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HPLC separation of human serum albumin isoforms based on their isoelectric points

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Abstract

Human serum albumin (HSA) is the most abundant protein in plasma. Cys34, the only free Cys residue, is the predominant plasma thiol and a relevant sacrificial antioxidant. Both *in vivo* circulating HSA and pharmaceutical preparations are heterogeneous with respect to the oxidation state of Cys34. In this work, we developed an external pH gradient chromatofocusing procedure that allows the analysis of the oxidation status of HSA in human plasma and biopharmaceutical products based on the different apparent isoelectric points and chemical properties of the redox isoforms. Specifically, reduced-mercury blocked HSA (HSA-SHg⁺), HSA with Cys34 oxidized to sulfenic acid (HSA-SOH) and HSA oxidized to sulfinate anion (HSA-SO₂⁻) can be separated with resolutions of 1.4 and 3.1 (first and last pair) and hence quantified and purified. In addition, an N-terminally degraded isoform (HSA₃₋₅₈₅) in different redox states can be resolved as well.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jchromb.2013.11.019>.

Confirmation of the identity of the chromatofocusing isolated isoforms was achieved by high resolution whole protein MS. It is proposed that the chromatofocusing procedure can be used to produce more exact and complete descriptions of the redox status of HSA *in vivo* and *in vitro*. Finally, the scalability capabilities of the chromatofocusing procedure allow for the preparation of highly pure standards of several redox isoforms of HSA

Keywords

Chromatofocusing; Human serum albumin; Redox isoforms; Plasma; Posttranslational modifications

1. Introduction

Human serum albumin (HSA) is normally secreted at a rate of 10-15 g/day into the circulation by hepatocytes as a mature native protein. The native form of this three domain protein consists of 585 residues (gi: 3212456) with 17 disulfide bonds and Cys34 as its single free thiol group (–SH), giving a theoretical average mass of 66438 Da [2]. The free Cys34 in HSA (HSA–SH) represents ~80% of the measurable thiol pool in plasma [3]. HSA is the most abundant protein in plasma (~0.65 mM) and functions as a key multispecific transport protein in the extracellular space, contributing also to the maintenance of the colloid osmotic pressure of plasma [2]. In addition, evidence has mounted that HSA–SH represents a relevant plasma scavenger of oxidant species. Accordingly, the thiol of Cys34 has been found in different oxidation states *in vivo* (for review see [4]). Previous chromatographic studies have shown that the HSA–SH fraction decreases with age ($76 \pm 4\%$ vs. $48 \pm 6\%$ of total HSA in young and elderly healthy male subjects respectively) [1,5]. A fraction of about 25% of HSA (160 μ M) circulates as mixed disulfides with low molecular weight thiols [1,5]. Sulfinate (HSA–SO₂[–]) and sulfonate (HSA–SO₃[–]) anions, the four and six electron oxidized products of Cys34, have also been reported *in vivo* [1,6–10]. It can be concluded that redox reactions involving Cys34 give rise to the most frequent cause of chemical heterogeneity in the circulating HSA pool. We have previously addressed questions about the kinetic mechanism of HSA–SH oxidation by H₂O₂ and ONOOH (Scheme 1). It has been shown that a relatively stable sulfenic acid (HSA–SOH) is formed by the two-electron oxidation of HSA–SH (pathway *a*). HSA–SOH can react with a low molecular weight thiol giving HSA–SSR (pathway *b*), which can react with a second low molecular weight thiol giving HSA–SH and the exchanged disulfide (pathway *c*). HSA–SOH can also be further oxidized (pathway *d*) giving HSA–SO₂[–] or can spontaneously decay (pathway *e*) [6,11,12] to a product that we called HSA–SX of yet unknown chemical identity and currently under study in our laboratory (unpublished work). Cys34 oxidation products may potentially induce significant structural and functional alterations and their levels correlate with the severity of several pathophysiological conditions associated with increased oxidative stress [4,8,13]. It has also been proposed that the N-terminus of HSA is prone to *in vivo* and *ex vivo* modifications that alter its ability to bind metals [14–17]. In fact, a small HSA fraction where the first two amino acids, Asp1 and Ala2, are missing (HSA_{3–585}) has been previously reported to be present in the circulation and in commercial HSA preparations [7]. Human plasma-derived HSA is used clinically to treat a restricted list

of severe acute and acute-on-chronic conditions associated with intravascular volume contraction and hypoproteinemia. Commercial HSA preparations (both clinical and for laboratory use) show a pronounced redox heterogeneity [4,7,18]. However, no tests or quality standards are required to ensure the redox integrity of commercial HSA and HSA isoforms remain as the main contaminants of native HSA in pharmaceutical preparations. Moreover, recombinant HSA preparations under development display a similar redox heterogeneity [19], highlighting a continuous need for improved preparations and appropriate quality controls.

