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Characterisation of human serum albumin heterogeneity by capillary zone electrophoresis and electrospray ionization mass spectrometry

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Abstract

Human serum albumin (HSA) preparations and HSA-containing recombinant human erythropoietin (rhEPO) formulations were analyzed by capillary zone electrophoresis. HSA was separated into several components by the addition of 1,4-diaminobutane to 20 m*M* sodium phosphate, pH 6.0 as electrophoretic buffer. Resolution was improved by increasing the buffer pH to 8.5. A comparative analysis of HSA preparations from three manufacturers provided evidence that this method can be used to differentiate individual preparations. The analysis of rhEPO formulations, composed largely of HSA, showed levels of heterogeneity comparable to that of HSA preparations. Electrospray ionisation mass spectrometry was used as an independent method to confirm the heterogeneous nature of HSA.

Keywords: Pharmaceutical analysis; Proteins; Albumin

1. Introduction

Capillary zone electrophoresis (CZE) has rapidly become a useful analytical tool for the study of proteins. In addition to providing exceptional separation efficiencies, it is a technique capable of rapid, automated and reproducible separations [1]. It is particularly well suited for the study of protein heterogeneity arising from post-translational modifications, degradation or genetic variation. However, protein separations on bare fused-silica capillaries have been problematic mainly because of protein interactions with the capillary inner walls. The use of buffer additives, such as diamines, has been shown to reduce protein–inner wall interactions and to increase the resolution of complex mixtures of closely related molecules [2,3].

Human serum albumin (HSA) is perhaps the most studied protein owing to its abundance, relative stability and commercial importance. It is a single chain protein of 585 amino acids containing a total of 17 disulphide bridges and a free Cys-34 residue [4]. Currently, HSA is prepared by the fractionation of donated plasma and is used in medical procedures such as the treatment of burns or as a blood expander. It is also widely used as an excipient in pharmaceutical preparations to stabilize drugs during processing and/or storage. Although HSA has been traditionally described electrophoretically as a single band with approximate molecular mass of 66 000, physical and chemical studies employing high resolution techniques have provided evidence that it is composed of multiple variants such as mercapto and non-mercapto [5], glycated [6] and polymerized [7,8] forms. As the gene coding for HSA is polymorphic [9], the pool of isolated HSA can be heterogeneous [10]. Conditions of albumin storage and manufacture can further increase the heterogeneity of the protein

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by introducing deamidation, oxidation or polymerization [8]. In a recent study a combination of three complementary techniques, size-exclusion HPLC, isoelectric focusing and immunoelectrophoresis, has been suggested for the quality control testing of HSA preparations [11].

Rapid progress in recombinant DNA technology has led to an increasing number of protein drugs reaching the pharmaceutical market. Typically, very small amounts of the active ingredient are required to attain the therapeutic dose, a situation that has led to the development of formulations containing large quantities of excipients to prevent loss through adsorption and degradation. Among others, protein excipients, such as HSA, are being used successfully. In the current regulatory context there is a need to develop high resolution physico–chemical methods for the determination of protein purity and the characterization of protein heterogeneity. In this study, we report on the utility of capillary zone electrophoresis (CZE) and electrospray ionisation mass spectrometry (ESI-MS) for the characterisation of HSA heterogeneity. Optimized CZE separation conditions were developed and used to analyze commercial HSA preparations and HSA-containing rhEPO formulations.

2. Experimental

2.1. Materials

Pentex HSA, fraction V, (lots 22 and 42) was obtained from Miles (Kankakee, IL, USA). Albuminar-25 HSA preparations (lots M65205, M66206 and M67007) were obtained from Armour Pharmaceuticals (Kankakee, IL, USA). Crystallized and lyophilized HSA (lot No. 126C-8070) was obtained from Sigma (St. Louis, MO, USA). Buffer salts and additives were HPLC or molecular biology grade reagents. Proteins used for the calibration of the mass spectrometer were hen egg lysozyme and horse heart myoglobin from Sigma. Recombinant human erythropoietin (rhEPO) formulations were obtained from the Bureau of Biologics (Health Canada). Fused-silica capillaries were from Beckman Instruments (Fullerton, CA, USA) [57cm (50 cm to the detector)×50 μm I.D.] and Polymicro Technologies (Phoenix, AZ, USA) [67 cm (60 cm to the detector)×52 μm I.D.).

