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Clinical assay of four thiol amino acid redox couples by LC–MS/MS: Utility in thalassemia☆

Jung H. Suh[a,*], Robert Kim[a], Burcu Yavuz[a], Daniel Lee[a], Ashutosh Lal[a], Bruce N. Ames[b], Mark K. Shigenaga[a,*]

[a] Nutrition and Metabolism Center, Children's Hospital Oakland Research Institute, 5700 Martin Luther King Jr. Way, Oakland, CA 94609, USA
[b] Department of Molecular and Cell Biology, University of California Berkeley, Berkeley, CA, USA

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ABSTRACT

The total concentrations of four sulfur amino acid (SAA) metabolite redox couples (reduced and oxidized forms of homocysteine, cysteine, glutathione, and cysteinylglycine) in human blood are assayed with a simple and sensitive method by liquid chromatography–electrospray positive ionization–tandem mass spectrometry. To prevent ex vivo thiol oxidation, iodoacetamide (IAM) is used immediately following the blood draw. To selectively enrich for *S*-carboxyamidomethylated SAA, and other cationic amino acids metabolites, proprietary strong cation-exchange solid phase extraction tips are used. Analytes are further derivatized with isopropylchloroformate (IPCF) to esterify the amino and the carboxylic groups. Double derivatization with IAM and IPCF improves the reverse phase liquid chromatography separation of SAA metabolites. The use of detection mode of multiple-reaction monitoring (MRM) allows sensitive and specific simultaneous detection of SAA. The internal standards used to account for the matrix effects of human plasma and erythrocytes were plant glutathione analogue, homoglutathione, and stable isotopes of cystine and homocystine. The method was validated for its linearity, accuracy, and precision. Excellent linearity of detection ($r^2 > 0.98$) was observed over relevant ranges for plasma and erythrocyte samples, and the limits of detection were established to be between 5 and 20 nM. Relative standard deviations were <9% for within-day variations and <15% for between-day variations. The method was used to assess thiol redox states in plasma and erythrocytes isolated from healthy subjects and thalassemia patients.

1. Introduction

Sulfur amino acid (SAA) metabolism plays an essential role in the regulation of oxidative stress in biological fluids, cells, and tissues [1–4]. Four closely integrated metabolic pathways – the transmethylation, transsulfuration, glutathione synthesis, and the glutathione catabolic pathways – produce several key metabolites that are essential for physiological redox regulation [1,5]. Four unique sulfur-containing redox couples, namely, homocysteine (Hcy)/homocystine (HcySS), cysteine (Cys)/Cystine (CysSS), glutathione (GSH)/glutathione disulfide (GSSG) and the cysteinylglycine (CysGly)/cysteinylglycine disulfide (CysGlySS) are produced by the above pathways. On a metabolic level, these SAA metabolites share tightly regulated precursor–product relationships. For example, Hcy serves as an important metabolic precursor for endogenous Cys synthesis by the transsulfuration pathway [5,6]; Cys is the rate-limiting substrate for GSH formation [7]; and, CysGly is formed during GSH-breakdown and subsequently can be recycled to yield cysteine in cells [8].

Aside from their metabolic linkages, these compounds also interact with each other and with other protein thiol targets chemically through thiol–disulfide exchange reactions that can ultimately influence cellular thiol/disulfide equilibrium. The specificity of their redox interactions are regulated by their individual concentrations and their specific standard redox potentials, which determine the thermodyamic feasibility for reduction/oxidation reactions between thiol redox couples and oxidants. For example, Hcy, which has a low reduction potential of −200 mV, exists predominantly as mixed disulfides and can act as a pro-oxidant [9]. In contrast, GSH has high reduction potential of −264 mV and exists predominantly in its reduced form even in the highly oxidizing extracellular environment. The steady-state redox states of these compounds appear to be discretely regulated. In human plasma, the Cys/CysSS redox couple is maintained at a more oxidized state than the GSH/GSSG redox couple, and upon exposure to oxidants, the oxidation of the Cys/CysSS precedes the oxidation of the GSH/GSSG redox couple [2–4,10]. Distinctive redox regulation of these com-

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* Corresponding authors. Tel.: +1 510 428 3885x2886; fax: +1 510 450 7910.
E-mail addresses: jsuh@chori.org (J.H. Suh), mshigenaga@chori.org (M.K. Shigenaga).

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pounds suggests that comprehensive analysis of all four oxidation states may provide more detailed information on systemic oxidative stress than a "snapshot" view of just a single redox couple [4].

Thalassemias are caused by defective synthesis of either α- or β-globin chains. In β-thalassemia, impaired β-globin synthesis causes an accumulation of unpaired α-globin protein [11]. As a consequence of disrupted globin synthesis, β-thalassemia patients suffer from ineffective erythropoesis and anemia, which necessitates lifelong blood transfusions [12]. Frequent blood transfusions cause secondary iron-overload in thalassemia patients, leading to excessive iron depositions in tissues such as the liver and the heart [13]. Increased availability of redox-active iron may cause oxidative damage to tissues and elevate the risks for cardiovascular and endocrine dysfunctions [12,14,15].

Iron-chelation therapy aimed at normalizing tissue iron status can lessen the incidence and the severity of secondary clinical complications in thalassemias [15,16]. However, owing to the lack of proximate sensitive and accessible markers of the consequences of iron-overload, the clinical management of iron-overload using chelation therapy remains imprecise [17,18]. Because SAA metabolites are sensitive targets of iron-dependent oxidative stress, alterations in SAA redox states in plasma or in erythroctyes may potentially serve as sensitive biomarkers of iron toxicity in thalassemias. While there are numerous analytical methods for quantifying SAA metabolites [19–28], existing methods have not been validated for measuring Hcy, Cys, GSH and CysGly redox states in thalassemia.

In the current work, we developed a simple and fast sample processing and analysis method for Hcy, Cys, GSH, and CysGly quantification. In this method, analytes are sequentially derivatized with iodoacetamide (IAM) and isopropylchlroformate (IPCF), and the derivatives are chromatographically resolved and detected by electrospray positive ionization–tandem mass spectrometry. Immediate mixing of blood samples with IAM minimizes the potential ex vivo oxidation in blood samples. This step is especially critical for thalassemia samples, where increased iron and hemoglobin availability increase the likelihood of thiol oxidation during plasma and erythroctye isolation from whole blood. The subsequent IPCF derivatization improves the chromatographic resolution of amino acid analytes [29,30]. For IPCF derivatization, a commercially available EZ-FAAST^{TM} Amino Acid LCMS kit is used. The EZ-FAAST kit includes a proprietary strong cation exchange resin filled solid-phase extraction (SPE) tips and reversed-phase HPLC column that greatly enhances analyte enrichment and improve chromatographic separation of analytes. The SPE tips included in this kit cannot be purchased individually and provides a convenient small volume analyte enrichment solution that cannot be easily replicated with standard SPE products. The combined usage of sequential IAM/IPCF derivation procedure with the proprietary SPE tips and HPLC column provided in the EZ-FAAST kit, efficiently minimized the ex vivo oxidation, improved the enrichment of low abundant oxidized disulfides, and the specificity of detection of the SAA metabolites.

