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Convenient high-resolution isoelectric focusing (IEF) method for the separation of alpha$_1$-proteinase inhibitor (A1PI) isoforms in A1PI concentrates

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Abstract

Currently, high-resolution separation of A1PI is done in highly specialized laboratories using gels made in-house. This paper presents a high-resolution method for the separation of A1PI concentrates and human plasma using commercially available gels. Hybrid IEF was performed with carrier ampholytes and the gels were stained with Coomassie Brilliant Blue G-250. In addition, a sensitive immunoblotting procedure is described. The IEF method allowed the reproducible and convenient determination of the IEF pattern of A1PI in concentrates including resolution of glycan-dependent isoforms and isoproteins with secondary modifications such a C-terminal Lys-truncation. Furthermore, a shift in the IEF pattern of A1PI occurring upon reduction could be detected. Finally, in combination with a sample pretreatment step, the method proved able to monitor complex A1PI isoform patterns in samples with low A1PI concentrations as present for example in bronchoalveolar lavage solutions.

Keywords: Alpha$_1$-antitrypsin; Isoelectric focusing; Isoform pattern; Alpha$_1$-antitrypsin concentrates

1. Introduction

Early work on the microheterogeneity of alpha$_1$-proteinase inhibitor (A1PI) described a combination of paper electrophoresis, agar-gel electrophoresis, and immunoelectrophoresis to analyze isoforms [1]. More recent work used starch gel electrophoresis, which led to the introduction of the multiallelic protease inhibitor (Pi) system [2,3]. These techniques were replaced by isoelectric focusing (IEF) in polyacrylamide gels [4–6], which results in a characteristic pattern of the normal M-type A1PI. This consists of the two main bands M4 and M6, and three minor bands, M2, M7 and M8 that all contribute to the microheterogeneity. The development of immobilized pH gradients (IPG) made it possible to separate A1PI variants that differ only slightly in their isoelectric points (pI), often caused only by deletion or exchange of a single charged amino acid [7–11]. These high-resolution IEF methods can usually only be established in few highly specialized laboratories and require laborious preparation of the gels.

Human A1PI [12] is the major serine protease inhibitor in human plasma. Its most important physiological function is the inhibition of neutrophil elastase, a potent serine protease able to destroy most forms of connective tissues [13]. The effect is seen in subjects with deficiency or functional impairment of this inhibitor who develop emphysema upon destruction of lung tissue [14,15]. For the substitution therapy, three plasma-derived A1PI concentrates – Prolastin [16], Aralast [17] and Zemaira – are available which differ in purity, concentration and content of active, inactive and polymerized A1PI. As recently shown [18] A1PI from each preparation differs in molecular terms including deamidation, cysteinylation and elimination of C-terminal lysine and, in fact, none of the three is identical to native A1PI from human plasma. Because these molecular alterations do not affect

Abbreviations: A1PI, alpha$_1$-proteinase inhibitor; DTE, dithioerythritol; IEF, isoelectric focusing; IPG, immobilized pH gradient; Pi, proteinase inhibitor; pI, isolectric point

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the active center of A1PI, the preparations do not differ in their inhibitory activity against elastase.

In human plasma, the mature 52 kDa highly polymorphic protein with at least 49 gene allelic variations [19] is found as a single 394-amino acid polypeptide chain with three complex type N-glycans. Variations in the N-glycan branching [20] and N-terminal truncation [21] lead to the microheterogeneity observed by isoforms within a given homozygotous phenotype of A1PI.

Here, a convenient high-resolution hybrid IEF method is described that has been developed for analysis of A1PI concentrates used to treat hereditary emphysema. This method uses commercially available IPGs and can be used in any good laboratory. The method offers the possibility of detecting the A1PI isoform pattern even in solutions with very low levels of A1PI. In addition, the method proved suitable for preparing samples for further detailed protein and N-glycan analysis of the separated bands with mass spectroscopy [6].