2.2. Methods

2.2.1. Capillary zone electrophoresis

Capillary zone electrophoresis (CZE) was performed on a P/ACE 5500 instrument (Beckman Instruments, Fullerton, CA, USA). Samples were injected using nitrogen pressure and peaks were detected on-column with a UV detector operating at 200 nm. Data were analyzed using the P/ACE Gold data analysis software ver 8.1. Conditions used in each experiment are indicated in the figure captions. A sequence of rinses starting with 1 *M* HCl (3 min), water (3 min), 1% NaOH (3 min), water (3 min) and buffer (5 min) was carried out after each run.

2.2.2. HSA purification procedure

HSA was dissolved in 8 *M* ultrapure urea previously acidified to pH 3.0. After stirring at room temperature for 2 h, the solution was dialysed against water in a stepwise fashion over a 24 h period at 4°C. The dialyzed sample was lyophilized and dissolved in 0.5% aqueous formic acid–acetonitrile (1:1) at a concentration of about 1 mg/ml.

2.2.3. Electrospray ionization mass spectrometry

Samples were analyzed on a VG Quattro (VG Biotech, Altrincham, UK) equipped with an electrospray interface, and a Hewlett-Packard 1090 HPLC system (Hewlett-Packard, Palo Alto. CA, USA). The instrument settings were as follows: source temperature 80°C, scan range M_r 268 to 1468, scan duration 9.59 s, capillary voltage 3.32 kV, HV lens 0.48 kV, cone 38 V, skimmer offset 5 V, skimmer 1.3 V, RF lens 0.2 V. The data were processed using the VG MassLynx ver. 2 software.

3. Results and discussion

3.1. Examination of HSA preparations by sodium sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and isoelectric focusing (IEF)

SDS–PAGE analysis of the commercial HSA preparations showed single bands of molecular mass

approximately $66 \cdot 10^3$, indicating that the products used in this study were relatively homogeneous (data not shown). Examination by IEF on a PhastSystem in the isoelectric point (p*I*) range 4–6.5 provided evidence of charge heterogeneity (Fig. 1). A cluster of bands appeared in the expected zone for HSA, that is, between p*I* 4.2 and 5.2. Similar results have been reported previously [11]. Band pattern differentiation was insufficient to distinguish between the individual HSA samples.

3.2. CZE separation of HSA on fused-silica capillaries

CZE separation of HSA was initially carried out using a 57 cm (50 cm to the detector)×50 μm fused-silica capillary and 20 m*M* sodium phosphate, pH 6.0 buffer at 25 kV and 25°C. Under these conditions, Pentex HSA migrated as a single peak (Fig. 2A). The addition of 1 m*M* 1,4-diaminobutane (putrescine) in the running buffer (Fig. 2B) produced the expected effect of significantly decreasing the electroosmotic flow (EOF) [2] and resulted in the appearance of shoulders on either side of the HSA peak. Increasing the putrescine concentration to 2.5 m*M* (Fig. 2C) further decreased the EOF and allowed separation of the shoulders into partially resolved peaks. The optimal resolution was attained at a putrescine concentration of 5 m*M* (Fig. 2D) where HSA was separated into one major and several minor components. Increasing the pH of the electrophoretic buffer to 8.5 had a marked effect on the separation of the HSA components. Under these conditions and in the presence of 5 m*M* putrescine, HSA was reproducibly separated into three major and several partially resolved minor components (Fig. 2E). HSA preparations from other manufactur-

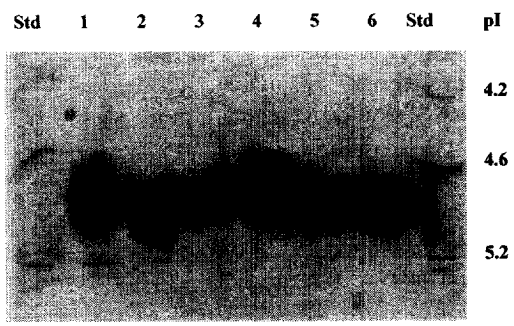

Fig. 1. Isoelectric focusing patterns obtained on pre-coated PhastGels, p*I* 4–6.5. Lanes 1 and 4, Sigma HSA; lanes 2 and 5, Armour HSA; lanes 3 and 6, Pentex HSA.

