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Brief reports

The presence of S-sulfonated transthyretin in commercial human serum albumin solutions: Potential contribution to neuropathy

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ABSTRACT

Background: Commercial solutions of human serum albumin (HSA) are administered to critically ill patients for the treatment of shock, restoration of blood volume, and the acute management of burns. Previously, conflicting results on the effects of HSA administration have been reported varying from a favorable increase in total plasma antioxidant capacity to a higher mortality rate in traumatic brain injury (TBI) patients. These results could be partially explained due to the known heterogeneity of HSA solutions. We report the discovery of S-sulfonated human transthyretin (hTTR) in HSA solutions.

Methods: Proteomics was performed on commercially available solutions of 5% HSA by LC-MS analysis. The MS charge envelope for hTTR was deconvolved to the uncharged native hTTR parent mass (13,762 Da). The parent mass was then integrated, and relative proportions of the 2 major species of hTTR, native and S-sulfonated hTTR (13,842 Da), were calculated.

Results: The majority of hTTR found in 5% commercial HSA solutions is in the S-sulfonated form regardless of the age of the HSA solution. S-sulfonation of hTTR at the free cysteine residue in position 10 appears to be the result of a mixed disulfide exchange possibly with S-cysteinylated hTTR or S-cysteinylated HSA. hTTR is a tetramer composed of four identical monomers each containing a reduced cysteine residue in position 10. S-sulfonation of hTTR at this cysteine residue can destabilize the hTTR tetramer, an important step in the formation of TTR-related amyloid fibrils.

Conclusions: Administration of a commercial HSA solution that already contains S-sulfonated hTTR could potentially contribute to the development of amyloid-related/polyneuropathy in the critically ill.

1. Introduction

The first commercially available solutions of human serum albumin (HSA) were developed in the 1940s using the cold ethanol fractionation technique created by Cohn [1]. Today, HSA is clinically used for the treatment of shock, burns, acute restoration of blood volume, hypoalbuminemia commonly observed in critically ill patients, in high volume paracentesis, and dialysis. The clinical use of commercial HSA solutions in the critically ill has been reported with mixed results. In a study involving 138 TBI patients, low HSA blood levels significantly correlated with poor outcome after 6 months suggesting a potential clinical benefit of albumin administration [2]. However, in a post hoc analysis of 460 TBI patients in the Saline versus Albumin Fluid Evaluation (SAFE) study, fluid resuscitation with HSA was associated with higher mortality rates versus saline [3]. In a follow-up analysis of 321 TBI patients enrolled in the SAFE study, the use of HSA for resuscitation was associated with increased intracranial pressure (ICP) during the first week suggesting that this is the most likely mechanism of increased mortality [4].