2. Materials and methods

2.1. A1PI concentrates and other A1PI samples

The following batches of the plasma-derived A1PI concentrates Aralast, Prolastin and Zemaira were used: Aralast batches nos. LH02031A, LH03002A and LH04039A (Alpha Therapeutics, now Baxter); Prolastin batches nos. 26N0361 and PR4HA43A (Bayer, now Talecris); Zemaira batch no. Y400207 (Aventis Behring, now ZLB Behring). The lyophilized preparations were reconstituted according to the manufacturers' instructions. Aliquots were stored frozen at −20 °C until analysis. A lyophilized reference plasma pool (lot no. 1R92) and several single plasma specimens were obtained frozen from Baxter BioScience, the A1PI phenotype controls MM, MZ and SZ were from Canterbury Scientific (Christchurch, New Zealand).

2.2. IEF method

For the optimal IEF method IPG Immobiline gels (pI 4.2–4.9) were re-hydrated for 60 min in 20% glycerol [22] and 1% Pharmalyte 4.2–4.9 (all from GE Healthcare Bio-Sciences, Uppsala, Sweden). The test samples were diluted with distilled water to 0.5 mg A1PI/ml and dithioerythritol (DTE) as reductant at a final concentration of 5 mM. IEF was run on Multiphor II (GE Healthcare Bio-Sciences) at +2 °C. As anodic and as cathodic solutions, 0.2 M H_3PO_4 and 0.2 M NaOH were used, respectively. After pre-focusing the gel for 30 min (3000 V, 150 mA, 5 W), 20 μl samples were applied close to the cathode. Focusing was achieved in a multiphase-step gradient: 15 min, 500 V (150 mA, 5 W); 15 min, 1000 V (150 mA, 5 W); 30 min, 2000 V (150 mA, 10 W); 180 min, 3000 V (150 mA, 15 W). Typically, a total of 11,000 Vh was reached. After fixation with a solution of 11.5% trichloroacetic acid and 3.45% sulfosalicylic acid for 60 min at room temperature, the staining with Coomassie Brilliant Blue G-250 (Bio-Rad, Vienna, Austria) was done as described [23]. Briefly, 160 ml 10% ammonium sulfate in 2% H_3PO_4 were mixed with 40 ml methanol. One-hundred and sixty microliters of a 5% Coomassie Blue G (Serva, Vienna, Austria) solution were then added and the gel was stained overnight under constant shaking at room temperature.

The following conditions were tested to optimize the method: (a) re-swelling in 20 mM DTE used also as anolyte and catholyte, no pre-focusing; (b) re-swelling in 2.5% Pharmalyte 4.2–4.9, 20% glycerol; as anode and cathode solution 40 mM glutamic acid and 0.1 M NaOH, respectively, pre-focusing; (c) 2.5% Pharmalyte 4.2–4.9, distilled water as anode and cathode solution, no pre-focusing. The running conditions were as described above.

2.3. Western blotting and A1PI immunostaining

After the IEF the gel was equilibrated for 10 min with the blotting buffer (25 mM Tris(hydroxymethyl)-aminomethane, 192 mM glycin; 20% methanol), cut from the support and overlaid with a 0.2-μ nitrocellulose membrane (Bio-Rad). Electroblotting at 15 °C and 40 V lasted 16 h. The membrane was incubated for 30 min with phosphate-buffered saline-milk-Tween solution (2 mg/ml skimmed milk, Maresi, Vienna, Austria; 0.05% Tween 20, Bio-Rad). This buffer was also used for diluting the primary antibody (rabbit anti-human alpha$_1$-antitrypsin, A-012; DakoCytomation, Glostrup, Denmark) and the detection antibody (goat anti-rabbit IgG-peroxidase, Bio-Rad). Both incubations were done at room temperature for 60 min. The staining kit Opti 4 CN (Bio-Rad) was used. Samples with low A1PI concentrations or with salt concentrations incompatible with IEF were concentrated and/or desalted as follows: 1 ml sample was mixed with 20 μl rabbit serum (Rockland, Philadelphia, Pennsylvania, USA) and 1 ml 50% (w/w) polyethylenglycol 6000 solution (VWR, Vienna, Austria). The mixes were kept at +4 °C overnight. The precipitate was recovered by centrifuging at 4000 × g at +4 °C, and, dissolved in 200 μl distilled water, used for IEF.