2. Experimental

2.1. Reagents and analytical standards

The following reagents of highest analytical grade were purchased from Sigma–Aldrich (St. Louis, MO): homocysteine (Hcy), homocystine (HcySS), cysteine (Cys), cystine (CysSS), glutathione (GSH), glutathione disulfide (GSSG), cysteinyl glycine (CysGly), cysteinyl glycine disulfide (CysGlySS), diethylene triamine pentaacetic acid (DTPA), dithiothreitol (DTT), Tris-base and iodoacetamide (IAM). Homoglutathione (hGSH) was purchased from BACHEM America Inc. (Torrance, CA). Stable isotopes, homocystine (3,3,3′,3′,4,4,4′,4′–d_8; HcySS–d_8), and cystine (3,3,3′3′–d_4; CysSS–d_4), were purchased from Cambridge Isotopes (Andover, MA). The isotopic purity of HcySS–d_8 and CysSS–d_4 were both >98%. Perchloric acid (PCA) and HPLC-grade solvents were purchased from Fisher scientific (Pittsburg, PA). The EZ-FAAST kit was purchased from Phenomenex (Torrance, CA).

2.2. Overview of analytical work flow

The analytical work flow for sample processing is shown in Fig. 1. Blood samples are divided into two aliquots. The first aliquot is used for quantifying total SAA metabolite (RSH) concentrations and the second aliquot is used for measuring the redox states of various SAA metabolites. To obtain total SAA concentrations, plasma samples are chemically reduced with DTT, prior to derivatization with IPCF. For thiol redox analysis, whole blood samples are immediately mixed with IAM and the plasma and the erythrocyte fractions are isolated by centrifugation. These samples are subsequently derivatized with IPCF.

For quantification purposes, reduced standards sequentially derivatized with IAM and IPCF and are used to generate standard curve used for SAA redox analysis. Similarly, reduced standards derivatized with IPCF alone are used for total SAA quantification. Oxidized disulfide derivatized with IPCF is used for quantifying disulfide forms of SAA metabolites in all samples.

2.3. Standard preparation

Two sets of reduced SAA external calibrant mixes were prepared. In the first set, Hcy, Cys, GSH and CysGly standards was prepared at 10 mM concentration by dissolving the standards in 5% PCA solution containing 0.5 mM DTPA, as a metal chelator. In the second set, 10 mM concentrations of the reduced SAA metabolites are dissolved in 20 mM IAM prepared in 100 mM Tris–HCl (pH 8.0) buffer.

The second set consisting of 10 mM HcySS, CysSS, GSSG, and CysGlySS was dissolved in PCA solution.

Plant glutathione analogue, homoglutathione (hGSH) and stable isotopes CysSS–d_4, and HcySS–d_8 were used as internal standards. Similar to reduced standards, the internal standard mixes were prepared at 10 mM concentrations and dissolved in either 5% PCA or in 20 mM IAM solutions.

Quality control (QC) samples were prepared by spiking 180 μl aliquots of plasma samples with 20 μl of external standard mixes to obtain the final concentrations of 0.5, 5 and 50 μM. 100 μl aliquot of the QC samples were immediately mixed with equal volume of 20 mM IAM dissolved in 100 mM Tris–HCl (pH 8) buffer. The remaining 100 μl aliquot of spiked plasma was stored and used as QC samples for IPCF reactions. All QC samples were stored at −80 °C and thawed on ice before use.

2.4. Human subjects

The Institutional Review Board at Children's Hospital Oakland Research Institute approved our study procedures for subject recruitment and consent. Twelve non-obese healthy normal controls (age 30–45 years old) were enrolled and blood samples were obtained after an overnight fast. Four β-thalassemia major patients (age 20–35 years old; 3 males and 1 female) undergoing blood transfusions were enrolled and their overnight fasting blood samples were obtained three weeks after their last transfusion. All thalassemia patients were receiving standard iron-chelation therapy. The average duration of transfusions in our subjects was 12.6 ± 5 years. Patients with acute illness, diabetes, liver dysfunc-

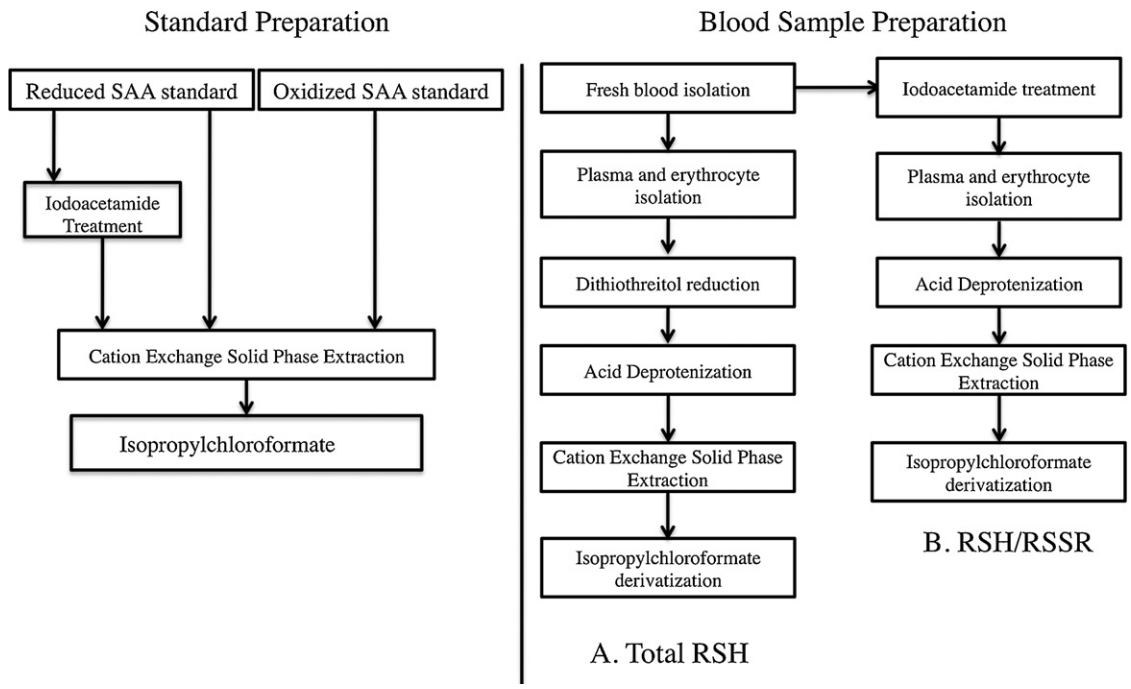

Fig. 1. Analytical work flow. Standard solution containing known amounts of reduced homocysteine, cysteine, glutathione and cysteinylglycine are sequentially derivatized with iodoacetamide (IAM) and isopropylchloroformate (IPCF) or with IPCF alone. Oxidized disulfides only react with IPCF. Standards derivatized with IPCF are used to generate calibration curves to quantify total SAA metabolite concentrations (A. Total RSH), where as, the standards sequentially derivatized with IAM and IPCF are used to quantify SAA metabolites redox states (B RSH/RSSR). To preserve the thiol redox states, freshly isolated whole blood is immediately treated with IAM following the blood draw. Plasma and erythrocyte fractions are isolated and acid deproteinated. The acid-soluble fraction is collected and processed with strong cation solid phase extraction (SPE) step to enrich for SAA metabolites and that are further derivatized with IPCF (A. Total RSH). For total RSH quantification (B RSH/RSSR), the samples are chemically reduced with DTT prior to the SPE and IPCF derivatization steps.

tions, and cardiac abnormalities at the time of blood draw were excluded from the study.