The analysis of the redox heterogeneity of HSA relies largely on MS [7,9,20] and HPLC studies. Era et al. [1] developed an HPLC procedure based on mixed-mode anion exchange–hydrophobic interaction (AEHI) chromatography that, according to the authors, allows the separation of HSA into three fractions: human mercaptalbumin (HMA), containing HSA–SH; human nonmercaptalbumin 1 (HNA1) containing mixed disulfides and human nonmercaptalbumin 2 (HNA2) including HSA oxidized to sulfenic, sulfinic and sulfonic acids [1]. This analytical procedure has been widely used to study the heterogeneity of HSA [5,21–32] and may be considered the gold standard test for the chromatographic assessment of the redox state of HSA. However, the composition of the eluting fractions has not been analyzed using mass spectrometric techniques. Herein, we report a new column liquid chromatography procedure that allows the separation of the different HSA isoforms according to their isoelectric point (pI) and well-known chemical properties. The proposed external pH gradient chromatofocusing (EPGC) procedure can be performed at analytic or preparative scales, using a high or medium resolution anion exchange DEAE matrix and a linear binary gradient of simple, low cost, volatile buffered aqueous solutions at low and constant ionic strength. The hypothesized identities of the purified fractions were validated by high resolution whole protein MS.

2. Materials and methods

2.1. Materials and solutions

Pharmaceutical grade HSA preparations were from three different vendors. Nanopure filtered (0.22 μm pore size) water was used in the preparation of all aqueous solutions. Hydrogen peroxide (30%) from J. T. Baker was diluted in water and its concentration was determined spectrophotometrically at 240 nm ($\epsilon = 43.6 \text{ M}^{-1} \text{ cm}^{-1}$) [33]. Ethanol, methanol and other organic solvents were HPLC ACS grade and purchased from Mallinckrodt and J. T. Baker. The remaining reagents were from Sigma–Aldrich. Hg(II) is a hazardous chemical, aqueous mercuric chloride stock solutions as well as contaminating chromatographic eluates were handled accordingly. The pH of buffer solutions were adjusted at 80% volume at room temperature using a glass calomel electrode and Sigma pH 4 and 7 standard solutions.

2.2. Pharmaceutical HSA solutions

HSA intended for clinical use was delipidated with activated charcoal according to Chen's procedure [34,35]. The reversibly oxidized thiol isoforms present in pharmaceutical grade HSA were reduced back to thiol by incubation with 10 mM 2-mercaptoethanol at pH 7.4

either for 6 h at room temperature or overnight at 4 °C. Excess reductant as well as additives already present in commercial HSA samples were eliminated by gel filtration using Hi-Trap or PD-10 desalting columns (GE Healthcare) equilibrated with Tris-HCl 20 mM, pH 7.4. The concentration of HSA was determined from the absorbance at 279 nm ($\epsilon = 0.531 \text{ (g/l)}^{-1} \text{ cm}^{-1}$) assuming a molecular mass of 66438 Da [2]. The thiol content was determined with DTNB [34,36] obtaining typically 0.85–0.95 SH/HSA. Pure HSA-SH was obtained from delipidated, reduced and desalted pharmaceutical grade HSA samples by external pH gradient chromatofocusing (EPGC, see below). To obtain HSA-SOH/HSA-SX in high yield, oxidation was performed by incubation of reduced HSA or pure HSA-SH (0.25 mM) with H_2O_2 (4 mM, 4 min, 37 °C, pH 7.4) [12]. HgCl_2 in 1.2–2-fold excess with respect to total HSA was added to HSA solutions for thiol blockage in agreement with previous reports [37,38]. In AEHIC experiments reduced HSA (600 μM) was oxidized with H_2O_2 (5 mM, 15 min, room temperature) in PBS. An aliquot (150 μM) was gel filtered to remove H_2O_2 and then treated with reduced glutathione (GSH, 50 μM , 10 min, room temperature).

2.3. Plasma proteins samples

After informed written consent and with the approval of the Institutional Review Board of the Universidad de la República, whole blood (5 ml) was collected from a healthy volunteer in a heparinized tube. The sample was centrifuged (10 min, $1000 \times g$, room temperature), the supernatant was separated from blood cells and centrifuged again (10 min, $1000 \times g$, room temperature). The supernatant was then filtered through a biocompatible 0.22 μm pore membrane and desalted in a PD-10 column pre-equilibrated with sodium chloride 0.15 M, aliquoted and kept at -20°C until analysis (performed within one week). Samples were centrifuged at $15,000 \times g$ for 10 min and then passed through 0.2 μm pore size syringe filter just before use. In plasma oxidation experiments, gel filtered plasma (HSA $\sim 260 \mu\text{M}$) was incubated with 15 mM H_2O_2 , 10 min at room temperature. The reaction was stopped with catalase and then the mixture was incubated or not with 1 mM N-acetylcysteine (NAC) for 60 min at room temperature.

