from ESI-MS showed good correlation with the percent HbA1c from conventional HPLC ($r=0.53$, $p<0.05$) (Table 2).

In vitro glutathionylation of normal human haemoglobin sample incubated with oxidized glutathione (GSSG) showed a pronounced peak for 16,171 Da, corresponding to glutathionylated beta chain (Fig. 4). Digestion of GS-Hb with chymotrypsin yielded a peptide fragment with mass of 2374 Da and a peak of low intensity having a mass of 2069 Da. The peptide of

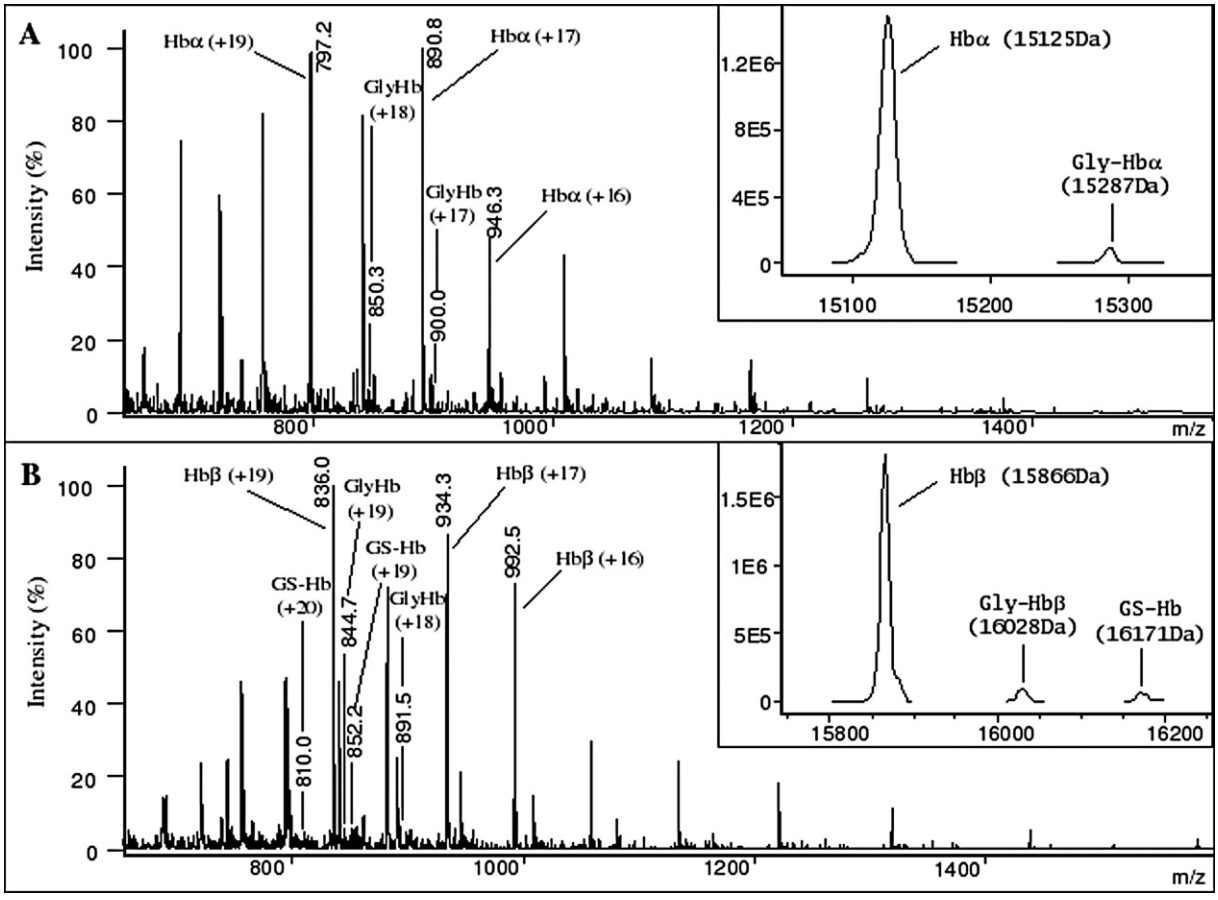

Fig. 1. ESI mass spectrum of haemoglobin sample from a normal subject: (A) α globin chain and its adduct with deconvoluted masses of α 15,125 Da, Gly-Hb_α 15,288 Da (inset). (B) β globin chain and its adducts with deconvoluted masses of β 15,866 Da, Gly-Hb_β 16,028 Da and GS-Hb 16,171 Da (inset).