2.5. Preparation of samples

Twelve hour fasting whole blood was collected by venipuncture to the antecubital vein using a 21-gauge butterfly needle attached to a 10 ml Vacutainer tube containing K_3-EDTA as an anticoagulant agent. Immediately following the blood draw, 1 ml aliquot of whole blood was gently mixed with 1 ml of 20 mM IAM dissolved in 100 mM Tris–HCl (pH 8) buffer. The IAM-diluted samples are then transferred into a 2 ml Eppendorf tubes and centrifuged at 10,000 × *g* for 1 min and the plasma and the erythrocyte fractions are collected into two separate tubes.

Another 1 ml aliquot of blood samples was centrifuged at ambient temperature at 10,000 × *g* for 1 min and the plasma and erythrocyte fractions were collected and stored in −80 °C. The exact dilution factors for IAM-diluted plasma were calculated by dividing the protein concentration in undiluted plasma sample with values obtained from IAM-diluted sample from the same individual. Because plasma and erythrocyte volumes may vary, the exact dilution of plasma sample needs to be calculated, when whole blood is diluted directly with IAM solution. For normalization of erythrocyte metabolites, hemoglobin concentrations were measured by Drabkin's method [31].

2.6. Sample derivatization of IAM treated standard and biological samples

2.6.1. Preparation of $KOH/K_2B_4O_7$ buffer

The $KOH/K_2B_4O_7$ buffer is prepared by dissolving 5.6 g KOH and 50 g $K_2B_4O_7$ in 100 ml of water. The supersaturated solution is allowed to stand overnight and the remaining salt removed by filtration [32].

2.6.2. Plasma and erythrocyte SAA redox analysis

Frozen IAM-diluted plasma and erythrocyte samples are thawed on ice. For plasma analysis, 200 μl of plasma sample is mixed with 200 μl of 10% PCA solution, while 40 μl of the packed erythrocyte sample is diluted in 360 μl of 5% PCA solution. Acidified samples are centrifuged and clear supernatants are collected for IPCF derivatization. External calibrant mix dissolved in 20 mM IAM solution is diluted in 5% PCA solution to achieve the final concentrations of 0.025, 0.05, 0.5, 5.0, 50 and 100 μM.

To 180 μl of the acidified samples and standards, 20 μl of 100 μM internal calibrant mix prepared in IAM solution was added. Following this step, samples are treated with 70 μl of $KOH/K_2B_4O_7$ buffer to precipitates out the excess perchlorate salt that interferes with the strong cation-exchange solid phase extraction. The KOH-treated samples are then centrifuged at 10,000 × *g* for 5 min. 200 μl of the clear supernatant is transferred into a glass vial for IPCF derivatization.

2.6.3. Plasma reduction with DTT and total SAA concentration analysis

150 μl of frozen plasma samples were thawed on ice and subsequently incubated with 150 μl of 50 mM DTT solution containing 1 mM potassium oxalate at room temperature for 10 min to completely reduce plasma mixed disulfides [30]. Upon completion of the DTT reduction step, 300 μl of 10% PCA solution is added, vortexed and centrifuged at 10,000 × *g* for 5 min. Clear acid-soluble supernatant is collected for derivatization and stored in −80 °C. External calibrant mix dissolved in 5% PCA solution is diluted with the same solution to achieve the final concentrations of 0.025, 0.05, 0.5, 5.0, 50 and 100 μM.

Table 1
Mean retention times and analyte-specific MS parameters.

Analyte	MW	Precursor ion $[M+H]^+$	Product ion	Retention time	Cone voltage	Collision energy
IAM + IPCF derivative						
Homocysteine	135.2	321.0	142.0	5.0 ± 0.5	28	15
Cysteine	121.2	307.0	116.0	4.7 ± 0.5	28	18
Glutathione	307.3	535.5	418.5	4.9 ± 0.4	28	18
Cysteinylglycine	178.2	364.0	116.0	5.7 ± 0.6	28	20
Homoglutathione	321.3	549.5	418.5	5.2 ± 0.5	28	18
IPCF derivative						
Homocysteine	135.2	350.0	204.0	10.9 ± 0.5	30	13
Cysteine	121.2	336.5	190.3	9.8 ± 0.4	30	12
Glutathione	307.3	564.5	162.2	8.2 ± 0.5	30	20
Cysteinylglycine	178.2	393.5	248.5	7.3 ± 0.4	30	17
Homoglutathione	321.3	578.5	162.2	9.6 ± 0.4	30	20
Homocystine	268.4	525.5	262.0	12.5 ± 0.5	35	15
Homocystine–d_8	276.4	533.5	266.5	12.5 ± 0.5	35	15
Cystine	240.3	497.5	248.5	10.6 ± 0.6	37	17
Cystine–d_4	244.3	501.5	250.5	10.6 ± 0.6	37	17
Glutathione disulfide	612.6	953.5	836.5	11.7 ± 0.5	58	20
Cysteinylglycine disulfide	297.4	611.5	363.5	9.7 ± 0.5	35	20

To 180 μl of the acidified samples and standards, 20 μl of 100 μM internal calibrant mix prepared in 5% PCA solution was added. Following this step, samples are treated with 70 μl of $KOH/K_2B_4O_7$ buffer and centrifuged at 10,000 × *g* for 5 min. 200 μl of the clear supernatant is transferred into a glass vial for IPCF derivatization.

The integrated peak area ratios of each SAA analyte (Ext) and their specific internal standards (IS) were plotted against the known amounts injected to generate the standard curves for each compound. The regression equation obtained was used to calculate the concentrations of analytes in plasma and erythrocyte samples.

2.6.4. IPCF derivatization

IPCF derivatization was performed using the EZ-FAAST LC/MS amino acid analysis kit according to the manufacturer's protocol. The EZ-FAAST kit is a commercial product from Phenomenez (Torrance, CA), which is designed for rapidly quantifying more than 50 different amino acid metabolites. IPCF derivatization protocol and analysis of IPCF-derivatized amino acid metabolites have been validated by Phenomenex (for list of metabolites detectable using this assay see the website: http://www.phenomenex.com/products/brands/view.aspx?id=178). Analyte's specific linearity of detection, detection limits and recovery in blood samples are summarized in Tables 2 and 3.