2.4. External pH gradient chromatofocusing

Liquid chromatography was performed using an Akta Purifier FPLC system equipped with an UPC-900 control unit (GE Health-care). A weak anion exchange high resolution commercial column (TSK DEAE 5PW glass 7.5 cm $\times$ 8 mm I.D., 10 μm) from Tosoh Biosciences LLC (column 1) with approximately 2.2 ml of dead volume was used for analytical and semi-preparative purposes. Buffers A and B consisted of 10 mM ammonium acetate to which glacial acetic acid was added to adjust the pH to 5.35 (buffer A) or 3.95 (buffer B). The column was equilibrated with buffer A. Elution was performed with a linear gradient of buffer B starting at $t = 5$ min, from 0% to 40% in 60 min at a flow rate of 1 ml/min. The column was washed with 7 ml of 100% B after each run. Commercial HSA ($\sim 350 \mu\text{g}$) and plasma proteins (10 μl , $\sim 350 \mu\text{g}$) samples were injected manually. Absorbance at 280 nm and pH were monitored at the outlet of the column. The pH electrode was calibrated using freshly prepared A and B buffers. The eluted fractions were collected and stored at -20°C for further characterization within one month. For semi-preparative purposes, TSK-Gel DEAE-5PW (20 μm) matrix from Tosoh Bioscience LLC was packed in a 55 ml bed volume column (column 2). 100–200 mg of HSA were typically injected. Buffers A and B were the

same as above and elution was achieved in a linear gradient of B from 0% to 40% in 180 min at a flow rate of 2.5 ml/min. Resolution was determined using the following equation:

$R = 2 R_V / (W_1 + W_2)$ where R_V is the difference in retention volumes between analyte 2 and 1 and W_1 and W_2 are the full width at baseline for the corresponding peaks.

2.5. Dual-mode anion exchange-hydrophobic interaction chromatography

This chromatography was performed on a Gilson HPLC system using a Shodex Asahipak ES-502N mixed-mode resin column (column 3) as reported by Era et al. [1]. Solution C consisted in 0.4 M sodium sulfate/0.05 M sodium acetate buffer, pH 4.85 and solution D was anhydrous ethanol. The column was equilibrated in solution C and kept in a column heater at 35 °C. Elution was performed with a linear gradient of solution D from 0 to 5% in 30 min at a flow-rate of 1 ml/min. HSA samples were injected manually and protein was fluorimetrically detected ($\lambda_{\text{exc}} = 280 \text{ nm}$, $\lambda_{\text{em}} = 340 \text{ nm}$). Resolution (R) was calculated as indicated above (Eq. (1)).

2.6. Mass spectrometry analysis

Eluted fractions were characterized by liquid-chromatography–high resolution mass spectrometry (LC–HRMS) using an LTQ Orbitrap analyzer. 10 μl of each sample in 1% formic acid (HSA–SH 570 μg , HSA–SO₂[−] 25.2 μg , HSA–S–Hg⁺ 11.3 μg) were loaded in a C3 reverse-phase column (4.6 mm $\times$ 150 mm, 5 μm , Zorbax 300 SB, Agilent) and resolved using a linear gradient from 97% solution A (water/0.1% formic acid) to 100% solution B (acetonitrile/0.1% formic acid) in 15 min at a flow rate of 250 $\mu\text{l}/\text{min}$. The ESI source parameters were as follows: source temperature, 450 °C; ion transfer capillary temperature, 270 °C; source voltage, 4.0 kV; sheath gas flow rate, 30%; auxiliary gas flow, 10; sweep gas flow, 15. Positive ion mass spectra were recorded over the range m/z 850–2000 with a resolution of 7500 using Xcalibur software (version 2.1, Thermo Fisher Scientific) followed by charge state distribution deconvolution using ProMass software (version 2.5 SR-1, Novatia, Monmouth Junction, NJ, USA) using the “large protein” default parameters and a background subtraction factor of 2.5.

3. Results

3.1. Separation of HSA redox isoforms using external pH gradient chromatofocusing

The EPGC technique was implemented as mentioned under Section 2. The pH of the eluate was monitored along with its absorbance at 280 nm (Fig. 1A, gray and black traces). The linear buffer gradient resulted in a non-linear pH time course and displayed a complex chromatogram when delipidated and reduced pharmaceutical HSA (350 μg) was applied to the column (Fig. 1A, black trace). A major protein peak was observed at pH 4.88 ± 0.06 and $B = 14.8 \pm 0.01\%$ (peak a in Fig. 1A). Similar chromatograms were obtained when injecting up to 10 mg of total HSA in column 1 and 100 mg in column 2 (data not shown). The chromatographic peak a was assigned to HSA–SH as a pre-reduced sample was used. Whole protein MS analysis of peak a yielded a mass of $66435 \pm 3 \text{ Da}$, in agreement with the expected mass for HSA–SH (66438 Da) confirming the peak assignment (Fig. 1B, left panel). This value for the HSA–SH mass represents the average $\pm$ standard deviation of the