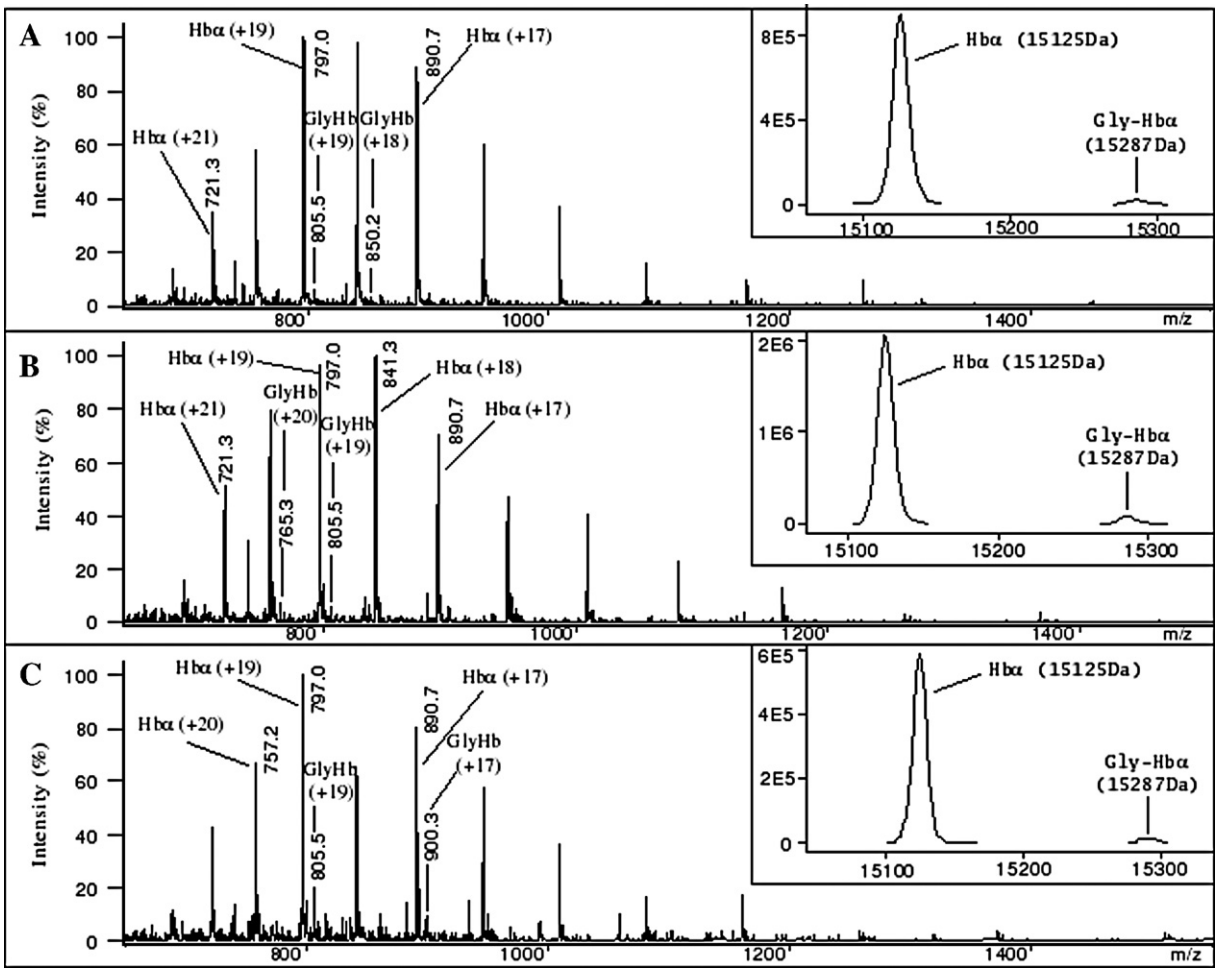

Fig. 2. ESI mass spectrum of haemoglobin sample from patients belonging to different renal replacement therapy groups. Panels A, B and C show mass spectrum of α globin chain and its adducts in a transplant, CAPD and HD patients with deconvoluted masses of α 15,125 Da and Gly-Hb$_\alpha$ 15,287 Da (inset).

mass 2069 Da may be assigned to the fragment residue 86–103 in beta globin chain of haemoglobin, which contains a Cys residue at position 93. The peptide of mass 2374 Da may be attributed to glutathionylation (ΔM=305 Da) of Cys 93 residue in 2069 Da fragment.

