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High-performance capillary electrophoretic separation of human serum albumin using a neutral coated capillary

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Abstract

A rapid, simple and highly resolving capillary electrophoresis technique is described for the separation of sub-species of clinical grade human serum albumin (HSA) using a neutral coated capillary. As the buffer pH approached the p*I* of HSA (5.2), eight peaks representing HSA sub-populations were resolved. On a non-coated capillary, no such resolution was obtained and buffers several pH units away from HSA's p*I* were required to prevent protein adsorption to the capillary wall. Some sub-species could be identified by mass spectrometric analysis as representing intact and amino-terminally damaged HSA with and without a blocked thiol at cysteine 34.

1. Introduction

Capillary electrophoresis (CE) is recognised as a powerful separation technique for the analysis of a diverse range of molecules [1–4]. High resolution, short analysis times and ease of use make it an ideal choice for monitoring therapeutic proteins and biopolymers. Separation efficiencies for CE are typically high (>100 000 theoretical plates). In both quality control (QC) and research environments, CE now stands alongside the more established high-resolution separation techniques such as HPLC and polyacrylamide gel electrophoresis (PAGE) [5,6].

An advantage of CE is the ability to separate molecules in free solution. Large proteins can be separated, but may suffer from a lack of resolution between native and modified forms (e.g., post translational modifications, single residue mutations). This lack of resolving power is often due to protein interaction with the walls of untreated fused-silica capillaries which act as cation exchangers towards negatively charged samples. This leads to band broadening, loss of sensitivity and/or loss of sample [7,8]. Normally, this can only be counteracted effectively by using either extremes of pH away from the protein's p*I*, high salt concentrations to compete with available binding sites or by addition of solvent modifiers such as detergents or ion-pairing reagents to reduce wall effects. However, these actions tend to reduce the separation capabilities of CE and the ability to differentiate between closely related sub-populations of the protein.

Approaches have been made to overcome these problems. A wide range of coatings have been linked covalently to the fused silica in order to reduce adsorption of biomolecules such as

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proteins to the capillary wall [7–9]. These coatings can confer further advantages such as an increase in the stability of the capillary, often at extremes of pH, and a potential increase in the capillary lifetime. Neutral coated capillaries can offer many of these benefits allowing sharp peaks to be resolved whilst using buffers near the p*I* of the protein [10]. Thus, short analysis times can be maintained and adsorption to the capillary wall is reduced significantly.

HSA exists in more than one form, as shown by the broad p*I* range quoted for this protein [11] and the band profile observed on an isoelectrofocusing (IEF) gel [12,13]. The ability to separate these different species in free solution by CE offers many advantages over alternative methods such as PAGE and IEF. CE is generally less time-consuming, less labour-intensive and allows rapid, quantitative separation of biomolecules such as proteins. The high-resolution separation of HSA is described using a new neutral coated capillary. The silica is bonded with polyacrylamide to give a neutral coating which reduces electroosmotic flow (EOF) and analyte/sample interactions with the capillary wall. Buffer conditions ensure that samples are negatively charged (just above the p*I* of the protein). As the EOF is negligible, electrophoretic migration is achieved through charge attraction of the negative protein to the anode. The separation of HSA sub-species using this neutral coated capillary is compared with that of a non-coated fused-silica capillary and the identification of the sub-species is discussed.

2. Experimental

2.1. Materials

Clinical grade HSA was obtained from a commercial supplier. Glycated HSA was purchased from Sigma (Poole, Dorset, UK). The neutral coated capillary and citrate 2-N-morpholino-ethanesulfonic acid (MES) buffers (kit part No. 477445) and non-coated capillaries (part No. 338451) were obtained from Beckman (High Wycombe, Buckinghamshire, UK). All other chemicals used were purchased from Sigma (analytical grade). Milli-Q grade water (Millipore, Watford, Hertfordshire) was used throughout.

2.2. Instrumentation

Capillary electrophoresis was performed on a P/ACE 2210 unit controlled by System Gold software (Beckman). Fixed-wavelength detection was set at 214 nm for the neutral capillary separations, as stipulated in the manufacturer's instructions, and 200 nm for non-coated fused-silica capillary separations for maximum sensitivity. In both cases, the data collection rate was 5 Hz and the capillary internal diameter (I.D.) was 50 μm; the length of the neutral coated capillary was 37 cm, that of the non-coated capillary was 43 cm. All separations were performed at 25°C.