deconvoluted mass spectra of ten samples (range 66431.9–66437.0 Da). The standard deviation is within the expected error according to the resolution of the equipment. The second peak in order of intensity (Fig. 1A, peak b) includes HSA-SO₂⁻ because it displayed an elution pH 0.10 to 0.16 pH units lower than peak a (pH 4.78 ± 0.06, *B* = 19.0 ± 0.01%) in agreement with an additional negative charge due to the expected p*K*_a for a solvent exposed sulfinic acid (p*K*_a = 2.2–2.3) [39] and it did not change after treatment with DTT or β-ME (not shown). Furthermore it increased at the expense of peak a under oxidizing conditions (Fig. 2C). Whole protein MS analysis allowed us to identify a major component of peak b with a mass of 66470 ± 1 Da (*n* = 5, range 66468.3–66470.3 Da), which corresponds to the mass increase expected for the addition of two oxygen atoms (32 Da) to native HSA (Fig. 1B, right panel). Since the only requisite for HSA isoforms to pertain to peak b is to have lost a positive or gained a negative charge in a way that is not reversible with reduced thiols, it is conceivable that peak b from commercial preparations could contain not only HSA-SO₂⁻ but also other modified isoforms of HSA as well. For this reason, EGCF-pure HSA-SH should be used for the preparation of EGCF-pure HSA-SO₂⁻. Finally, two minor peaks (a' and b') can be observed in Fig. 1A, eluting at pHs a slightly higher than peaks a and b. Noticeable, the proteolysis of the terminal Asp1-Ala2 observed in HSA-SH and HSA-SO₂⁻ results in the net loss of a negative charge, thus giving rise to products identified as peaks a' and b' in Fig. 1A that eluted at higher pH's. Whole protein MS analysis on the HSA samples used in this studies confirmed the presence of this fraction in reduced and delipidated HSA (not shown) supporting the assignment of peaks a' and b' in Fig. 1A to HSA₃₋₅₈₅-SH and HSA₃₋₅₈₅-SO₂⁻ (also present in Figs. 2 and 6).

The thiol of HSA can be selectively oxidized by two electrons with H₂O₂ as oxidant leading to the formation of HSA-SOH [6,11,12]. Nevertheless, using the proposed EPGC procedure, HSA-SOH, HSA-SX and HSA-SSR (if R is neutral) cannot be directly resolved from HSA-SH, reasonably because no net charge change occurs on the protein's surface because of these covalent modifications. Therefore, an alternative sample preparation procedure was developed based on the fact that Hg(II) ions react only with HSA-S⁻ leading to the formation of a thiol-blocked HSA (HSA-SHg⁺) with expectedly extra positive charge at the protein surface. Treatment of reduced HSA with Hg(II) immediately before analysis showed the formation of a new peak, peak c (Fig. 2A, gray trace) at the expense of peak a and absent in the sample without treatment (Fig. 2A, black trace), eluting at pH 5.01 and *B* = 12.7% (0.06–0.10 pH units higher than peak a). It is noteworthy that peak b did not change with this treatment, again showing that it did not contain a reduced thiol group and also that the transformation of peak a in peak c was almost quantitative. When peak c was analyzed by MS we observed a mass of 66661 ± 2 Da (*n* = 7, mass range 66658.0–66665.1 Da) (Fig. 2B). The expected mass corresponding to reduced thiol with one bound mercuric ion is 66637.6 Da. The +23 Da difference observed is probably due to the substitution of one proton by one sodium ion (22 Da mass difference). When reduced HSA was oxidized in conditions where HSA-SOH and HSA-SX are formed (H₂O₂ 4 mM, 4 min, 37 °C, pH 7.4) [12] only a small increase in peaks b and b' was observed with almost no change in peaks a and a' (compare black traces in Fig. 2A and C) consistent with the formation of a small amount of HSA-SO₂⁻ and HSA₃₋₅₈₅-SO₂⁻. When the oxidized sample was treated with Hg(II), a partial decrease in peak a was observed leading to the appearance of peak c (HSA-

SHg⁺). Thus in this condition, where excess Hg(II) with respect to total HSA was used, the remaining of peak a should represent HSA–SX or HSA–SOH (Fig. 2C). We are currently further investigating the chemical and structural nature of this isoform. With the employed gradient, HSA–SHg⁺ is partly separated from HSA–SOH/SX with $R \sim 1.4$ (Eq. (1)).

