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# Enrichment of cysteinyl adducts of human serum albumin

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ABSTRACT

We report a method to enrich cysteinyl adducts of human serum albumin (HSA), representing biomarkers of exposure to systemic electrophiles. Because the major site of HSA adduction is the single free sulfhydryl group at Cys34, we used thiol-affinity resins to remove mercaptalbumin (i.e., unadducted HSA) from the cysteinyl adducts. Electrospray ionization mass spectrometry was used to detect mercaptalbumin and HSA–Cys34 modifications before and after enrichment of HSA. Differences in adduct content were detected across samples of freshly isolated, archived, and commercial HSA. Cysteinylated and glycosylated adducts were present in all samples, with abundances decreasing in the following order: commercial HSA > archived HSA > fresh HSA. After enrichment of HSA, mercaptalbumin was no longer observed in mass spectra. The ratios of HSA adducts post-/preenrichment, quantified via the Bradford assay and gel electrophoresis, were 0.029 mg adducts/mg HSA in fresh HSA and 0.323 mg adducts/mg HSA in archived HSA. The apparent elevation of adduct levels in archived samples could be due to differences in specimen preparation and storage rather than to differences in circulating HSA adducts. We conclude that thiol-affinity resins can efficiently remove mercaptalbumin from HSA samples prior to characterization and quantitation of protein adducts of reactive systemic electrophiles.



It has been argued that biomarkers of exposure should be used in studies of the effects of toxic chemicals on human health [1]. Biomarkers can also provide a more precise estimate of internal dose, which increases the power to detect relationships between adverse health effects and causal agents [2]. However, because environmental toxicants are often chemically reactive electrophiles, their short life spans in vivo make them difficult to measure directly in biological media. This has motivated the use of stable adducts of these electrophiles with abundant blood proteins, notably hemoglobin and human serum albumin (HSA)[1], as exposure biomarkers (reviewed by Tornqvist et al. [3] and Rubino et al. [4]). Stable adducts accumulate over the mean residence time of a protein and thereby reflect the integral of the blood level of the reactive species over, for example, 28 days for HSA and 60 days for hemoglobin. This makes protein adducts potentially more useful measures of exposure for retrospective epidemiological studies than environmental measurements or short-term biomarkers, such as urinary metabolites and blood levels of parent compounds, which have residence times of hours or days [2].

HSA contains 35 cysteine residues, 34 of which are bound as intramolecular disulfides. Although the remaining cysteine, Cys34, has the only free sulfhydryl group in HSA, it represents the largest fraction of thiols in serum [5], where it has been estimated to account for approximately 80% of the antioxidant capacity [6,7]. Within the tertiary protein structure of HSA, Cys34 resides in a unique microenvironment close to three ionizable residues: Asp38, His39, and Tyr84 [8]. As a result, Cys34 has an unusually low p$K_a$ (<6.7 vs. ~8.0–8.5 for thiols in most other proteins and peptides) and exists primarily in the highly nucleophilic thiolate form [8]. Examples of the many chemical species that form adducts with Cys34 include oxirane and quinone metabolites of benzene, naphthalene, and pentachlorophenol [9–13]; nitrogen mustards [14]; 4-hydroxy-*trans*-2-nonenal, 2-propenal, malondialdehyde, glyoxal, and other products of lipid peroxidation [15–17]; metal ions such as $Ag^+$, $Hg^{2+}$, and $Au^+$; and a host of drugs such as auranofin, D-penicillamine, ethacrynate, and cisplatin (reviewed in Ref. [18]). Yet although some HSA–Cys34 adducts have been detected in studies of humans, the analytical matrix is complex and the levels of individual adducts have been very low (<1 in 1 million unadducted protein molecules [4]). The combination of matrix effects

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[1] *Abbreviations used:* HSA, human serum albumin; DTT, dithiothreitol; ESI, electrospray ionization; MS, mass spectrometry; TCEP, *tris*(2-carboxyethyl)phosphine; IAA, iodoacetamide; MWCO, molecular weight cutoff; FWHM, full-width at half-maximum; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; LC, liquid chromatography; HSA + Cys, cysteinylated HSA; HSA + Gluc, glycosylated HSA; NO, nitric oxide; SD, standard deviation.

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plus low abundance has made it difficult to simultaneously measure numerous HSA adducts in samples of human serum or plasma and to identify unknown adducts.

Here we report a method to selectively enrich Cys34 adducts in fresh or archived samples of HSA. The enrichment approach takes advantage of the propensity of HSA containing unmodified Cys34 (mercaptalbumin) to bind with thiol-affinity resins [19]. Because adducted HSA is composed primarily of small mixed disulfides that exhibit reversible binding with HSA–Cys34, disulfide adducts were reduced to mercaptalbumin by dithiothreitol (DTT) prior to treatment with thiol-affinity resins. Using electrospray ionization mass spectrometry (ESI–MS), we characterized intact HSA from various sources and investigated the effects of reduction of mixed disulfides on the enrichment process. Enrichment was assessed both by MS of the intact proteins and by quantification of the amounts of HSA via the Bradford assay and gel electrophoresis before and after enrichment via thiol-affinity resins.

## Materials and methods

### Chemicals

DTT, *tris*(2-carboxyethyl)phosphine (TCEP), iodoacetamide (IAA), phosphate-buffered saline, Activated Thiol Sepharose 4B, sequence-grade trypsin, sodium chloride, calcium chloride, ammonium sulfate, nitrogen, Bio-Rad reagent dye, sodium dodecyl sulfate, BioSafe Coomassie G-250 dye (Bio-Rad, Hercules, CA, USA), ammonium sulfate, Tris base, ProteaseMAX, acetonitrile (Fisher Optima grade, 99.9%), and formic acid (Pierce, 1-ml ampoules, 99+%) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Commercial HSA was obtained from Sigma–Aldrich (St. Louis, MO, USA). Water was purified to a resistivity of 18.2 MΩ·cm (at 25 °C) using a Milli-Q Gradient ultrapure water purification system (Millipore, Billerica, MA, USA).