2.7. HPLC condition

Liquid chromatography was performed on a Shimadzu LC-10AD HPLC system equipped with a SIL-10 AVP autosampler, and LC–AD pumps. The chromatographic separation was performed using an EZ:FAAST 3 μm AAA–MS 150 mm × 3.0 mm HPLC col-

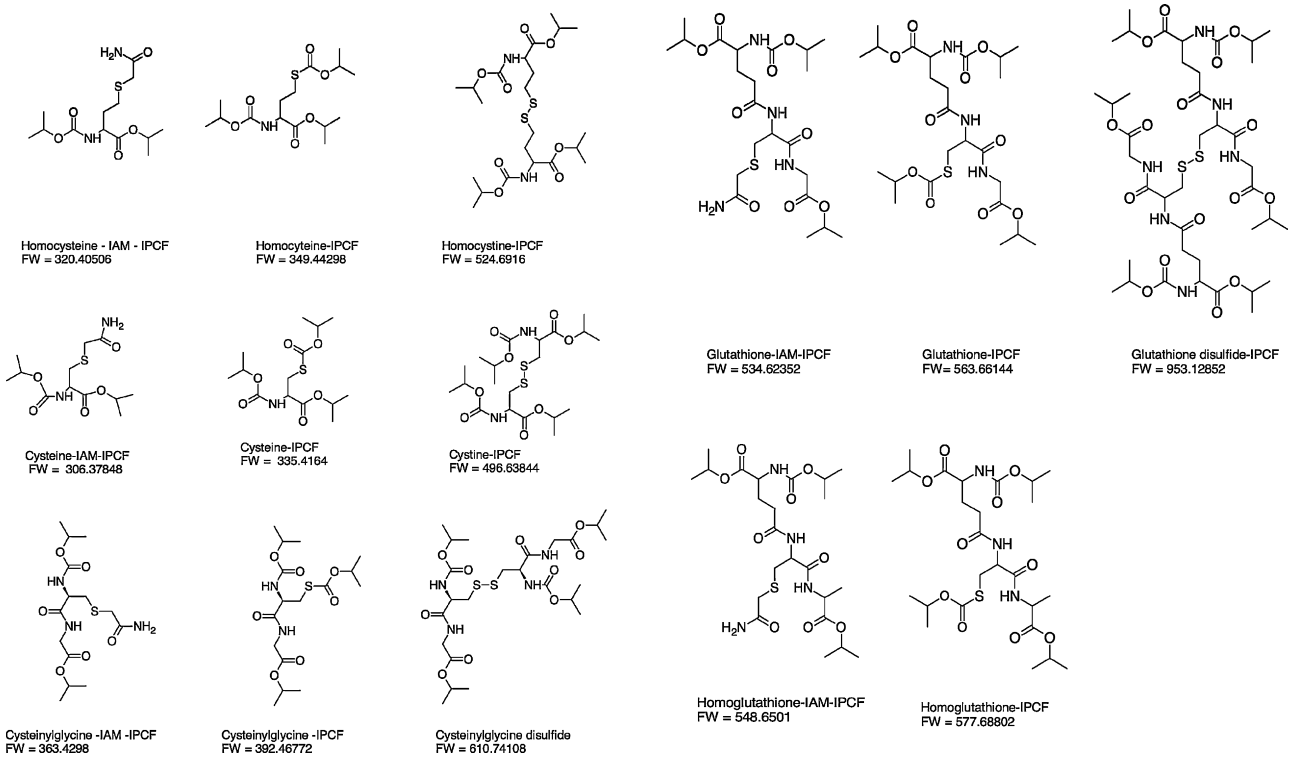

Fig. 2. The structures of sulfur amino acid metabolites derivatized with iodoacetamide and isopropylchloroformate or with isopropylchlorofomate.

Table 2
The regression equations and limits of detection and quantitation of analytes.

Analyte	Internal standard	Regression equation	Linear range	r^2	LOQ (nM)	LOD (nM)
IAM + IPCF derivative						
Homocysteine	Homoglutathione	$y = 0.13x - 0.02$	0.05–100	0.999	10	0.5
Cysteine	Homoglutathione	$y = 0.04x + 0$	0.05–100	0.986	20	0.5
Glutathione	Homoglutathione	$y = 0.25x + 0.05$	0.05–100	0.999	10	0.5
Cysteinylglycine	Homoglutathione	$y = 0.02x - 0.002$	0.05–100	0.999	20	0.5
IPCF derivative						
Homocysteine	Homoglutathione	$y = 0.06x - 0.01$	0.05–100	0.999	10	0.5
Cysteine	Homoglutathione	$y = 0.03x - 0.02$	0.05–100	0.992	20	0.5
Glutathione	Homoglutathione	$y = 0.32x + 0.10$	0.05–100	0.999	10	0.5
Cysteinylglycine	Homoglutathione	$y = 0.09x + 0.02$	0.05–100	0.988	20	0.5
Homocystine	Homocystine–d_8	$y = 0.13x + 0.11$	0.05–100	0.999	20	0.5
Cystine	Cystine–d_4	$y = 0.18x + 0.05$	0.05–100	0.999	5	0.5
Glutathione disulfide	Cystine–d_4	$y = 0.54x - 0.07$	0.05–100	0.999	5	0.25
Cysteinylglycine disulfide	Cystine–d_4	$y = 0.77x + 0.22$	0.05–100	0.999	5	0.25

r^2 denotes the correlation coefficient for linear regression equations. LOQ denotes limits of quantification; LOD denotes limits of detection; IAM = iodoacetamide; IPCF = isopropylchloroformate.

umn supplied in the EZ-FAAST kit. Mobile phase was composed of methanol–water (80:20, v/v) containing 1 mM ammonium formate at a flow rate of 0.2 ml/min. Each sample was injected in a volume of 20 μl via an autosampler and separated by isocratic elution for 15 min. Sample solutions were maintained at 4 °C in the autosampler prior to injection.

2.8. Mass spectrometry condition

Samples were monitored using a Waters Micromass Quattro LC triple quadrupole mass spectrometer (Waters, Milford, MA) equipped with an electrospray ionization (ESI) source. Masslynx and Quanlynx software 3.3 (Waters, Milford, MA) were used for system control and data processing. Capillary voltage was set to 3 kV, source temperature to 130 °C, and nebulizer gas temperature to 400 °C, respectively. The nebulizer and analysis cell gas flows (both nitrogen) was set at 80 and 800 l/h, respectively. The low and high mass resolutions were fixed at 15 for both the first and third quadropole mass analyzers. The collision gas (argon) pressure in the second quadropole was set at 1.5 mbar. The photomultiplier was fixed at 650. The dwell time and the interchannel delay were fixed at 0.2 and 0.01 s, respectively. The mass spectrometer was operated in positive ion multiple-reaction monitoring mode. The analyte specific chromatographic retention times, mass transitions and optimized cone and collision energy settings are listed in Table 1. The chemical structures of IAM–IPCF or IPCF derivatized compounds are shown in Fig. 2. The chromatogram peak area for each MRM transition was automatically integrated using Quanlynx software.

2.9. Method validation

The precision and accuracy studies were performed by calculating the relative standard deviation (RSD) and recoveries obtained from the QC samples. Intra-day precision (each $n = 5$) was evaluated by analysis of QC samples at different times of the same day. Inter-day precision ($n = 5$) was determined by repeated analysis of QC samples three times over a 2-month period. The calibration curves were generated on the day of analysis. Limits of detection (LOD) are defined as the lowest concentration significantly different from the blank with a minimum signal that is at least three times greater than the baseline noise value. The analyte's specific linearity of detection, precision, and accuracy parameters are listed in Tables 2 and 3.

2.10. Plasma SAA redox state quantitation

The redox potential for the Cys, GSH, and CysGly redox couples in plasma were calculated using the Nernst equation; E_h = (redox potential at pH 7.4) + 30 log ([oxidized disulfide]/[reduced thiol]^2). The standard redox potential used for the Cys, GSH, and CysGly redox couples at pH 7.4 were −246, −264, and −272 mV, respectively [2,33].

3. Results and discussion

3.1. Optimization of derivatization procedure

In the current method, thiol containing SAA metabolites stabilized with IAM prior to acidification and *S*-carboxyamidomethylated thiols were further derivatized with IPCF. The optimal condition for *S*-carboxyamidomethylation was to maintain plasma pH at 8.0 and use 20 mM concentrations of IAM. This is achieved by mixing equal volume of plasma with 20 mM solution of IAM dissolved in 100 mM Tris–HCl, pH 8.0 solution.