3. Results

The method was developed with samples of A1PI concentrates but also allowed the analysis of A1PI in plasma milieu and even in bronchoalveolar lavage fluids without loosing any resolution power. Fig. 1a shows the gel re-hydrated in 20 mM DTE which was used also as electrode solution. All A1PI isoforms separated to a high-resolution especially visible in the range of the isoform M4. The Aralast concentrate displayed a second more intense band, which focused more cathodically than the M4 band in plasma and Prolastin or Zemaira. In addition, a band was observed in Aralast that focused in the acidic M2 region and was not found in such intensity in the other concentrates. The A1PI protein appeared to migrate on the surface of the gel only, resulting in faintly stained bands. Adding carrier ampholytes did result in higher resolution, but increasing the separation time (data not shown) did not (Fig. 1b–d). The amount of carrier ampholytes added however appeared not to be important because there was no difference when 2.5 or 1% were added to the rehydration cocktail. Consequently, the electrode solutions influenced the separation. Using just water resulted in

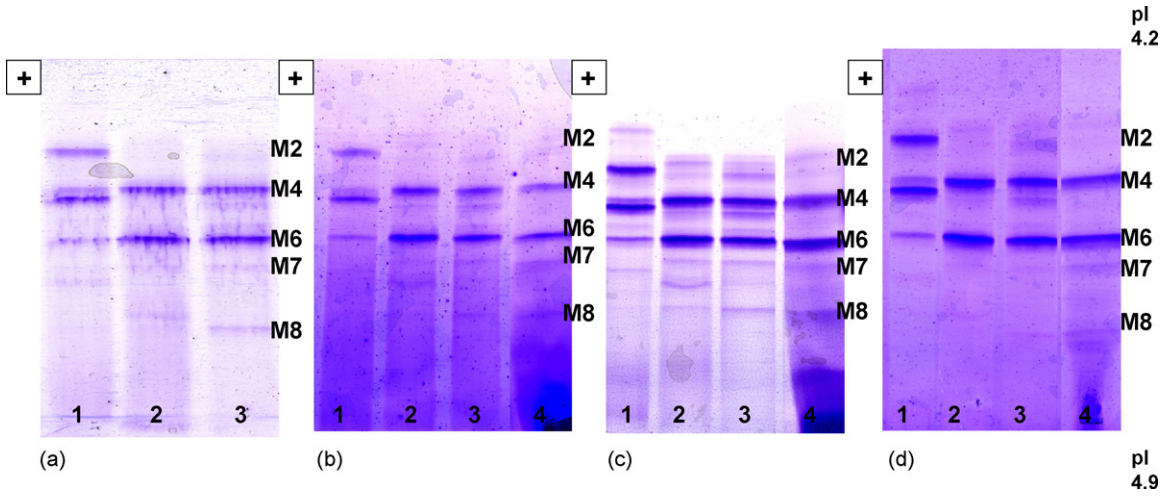

Fig. 1. Optimization of the method (1) Aralast LH02031A, (2) Prolastin 26N3061, (3) Zemaira Y400207, (4) reference plasma pool 1R92. Anode is on the top and the gels were run to yield a total of 4000 Vh. The following conditions were applied: (a) re-swelling in 20 mM DTE, anolyte and catholyte 20 mM DTE; no pre-focusing, (b) 2.5% Pharmalyte 4.2–4.9, 20% glycerol; pre-focusing; anolyte 40 mM glutamic acid, catholyte 0.1 M NaOH, (c) 2.5% Pharmalyte 4.2–4.9, anolyte and catholyte distilled water and (d) 1.0% Pharmalyte 4.2–4.9, 20% glycerol; pre-focusing; anolyte 0.2 M orthophosphoric acid, catholyte 0.2 M NaOH. The gels were adjusted for the position of the band M6.

a good resolution, but the resolution even improved when ionic electrode solutions and pre-focusing was used. When we strictly followed the conditions described by the manufacturer of the gel, we found on analysis of plasma much protein which did not focus properly, but distributed all over the gel and when loading A1PI concentrates band broadening occurred reducing the resolution of the method. In addition, there was a lot of protein which seemed to precipitate on the gel. Finally, the conditions used in Fig. 1d were selected because they produced the best resolution in the M4 region. In this pI range the A1PI concentrate Aralast displayed a second band. Mass spectrometric analysis of tryptic peptides derived from this band focusing slightly more cathodically than the plasma M4 band revealed that this difference in pI was caused by the removal of the C-terminal amino acid lysine [18] and was not related to an altered glycosylation of A1PI.