Although the current manufacturing process of commercial

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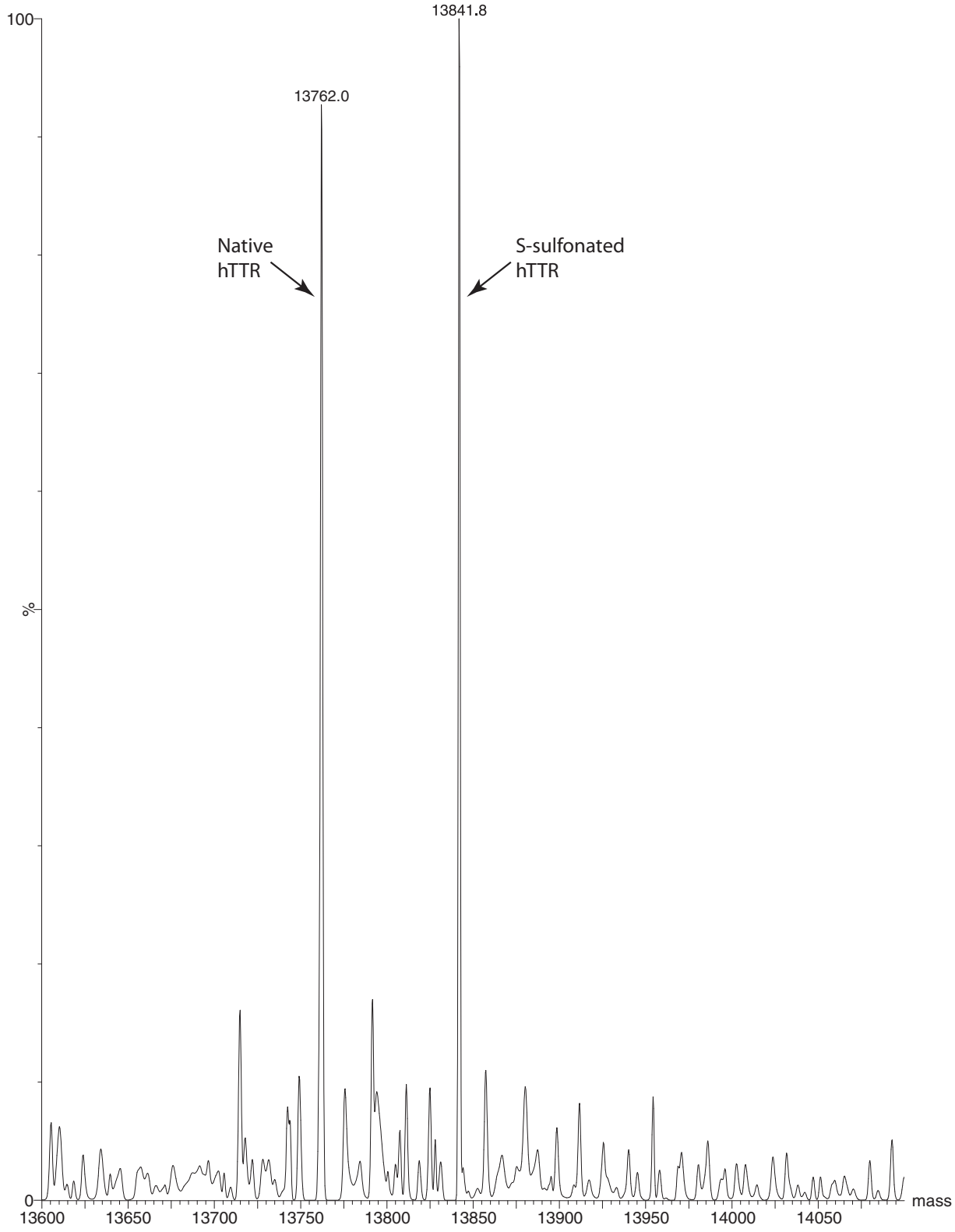

Fig. 1. Representative LCMS spectra of hTTR in a 5% commercial HSA solution.

solutions of HSA results in > 95% purity, other factors can potentially contribute to the poor outcomes in TBI patients. For example, the majority of HSA was oxidized in 6 commercially available HSA preparations tested suggesting potential clinical consequences due to the heterogeneity of HSA solutions [5]. Additionally, a total of 1219 peptides corresponding to 141 proteins (not originating from HSA) were identified suggesting that characterization of these proteins and peptides may be important in order to understand the therapeutic and possible deleterious effects of albumin therapies [6]. One of these proteins that is co-eluted with HSA during the Cohn fractionation process is human transthyretin (hTTR). Like HSA, hTTR is an indicator of nutrition status, a negative acute phase reactant, and a possible

Table 1
Relative percentages of native hTTR, sulfonated hTTR, and cysteinylated hTTR in commercial HSA solutions.

HSA Solution[a]	Native hTTR	Sulfonated hTTR	Cysteinylated hTTR	Unidentified PTM
1	41.9%	37.5%	3.1%	17.4%
2	37.9%	41.3%	2.0%	18.8%
3	40.9%	41.3%	5.5%	12.3%
4	41.7%	41.2%	3.5%	13.5%
5	41.3%	42.4%	0.0%	16.3%
6	49.6%	38.7%	8.4%	3.3%

[a] HSA solutions listed from oldest (#1) to newest (#6).

predictor of poor outcomes in TBI [2].

Currently, there is very little scientific understanding of the oxidation status of hTTR in commercial solutions of HSA much less the contribution of hTTR to clinical outcomes. In this study, we perform LC-MS proteomics on hTTR found in commercially available solutions of HSA. Like HSA, hTTR has a free cysteine residue which is in position 10 (Cys10). The oxidation status of Cys10 is important in the stability of the hTTR tetramer with certain types of Cys10 oxidation resulting in tetramer destabilization and potential hTTR-related amyloidosis [7–9].

2. Materials and methods

2.1. Materials

Six different manufactured lots of commercially available 5% human serum albumin (HSA) solutions (Octapharma) of varying shelf life were used in the study. All other reagents were from Sigma-Aldrich. Total hTTR was analyzed with the PREALB in vitro diagnostic test method (Siemens) using the Dimension Vista® System with a Flex® reagent cartridge.