The reaction sequence of IPCF derivatization of IAM-modified Cys is shown as an example in Fig. 3A. The initial *S*-carboxyamidomethylation reaction with IAM adds 57 mass units to the parent mass of 121 of cysteine. The subsequent reactions with IPCF add 86 and 42 mass units to the amine and carboxylic functional groups. The IPCF derivatization converts amines to their corresponding carbamates and esterifies the carboxylic functional group of amino acids (Fig. 3A). The predicted mass of the IAM and IPCF derivatized Cysteine is 306 (121 + 57 + 86 + 42) and was confirmed by using precursor ion scanning mass spectrometry (Fig. 3B). The positive ion electrospray ionization (ESI) precursor ion spectrum in Fig. 3B shows intense $[M+H]^+$ ion of IAM and IPCF derivatized cysteine at *m*/*z* 307. We also observed a minor unidentified product ion at 329. The product ion scan in Fig. 3B shows the collision induced dissociation (CID) fragmentation pattern from 307 precursor ion. The most abundant product ion at *m*/*z* 116 corresponding to the fragment sites shown in Fig. 3A was detected.

Fig. 3C shows the reaction sequence of IPCF derivatization of Cys. In this reaction, IPCF converts the sulfhydryl group of Cys to its *N*,*S*-diisopropoxycarbonyl methyl ester derivative, resulting in an addition of 86 mass units to one free sulfhydryl group. With an additional modification of the amine and the carboxylic groups, the predicted parent mass of IPCF-derivatized Cys is 335 (121 + 86 + 86 + 42). The precursor and the product ion mass scan of final derivatized product of cysteine showed the formation of predicted precursor $[M+H]^+$ ion at *m*/*z* 307 and a dominant product ion at *m*/*z* at 190 (Fig. 2D). The chemical structures and their predicted mass of IAM and/or IPCF derivatized SAA metabolites are shown in Fig. 2.

Table 3
Precision and accuracy of thiol redox analysis.

IAM + IPCF derivative		Spiked amount				IPCF derivative		Spiked amount			
Analyte	Parameter	0 μM	0.5 μM	5 μM	50 μM	Analyte	Parameter	0 μM	0.5 μM	5 μM	50 μM
Homocysteine	Mean	0.63	1.30	5.74	50.80	Homocysteine*	Mean	8.30	9.10	13.80	59.50
	Recovery (%)	97.00	115.04	101.95	100.34		Recovery (%)	98.00	103.41	103.76	102.06
	Intra-day precision RSD (%)	8.70	8.90	7.80	6.50		Intra-day precision RSD (%)	7.50	7.50	5.80	5.80
	Inter-day precision RSD (%)	16.00	15.40	12.50	10.50		Inter-day precision RSD (%)	12.50	13.20	8.50	8.50
Cysteine	Mean	22.00	23.20	26.50	72.50	Cysteine*	Mean	210.60	212.50	216.00	261.50
	Recovery (%)	98.00	103.11	98.15	100.69		Recovery (%)	102.50	100.66	100.19	100.35
	Intra-day precision RSD (%)	5.40	4.50	4.00	5.20		Intra-day precision RSD (%)	5.70	5.70	5.00	5.60
	Inter-day precision RSD (%)	8.50	9.20	8.50	7.50		Inter-day precision RSD (%) 14.50	12.50	9.80	10.50	
Glutathione	Mean	3.90	4.70	9.00	54.20	Glutathione*	Mean	5.50	6.40	11.00	57.50
	Recovery (%)	96.90	106.82	101.12	100.56		Recovery (%)	103.60	106.67	104.76	103.60
	Intra-day precision RSD (%)	8.40	8.70	6.50	5.50		Intra-day precision RSD (%)	8.50	8.50	6.40	5.70
	Inter-day precision RSD (%)	12.50	14.00	8.50	7.50		Inter-day precision RSD (%)	12.70	14.50	10.50	10.40
Cysleinylglycine	Mean	2.25	2.85	7.50	53.50	Cysteinylgly cine*	Mean	35.90	37.20	41.20	86.50
	Recovery (%)	95.00	103.64	103.45	102.39		Recovery (%)	105.50	102.20	100.73	100.70
	Intra-day precision RSD (%)	7.56	8.40	6.50	5.20		Intra-day precision RSD (%)	6.70	7.50	5.60	6.20
	Inter-day precision RSD (%)	11.70	12.60	10.80	9.80		Inter-day precision RSD (%)	12.50	13.50	10.50	11.20

IPCF derivative—oxidized disulfides		Spiked amount						Spiked amount			
Analyte	Parameter	0 μM	0.5 μM	5 μM	50 μM	Analyte	Parameter	0 μM	0.5 μM	5 μM	50 μM
Glutathione disulfide	Mean	0.07	0.72	5.50	51.50	Homocystine	Mean	0.08	0.62	5.20	50.00
	Recovery (%)	95.00	115.04	108.48	102.86		Recovery (%)	89.00	106.90	102.36	99.84
	Intra-day precision RSD (%)	8.80	7.50	6.80	6.20		Intra-day precision RSD (%)	8.80	9.20	6.50	5.80
	Inter-day precision RSD (%)	12.50	13.50	11.50	10.50		Inter-day precision RSD (%)	18.00	15.00	12.50	10.50
Cysleinylglycine disulfide	Mean	0.80	1.32	6.10	51.00	Cystine	Mean	53.20	54.20	58.70	103.00
	Recovery (%)	97.00	115.04	105.17	100.39		Recovery (%)	98.00	115.04	100.86	99.81
	Intra-day precision RSD (%)	8.30	7.90	7.00	6.50		Intra-day precision RSD (%)	8.00	8.50	6.40	6.40
	Inter-day precision RSD (%)	15.00	13.50	11.50	9.50		Inter-day precision RSD (%)	12.00	13.50	10.50	10.50

1. Fresh plasma sample from a single donor was either left untreated or spiked with 0.5, 5.0, or 50 μM olanalyles and data are presented as mean ± SD.
2. IAM + IPCF derivatization measures plasma free thiols.
3. IPCF derivatization measure total plasma thiols following DTT reduction.
4. IPCF derivatives of oxidized disulfides were quantified in plasma samples pretreated with IAM.
5. Intra- and inter-day precision over a 2-month period was calculated based of 5 repetitive measurements.