3.2. Analysis of HSA in plasma

HSA in the circulation is mostly in native reduced (HSA–SH) and mixed disulfides states (chiefly HSA–SSC). When the developed chromatographic procedure was applied to plasma protein samples (Fig. 3) a major peak at pH 4.83 and 15.1% B was observed (Fig. 3), in perfect agreement with peak a from Figs. 1 and 2. A small peak at pH 4.88 and 13.4% B (peak a') assigned to HSA_{3–585}–SH was also observed. Furthermore, a minor peak at pH 4.73 and 19.3% B was observed consistent with peak b which corresponds to HSA–SO₂[–]. By measuring the peak areas we estimated that the amount of HSA–SO₂[–] and HSA_{3–585}–SH in circulating plasma from this healthy donor was ~2.3% and ~6.1% of total HSA (Table 1) in agreement with previous reports [7]. Analysis of fresh or frozen plasma proteins samples yielded indistinguishable results (not shown).

3.3. Analysis of pharmaceutical preparations of HSA

We applied the EPGC method to the analysis of samples of untreated pharmaceutical HSA preparations from three different commercial sources (Fig. 4). A large variation was observed in the fraction of irreversibly oxidized HSA between untreated samples from different laboratories (Table 1). However, the three preparations were compliant with both the European Pharmacopoeia and the United States Pharmacopeia and The National Formulary [40,41] (Fig. S1, Table S1).

3.4. External pH gradient chromatofocusing compared with anion exchange-hydrophobic interaction chromatography

Although HSA–SOH is a key intermediate in HSA–SH oxidation [12], no direct evidence of HSA–SOH formation *in vivo* or *ex vivo* has been provided using the AEHIC procedure reported by Era et al. [1]. In addition, HSA–SH and HSA–SOH/SX are similar with respect to their apparent isoelectric points (Fig. 2A and C, black traces). Therefore, we speculated that HSA–SOH co-elutes with HSA–SH as part of the so called human mercaptalbumin (HMA) fraction. The study of the oxidation products of HSA in conditions where HSA–SOH and HSA–SO₂[–] are formed [12] using the reported AEHIC procedure (see Materials and Methods section) showed that HSA–SH oxidation leads to a decrease of the HMA fraction accompanied by an increase in HNA2 fraction (using Era's nomenclature). Since treatment with DTT did not decrease the HNA2 peak it can be concluded that the increase in this fraction was due to the oxidation of HSA–SH by 4 or 6 electrons to HSA–SO₂[–] or HSA–SO₃[–] and that HSA–SOH was not present in the HNA2 fraction (Fig. 5A). When an oxidized HSA sample was exposed to substoichiometric amounts of reduced glutathione (150 μM HSA and 50 μM GSH) a shoulder in the chromatogram (herein denoted as HNA3) appeared due to the formation of the mixed disulfide HSA–SSG at the expense of the HMA fraction with no changes in the HNA2 (Fig. 5B), demonstrating that HMA previously taken as pure HSA–SH can contain HSA–SOH/SX. Differently, reduced HSA incubated with

GSH showed the same chromatogram as reduced HSA (not shown). Noteworthy, blockage of HSA-SH using Hg(II) was not useful to accomplish the separation of HSA-SH and HSA-SOH/SX using Era's procedure (data not shown) as it was by our method.

The proposed EPGC method was then used to reveal the presence of HSA-SOH/-SX in oxidized protein plasma samples through the reaction of HSA-SOH/-SX with low molecular weight thiols that add a negative charge to the mixed disulfide (Fig. 6). Instead of using GSH, we decided to employ N-acetylcysteine (NAC), which should affect the isoelectric point of the macromolecule in the same way as GSH but the former has the analytical advantage that it is not a biosynthetic product. The incubation time was adjusted to allow the reaction to reach nearly 95% completeness. Plasma sample oxidation by H₂O₂ caused a decrease in peak a and a' with a concomitant increase in peak b and b' (Fig. 6A), consistent with the formation of HSA-SO₂⁻. Addition of NAC after treatment with H₂O₂, led to the appearance of a new peak (peak d, HSA-SSNAC) characterized by elution at pH 4.73 (Fig. 6B), formed at the expense of peak a as expected. Therefore, HSA-SOH/-SX coelute with HSA-SH using Era's or the EPGC procedure, but the last procedure can be used in conjunction with the selective blockage by Hg(II) of free Cys34 to resolve HSA-SHg⁺ from the reversibly oxidized products HSA-SOH/-SX and HSA-SSC if present. In fact, the resolution between HSA-SOH/-SX/-SH and HSA-SO₂⁻ achieved with the herein proposed EPGC procedure doubles that of the AEHC procedure ($2.8 R_{\text{EPGC}} \text{ vs. } 1.4 R_{\text{AEHC}}$).

