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Biochemical comparison of four commercially available human α_1-proteinase inhibitors for treatment of α_1-antitrypsin deficiency

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ABSTRACT

Intravenous therapy with purified plasma-derived alpha$_1$-proteinase inhibitor (α_1-PI) concentrates is the only specific treatment for α_1-PI deficiency. For the therapy to be safe and efficacious, α_1-PI concentrates should be highly pure and contain high amounts of functional protein. This study compared the four plasma-derived α_1-PI products commercially available in Europe (Respreeza, Prolastin, Alfalastin, Trypsone) by biochemical methods with respect to function, purity, structure, and chemical modifications. Respreeza had the highest level of functional protein (48.8 mg/mL) and the highest specific activity (0.862 mg active α_1-PI per mg total protein). By size exclusion chromatography, Respreeza was 97.4% pure, followed by Alfalastin 88.1%, Prolastin 76.9%, and Trypsone 70.8%. By reversed phase chromatography, Respreeza had an α_1-PI purity of 97.7%, followed by Trypsone 88.0%, Prolastin 78.0%, and Alfalastin 69.5%. The main protein band by sodium dodecyl sulphate-polyacrylamide gel electrophoresis was found for all products at approximately 50 kDa. Additional protein bands were found for Prolastin, Alfalastin, and Trypsone. The α_1-PI products differed in cysteine oxidation state and C-terminal lysine status. α_1-PI products tested differ in purity, concentration, and chemical variation. Respreeza has the highest level of purity. The impact of the non-therapeutic proteins identified has not been evaluated.

1. Introduction[1]

α_1-Antitrypsin deficiency is an autosomal codominant condition that causes defective production of functional α_1-proteinase inhibitor (α_1-PI) and represents one of the most common genetic respiratory disorders worldwide [1,2]. α_1-PI is one of the major circulating anti-proteases and an acute phase protein, with increased release during the course of an acute inflammation. One of the main functions of α_1-PI is to protect tissues containing elastin, most notably lung parenchyma, from degradation by serine proteases like neutrophil elastase [3,4]. Individuals with severe α_1-antitrypsin deficiency present with serum α_1-PI concentrations below 11 μM and can develop pulmonary emphysema at a relatively early age by the fourth or fifth decade of life [5]. To slow down the progression of emphysema in patients with α_1-PI deficiency, the protease to anti-protease balance is restored by lifelong augmentation therapy with intravenous infusions of purified plasma-derived α_1-PI concentrates [2,6]. Large observational studies have shown that augmentation therapy is well tolerated, safe, and can preserve lung function in some patients as measured by forced expiratory volume in 1 s (FEV_1) [7,8]. However, placebo-controlled studies proving efficacy of intravenous α_1-PI therapy were still lacking until the recently published RAPID trial [9]. The RAPID trial used as primary endpoint computed tomography lung densitometry, which is endorsed by medical authorities like the Food and Drug Administration and the European Medicines Agency as appropriate, clinical meaningful and a most sensitive endpoint to assess efficacy of intravenous α_1-PI therapy in α_1-antitrypsin deficiency-associated emphysema [10,11]. This measure has been shown to correlate to lung function parameters such FEV_1 [12,13]. A

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[1] **Abbreviations:** α_1-PI = alpha1-proteinase inhibitor; FEV_1 = forced expiratory volume in 1 s; IgA = immunoglobulin A; LC-MS = liquid chromatography-mass spectrometry; LC-MS/MS = liquid chromatography-tandem mass spectrometry; RP-HPLC = reversed phase-high performance liquid chromatography; SDS-PAGE = sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SEC-HPLC = size exclusion-high performance liquid chromatography; WHO = World Health Organization.

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statistically significant disease modifying effect was shown by the main study in combination with a 2-year open-label extension study using Respreeza [14,15].

Human α_1-PI is a single-chain, 394 amino acid glycoprotein containing three N-linked glycans, a single cysteine at position 232, and an apparent molecular weight of approximately 52 kDa [16]. It has a significant structural similarity to other members of the serine protease inhibitor family, with a secondary structure consisting primarily of α-helix and β-sheet motifs. One notable structural element is the reactive center loop, encompassing residues 344–368. This unstructured loop extends into the solvent and contains the cleavable bond (between residues 358 and 359) integral to protease inhibition [17].

Glycosylation of the α_1-PI molecule consists of three N-linked oligosaccharides, which are attached to asparagine residues at positions 46, 83, and 247. The glycans are mainly diantennary but also tri- and tetraantennary [18]. The glycans may or may not be fucosylated. Sialic acid residues on the N-glycans give α_1-PI a high negative charge. In an electric field, multiple isoforms of α_1-PI (M2-M8) are separated mainly due to the different numbers of sialic acids residues on the N-glycans.

In addition to glycosylation, the α_1-PI molecule may contain a number of other potential modifications. Amino acid side chain modifications may include methionine oxidation and asparagine deamidation. Oxidation of two methionines in particular, residues 351 and 358 located in the reactive center loop, is known to have functional implications for the molecule [19]. Proteolytic cleavage of the five N-terminal residues and the C-terminal lysine has also been reported [20,21].