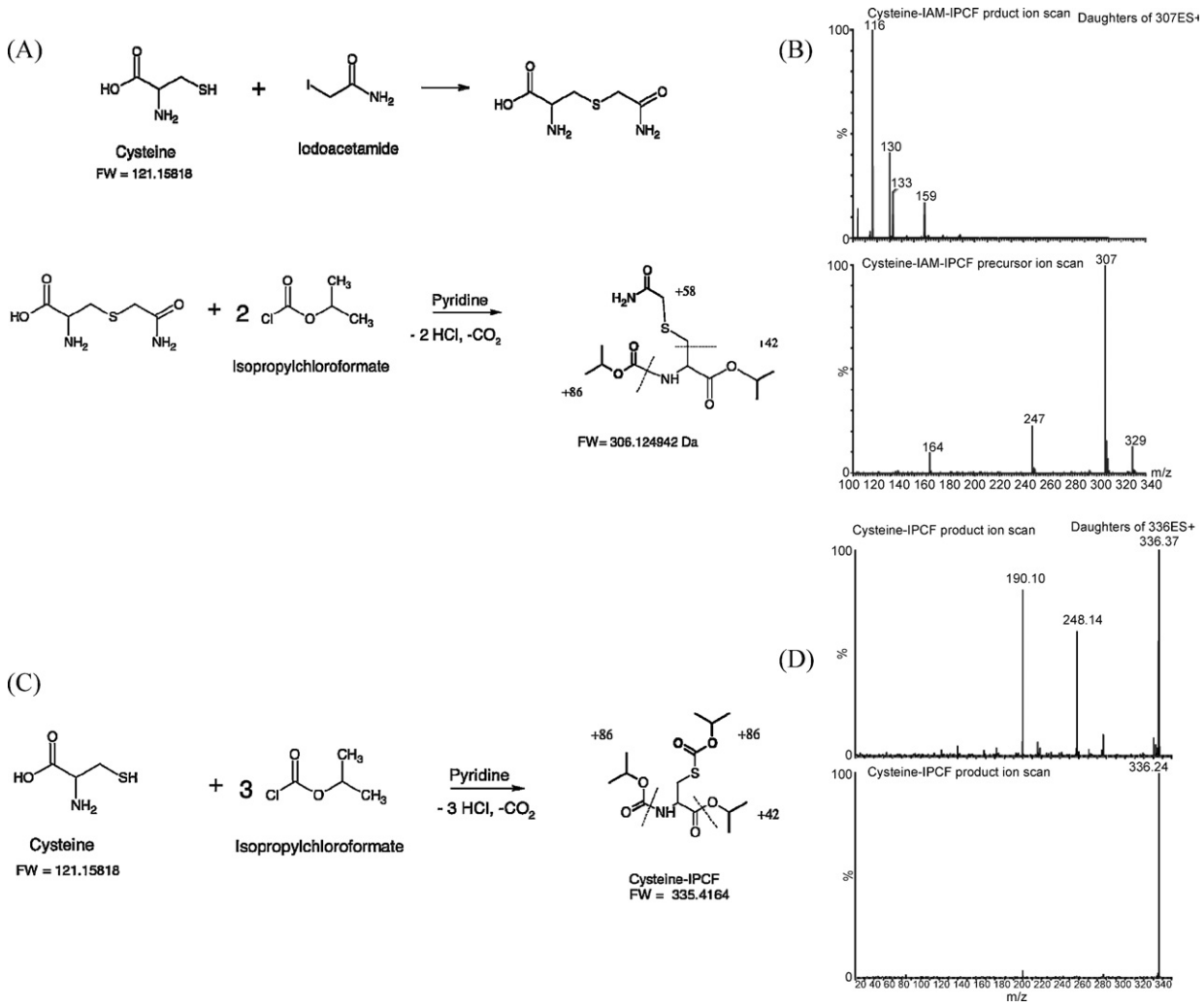

Fig. 3. Cysteine derivatization with iodoacetamide and isopropylchloroformate. The reaction schemes for cysteine derivatized with both iodoacetamide and isopropylchloroformate (A) or with isopropylchloformate alone (C) are shown. Panel B and Panel D shows the precursor and the product ions generated from cysteine–IAM–IPCF and cysteine–IPCF derivatives, respectively. Masses associated with each species represent the $[M+H]^H$ ion and the protonated mass of the fragment ion. The collision-induced dissociation (CID) fragmentation sites are indicated by dotted lines in A and C.

3.2. Optimization of solid phase extraction (SPE)

The proprietary strong cation-exchange solid phase extraction (SPE) tips included in the EZ-FAAST kit are used to selectively concentrate positively charged amino acid metabolites. Acidification with PCA, however, can interfere with the SPE step because the excess perchlorate salt in the PCA supernatant can lower the interactions between positively charged cationic metabolites with the SPE matrix. To minimize this effect, excess perchlorate salt is precipitated out prior to the SPE step by treating the samples with concentrated $KOH/K_2B_4O_7$ solution to achieve the final pH of 1.5. Our titration experiments indicate that 35 μl of $KOH/K_2B_4O_7$ per 100 μl of 5% PCA solution was sufficient to remove interfering perchlorate salt and regain efficiency of SPE analyte enrichment step.

3.3. Optimization of chromatographic separation

Standard reversed-phase chromatography yields little retention for amino acids because of their hydrophilic and ionic characteristics. By derivatizing with IAM and IPCF, amino acids can be more efficiently separated using reversed-phase liquid chromatography (LC). We optimized the chromatographic conditions for resolving various IPCF and IAM/IPCF derivatized SAA analytes using the proprietary reverse phase HPLC column included in the EZ-FAAST kit. This column performed better than standard C18 reversed-phase column in resolving IPCF and IAM/IPCF derivatized analytes. Under isocratic elution conditions, baseline separation of analytes was achieved with the EZ-FAAST reversed-phase column, but not with a standard C18 column. However, when running a linear gradient, the EZ-FAAST column took longer time to re-equilibrate between runs than a typical C18 column. To remove the requirement for between run re-equilibration and shorten the sample analysis time, and isocratic elution method was developed. We found that analyte elution with a mobile phase composed of methanol:water (80:20 v/v) containing 1 mM ammonium formate was optimal for resolving our target analytes within 15 min. The analyte specific retention times are shown in Table 1. Under the above chromatographic conditions, the IAM and IPCF derivatives of SAA metabolites eluted between 3 and 6 min, while the IPCF derivatives of the same analytes were retained for longer time. The shorter retention time of IAM/IPCF derivatives maximized the chromatographic separation of reduced thiols from their corresponding oxidized disulfide forms.

3.4. Assay validation

The method was validated by determining the linearity ($r^2 > 0.98$), sensitivity (limits of detection ranged from 0.25 to 0.5 nM), intra- and inter-day precision (RSD < 8.5 and 15.4%, respectively; Table 3). Accuracy for all analytes detected was greater than 84% and the average relative standard deviations (RSD) for the intra-day and inter-day precision calculated over a 2-month period, were below 15% and 20%, respectively.

Limits of detection (LOD) are defined as the lowest concentration significantly different from the blank with a minimum signal that is at least three times greater than the baseline noise value, where as the limit of quantification (LOQ) refers to the lowest concentration of the standard curve that can be measured with acceptable accuracy and precision (RSD <10%). Table 2 lists the value of these two parameters for all analytes.

3.5. Determination of absolute matrix effect (ME)

Matrix effects, or ion suppression, can be defined as the change in response observed for a given concentration of target analyte in the presence of other sample components. Matrix effects are concern in the preparation of biological samples prior to LC/MS/MS analysis. However, the use of optimized internal standards can compensate for the effects of biological matrices. To determine whether our internal standardization was effective in compensating for the matrix effects, we determined the matrix effects of plasma and erythrocytes using the strategy applied by Matuszewski et al. [34]. MS/MS areas generated from known amounts of standards (A) were compared with those obtained from PCA extracts spiked with the same amount of analyte (B). The ratio ($B/A \times 100$) is defined as the absolute matrix effect (ME%). A value of 100% indicates that there is no absolute matrix effect. There is signal enhancement if the value is >100% and signal suppression if the value is <100%. Plasma samples were spiked to obtain a concentration of 50 μM of each analyte. Absolute ME effect calculated based on absolute area shows significant ion suppression caused by both plasma and erythrocyte matrix (~49%; Table 4). Similar ME calculations were performed using the area ratios of external/internal standard compound. As summarized in Table 4, the ME effects of plasma and erythrocyte matrix were significantly minimized when area ratios were used in our calculation. hGSH, Cys–d_4, and HcySS–d_8 were chosen based on their structural and chemical similarities to our target analytes and chromatographic retention times for these standards were close to our target analytes (Fig. 4). Our results in Table 4 suggest that our internal standardization performed well in accounting for the matrix effects of human plasma and erythrocytes.