The tandem mass spectrum of the precursor peptide ion having a mass of 2373.97 Da consists of a series of 'b' and 'y' ions (Fig. 5). Assignment of these molecular ions along with their neutral losses, followed by *de novo* sequencing, confirms that the peptide fragment corresponds to the residue 86–103 of Hb β-chain. The resulting mass difference of 408 between b7 and b8 in 'b' ion series and y10 and y11 in 'y' ion series confirms that Cys-93 of the beta globin chain is the site of glutathione attachment via a disulfide bridge.

Discussion

S-Glutathionylated proteins have been investigated as possible biomarkers of oxidative stress in correlation with disease. The variations in glutathionylated proteins, caused by pathophysiological conditions, can make them mediators of important functions or parameters of clinical significance [23]. Though earlier studies have indicated elevated levels of GS-Hb in uremic patients [19], our study assumes importance as we have tried to assess the utility of this marker in dialysis as well as transplant patients in conjunction with other markers of oxidative stress.

Oxidative stress has long been implicated as one of the co-morbid factors leading to premature mortality in relation to dialysis [24,25]. Persistence of oxidative stress after successful transplantation was reported by several authors [26–29]. Maintenance treatment with immunosuppressive drugs like cyclosporine (CsA) may enhance ROS production in transplant patients [30,31]. Increased erythrocyte TBARS levels and decreased GSH levels in dialysis group compared to the transplant group indicates an increased production of ROS and impairment of the antioxidant defence system. This is supported by a negative correlation between GSH and TBARS. The trend observed with GS-Hb was similar to the above two parameters. Significantly elevated GS-Hb levels in the dialysis group could be due to oxidative perturbations arising out of substitutive therapies. In case of HD patients, as mentioned earlier, it could be due to repeated blood loss, contact with dialyzer membranes, loss of vital soluble antioxidants like vitamin C during dialysis, malnutrition, intravenous iron supplementation and inflammation (as evidenced by elevated CRP levels; Table 2) all of which could be responsible for high oxidative stress.