A number of α_1-PI preparations, purified from pooled human plasma, are currently available in Europe for therapy: Respreeza (CSL Behring, Kankakee, IL USA, sold as Zemaira in the US), Alfalastin (LFB Biomedicaments, France), Prolastin (Grifols, Clayton, NC USA), and Trypsone (Grifols, Spain). While all four products elevate α_1-PI levels, there are differences in upstream manufacturing and downstream purification processes, including pathogen reduction steps.

The aim of this study was to compare multiple batches of the four α_1-PI products available in Europe by a series of biophysical and biochemical methods with respect to function, purity, structure, and chemical modifications.

2. Materials and methods

2.1. α_1-PI products: starting material and manufacturing process

The analysis included commercially available batches of four plasma-derived α_1-PI products (3 batches of Respreeza, 3 batches of Prolastin, 2 batches of Alfalastin, and 3 batches of Trypsone). All batches were used within their shelf life (Supplementary Table 1).

The lyophilized vials of the products were reconstituted with sterile water according to the manufacturers' instructions, resulting in different α_1-PI concentrations as declared (Respreeza, 50 mg/mL; Prolastin, 25 mg/mL; Alfalastin, 33.33 mg/mL; Trypsone, 20 mg/mL). For all testing, the material was aliquoted and frozen at −80 °C until the time of testing. Before analysis, the samples were thawed at room temperature.

2.2. Functionality of α_1-PI protein

The amount of functional α_1-PI protein was determined by the existing Respreeza potency assay, which measures the elastase inhibitory capacity of α_1-PI using a chromogenic substrate. This assay used an α_1-PI standard calibrated against the World Health Organization (WHO) international standard [22]. The WHO standard-derived value was then adjusted by dividing it by 1.089 to maintain consistency with internal results prior to the WHO standard release.

2.3. Overall composition and purity of α_1-PI products

2.3.1. Total protein and specific activity

The total amount of protein per vial was determined for each product with the Bradford method [23], using a Bio-Rad Coomassie Brilliant Blue G-250 solution (Bio-Rad, Hercules, California USA). Samples were diluted to approximately 150 μg/mL and analyzed against a standard curve consisting of pure α_1-PI (a Respreeza batch) from 50 to 250 μg/mL. The protein concentration of the α_1-PI standard was determined using absorbance at 280 nm and an extinction coefficient of 0.433 $(mg/mL)^{-1}cm^{-1}$.

The specific activity, as a measure of protein purity, was calculated as the ratio of α_1-PI potency to total protein amount (as determined by the Bradford method).

2.3.2. Purity

Immunonephelometry on a BNII instrument (Siemens Healthcare, Malvern, PA USA) was used to measure impurities of α_1-PI products following the manufacturer's instructions. Results of immunonephelometry testing were normalized by dividing the impurity concentrations by the total protein concentration determined by the Bradford method. The following proteins were assayed: albumin, α_1-acid glycoprotein, α_2-macroglobulin, apolipoprotein A-I, antithrombin-III, ceruloplasmin, haptoglobin, immunoglobulin A (IgA), immunoglobulin G, and transferrin.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to identify purity and degradation state of α_1-PI products. It was performed according to Lämmli [24], using 4–12% gradient precast polyacrylamide gels (Invitrogen, Thermo Fisher Scientific, Waltham, MA USA) under reduced conditions. Each sample was diluted with Bio-Rad 2X Lämmli Sample Buffer, adjusting the total amount of active α_1-PI to 2 μg/lane. A prestained molecular weight marker (Bio-Rad) was run in parallel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250. For the quantification of the SDS-PAGE results, 4–20% gradient precast polyacrylamide gels (Invitrogen) were used with loads of 9, 11, and 13 μg protein/lane. The percent main band (α_1-PI) values were calculated by comparing the density of the α_1-PI band to the total band density based on densitometry (Appraise Densitometer, Beckman, Indianapolis IN USA). The results from the three protein loads were averaged.

Size exclusion-high performance liquid chromatography (SEC-HPLC) was performed to separate the molecules in the final α_1-PI products and to determine the amount of high and low molecular weight proteins (including potential α_1-PI protein aggregates and fragments). For SEC-HPLC, approximately 200 μg of α_1-PI was applied to a TSK gel G3000SWxl column (Tosoh Biosciences, King of Prussia, PA USA) using a buffer that contained potassium phosphate, potassium chloride, and sodium azide at pH 6.8 for isocratic elution at room temperature.

For each product, the areas of the observed peaks were calculated and compared. Purity was evaluated for all α_1-PI products based on the peak area of α_1-PI relative to the total area of all integrated peaks.

Reversed phase-high performance liquid chromatography (RP-HPLC) provided a quantitative evaluation of the overall purity of α_1-PI products. It was carried out on an Alliance HPLC System, utilizing Empower software (Waters, Milford, MA USA). The separation was performed on a Jupiter 5μ C4 300 Å, 250 × 4.6 mm column (Phenomenex, Torrance, CA USA) with a water/acetonitrile + 0.1% trifluoroacetic acid mobile phase system.

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