3.6. Analysis of human plasma and erythrocyte samples

We applied our optimized protocol to analyze the total SAA concentrations and their redox states in erythrocyte and plasma samples obtained from twelve healthy controls and four β-thalassemia major patients. All of the patients recruited for this study were undergoing blood transfusions and iron-chelation treatments at the Children's Hospital Oakland Research Institute. Overnight fasting blood samples were collected from healthy controls and from thalassemia patients at three weeks following their previous transfusion.

To first confirm the applicability of our method in analyzing thalassemia blood samples, recovery efficiencies were determined using pooled plasma samples obtained from four thalassemia patients. For this experiment, pooled plasma samples were either left untreated or spiked with 5 μM concentration of Hcy, Cys, GSH and CysGly and corresponding recovery efficiencies were calculated. Our results showed that the recovery efficiencies of the four thiol compounds were > 92% are similar to the values obtained using normal plasma samples. A lack of significant oxidation of spiked thiol compounds indicates that the IAM preservation was sufficient to prevent ex vivo thiol oxidation in thalassemia plasma. The recoveries of internal standards in plasma were also similar between thalassemia and controls.

Table 4
Absolute matrix effect (ME) based on areas and area ratios for the analytes in human plasma and erythrocytes.

Analyte	Matrix	ME without internal Std (%)	ME with Int. Std. (%)
Homocysteine	Plasma	49	81
	Erythrocytes	50	97
Cysteine	Plasma	43	107
	Erythrocytes	40	90
Glutathione	Plasma	60	89
	Erythrocytes	50	116
Cysteinylglycine	Plasma	47	83
	Erythrocytes	42	113
Homocystine	Plasma	46	85
	Erythrocytes	47	89
Cystine	Plasma	65	129
	Erythrocytes	48	103
Glutathione disulfide	Plasma	43	97
	Erythrocytes	45	99
Cysteinylglycine disulfide	Plasma	53	82
	Erythrocytes	58	87

1. Homoglutathione, cystine–d_4, and homocystine–d_8 was used as internal standards.
2. MS/MS areas of known amounts of standards in water were compared with those measured in plasma and erythrocyte samples, after extraction spiked with the same analyte amount.
3. Signal from 5 and 50 μM concentrations of analytes were compared.

A representative extracted ion chromatograms of analytes are shown in Fig. 4 and the plasma values of Hcy, Cys, GSH and CysGly and their redox states are summarized in Table 5. The total concentrations of plasma Hcy, Cys, GSH and CysGly were quantified following DTT reduction step.

The average total concentrations of Hcy, Cys, GSH and CysGly in healthy controls were within the published normal reference range (Hcy reference 3–12 μM, Cys reference 100–400 μM, GSH reference 2–10 μM and CysGly reference 30–60 μM) [35–39]. In thalassemia patients, however, the average concentrations of Hcy, Cys, GSH and CysGly were significantly elevated relative to controls. Among these metabolites, the largest increase in concentration was observed with cysteine, where the levels in thalassemia patients were ~200 μM higher than normal control values.

Using the values obtained from IAM preserved samples, the redox states of Cys, GSH and CysGly were calculated using the Nernst equation. The calculated redox potential of plasma Cys/CysSS, GSH/GSSG, and CysGly/CysGlySS redox couples in our control subjects were -76 ± 11, -137 ± 4.9, -129.3 ± 19 mV, respectively. In thalassemia patients, the plasma redox potentials for Cys and CysGly were more reduced when compared to controls. The reduction of Cys and CysGly redox couples were driven primarily by the increases in Cys and CysGly. In contrast, the GSH redox potential became more oxidized in thalassemia patients relative to control subjects. The oxidation of GSH redox couple was caused by higher accumulation of GSSG rather than a decrease in GSH. Higher availability of Cys and CysGly may be indicative of physiological compensatory response to the increased oxidative stress seen in our thalassemia subjects. The higher accumulation of GSSG in thalassemia patients suggests that the