### Isolation of HSA

Whole blood (4 ml) was obtained from a healthy Asian subject (34 years of age) by venipuncture in heparin. The blood was immediately centrifuged for 15 min at 3000*g*. The plasma layer was transferred to a 15-ml centrifuge tube, and saturated ammonium sulfate was added dropwise to a final concentration of 60% ammonium sulfate. The plasma was vortexed briefly and centrifuged for 30 min at 3000*g* to pellet the precipitated protein. The supernatant, containing HSA, was transferred to 10-kDa molecular weight cutoff (MWCO) spin columns (Amicon Ultra-4, Millipore), and the protein was desalted using 5 × 15-ml volumes of deionized water. The isolated HSA was immediately aliquoted and frozen at −80 °C. Archived HSA from 40 subjects (pooled and balanced from males/females, smokers/nonsmokers, and black/white subjects with 5 subjects per pool) had previously been isolated according to a similar protocol [20], which included dialysis to remove small molecules, lyophilization to constant weight, and dissolving the purified HSA in deionized water at 50 mg/ml. Archived samples were stored at −80 °C prior to processing in the current study.

### MS of HSA

The heterogeneity of HSA with respect to Cys34 adduct status was evaluated in freshly isolated and archived HSA (described above) and commercial HSA from Sigma–Aldrich. Protein samples were analyzed using an Agilent 1200 series liquid chromatograph (Santa Clara, CA, USA) that was connected in-line with an LTQ Orbitrap XL hybrid mass spectrometer equipped with an Ion Max ESI source (Thermo Fisher Scientific, Waltham, MA, USA). The liquid chromatograph was equipped with C8 guard (Poroshell 300SB-C8, 5 μm, 12.5 × 2.1 mm, Agilent) and analytical (75 × 0.5 mm) columns and a 100-μl sample loop. Solvent A was 0.1% formic acid/99.9% water, and solvent B was 0.1% formic acid/99.9% acetonitrile (v/v). For each sample, approximately 50–100 pmol of protein analyte was injected onto the column using an Agilent 1200 autosampler. Following sample injection, analyte trapping was performed for 5 min with 99.5% A at a flow rate of 90 μl/min. The elution program consisted of a linear gradient from 25% to 95% B over 34 min, isocratic conditions at 95% B for 5 min, a linear gradient to 0.5% B over 1 min, and then isocratic conditions at 0.5% B for 14 min at a flow rate of 90 μl/min. The column and sample compartments were maintained at 35 and 10 °C, respectively. Solvent (Milli-Q water) blanks were run between samples, and the autosampler injection needle was rinsed with Milli-Q water after each sample injection to avoid cross-contamination between samples. The mass spectrometer ESI source parameters were as follows: ion transfer capillary temperature, 275 °C; normalized sheath gas (nitrogen) flow rate, 25%; ESI voltage, 2.0 kV; ion transfer capillary voltage, 33 V; and tube lens voltage, 125 V. Positive ion mass spectra were recorded over the range $m/z$ 500–2000 using the Orbitrap mass analyzer, in profile format, with full MS automatic gain control target settings of $3 \times 10^4$ and $5 \times 10^5$ charges for the linear ion trap and Orbitrap, respectively, and an Orbitrap resolution setting of $6 \times 10^4$ (at $m/z$ 400, full-width at half-maximum [FWHM]). Raw mass spectra were processed using Xcalibur software (version 4.1, Thermo Fisher Scientific), and measured charge state distributions were deconvoluted using ProMass software (version 2.5 SR-1, Novatia, Monmouth Junction, NJ, USA) using the default "large protein" parameters and a background subtraction factor of 1.5. Reproducibility of the measured masses of intact, adducted HSA proteins was within ±3 Da (calculated from the repetitive measurements of the deconvoluted mass spectra of HSA samples over 2 weeks).

### Reduction of HSA mixed disulfides

The efficiency of reducing small mixed disulfides bound to HSA–Cys34 was investigated with both DTT and TCEP. Commercial HSA (1-mg portions) was treated with 1 of 11 different concentrations of DTT or TCEP that ranged from equimolar to 270-fold molar excess, with reaction times ranging from 5 to 60 min. All experiments were conducted at room temperature in phosphate buffer (pH 7.4). Following protein reduction, samples were reacted with IAA and the degree of reduction was assessed by monitoring the number of IAA additions (+57 Da per modification) above the measured mass of HSA (66,436 ± 3 Da) [21,22]. Reduction status was assessed before and after reduction using an LTQ Orbitrap XL hybrid mass spectrometer under the same MS conditions described above. Reduction conditions were considered as optimal when they were stringent enough to reduce the Cys34 mixed disulfides while also preserving the intramolecular disulfide linkages.

For enrichment experiments, 2-mg portions of fresh HSA ($n = 6$) and 0.5-mg portions of pooled archived HSA ($n = 16$) were reduced using a 2.7-fold molar excess of DTT to release Cys34-bound mixed disulfides in 1- and 0.5-ml volumes, respectively. This concentration of DTT had been found to be optimal in preliminary experiments with commercial HSA (described above).

### Using IAA-modified HSA as a positive control

IAA was reacted with HSA to create a Cys34 adduct that would serve as a positive control for the enrichment experiments. Commercial HSA was treated with a 2.7-fold molar excess of DTT and was reacted with 150 mM IAA in phosphate buffer (pH 7.4) at 37 °C for 1 h. After removing excess IAA and buffer salts with a