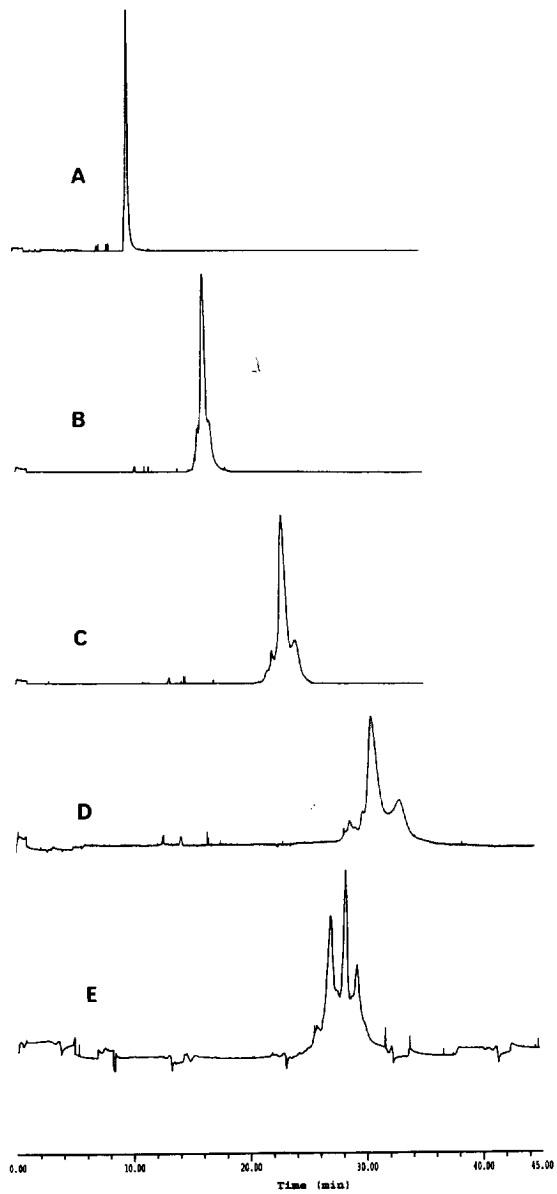

Fig. 2. CZE separation of Pentex HSA (2 mg/ml) obtained on a 57 cm×50 μm I.D. fused-silica capillary at 25 kV, 25°C, 10 s pressure injection with detection at 200 nm. The electrophoretic buffer consisted of 20 m*M* sodium phosphate at (A) pH 6.0, (B) pH 6.0, 1 m*M* putrescine, (C) pH 6.0, 2.5 m*M* putrescine, (D) pH 6.0, 5 m*M* putrescine and (E) pH 8.5, 5 m*M* putrescine.

ers were analyzed under identical separation conditions and also showed appreciable heterogeneity (Fig. 3). The peak pattern for the HSA obtained from Armour (Fig. 3A) was qualitatively similar to that obtained from Pentex (Fig. 3C), which showed the three major components, although peak heights were proportionally different. On the other hand, the product from Sigma (Fig. 3B) presented a pattern unlike that of the other two products. It migrated as a broad cluster with three summits.

In order to ascertain that the peaks observed were protein components and not simply contaminants present in the preparations or liberated during the separation, electropherograms of the Armour product were monitored simultaneously at 200 and 280 nm using a diode-array detector (Fig. 4). While the electropherogram at 280 nm (Fig. 4A) showed the expected lower absorbance than at 200 nm (Fig. 4B), the peak patterns were essentially identical strongly suggesting that the peaks observed were protein-related. The poorer resolution obtained in these electropherograms was a result of having to overload the capillary using a 10 mg/ml HSA solution in order to get acceptable detection at 280 nm.