The applicability of the method to the typing of genetic variants of A1PI and, thus, to the analysis of single plasma specimens is shown in Fig. 2. Also in plasma, the method provided a clear separation of the A1PI isoforms and the most important pathological genetic A1PI variants S and Z were undoubtedly identified as shown by the analysis of the heterozygotous A1PI control samples MZ and SZ. The slow moving band was clearly recognizable in the MZ and SZ control and in the latter, the S6 and Z4 bands are resolved clearly. Furthermore, even though not within the primary scope of the method, different M subtypes were also discriminated to a certain extent. Thus, sample 1 is a M2S subtype, whereas sample 2 appears to be M1M2 and sample 4 either M1M4 or M1M3. None of the plasma specimens showed an IEF pattern similar to that of the A1PI concentrate Aralast, which resembles that of the non-pathological A1PI phenotype M1E [24–26]. The pI shift of this A1PI variant is also caused by an alteration, which affects one lysine residue only, although this residue is situated within the polypeptide and not at the C-terminus.

By analysing A1PI concentrates, we could detect an influence of the reduction on the IEF pattern of A1PI (Fig. 3). Reduction with 5 mM DTE shifted the whole pattern of the concentrates Aralast and Prolastin and of plasma slightly towards the anode equally affecting all A1PI bands. In contrast, reduction did not affect the IEF pattern of Zemaira suggesting differences amongst the concentrates related to the single cysteinlation of Cys232 in the A1PI molecule.

Fig. 4 shows the results of the sensitive immunostaining after IEF for the two A1PI concentrates Aralast and Prolastin, which were loaded at different concentrations. Their specific IEF pattern could be detected at concentrations as low as 0.5 μg A1PI/ml. The separation performance was not affected. Even at the low A1PI concentration the well-separated double band focusing in the M4 range was still clearly detectable in Aralast. The minor isoforms M7 and M8 as well as the acidic iso-

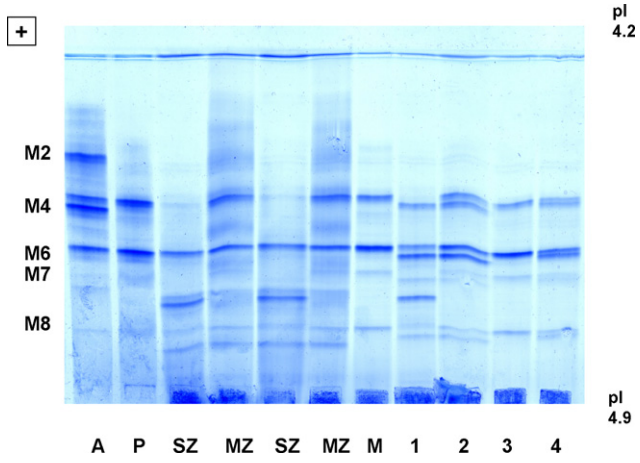

Fig. 2. Analysis of single plasma specimens and MZ and SZ controls: A, Aralast; P, Prolastin; SZ, MZ, M, IEF standards, from Canterbury Scientific; 1, 2, 3, 4, single plasma specimens. Anode is on the top.