2.3. Preparation of samples

HSA, glycated HSA and other albumin samples were diluted to 1 mg/ml in water prior to separation. Dilution into one tenth concentration of appropriate buffer did not affect the separation achieved and the use of water greatly facilitated method development. Reduction of HSA was performed by adding 2m*M* dithiotrietol (DTT) to the sample vial and incubating in the autosampler for 1 h prior to injection. Amino-terminal degradation of HSA was increased by incubating the protein at 37°C for 8 weeks [14]. The reference marker Orange-G was included in samples separated on the neutral coated capillary.

2.4. Separation conditions

The neutral coated capillary was used with separation buffers of 20 m*M* citrate MES at pH 5.2, pH 5.5 and pH 6.0. To achieve separation of HSA at these pH values the instrument polarity was reversed (with the anode at the capillary outlet). Prior to separation the capillary was rinsed for 2.5 min with the appropriate buffer. Sample was introduced using a 3-s pressure

injection. Electrophoresis was performed at a constant voltage of 18.5 kV (500 V cm^{-1}). The non-coated capillary was used with separation buffers of 50 m*M* phosphate at pH 1.6, pH 5.2, pH 6.5 and pH 8.0 and the instrument polarity was normal (with the cathode at the capillary outlet). Prior to separation the capillary was rinsed firstly with 0.1 *M* NaOH for 1 min then with the appropriate buffer for 2 min. A 3-s pressure injection of the sample was followed by constant voltage separation at 20 kV (465 V cm^{-1}).

2.5. Electrospray mass spectrometry (ESMS)

HSA was prepared for ESMS by reverse-phase HPLC and subjected to mass analysis on a VG Quattro mass spectrometer which had been calibrated over the *m/z* range 950–1750 Da/e using horse heart myoglobin.

3. Results

3.1. A comparison of neutral coated with non-coated silica capillaries for the separation of HSA

The separation of HSA sub-populations using a neutral coated capillary is demonstrated (Fig. 1) and shown to improve as the pH approached the p*I* of HSA. Six poorly separated peaks identified using a buffer at pH 6 (Fig. 1c) were resolved into eight well defined peaks at pH 5.2 (Fig. 1a). The migration time increased as the pH neared the p*I* of HSA but there was no evidence of loss of protein at any of the pH values tested. Using buffers further away from the p*I* of HSA (pH 2 or pH 7) to achieve separation offered no benefits using the neutral coated capillary. A comparison of the separation of HSA using fused-silica capillaries can be seen in Fig. 2. Here, in contrast to the neutral coated capillary separation, buffers with pH extremes were required to prevent adsorption of the protein to the capillary wall. The best separation of HSA was found using buffers at pH <3 or >7. Only single major peaks were obtained at

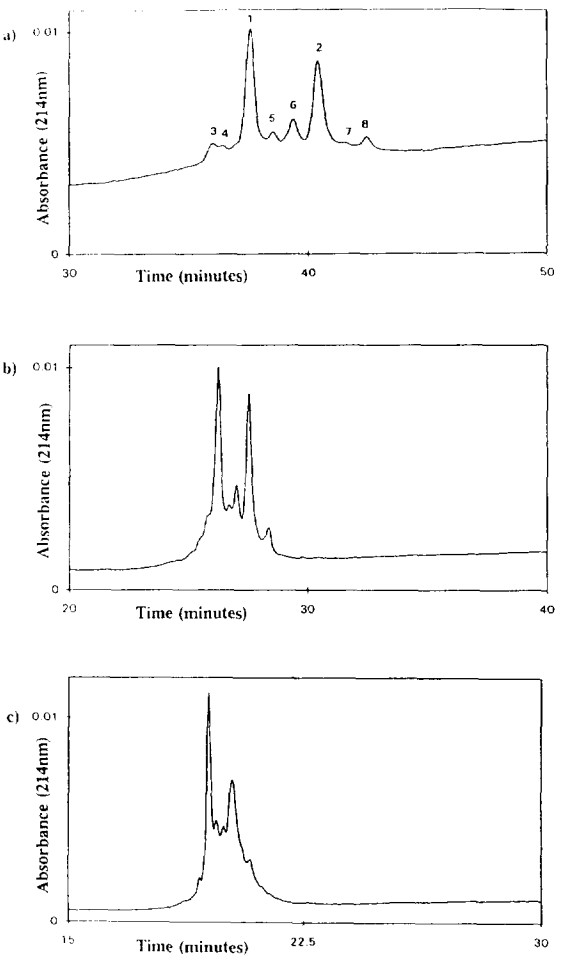

Fig. 1. Separation of HSA on a neutral coated capillary. HSA was separated at pH 5.2 (a), pH 5.5 (b) and pH 6.0 (c). Eight peaks (1–8) were resolved at pH 5.2.

these pH values with no significant resolution of the sub-populations. As the pH neared the p*I* of HSA, rather than observing an increase in resolution, the response decreased, the peaks broadened and migration times varied due to excessive interaction between the protein and the capillary wall (Fig. 2b). The inclusion of additives such as ion-pairing reagents (1,3-diaminopropane) or detergents (sodium dodecyl sulfate) failed to improve resolution (results not shown). These additives decreased the protein–wall interactions but did not allow high peak resolution.