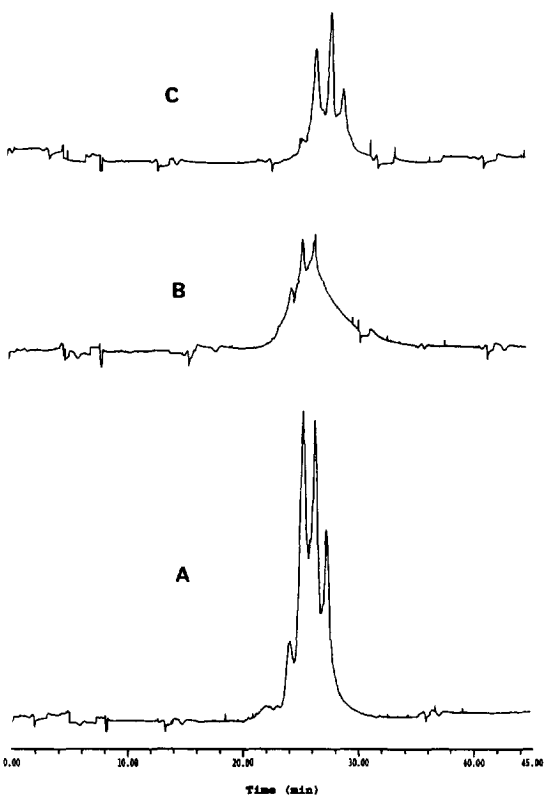

Fig. 3. CZE separation of (A) Armour HSA (3 mg/ml) and (B) Sigma HSA (2 mg/ml) and (C) Pentex HSA (2 mg/ml). Separation conditions as in Fig. 2E.

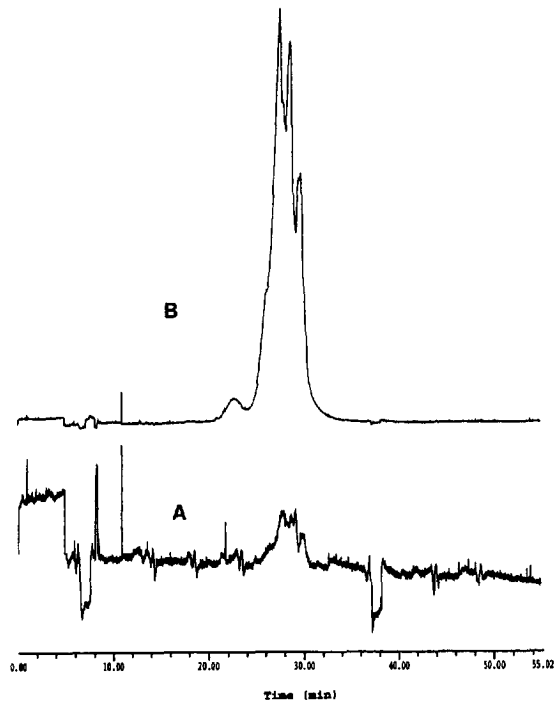

Fig. 4. CZE separation of Armour HSA (10 mg/ml) with detection at (A) 280 and (B) 200 nm. Separation conditions as in Fig. 2E.

The reproducibility of the separation was verified by performing separations on a fused-silica capillary from a different manufacturer (Polymicro Technologies). Virtually identical profiles were obtained on the two capillaries (data not shown). Table 1 presents the within-day precision of peak migration times and area responses for replicate injections of Pentex HSA using the conditions at pH 8.5 and 5 m*M* putrescine. The precision of peak migration times was consistently better than that of peak area responses, a situation that likely reflects the tendency of HSA to adsorb to the silica surface. A comparative analysis of three lots of the Armour HSA

Table 1
Precision data for migration times and peak area response for Pentex HSA components

	Migration time (min)			Peak area		
	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3
Mean (n=5)	29.42	30.44	31.80	11.7529	10.6651	3.0542
S.D.	0.24	0.23	0.25	0.3446	0.3810	0.5449
R.S.D.(%)	0.83	0.76	0.78	2.93	3.57	17.84

CZE conditions as in Fig. 2E.

preparation showed essentially identical peak profiles with slight variations in the relative proportions of individual components (data not shown).