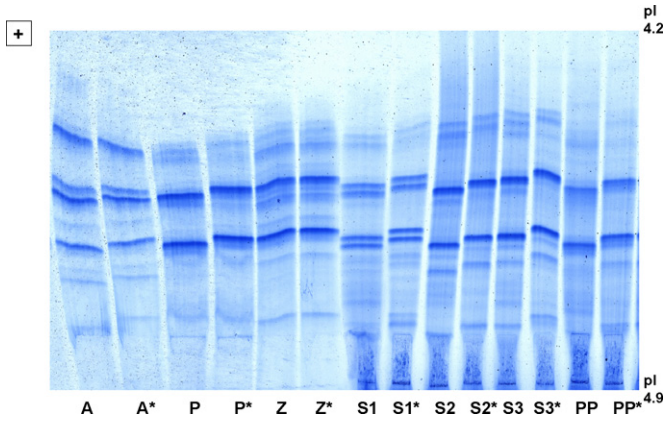

Fig. 3. IEF of A1PI concentrates and plasma specimens; high-resolution IEF of non-reduced and reduced samples of the A1PI concentrates Aralast (A), Prolastin (P) and Zemaira (Z), human plasma specimens S1, S2 and S3 (ER200544400, ER200544524 and 0064350) and a human plasma reference pool (PP). Anode is on top of the gel. Samples reduced with a final concentration of 5 mM DTE are indicated by a star. An anodal shift of the whole banding patterns caused be mild reduction is seen in Prolastin, Aralast and normal plasma.

forms M2 and M0/M1 [27] were visible in both concentrates, too.

4. Discussion

Conventional IEF has been used to identify various Pi M A1PI subtypes and variants with different pI. The method was modified in 1982 by the introduction of stable pH gradients [28]. Due to the advent of the DNA-based methodology IEF has lost its importance for the A1PI phenotyping of individuals. There is a large body of data on serum analyses, but only few data on the analyses of A1PI concentrates, probably due to the lack of a convenient high-resolution method capable for use in routine analysis in non-specialized laboratories.

This is also suggested by the fact that in the two recent papers dealing with the IEF pattern of the three US-licensed A1PI concentrates the authors did not use high-resolution IEF and, therefore, described only an anodal shift of the banding pattern

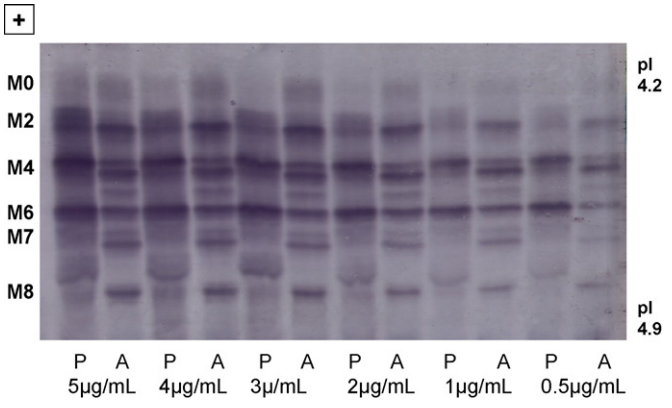

Fig. 4. Sensitive immunoblotting of A1PI concentrates; Prolastin (P) and Aralast (A). Anode is on the top. A1PI concentrate samples were diluted to A1PI concentrations indicated with 0.9% NaCl solution.

for Aralast [29,30]. Thus, the bands M4 and M2 showed higher intensities in Aralast than the corresponding bands in plasma or in the other A1PI concentrates investigated. However, the methods applied in these papers used commercially available IEF gels that were insufficient to detect the presence of two bands in the M4 region or the slightly modified pI of the more intense band compared with the corresponding band found in plasma.

Therefore, a robust and convenient high-resolution IEF method useful for the reproducible analysis of A1PI concentrates and plasma samples was required. The method developed used commercially available IPG gels and, therefore, did not require the handling of toxic acrylamide or the time-consuming preparation of gels necessitating experimental skill. The use of commercially available IPG gels together with carrier ampholytes enhanced the solubility of A1PI and allowed the high, robust separation performance.