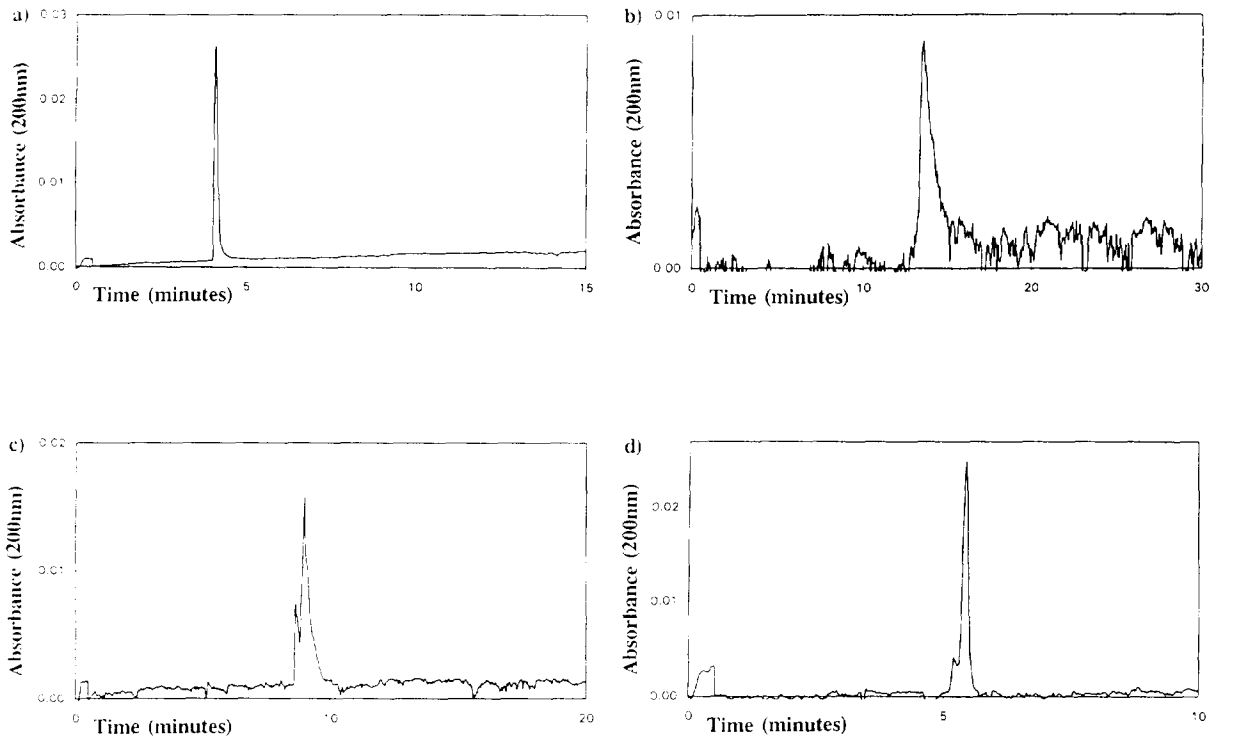

Fig. 2. Separation of HSA on a non-coated capillary. HSA was separated at pH 1.6 (a), pH 5.2 (b), pH 6.5 (c) and pH 8.0 (d). At the two pH extremes one peak was resolved whilst closer to HSA's p*I* poor/variable resolution was achieved.

3.2. Identification of HSA sub-species separated on the neutral coated capillary

HSA is regarded as a heterogeneous protein [15] which can be confirmed by ESMS (Fig. 3). The main species were identified from their relative masses to be HSA monomer with a free thiol at cysteine 34 (Fig. 3, peak A), HSA monomer with the thiol at cysteine 34 blocked with cysteine (Fig. 3, peak B) and amino-terminally degraded HSA (Fig. 3, peak C) [14]. The first major peak on the CE electropherogram (Fig. 1a, peak 1) represents HSA with a free thiol group and the second major peak (Fig. 1a, peak 2) represents HSA with a blocked thiol group. This was verified after reduction of the sample with DTT which resulted in the disappearance of peak 2 with a concomitant increase in peak 1 (Fig. 4).