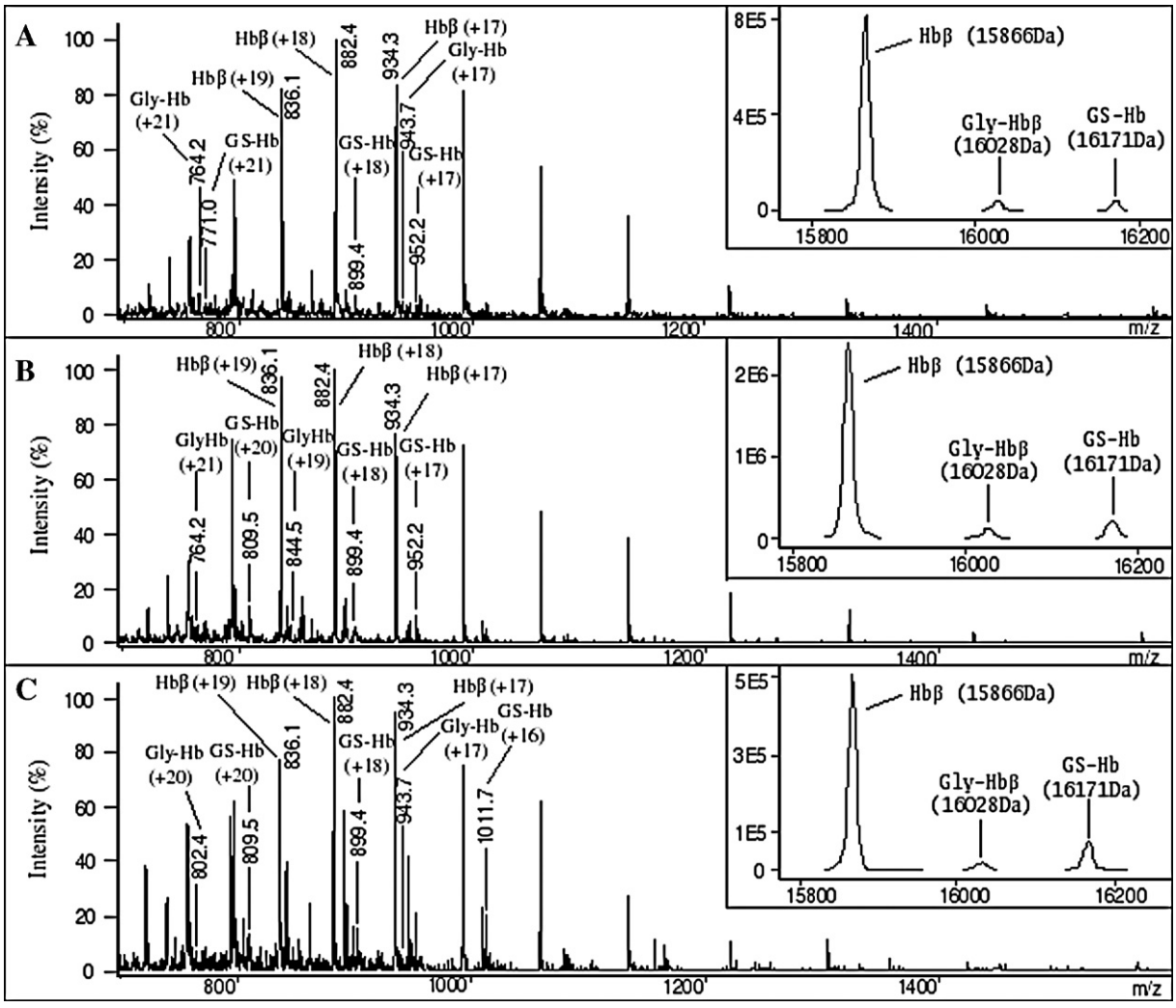

Fig. 3. ESI mass spectrum of haemoglobin sample from patients belonging to different RRT groups. Panels A, B and C show mass spectrum of β globin chain and its adducts in a transplant, CAPD and HD patients with deconvoluted masses of β 15,866 Da, Gly-Hb_{β} 16,028 Da and GS-Hb 16,171 Da (inset).

In CAPD though the exchange takes place *in vivo*, inflammation of the peritoneum, malnutrition and hyperglycaemia from the dialysate solution could contribute to the oxidative burden. Previous studies have indicated increased lipid peroxidation and reduced GSH in erythrocytes and mesangial cells subjected to hyperglycaemia [21,32]. The reduced GSH levels are accompanied by decreased activity of gamma-glutamylcysteine synthetase, the rate-limiting enzyme in the *de novo* synthesis of GSH as well as a reduced glucose-6-phosphate dehydrogenase (G-6-PD) activity, which supplies NADPH for glutathione reductase to reduce GSSG inside the erythrocytes, as indicated by previous studies in uremic population [33–35]. Reduced levels of GSH in the dialysis group may represent an imbalance of redox signalling, favouring increased formation of GSSG and subsequent protein glutathionylation which is in agreement with our findings. Hence, increased GSSG as a consequence of decreased glutathione reductase activity and G-6-PD activity could be responsible for increased erythrocyte GSSG concentration, favouring GS-Hb formation.

In case of transplant subjects the intracellular GSH levels are comparable to that of controls. These findings indicate that oxidative stress may induce post-translational changes in proteins, which might play an important role in the maintenance and progression of disease. Protein glutathionylation promises to

Table 2

Hb adducts	Category			
	Control group ($n=15$)	Transplant group ($n=15$)	Peritoneal dialysis group ($n=12$)	Hemodialysis group ($n=15$)
GS-Hb (%)	3.15±1.99	4.69±3.19	7.63±1.81[a]	9.99±2.97[a]
Gly-Hb_{β} (%) (ESI-MS)	5.08±0.89	7.65±3.78	7.77±1.37	6.92±2.27
Gly-Hb_{α} (%) (ESI-MS)	4.09±1.77	5.06±1.71	5.81±1.52	5.06±2.9
Average glycation (%) (ESI-MS)	4.60±1.33	6.35±2.74	6.8±1.44[a]	5.99±2.58
HbA1c (%) (HPLC)	5.2±0.35	6.12±0.96	6.3±0.49[a]	5.71±0.61

Glutathionylated Hb and glycated adducts of β and α subunits by ESI-MS and HbA1c in control and renal replacement therapy group.
Values are mean±SD. n=number of subjects.
[a] $p<0.05$.