4. Discussion

In this work we report on a new method for the analysis and purification of HSA isoforms based on external pH-gradient chromatofocusing (EPGC). In EPGC analyte elution occurs by changes of its surface charge brought about by changes in the pH of the low ionic strength mobile phase. The EPGC technique cannot be widely applied since it requires working near the isoelectric point at ionic strengths typically below optimal solubility values. Proteins that need ionic strengths >50 mM may precipitate in the conditions required for high resolution chromatofocusing. In other words, the higher the solubility of the protein in water, the higher the resolution that can be achieved with this technique. HSA has a very high solubility in pure water, even near its minimum as the pH gets close to its isoelectric point [2]. Reported isoelectric points for HSA-SH vary between 5.67 and 5.9 when estimated using theoretical calculations [42,43] (Fig. S2) and 4.7 and 5.8 when measured experimentally [2]. As established by Sluyterman and Elgersma [44], when performing EPGC a difference between the pH of the mobile phase and the stationary phase is expected due to the fixed charges in the latter. The observed elution pH is likely to be lower than the real pI of the analyte when an anion exchanger is used, an event that should also prevent protein precipitation. With this method an apparent isoelectric point of 4.88 ± 0.06 for HSA-SH was obtained. Since MS analysis unequivocally confirmed the nature of HSA-SH, we conclude that the real pI of HSA-SH should be higher than 4.9. From the methodological point of view, the most relevant antecedent of this method applied to serum albumins is the report on column isoelectrofocusing of bovine serum albumin (BSA) in water and urea 6 M by Wallevik [45]. This previous report and 2-dimensional polyacrylamide gel

electrophoresis practices suggest that the EPGC procedure could be performed in denaturing conditions too.

The proposed EPGC method is able to separate HSA-SH (in the form of HSA-SHg⁺) from HSA-SO₂⁻, HSA-SOH/-SX and HSA-SSR (if R is negatively charged). Indeed, whole protein high resolution MS confirmed the presence of four isoforms in previously reduced H₂O₂-oxidized HSA: HSA-SH (which reacts very fast with Hg(II) ions) and three oxidation products of HSA-SH, HSA-SOH (which can decay to HSA-SX or rapidly react with thiols [12]), HSA-SX and HSA-SO₂⁻ (Table 2). We clearly confirmed the formation of HSA-SHg⁺ and HSA-SO₂⁻ by EPGC followed by MS (Figs. 1B, right panel and 2B). We could not detect HSA-SOH by MS likely because it had already decayed to HSA-SX before analysis. Nevertheless we used NAC to reveal its presence (Fig. 6). Regrettably, NAC and Hg(II) cannot be used together on oxidized HSA samples, because they interfere with each other (NAC can displace HSA-S⁻ from HSA-SHg⁺ or form HSA-SHgNAC and Hg(II) ions react with free NAC). It must be stressed that a further improvement to this technique would be the replacement of Hg(II) by a non-toxic positively charged thiol reactant.

In the previously reported AEHIC procedure HSA-SOH coelutes with HSA-SH (Fig. 6), HSA-SHg⁺ and HSA₃₋₅₈₅-SH, taking part of the fractions assigned to human mercaptalbumin (HMA) [1] (Table 2). Furthermore, implementing the AEHIC procedure requires working above room temperature (35 °C) and the elution is performed in an ethanol gradient over a concentrated buffer reaching a point close to the solubility limit of the component salts. In addition, the high salinity of the eluate hampers its direct employment in LC-MS, which would be highly desirable in order to increase measurement sensitivity and specificity. Finally, it does not allow the measurement of HSA₃₋₅₈₅. However, since the AEHIC procedure partially resolves HSA-SSR (R neutral, *i.e.* HSA-SSC) from HSA-SH, HSA-SOH/-SX and HSA₃₋₅₈₅-SH, both HPLC procedures are complementary in principle.

Other than the herein proposed modifications of Cys34 can take place in HSA. Indeed Table 2 is not exhaustive, and the list of HSA isoforms should be considered open and will be enlarged as deeper analyses are performed. An increase in HNA2 and a decrease in HMA fractions under certain pathological conditions has been widely reported (for review see [4]). Considering our results (Fig. 5B), these observations mean that HSA-SO₂⁻ and/or HSA-SO₃⁻ are formed at the expense of HSA-SH, but nothing is known about HSA-SOH/-SX *in vivo* due to the fact that they coelute with HSA-SH using the previously reported AEHIC procedures (Figs. 2C and 5, respectively). Thus, these two isoforms may have passed overlooked in previous reports, possibly leading to an inexact evaluation of oxidative stress in plasma and other extracellular fluids. Furthermore, a recent report by Grigoryan et al. suggests the formation of a new HSA redox isoform characterized by a sulfinamide linkage between oxidized Cys34 and Gln33 and a mass difference of +14 [46].