The assignment of the other peaks was more difficult. The mass difference between the degraded HSA (Fig. 3, peak C) observed in the mass spectrum and HSA represents the loss of the amino-terminal residues aspartic acid and alanine. After reduction, the electropherogram shows a drop in the height of peak 8 with a corresponding increase in the height of peak 6 (Fig. 4). These two peaks could represent amino-terminally damaged HSA with free (peak 6) and blocked (peak 8) thiol groups. Furthermore, it has been shown that there is an increase in amino-terminal degradation of HSA when incubated at elevated temperatures (e.g. >30°C) [14] which can be monitored by mass spectrometric analysis (data not shown). The separation of HSA after incubation at 37°C shows increased levels of peaks 6 and 8, with corresponding drops in the heights of the main peaks (1 and 2) when compared to non-incubated HSA (Fig. 5), providing further evidence of the nature of these species. Peak 3, which was not affected by reduction with DTT, also increased after incubation at elevated temperatures (Fig. 5). This could represent a further degradation prod-

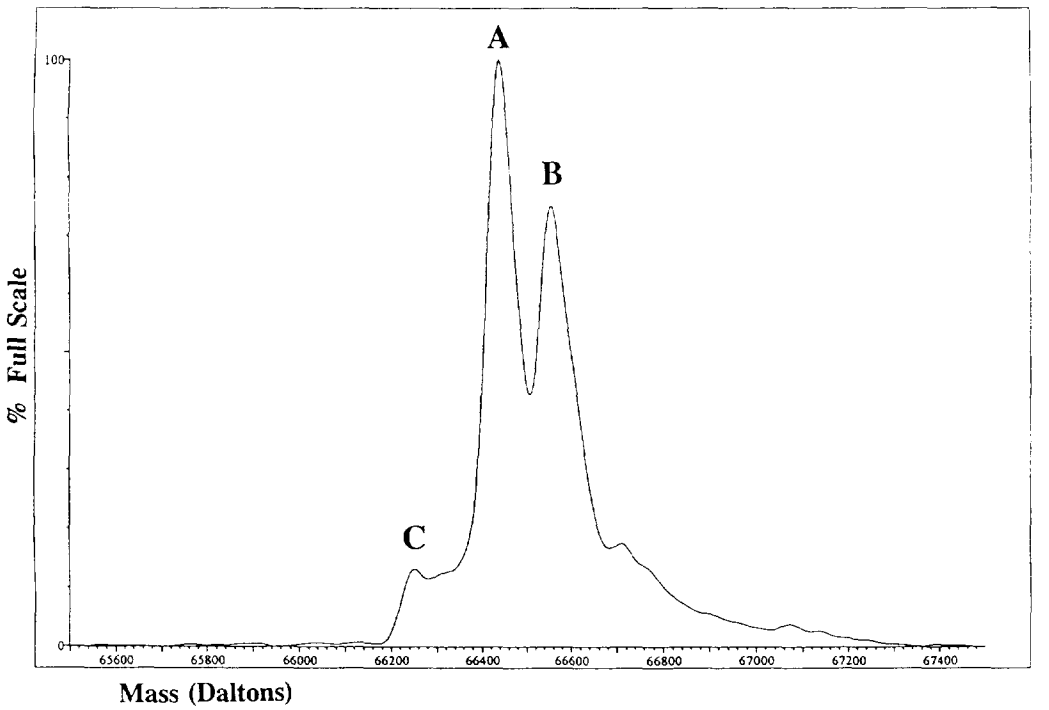

Fig. 3. Electrospray mass spectrometry of HSA. Three masses for HSA were observed; monomer, free thiol group (A, M_r 66 437); monomer, blocked thiol group (B, M_r 66 555); and amino-terminally degraded HSA (C, M_r 66250).