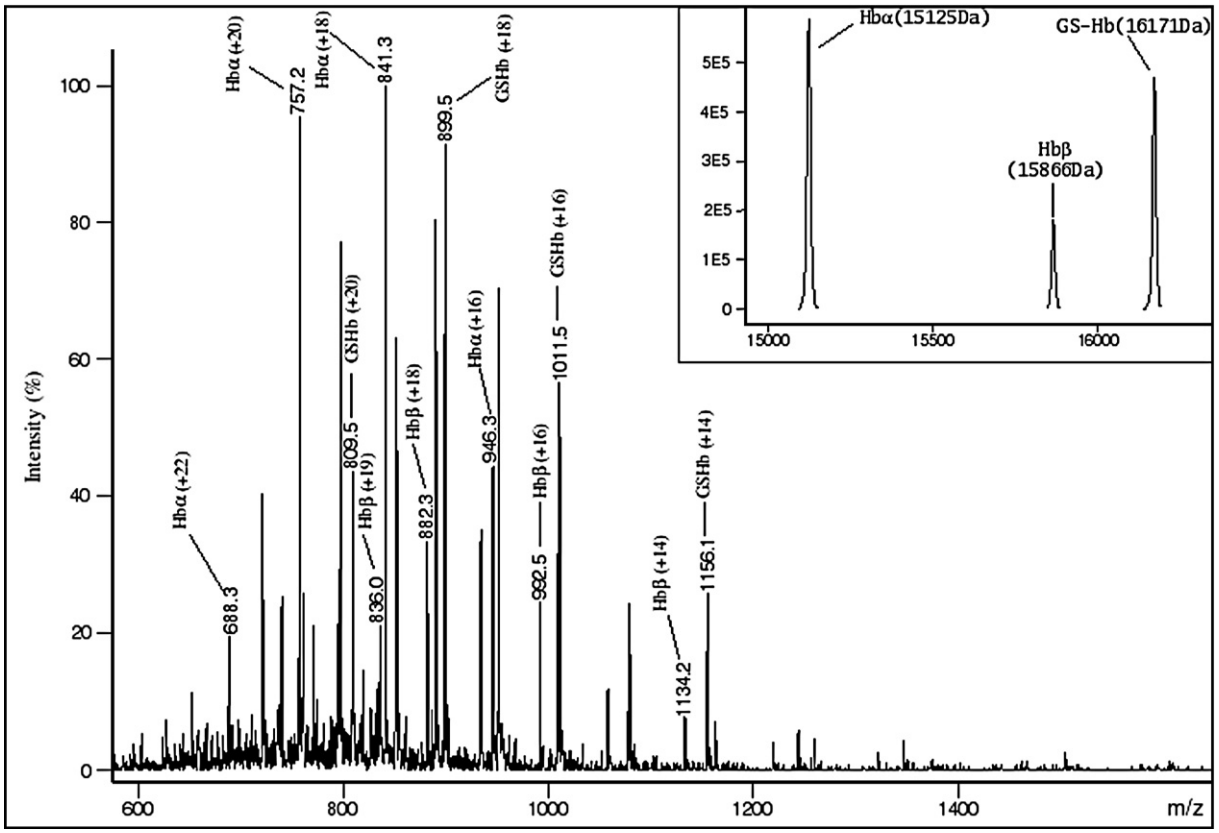

Fig. 4. ESI mass spectrum of *in vitro* glutathionylated human haemoglobin with α, β and glutathionylated β globin chain with deconvoluted masses of α 15,125 Da, β 15,866 Da and an intense peak for GS-Hb 16,171 Da.