The above-mentioned anodal shift of the A1PI banding pattern in the concentrate Aralast could be attributed to the appearance of additional, clearly resolved bands focusing in the M4 and M2 region of plasma A1PI, only when the high-resolution IEF method was used. We recently explained that these additional bands are caused by the loss of the C-terminal amino acid, Lys394 [18]. This loss of the C-terminal amino acid affected all isoforms. Thus, the IEF pattern of Aralast demonstrated additional complexity to that of normal plasma M-type A1PI. In particular, the bands focusing in the M4 region represented the isoform M6 with six sialic acids, but missing the C-terminal lysine and the isoform M4 with seven sialic acids. This complex pattern is similar to that seen in the very rare, but non-pathological A1PI phenotype M1E, in which a lysine is deleted or substituted. Because isoform M4 was also affected by the Lys-truncation, a new band with no equivalent in plasma appeared in the M2 region on IEF of Aralast.

Basically, the IEF analysis of A1PI concentrates is hampered by the mixture of A1PI variants present in the large plasma pools which are used as starting material. Therefore, clear separations similar to those obtained by analysis of single plasma specimens are difficult to obtain for concentrates. However, the method resulted in the separation of the M-types of single plasma specimens and of MZ and SZ controls. According to common practice [6] the difference of 0.01 pH unit should represent 1–2 mm on the gel. Usually, such a resolution is only obtained by gels made in-house. To evaluate the separation performance we used the charge difference introduced by one sialic acid, which represents a difference of 0.06–0.07 pH units [31]. On the gels obtained by the method described here, an average distance of 10–12 mm between the main isoforms M6 and M4 differing in one sialic acid was measured. This complies with the criterion specified to indicate a high-resolution. In addition, the method also resolved the bands Z4 and S6 differing only marginally in their pIs, indicating its high separation power not only in the more acidic M4 range.

Furthermore, it was shown that reduction influenced the IEF pattern of A1PI in plasma and in A1PI concentrates. A1PI contains a single Cys residue at position 232. In the concentrates

Aralast and Prolastin reduction with 5 mM DTE resulted in a small shift of the whole banding pattern towards the anode similar to the shifting observed for plasma A1PI. All isoforms were affected. In the concentrate Zemaira however, these slight alterations in migration were not detected under these conditions. Several reports [32,33] indicated that Cys232 in plasma A1PI is substituted by cysteine. Thus, the reductive removal of the uncharged amino acid Cys resulted in a small but distinct and reproducible anodic shift of the total IEF banding pattern.

Finally, a sensitive method to reveal the IEF pattern of A1PI in dilute solutions, as present for example in bronchoalveolar lavage fluid was described. Only a few reports deal with the analysis of these dilute solutions [34–36], with IEF rarely used. Currently, immunoblotting after IEF is used for identifying questionable faint bands in serum samples using the so-called immunoprinting procedure [10]. The method described here included concentrating and/or desalting the samples by polyethylenglycol precipitation followed by an electroblotting procedure. We tried to mimic solutions obtained after bronchoalveolar lavage in a dilution series done with the two concentrates Aralast and Prolastin, in which A1PI concentrations ranging from 5 to 0.5 μg/ml were prepared. The results obtained confirmed that the procedure was suitable for revealing complex A1PI IEF patterns like that of Aralast in a low A1PI concentration range.

Overall, we describe a high-resolution, robust hybrid IEF method, which was applied to investigate the IEF pattern of A1PI in A1PI concentrates. Commercially available gels and reagents were used and with the described sample pretreatment the method was also shown to be useful for the sensitive detection of A1PI isoforms, when present only at low concentrations.

Disclosure

AW, AE, HPS and PLT are employees of Baxter BioScience. MCO's company, Canterbury Scientific Limited, supplies IEF standards on a commercial basis.