3.3. CZE separation of HSA in rhEPO formulations

rhEPO formulations are typically prepared in doses ranging from 3000 to 10 000 IU per vial and contain large quantities of HSA (usually 1 mg HSA per vial). In terms of mass content, these doses correspond to a range of approximately 3–10% (w/w) rhEPO relative to HSA, that is, 30–100 μg rhEPO/1 mg of HSA as determined from standard curves obtained under CZE conditions reported elsewhere [12]. rhEPO formulations were analyzed by CZE using the 5 m*M* putrescine (pH 8.5) conditions to determine the potential of this method for the characterization of the HSA present within a pharmaceutical preparation. Fig. 5 shows typical electropherograms of rhEPO formulations from two manufacturers (Fig. 5A and B). As was the case for the HSA preparations, the observed profiles were highly reproducible. Although the two products were essentially HSA solutions, they exhibited significantly different electrophoretic profiles. Whereas the product shown in Fig. 5B showed a close similarity to that of Pentex HSA (Fig. 5C), the product in Fig. 5A showed considerably more heterogeneity, with a peak profile unlike that of the other HSA preparations examined above.

3.4. ESI-MS analysis of HSA

The heterogeneity of HSA was confirmed by ESI-MS analysis. Although mass spectrometry has traditionally been used for the study of small molecules, it is gaining widespread acceptance in the field of protein chemistry [13]. The instrument was calibrated with hen egg lysozyme which gave a molecular mass of 14 303.4±0.46 (calculated value 14 305) and horse heart myoglobin which gave a molecular mass of 16 951.5±1.4 (calculated value 16 951.5). Initial experiments using untreated Pentex HSA were unsuccessful owing to the presence of salts associated with the protein. This gave rise to an unresolved multiplicity of peaks in the ESI-MS spectrum. A purification procedure involving denaturation of the protein in 8 *M* urea under acidic conditions and renaturation [10] through stepwise removal of the denaturant by dialysis was carried out to remove the cations associated with HSA (see Materials and Methods). Analysis by CZE using the 5 m*M* putrescine (pH 8.5) conditions of the Pentex HSA following purification (Fig. 6A) showed an electrophoretic profile similar to that of untreated Pentex HSA (Fig. 6B), indicating that the purification procedure did not significantly alter the eletrophoretic mobility of HSA.

The continuum data and the maximum entropy (ME) transform from the ESI-MS analysis of purified HSA are shown in Fig. 7A and B respectively. Two major and at least ten minor components, ranging in mass from 66 435 to 66 756, were observed in the spectrum. The predominant peak has a molecular mass of 66 438±5 which closely matches the value of 66 437 obtained for recombinant HSA [14]. The major component also has a peak width very close to the theoretical value of 16.5, whereas the peak at 66 550 has a peak width at half height of 26, indicating that this peak is an unresolved mixture. The additional compounds, each with an M_r uncertainty of approximately ±20, may be accounted for in the following manner; cysteinylation, or the