In addition to Cys34 modifications, other modifications possibly redox-related that alter the isoelectric point of HSA have been described [15,47]. That could be the case of HSA₃₋₅₈₅ isoforms reported herein (Figs. 1–4 and 6). The N-terminal region of HSA is involved in Cu(II), Ni(II) and Co(II) metal ions binding [2]. It has been proposed that HSA isoforms lacking the first two N-terminal amino acids or with an altered ability to bind Co(II), called

Ischemia Modified Albumin (IMA) are formed *in vivo* [15]. Furthermore, these isoforms have been proposed as biomarkers of cardiac ischemia [14,16]. However the mechanism of its action remains controversial. The proposed EPGC procedure may help to evaluate the extent of the loss of this N-terminal dipeptide.

When comparing the redox status of pharmacological HSA preparations we observed important differences in the percentage of irreversibly oxidized HSA (chiefly HSA-SO₂⁻) between different commercial sources (Table 1), despite the fact that they all fulfill the quality requirements established in the European Pharmacopoeia [40] and the United States Pharmacopoeia and The National Formulary [41] (Fig. S1, Table S1). Well designed studies aimed at confirming and extending these observations and a better understanding of the mechanism of oxidation of Cys34 in these preparations should be useful to design strategies to prevent this irreversible modification and to eventually establish new quality standards. It has been reported that the intravenous administration of HSA can influence the redox state of plasma [48]. Nevertheless, it is unknown if it depends on the redox state of the pharmaceutical HSA in use. Given that increased concentrations of non-native isoforms of HSA correlate with human disease and ageing. The pharmacological and clinical consequences of the alteration of the redox state of pharmaceutical HSA preparations, particularly the abundance of irreversibly oxidized isoforms, should be further explored and taken into consideration.

5. Conclusions

Different thiol-oxidized derivatives of HSA can be separated by this novel external pH-gradient chromatofocusing (EPGC) method. In general, the technique can be used as the simplest way to evaluate or prepare any modified derivative of HSA that differs in isoelectric point with the starting material. Furthermore, this separation method can be directly coupled to MS. This chromatographic procedure may find a wide range of applications in the evaluation of the redox status of HSA in plasma and other extracellular fluids. Detailed evaluations of pharmaceutical preparations of HSA can be accomplished as well.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

EPGC	external pH gradient chromatofocusing
AEHIC	anion exchange-hydrophobic interaction chromatography
DTNB	5,5'-dithiobis-(2-nitrobenzoate)
DEAE	diethylaminoethyl
pI	isoelectric point
GSH	reduced glutathione
NAC	N-acetylcysteine
HSA	human serum albumin
HSA-SH	reduced native HSA
HSA-SOH	HSA with Cys34 oxidized to sulfenic acid
HSA-SO₂⁻	HSA with Cys34 oxidized to sulfinate anion
HSA-SO₃⁻	HSA with Cys34 oxidized to sulfonate anion
HSA-SX	spontaneous decay product of HSA-SOH
HSA-SHg⁺	HSA-SH with Cys34 blocked by a Hg(II) ion
HSA₃₋₅₈₅	HSA lacking the amino terminal dipeptide Asp1-Ala2
SH/HSA	mol of thiol per mol of albumin
HSA-SSR	mixed disulfides of HSA and low molecular weight thiols
HSA-SSG	mixed disulfide of HSA with glutathione
HSA-SSC	mixed disulfide of HSA with cysteine
HSA-SSNAC	mixed disulfide of HSA with N-acetylcysteine
HMA	human mercaptalbumin
HNA1 and 2	human nonmercaptalbumin 1 and 2, according to the AEHIC fractions reported by Era et al. [1]

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Scheme 1.

Fig. 1.

External pH-gradient chromatofocusing analysis of reduced pharmaceutical HSA. (A) Reduced HSA (0.85 SH/HSA) was injected manually (350 μ g) in a weak anion exchange high resolution commercial column (column 1: TSK DEAE 5PW glass 7.5 cm $\times$ 8 mm I.D., 10 μ m) and eluted at a flow rate of 1 ml/min with a linear pH gradient from 0% to 25% of solution B in 38 min starting 1 min after injection. Absorbance at 280 nm (black trace), pH (gray trace) and % B (dashed trace) were measured. Peak a corresponds to HSA-SH (pH 4.91, B = 14.9%), b to HSA-SO₂⁻ (pH 4.79, B = 19.2%), a' to HSA₃₋₅₈₅-SH (pH 4.95, B = 13.3%) and b' to HSA₃₋₅₈₅-SO₂⁻ (pH 4.84, B = 17.2%). (B) Off-line whole protein MS analysis of peaks a (left panel) and b (right panel).

Fig. 2.