2.2. LCMS proteomics

Proteomics were performed using an Acquity UPLC (Waters) coupled to a Xevo G2-S positive electrospray ionization time of flight mass spectrometer (Waters). Samples were collected by syringe and diluted 1:10 with dH_2O prior to LC-MS analysis. Two microliters of diluted sample was injected onto an Acquity UPLC Protein BEH C4 column (2.1 mm × 50 mm, 300 Å, 1.7 µm, Waters) heated to 50 °C using a linear gradient. At a flow rate of 0.8 mL/min, solvent A (dH_2O with 0.1% trifluoroacetic acid (TFA)) was initially started at 85% versus solvent B (acetonitrile with 0.1% TFA). Solvent B was increased to 90% during the 4-min run allowing for a 1-min equilibration back to starting conditions. MS conditions were as follows: scan range of 100–4000 Da, cone voltage of 50 eV, desolvation temperature of 550 °C, and collision energy of 6 V.

2.3. S-sulfonation of hTTR

In vitro S-sulfonation of hTTR was performed using a modified process for the reaction of sulfite with proteins previously described by Bailey et al. [10]. Briefly, heparinized plasma from a healthy volunteer was incubated with 1 mM sodium hydrosulfite or 10 mM D, L-dithiothreitol (DTT) in 1 × PBS (pH 7.4) for 3 h at 37 °C. All incubations were analyzed using the LCMS method described above and compared to the $t = 0$ control solution (5% HSA in 1 × PBS). All incubations were assayed for total [hTTR] using the PREALB in vitro diagnostic test method.

2.4. Data analysis

LCMS spectra were deconvolved using MaxEnt 1 (Waters) with an input range of 900 to 1250 Da and output range of 13,500 to 14,500 Da with simulated isotope pattern spectrometer blur width of 0.35. Deconvolved spectra were integrated by area and percent was calculated by dividing peak area by the sum of the areas of detected peaks.

3. Results

A total of 6 lots of commercial HSA solutions were analyzed by LCMS proteomics. Using the LCMS method described above, both HSA and hTTR have a retention time of ~1.8 mins. For HSA, the dominant species were native HSA and cysteinylated HSA (data not shown). For hTTR, the most prevalent species were native hTTR and sulfonated hTTR (Fig. 1). The amount of S-sulfonation of hTTR did not correlate with the age of the HSA solution as sulfonated TTR levels remained around 40% for all HSA solutions tested (Table 1). The newest HSA solution had a higher amount of native and cysteinylated hTTR than the other 5 HSA solutions. Additionally, there was less of the unidentified post-translation modification (PTM) hTTR species in the newest HSA solution. These unidentified PTM species correspond to N-terminal or C-terminal truncated hTTR although confirmation via trypsin digestion mapping is necessary for definitive identification.

All 6 HSA solutions had a total hTTR concentration of 10–15 mg/dl as measured by the PREALB in vitro diagnostic test (data not shown). Normal human hTTR blood levels range from 15 to 30 mg/dl. This suggests that the manufacturing of commercial solutions of HSA results in a ~50% recovery of hTTR from donor plasma.

In vitro S-sulfonation of hTTR was successfully achieved in normal plasma using sodium hydrosulfite with > 30% conversion of native hTTR to S-sulfonated hTTR after a 3-h incubation at 37 °C (Fig. 2A). The addition of DTT resulted in the reduction of the Cys10 residue and conversion to > 90% native hTTR (data not shown). Incubation ($t = 3$ h at 37 °C) of the control solution (normal plasma in 1 × PBS) caused a significant increase in the formation of S-cysteinylated hTTR (Fig. 2B) versus the $t = 0$ control solution (Fig. 2C).

4. Discussion

Human transthyretin (hTTR), initially known as prealbumin, is a carrier protein associated with the transport of thyroxine and the complex of retinol and retinol-binding protein (RBP) in plasma [11]. hTTR, formed mainly in the liver and choroid plexus, is a 55 kDa homotetrameric protein with each subunit having a mass of 13.7 kDa and composed of 127 amino acids [12]. Deposition of wild type hTTR appears to be associated with senile systemic amyloidosis (SSA) and familial amyloidotic polyneuropathy (FAP) [13]. Possible causes of amyloidosis are oxidation of the lone cysteine residue at position 10 (Cys10) of the hTTR monomer. The types of oxidation of Cys10 include S-cysteinylation and S-sulfonation.