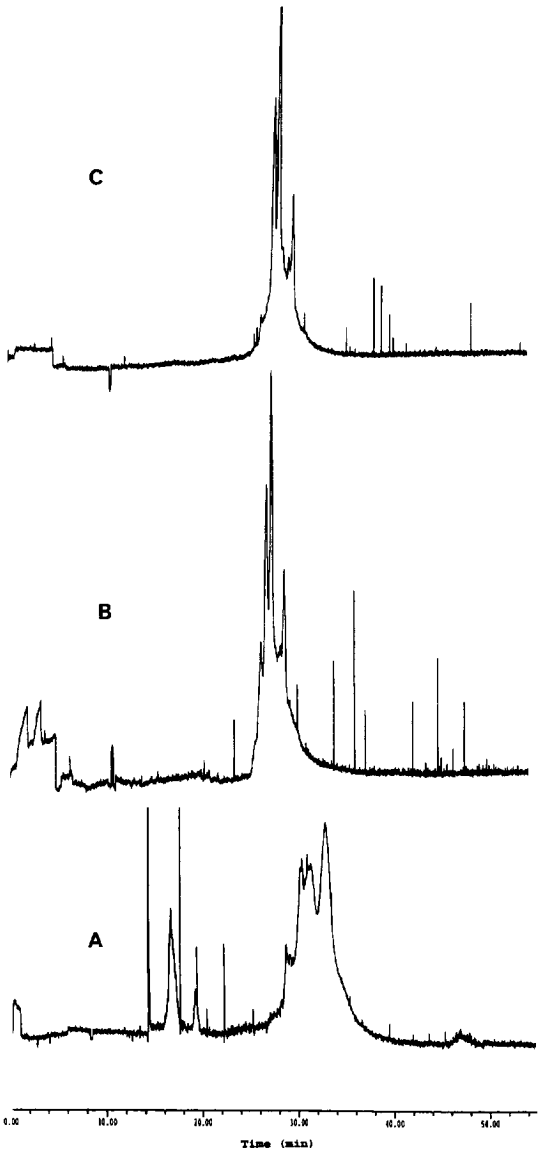

Fig. 5. CZE separation of (A) rhEPO formulation from manufacturer I, (B) rhEPO formulation from manufacturer II and (C) Pentex HSA (2 mg/ml). Separation conditions as in Fig. 2E.

addition of sugars such as hexosamine, N-acetylhexosamine, or sialic acid. The nature of the experiment, i.e., acid denaturation/renaturation followed by ionization and vaporization in the presence of trifluoroacetic acid (TFA), made it unlikely that any non-covalently bound ligand would remain bound to the HSA. Therefore the populations of HSA observed in

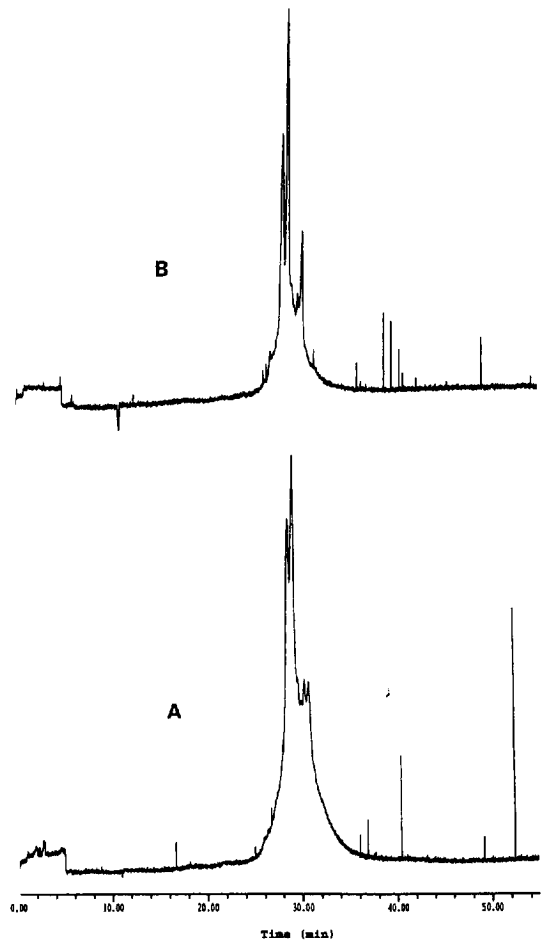

Fig. 6. CZE separation of Pentex HSA (A) purified by denaturation/renaturation and (B) untreated. Separation conditions as in Fig. 2E.

the ESI-MS spectrum are unlikely to be a result of non-covalent protein–ligand complexes.