External pH-gradient chromatofocusing analysis of reduced pharmaceutical HSA treated with Hg(II) and/or H₂O₂. (A) Reduced and delipidated pharmaceutical HSA treated (gray traces) or not (black traces) with 1.2 excess of mercuric chloride were injected manually (350 µg) into column 1 and the elution was performed as in Fig. 1. Absorbance at 280 nm (black trace), pH (not shown) and % B (not shown) were measured. In this conditions peak a corresponds to HSA-SH (pH 4.94, *B* = 14.7%), b to HSA-SO₂⁻, c to HSA-SHg⁺ (pH 5.01, *B* = 12.7%), a' to HSA₃₋₅₈₅-SH (pH 4.99, *B* = 13.2%), b' to HSA₃₋₅₈₅-SO₂⁻ (pH 4.87, *B* = 17.2%) and c' to HSA₃₋₅₈₅-SHg (pH 5.05, *B* = 11.6%). (B) Off-line whole protein MS analysis of peak c. (C) Delipidated and reduced HSA was oxidized by incubation with H₂O₂ (4 mM, 4 min, 37 °C, pH 7.4), treated or not with HgCl₂ (1.2-fold excess) and then subjected to EPGC as in (A) Under H₂O₂-oxidation and Hg(II)-blocking conditions peak a would correspond to HSA-SOH/SX (see the text for further details).

Fig. 3.

Analysis of fresh plasma. 10 μ l plasma samples prepared as described under the Materials and Methods section were injected directly into the column with no further purification. The elution was performed as in Fig. 1.

Fig. 4. Analysis of pharmaceutical grade HSA preparations. Commercial HSA (350 μ g) from three different laboratories was injected manually without any previous treatment. The elution was performed as in Fig. 1. Samples from Labs 1, 2 and 3 were analyzed.

Fig. 5.

Detection of HSA-SOH/SX by anion exchange-hydrophobic interaction chromatography in oxidized pharmaceutical HSA. HSA samples were analyzed as described under the Materials and Methods section using the dual-mode Shodex Asahipak ES-502N HPLC column (column 3). (A) Reduced HSA (600 μ M, solid line) was oxidized with H_2O_2 (5 mM, 15 min, room temperature) in PBS (dashed line). (B) An aliquot of this oxidized HSA was gel filtered to remove H_2O_2 and the resulting sample (150 μ M, solid line) was treated with 50 μ M glutathione for 10 min at room temperature (dashed line).

Fig. 6.

Detection of HSA-SOH/SX in oxidized plasma by external pH gradient chromatofocusing. (A) Gel filtered plasma (HSA ~ 260 μ M) was incubated with 15 mM H_2O_2 , 10 min, at room temperature, enough catalase was added to stop the reaction in 10 s and then injected (~350 μ g HSA) and analyzed as described in Fig. 1. (B) An aliquot of the oxidized sample was incubated with 1 mM N-acetylcysteine for 60 min at room temperature and then ~350 μ g of HSA were injected and analyzed.

Table 1

Analysis of redox isoforms in pharmaceutical grade HSA preparations and plasma.

Laboratory	Shelf-time ^a	% a ^b	% a ^b	% b ^b	% b ^b
1	2	76	8	11	5
2	8	72	6	17	5
3	9	46	4	39	11
Plasma		89	6	2	–

^aTime in months between packing and chromatofocusing analysis.

^bPercentages were obtained from the area under the peaks from Figs. 4 and 5.

Table 2

Assignment of HSA isoforms to AEHIC and EPGC fractions.

Chromatography	Fraction	Isoforms
AEHIC	HMA ^a	HSA-SH, HSA-SOH/SX, HSA-SHg ⁺ , HSA ₃₋₅₈₅ -SH
	HNA1 ^b	HSA-SSR
	HNA2 ^c	HSA-SO ₂ ⁻ , HSA-SO ₃ ⁻
	HNA3 ^d	HSA-SSR ⁻
EPGC	a	HSA-SH, HSA-SOH/SX, HSA-SSR
	a'	HSA ₃₋₅₈₅ -SH, HSA ₃₋₅₈₅ -SOH/SX, HSA ₃₋₅₈₅ -SSR
	b	HSA-SO ₂ ⁻
	b'	HSA ₃₋₅₈₅ -SO ₂ ⁻
	d ^d	HSA-SSR ⁻
	a ^e	HSA-SOH/SX, HSA-SSR
	a' ^e	HSA ₃₋₅₈₅ -SOH/SX, HSA ₃₋₅₈₅ -SSR
	b	HSA-SO ₂ ⁻
	b'	HSA ₃₋₅₈₅ -SO ₂ ⁻
	c	HSA-SHg ⁺
	c'	HSA ₃₋₅₈₅ -SHg ⁺

^aCan contain HMA and HNA.^bContains mixed disulfides with Cys and other neutral thiols.^cDoes not contain HSA-SOH.^dContains mixed disulfides with GSH, NAC or other negatively charged thiols.^eif enough Hg²⁺ is added to the sample.