In our proteomics study of 5% commercial solutions of human serum albumin (HSA), we detect the wild type hTTR monomer (13,762 Da) which is dissociated from the tetramer due to the LC-MS conditions. The other dominant species of hTTR is the sulfonated form which has a mass of 13,842 Da via the addition of an $-SO_3$ molecule (+80 Da) to Cys10. Albumin, in general, has multiple sulfur binding sites that can interact with proteins in various ways, preventing aggregation and oxidation, reducing surface adsorption and improving solubility and batch-to-batch consistency [14]. It is possible that, during the pasteurization process (heating at 60 °C for 10–11 h) of commercial HSA, sulfur-containing molecules are released from HSA that could be the source of hTTR sulfonation. Very little S-cysteinylation of Cys10 on hTTR was observed in the 5% HSA solutions. Levels of sulfonated hTTR did not change during the shelf life of the 5% HSA solutions. This contrasts with the increase in oxidized HSA (cysteinylated HSA) over time observed in this study (data not shown) as well as our previous study on the heterogeneity of HSA solutions [5].

Disulfide bond formation on free cysteine residues within proteins

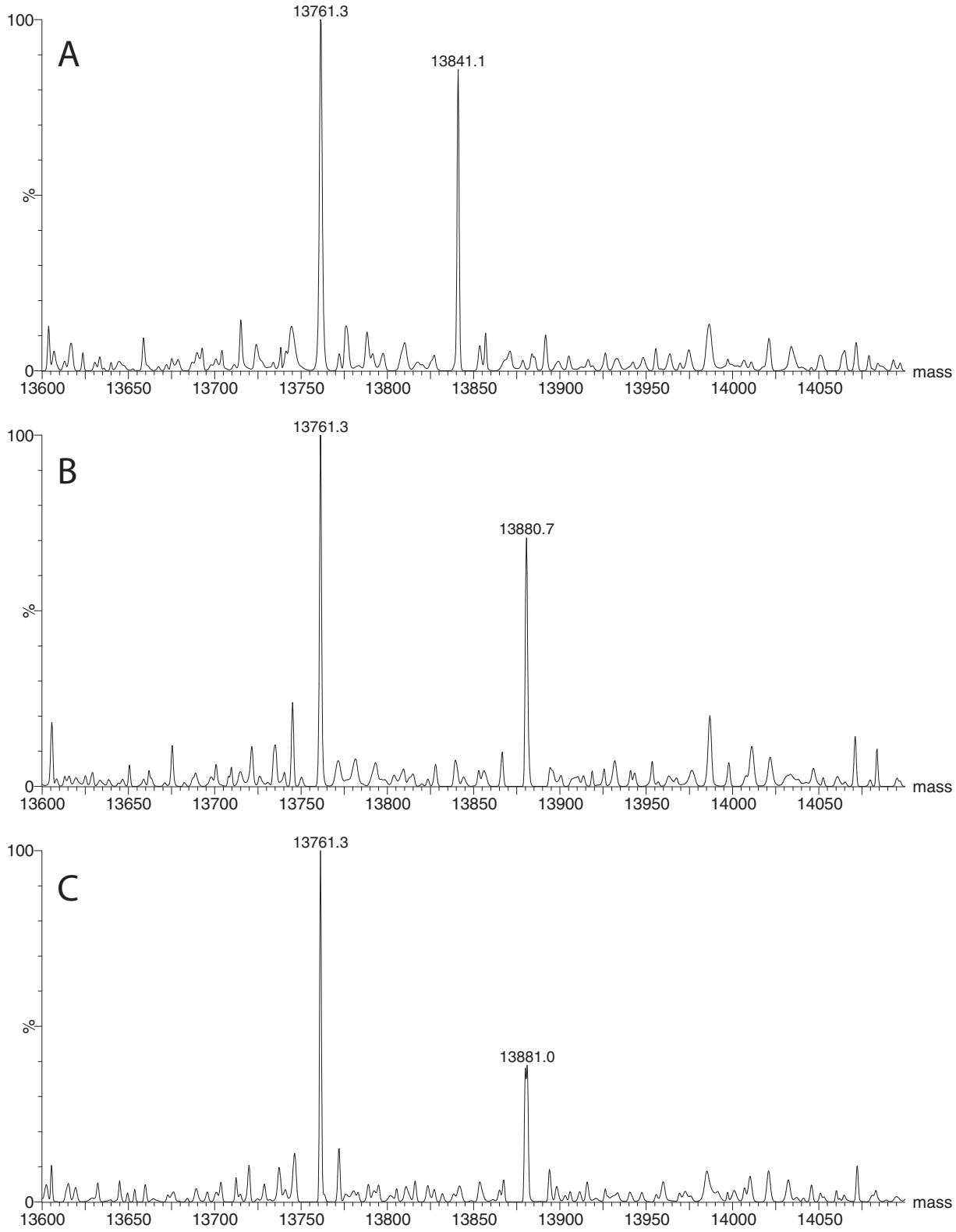

Fig. 2. LCMS spectra of hTTR following in vitro treatment of heparinized plasma from a healthy volunteer. Plasma was incubated for 3 h at 37 °C following treatment with 1 mM sodium hydrosulfite (A) or untreated in 1 × PBS (B). For comparison purposes, plasma in 1 × PBS at $t = 0$ is included (C). The major hTTR species include native hTTR (13,762 Da), S-sulfonated hTTR (13,842 Da), and S-cysteinylated hTTR (13,881 Da).

such as HSA and hTTR are obviously indicative of redox processes occurring. Higher oxidation of HSA has been observed in severe TBI patients versus control groups resulting in a positive correlation with overall oxidative stress by oxidation-reduction potential (ORP) measurement [15]. For hTTR, a transient increase in oxidized hTTR products was observed in severely injured patients (ISS = 34–66) admitted to the ICU [16]. It has been suggested that hTTR is a potential biomarker of several human diseases linked with oxidative stress and

inflammation [17]. Finally, any destabilization of the hTTR tetramer could lead to the formation of the hTTR monomers causing the production of reactive nitrogen species and neurotoxicity [18]. A cell culture model using a human neuroblastoma cell line demonstrates that the monomer rapidly aggregates to form transient low molecular mass assemblies (< 100 kDa) that are highly cytotoxic [19].