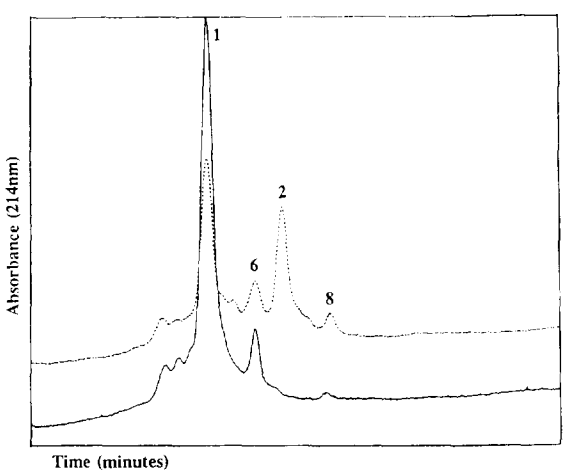

Fig. 4. Separation of reduced and non-reduced HSA on a neutral coated capillary. The reduced sample (——) showed an increase in peaks 1 and 6 (intact and amino-terminally degraded HSA with a free thiol group respectively) with corresponding decreases in peaks 2 and 8 (intact and amino-terminally degraded HSA with a blocked thiol group respectively) when compared to the non-reduced state (– – –).

uct of HSA, deamidation or dimerisation/polymerisation of the protein. Further analysis is required to identify this species.

HSA is glycated [16,17] and it would seem probable that one of the resolved peaks could be due to a glycated form. Analysis of glycated HSA on the neutral coated capillary demonstrated that the neutral species bound to the protein did affect the resolution but not the profile of the separation, as the same peaks were obtained as for HSA, but significantly broader (data not shown). As previously described, separation at pH values above the p*I* of the protein is dependent on negative charges. Sugars such as glucose and mannose bind to lysine residues (lysine 525 of HSA is a major site for glycation) which would not affect the neutral overall charge of the protein under these conditions and thus not the separation. Therefore, it would be expected that separation at pH values value below the p*I* of HSA may differentiate between glycated and non-glycated species. However, the

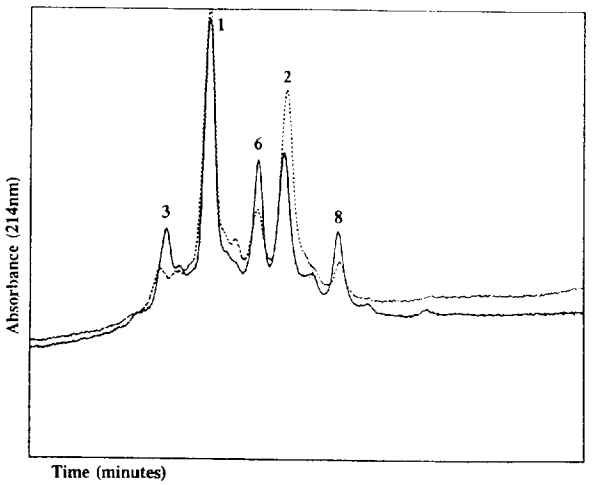

Fig. 5. Separation of HSA and HSA after incubation at 37°C on a neutral coated capillary. The 37°C incubated sample (—) demonstrated accelerated amino-terminal degradation by increases in peaks 6 and 8 (amino-terminally degraded HSA with free and blocked thiol groups respectively) with corresponding decreases in peaks 1 and 2 (intact HSA with free and blocked thiol groups respectively) compared to the non-incubated sample (– – –). The unidentified peak 3 also increased on incubation.

separation was found to be similar to that above the p*I* of HSA (the profile for glycated HSA constituted the same peaks as for HSA but broader and less well resolved). Therefore, we have been unable to demonstrate that a glycated form of albumin is separated by the neutral coated capillary. Further analyses are required to verify the identity of the remaining species resolved by this technique.

4. Discussion

High-resolution separation of clinical grade HSA and many other proteins is difficult using convential non-coated capillaries, especially with buffers near to the p*I* of the protein. This is demonstrated in this study where adequate HSA separation and limited resolution was only achieved at extremes of pH. However, the utilisation of a neutral coated capillary reversed this situation. Separation of HSA can be performed near to its p*I* and, because wall effects are greatly reduced, with high resolution. The resolving power of this technique could possibly be achieved or improved upon using IEF–PAGE, a method involving the casting of gels and staining of the proteins for identification and quantitation. However, this method requires the use of highly pure and expensive reagents to guarantee accuracy and reproducibility. To date, we have been unable to achieve a comparable separation using CE–IEF. The use of a neutral coated capillary, therefore, offers several advantages for the researcher or QC analyst for monitoring protein quality, even with large proteins such as HSA. The method is simple, requires no buffer additives, and allows for further method development depending on the individual protein being analysed. The method also has the capability to be micro-preparative if instrumentation allowing fraction collection is used. Resolved peaks can then be collected and subjected to further analysis. Potentially, the combination of this technique with electrospray mass spectrometry is an ideal method for further characterisation of sub-populations of proteins (as is the case with clinical HSA), the monitoring of minor specific protein modifications and for use in protein stability studies.

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