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References

[1] C.-B. Laurell, S. Eriksson, Scand. J. Clin. Invest. 15 (1963) 132–140.
[2] M. Fagerhol, M. Braend, Science 419 (1965) 986–987.
[3] U. Axelsson, C.-B. Laurell, Am. J. Human. Gen. 17 (1965) 466–472.
[4] J.A. Pierce, J.-O. Jeppsson, C.-B. Laurell, Anal. Biochem. 74 (1976) 227–241.
[5] F. Kueppers, J. Lab. Clin. Med. 88 (1976) 151–155.
[6] J.-O. Jeppsson, B. Franzen, Clin. Chem. 28 (1982) 219–225.
[7] S. Weidinger, Electrophoresis 13 (1992) 234–239.
[8] I. Yuasa, K. Suenaga, K. Umetsu, et al., Electrophoresis 9 (1988) 151–153.
[9] M.D. Holmes, M.L. Brantly, D.T. Curiel, et al., Am. J. Hum. Gen. 46 (1990) 810–816.
[10] M.L. Brantly, in: R.G. Crystal (Ed.), Alpha$_1$-Antitrypsin Deficiency. Biology Pathogenesis Clinical Manifestations Therapy, Marcel Dekker Inc., New York/Basel/Hong Kong, 1996, pp. 211–226.
[11] E.J. Campbell, Respir. Med. 94 (2000) S18–S21.
[12] R.W. Carrell, J.O. Jeppsson, C.-B. Laurell, et al., Nature 298 (1982) 329–334.
[13] J. Travis, G.S. Salvesen, Ann. Rev. Biochem. 52 (1983) 655–709.
[14] C.-B. Laurell, in: D.H. Bing (Ed.), The Chemistry and Physiology of the Human Plasma Proteins, Pergamon Press, New York, 1979, pp. 329–340.
[15] M.R. Norman, A.P. Mowat, D.C.S. Hutchison, Ann. Clin. Biochem. 34 (1997) 230–246.
[16] D.A. Lomas, P.R. Elliott, R.W. Carrell, Eur. Respir. J. 10 (1997) 672–675.
[17] J.K. Stoller, F. Rouhani, M. Brantly, et al., Chest 122 (2002) 66–74.
[18] D. Kolarich, P.L. Turecek, A. Weber, et al., Transfusion 46 (2006) 1959–1977.
[19] M. Brantly, in: R.G. Crystal (Ed.), Alpha$_1$-Antitrypsin Deficiency. Biology Pathogenesis Clinical Manifestations Therapy, Marcel Dekker Inc., New York/Basel/Hong Kong, 1996, pp. 45–59.
[20] D. Kolarich, A. Weber, P.L. Turecek, et al., Proteomics 6 (2006) 3369–3380.
[21] J.-O. Jeppsson, H. Lilja, M. Johansson, J. Chromtogr. 327 (1985) 173–177.
[22] A. Alonso, Electrophoresis 10 (1989) 513–519.
[23] V. Neuhoff, N. Arold, D. Taube, et al., Electrophoresis 9 (1988) 255–262.
[24] D.W. Cox, S. Smyth, G. Billingsley, Hum. Genet. 61 (1982) 123–126.
[25] Y. Qi-long, L. Zhi-quan, Z. Mei-lin, et al., Chin. Med. J. 97 (1984) 211–214.
[26] I. Yuasa, K. Umetsu, K. Ago, et al., Legal Med. 3 (2001) 213–219.
[27] K. Mills, P.B. Mills, P.T. Clayton, et al., Clin. Chem. 47 (2001) 2012–2022.
[28] B. Bjellqvist, K. Ek, P.G. Righetti, et al., J. Biochem. Biophys. Methods 6 (1982) 317–339.
[29] D.I. Cowden, G.E. Fisher, R.L. Weeks, Curr. Med. Res. Opin. 21 (2005) 877–883.
[30] E. Marszal, A. Shrake, Transfusion 46 (2006) 872–873.
[31] J.-O. Jeppsson, C.-B. Laurell, M. Fagerhol, Eur. J. Biochem. 83 (1978) 143–153.
[32] C.B. Glaser, L. Karic, T. Huffaker, et al., Int. J. Pept. Protein Res. 20 (1982) 56–62.
[33] S.C. Tyagi, S.R. Simon, Biochemistry 31 (1992) 10584–10590.
[34] A. Capelli, C.G. Cerutti, M. Lusuardi, et al., Clin. Chem. 39 (1993) 2201–2202.
[35] G.N. Olsen, J.O. Harris, J.R. Castle, et al., J. Clin. Invest. 55 (1975) 427–430.
[36] J. Wu, M. Kobayashi, E.A. Sousa, et al., Mol. Cell. Proteomics 9 (2005) 1251–1264.