The formation of amyloid fibrils based on the type of Cys10 oxidation (S-cysteinylation versus S-sulfonation) on hTTR remains highly controversial in the scientific literature. For example, multiple studies have demonstrated an increase in tetramer destabilization and amyloid fibril formation when hTTR is sulfonated at Cys10 [9,20,21]. However, another study demonstrated that S-sulfonation stabilized the hTTR tetramer by a factor of 7, whereas S-cysteinylation enhanced dissociation by 2-fold with respect to the unmodified form [7]. Additionally, under mildly acidic conditions (pH 4.8), the amyloidogenesis rates of cysteinylated hTTR are much faster than sulfonated hTTR which is less amyloidogenic and forms fibrils more slowly [8]. It has been proposed that treatment with sulfite which causes S-sulfonation of hTTR might be beneficial to SSA and FAP patients due to tetramer stabilization [22,23]. Currently, it remains speculative as to whether acute changes in TTR oxidation is indicative of an individual susceptibility to destabilizing modifications in TTR, or whether transient changes might be of long term consequence for the affected ICU patient with regard to amyloidosis, Alzheimer's disease, or the occurrence of other neurological disorders [16]. The development of a neurological disease from amyloid may be more likely if the patient has a mutation in the gene that encodes the amyloid precursor protein or apolipoprotein E [24,25].

Limitations to our study include a lack of a known correlation between HSA administration to critically ill patients and the development of a polyneuropathy versus similar patients not administered HSA. This type of study should be very extensive and address both short-term (< 1 month) and long-term (> 5 years) neurological events. Also, the controversy regarding whether S-sulfonation or S-cysteinylation is more detrimental to tetramer stability needs to be addressed. The studies contributing to this controversy cover a wide pH range where only a physiological pH would be necessary to possibly determine the important oxidation type in hTTR tetramer destabilization. Perhaps a direct binding assay of amyloid with wild type hTTR, sulfonated TTR, and cysteinylated TTR at physiologic pH is warranted. In the present study, 5% commercial HSA solutions contain mostly sulfonated hTTR with native hTTR being the other major species. It remains to be seen if administering HSA solutions containing significant levels of sulfonated hTTR to critically ill patients is safe due to potentially amyloid fibril-producing hTTR monomers. Also, the type of injury (brain, cardiovascular, etc.) and HSA administration might be an important determinant in the short- or long-term production of amyloidosis, Alzheimer's disease, or the occurrence of or possible protection from other neurological disorders.

In conclusion, the majority of hTTR in commercial solutions of HSA is S-sulfonated on the cysteine at position 10. The amount of S-sulfonation does not change with age of the HSA solution. Various studies have shown that the oxidation of cysteine in position 10 of hTTR via S-sulfonation or S-cysteinylation can destabilize the hTTR tetramer leading to the development of amyloid-related polyneuropathy. However, future clinical studies will have to be conducted in order to confirm the development of amyloid-related polyneuropathy after HSA administration.

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