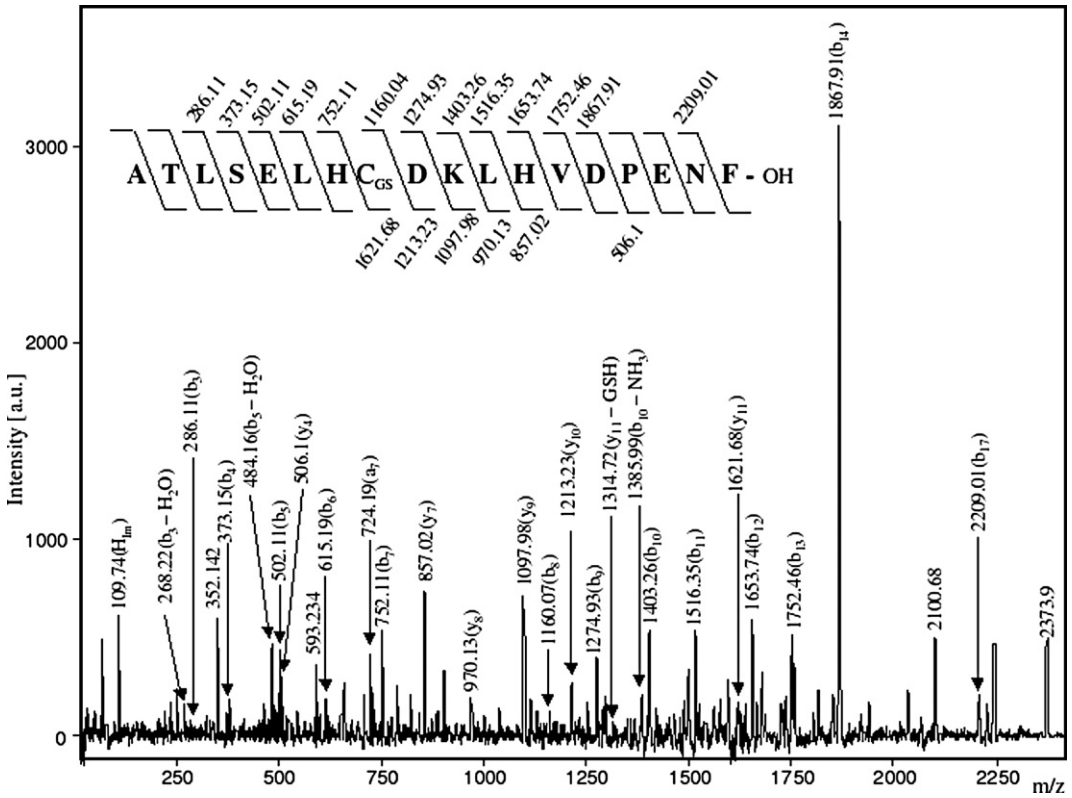

Fig. 5. Tandem mass spectra of precursor peptide 2373.97 Da. Series of 'b' and 'y' ions are labeled. *De novo* sequence of peptide is shown (inset).

be a sensitive indicator of intracellular redox changes in chronic stressed conditions like uremia and associated complication arising out of different RRT.

The utility of glutathionyl haemoglobin as a clinical marker can only be judged if studies are extended to a much larger group of chronic renal failure patients. In the design of our study, we have deliberately excluded diabetic patients from our sample because increased glutathionyl haemoglobin level has been previously reported in the case of diabetes. Restrictions imposed by limiting the study to a non-diabetic patient group limit the number of subjects who can be recruited into such a study. It would therefore be premature to suggest that glutathionyl haemoglobin can be used in clinical evaluation in the case of chronic renal failure patients who are under specific treatment regimens.

The average levels of glycated haemoglobin measured by mass spectrometry showed good correlation with HbA1c determined by HPLC across the RRT groups with a slight decrement in the control group. Quantitation of glycated species served to substantiate the fact that GS-Hb quantitation by mass spectrometry is reliable. Extensions to other haemoglobin adduct characterization and quantitation merits further study.

Human adult haemoglobin is a tetramer molecule ($\alpha_2\beta_2$) with one Cys residue in the alpha chain and two in the beta chain. Efficient *in vitro* glutathionylation of haemoglobin establishes the fact that Cys-93 of beta globin chain is the preferred site for non-enzymatic attachment of glutathione leading to the formation of GS-Hb, which is in agreement with the surface accessibility of beta Csy-93 residue from previous studies [36,37].

In conclusion, intracellular GSH redox balance is a key factor that determines the protein functionality with respect to oxidative stress. Glutathionylated proteins like GS-Hb can serve as good clinical markers of oxidative stress in chronic debilitating substitutive therapies like dialysis and transplantation. Whether GS-Hb merely serves as an indicator or is associated with the pathogenesis of RRT associated complications needs to be evaluated.

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