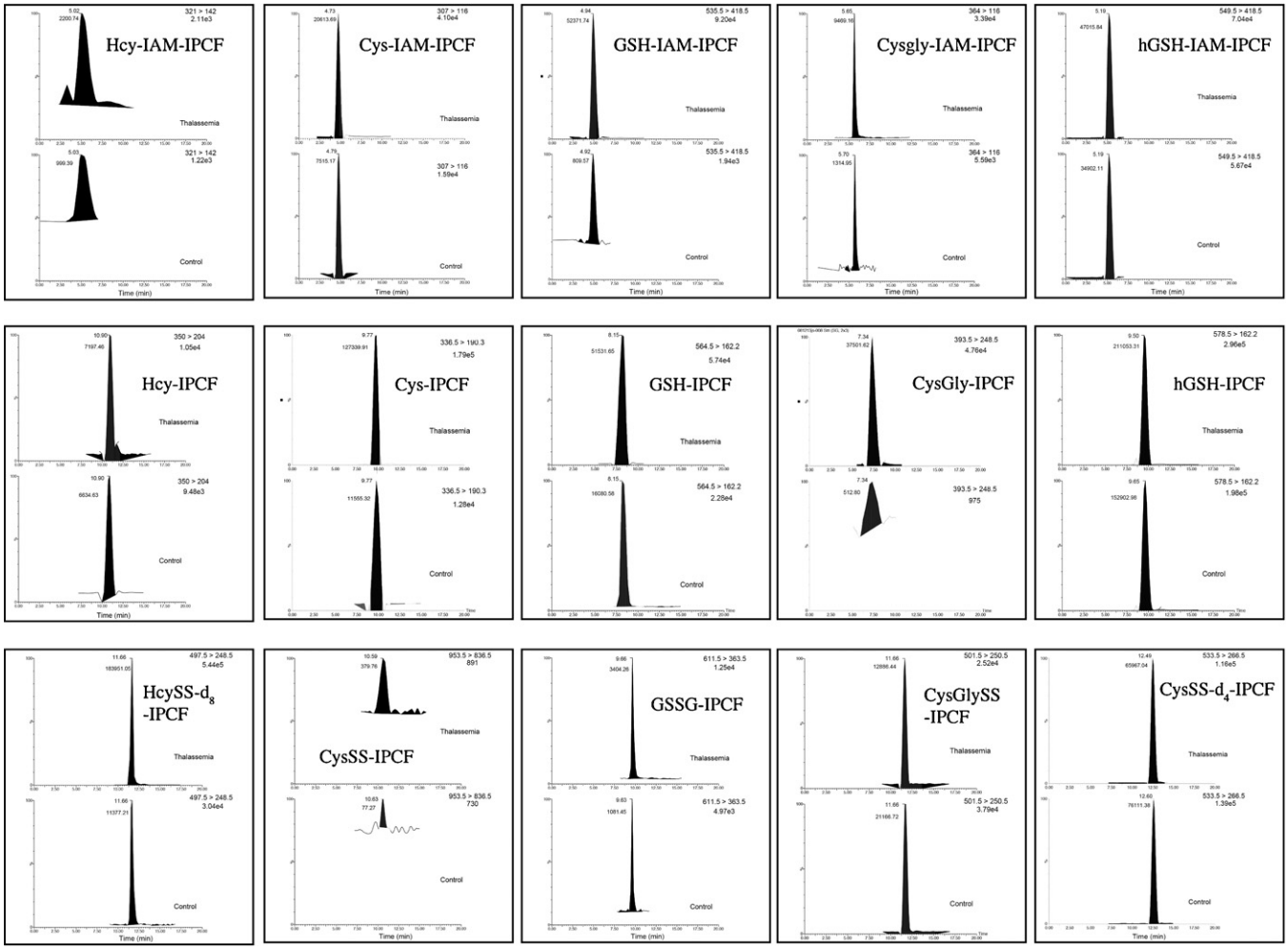

Fig. 4. Extracted ion chromatograms of fifteen analytes in the plasma of control and thalassemia case. X–IAM–IPCF denotes reduced SAA metabolites derivatized with both iodoacetamide (IAM) and isopropylchloroformate (IPCF), where as X–IPCF denotes analytes derivatized with IPCF alone. *Abbreviations*: homocysteine = Hcy, homocystine = HcySS, cysteine = Cys, cystine = CysSS, glutathione = GSH, glutathione disulfide = GSSG, cysteinylglycine = CysGly, cysteinylglycine disulfide = CysGlySS, homoglutathione = hGSH, cystine–d_4 = CysSS–d_4, homocystine–d_8 = HcySS–d_8–IPCF.

rate of GSH reduction may be a limiting factor in thalassemia patients.

We further characterized thalassemia-dependent changes in erythrocyte thiol redox status. In contrast to plasma redox changes, the total concentrations and the redox states of Cys were similar between controls and thalassemia subjects. The average GSH concentrations in healthy controls was 9.51 ± 2.97 μmol/g Hb, which was equivalent to 2.37 mM. The average GSSG concentration in health controls was 0.014 ± 0.05 μmol/g Hb, which was equivalent to 3.5 μM. In normal samples, the percent of GSSG present in the erythrocytes was ~0.3%, similar to values previously published values [36]. The erythrocyte GSH and CysGly concentrations were significantly higher in the thalassemia patients relative to controls (Table 6). Erythrocyte GSH concentrations were on average

Table 5
Plasma values in health controls and in thalassemia patients.

	Compound	Total RSH	Free RSH	Free RSSR	$E_{7.4}$ (mV)	E_h (mV)
	Controls					
1	Homocysteine	9.52 ± 3.40	0.60 ± 0.10	ND	−200	ND
2	Cysteine	198.80 ± 37.80	13.40 ± 5.00	78.10 ± 30.10	−247	−76 ± 10.80
3	Glutathione	5.20 ± 1.60	3.35 ± 1.40	0.05 ± 0.10	−264	−137 ± 4.90
4	Cysteinylglycine	33.80 ± 7.50	1.65 ± 0.60	1.40 ± 0.50	−272	−129.3 ± 19.0
	Thalassemia					
1	Homocysteine	14.20 ± 3.20	0.60 ± 0.10	ND	−200	ND
2	Cysteine	402.00 ± 38.00*	52.00 ± 2.70	108.30 ± 15.00*	−247	−108.00 ± 3.10*
3	Glutathione	8.20 ± 1.70*	3.80 ± 0.90	0.40 ± 0.20*	−264	−114.50 ± 5.00*
4	Cysteinylglycine	74.00 ± 7.24*	25.30 ± 4.80*	1.70 ± 0.20	−272	−168.80 ± 5.40*

All units are in μM and data are presented as mean ± SD.
$E_{7.4}$ (mV) is standard reduction potential at pH 7.4.
E_h is the calculated redox potential for specific redox couples.
* Denotes significant ($p < 0.05$) deviations from control mean values.

Table 6
Red blood cell values in health controls and in thalassemia patients.

Analyte	Control	Thalassemia
Homocysteine	0.020 ± 0.001	0.007 ± 0.002*
Cysteine	0.270 ± 0.160	0.240 ± 0.040
Glutathione	9.510 ± 2.97	11.620 ± 0.820
Cysteinylglycine	0.005 ± 0.001	0.022 ± 0.014*
Homocystine	BD	BD
Cystine	0.004 ± 0.004	0.05 ± 0.006
Glutathione disulfide	0.014 ± 0.047	0.054 ± 0.035*
Cysteinylglycine disulfide	BD	BD

All units are in μmol/g Hb and data are presented as mean ± SD.
BD = denotes below detection limit.
* Denotes significant change from mean control values at $p < 0.05$.

2 μmol/g Hb higher than the control values. Interestingly, erythrocyte GSSG concentrations also increased in thalassemia patients and accounted for ∼1% of the total GSH pool. This suggests that erythroctyes from thalassemia patients experience higher oxidative stress burden, which is compensated by increased GSH synthesis. The increased plasma availability of Cys and CysGly may in part serve as a mechanism for augmenting cysteine supply to erythrocytes in these patients.

Because of the ineffective erythropoesis and chronic anemia, thalassemia major patients are frequently treated with blood transfusions that lead to iron-overload in these patients. Several studies have shown that the concentrations of oxidatively modified protein [40], lipid [41] and DNA [42] are higher in thalassemia patients. Increased redox-active iron and hemoglobin availability in the plasma and erythrocyte makes redox analysis challenging for these patients. To minimize the potential for ex vivo oxidation, IAM was used to derivatize the labile thiol groups of SAA metabolites and was effective in minimizing ex vivo oxidation of SAA metabolites. Similar recoveries of reduced SAA metabolites from normal and thalassemia plasma matrix suggest that our assay effectively minimized potential oxidation during sample processing. The current assay is sufficiently sensitive for quantifying the redox states of Cys, GSH and CysGly in plasma and in erythrocytes and provides a convenient method to study SAA metabolism in clinical studies in thalassemia. Further studies are needed to assess the sensitivity of SAA based biomarkers to accurately determine iron-toxicity in thalassemia patients and validate their use as proximate markers of oxidative stress in this patient group.

4. Conclusion

A LC/MS/MS method was developed and validated for the quantification of major SAA metabolites. The sample processing involves sequential derivatization with IAM and IPCF, using the commercially available EZ-FAAST LCMS amino acid analysis kit. The three main advantages of using the EZ-FAAST kit are (1) the convenience and the efficiency of their proprietary SPE tips for sample enrichment, (2) the improved performance of their proprietary reversed-phase column and (3) the ability to standardize the IPCF derivatization process across different study sites. We had successfully used the EZ-FAAST kit in our previous study examining amino acid metabolism in sickle cell patients [43]. Our current method improves upon this assay by allowing accurate quantification of thiol redox states in biological fluids and cells. While the IPCF chemistry can be cheaply reproduced in the lab, the proprietary strong cation exchange solid phase exchange (SPE) tip and the reversed-phase HPLC column included in this kit are not available as individual products and therefore necessitate using this kit for performing this assay.

Acknowledgements

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