4. Conclusion

The results of this study have demonstrated the usefulness of CZE on fused-silica capillaries for the analysis of HSA preparations. The addition of putrescine to the phosphate buffer was found to greatly improve the resolution of several components present in the purified commercial HSA preparations. Electrophoretic profiles were highly reproducible and could be used to differentiate products. HSA hetero-

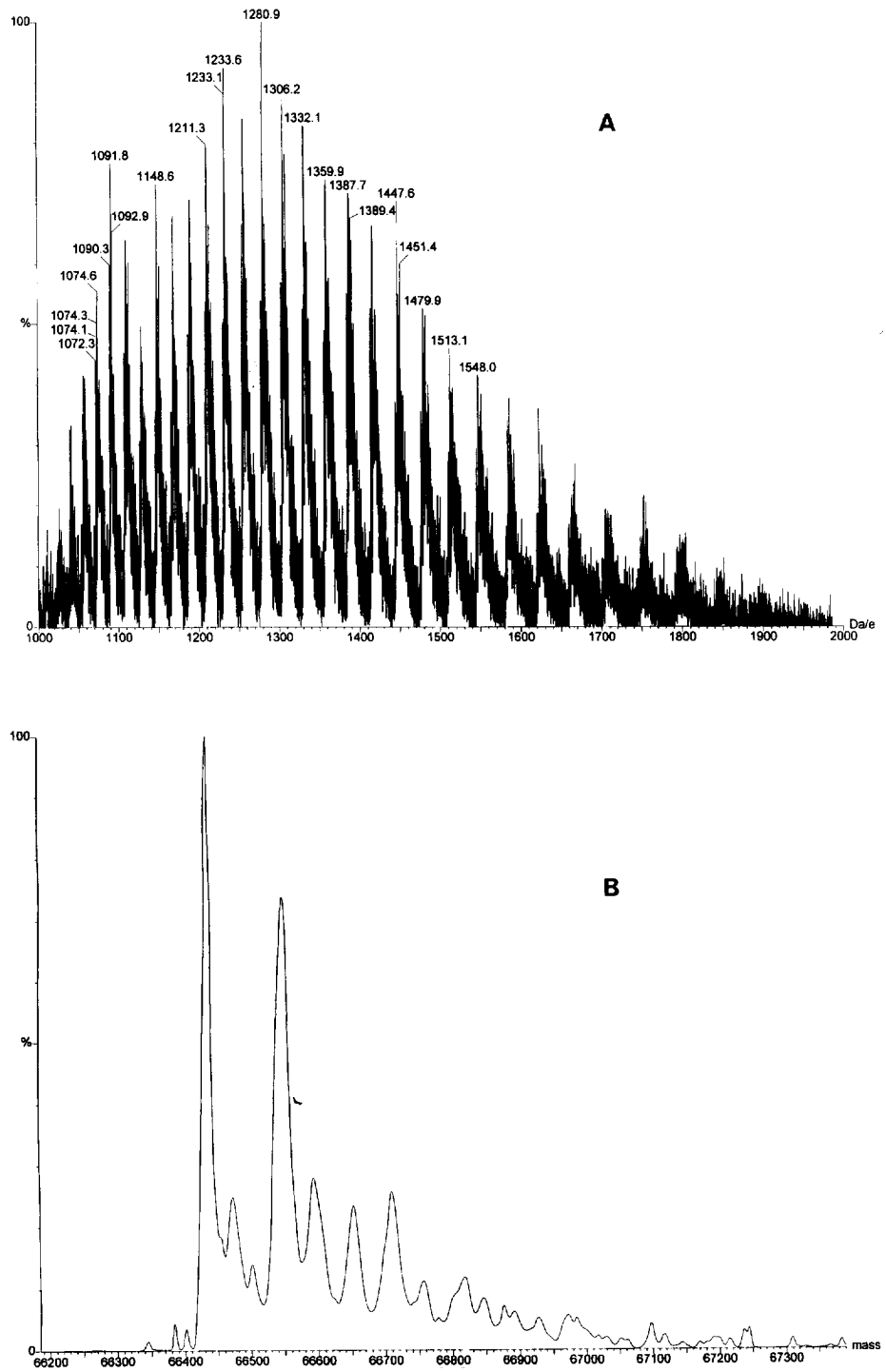

Fig. 7. ESI mass spectrum of purified Pentex HSA [3×10^{-5} *M*, in acetonitrile–water (1:1) with 0.2% formic acid]. (A) Continuum data and (B) maximum entropy transform using the MaxEnt program.

geneity was also shown to occur in HSA-containing rhEPO formulations. The study also demonstrated that ESI-MS can be successfully used to characterize HSA heterogeneity.

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