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Capillary zone electrophoresis–ionspray mass spectrometry of a synthetic drug–protein conjugate mixture

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ABSTRACT

Low-molecular-mass proteins, such as lysozyme, may be suitable carriers to target drugs to the kidney. Naproxen, an anti-inflammatory drug, has been conjugated with lysozyme via a covalent amide bond formed between the carboxylic acid function of naproxen and the amino group of one of the lysines in lysozyme. The reaction products were analysed by capillary electrophoresis–ionspray mass spectrometry. Native lysozyme and its conjugates with one, two and three naproxen molecules were separated and their identities were confirmed by mass spectrometry. The ion current profiles of the individual conjugates showed pH-dependent tailing and adsorption–desorption phenomena in the capillary electrophoresis column not observed in the total ion current profiles and not observable by UV detection.

INTRODUCTION

Research on new drugs requires well characterized materials. The combination of an efficient separation method with a mass spectrometer can be used for quality control of pure drugs and for the structure elucidation of individual components in a reaction mixture obtained in drug synthesis.

One-line gas chromatography–mass spectrometry (GC–MS) is used routinely in biomedical research. Liquid chromatography–mass spectrometry (LC–MS) has matured over the past 10 years and is now indispensable in the investigation of drug synthesis and drug metabolism. The obvious benefits of GC–MS and LC–MS have spurred the development of capillary electrophoresis–mass spectrometry (CE–MS). CE–electrospray MS and CE–ionspray MS have been shown to be powerful techniques in the separation and analysis of large proteins [1,2]. The recently introduced LC–MS interfaces, continuous-flow fast atom bombardment (CF-FAB) [3], electrospray [4,5] and ionspray [6] (pneumatically assisted electrospray), are suitable techniques for the combination of CE with MS [7,8,9]. However, FAB is not a suitable ionization technique for large biomolecules, whereas molecules of $M_r > 100\,000$ can be analysed using the electrospray technique [10].

Low-molecular-mass proteins, such as lysozyme, may be suitable carriers to target drugs to the kidney. Naproxen, an anti-inflammatory drug, has been conjugated with lysozyme via a covalent amide bond formed between the carboxylic acid function of naproxen and the amino group of one of the lysines in lysozyme, and the pharmacokinetics of the conjugate have been

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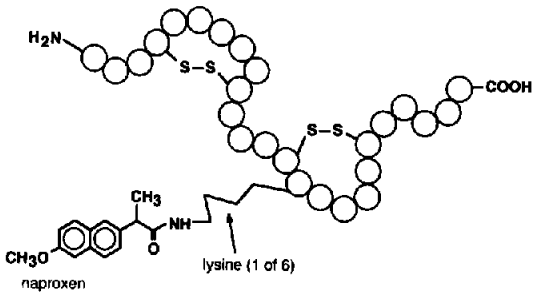

studied [11,12]. The synthetic 1:1 conjugate was purified by preparative ion-exchange fast protein liquid chromatography (FPLC). Under the conditions chosen for preparative FPLC, native lysozyme was separated from the 1:1 conjugate. Higher conjugates of naproxen with lysozyme (2:1, 3:1, etc.) were expected but not observed. In this study we apply CE–ionspray MS to the identification of the conjugates.

EXPERIMENTAL

Naproxen (Sigma) was coupled to lysozyme (Sigma) by the N-hydroxysuccinimide method described in detail previously [11,12]. The raw product (1 mg) was dissolved in the buffer (1 ml) used in CE. Injections were made with a Prince (Lauerlabs, Emmen, Netherlands) microprocessor-controlled injector using electrokinetic injection at 30 kV for 9 s. The electrophoresis voltage of 30 kV was provided by a Glassman (Whitehouse Station, NJ, USA) EH30R00.5-22 power supply controlled by the Prince. The fused-silica capillary (deactivated, 60 cm × 50 μm I.D., part No. 062804) was obtained from SGE (Melbourne, Australia). Unfortunately, the manufacturer does not provide information about the nature of the deactivation. The buffer solution used was 100 m*M* ammonium acetate in water purified using a Milli-Q system (Millipore) and the pH was adjusted to 4.6 and 5.6 with acetic acid.

The fused-silica capillary was inserted into a coaxial ionspray interface (Fig. 1). A voltage of 3 kV was applied to the stainless-steel tube (10 cm × 0.4 mm I.D.). The nebulizing gas (99.9% nitrogen) pressure was 3 bar. A make-up flow (5 μl/min) was provided by a Micro Gradient

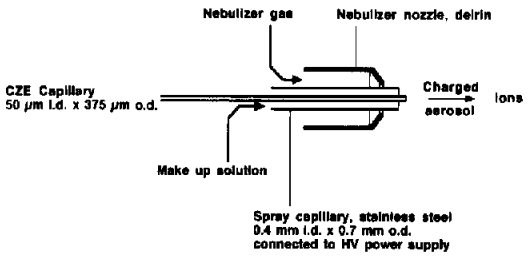

Fig. 1. Schematic illustration of the coaxial interface for combined capillary electrophoresis–ionspray mass spectrometry. HV = High voltage.

System syringe pump (ABI/Brownlee, Santa Clara, CA, USA). The make-up solution was water–methanol–acetic acid (40:60:1, v/v/v).

The mass spectrometer was a Nermag R 3010 (Delsi-Nermag, Argenteuil, France) equipped with a custom-built prototype atmospheric pressure ionization (API) source described previously [13]. Nitrogen (99.9%) was used as a curtain gas. The spectra were recorded in the positive-ion mode with a nozzle–skimmer voltage difference of 120 V. The mass spectrometer was scanned from m/z 1200 to 2000 with a cycle time of 1.5 s per scan. Interface and ion source tuning was done by means of the Prince by continuous pressure feed (50 mbar) of a sample solution via the CE capillary. The sample solution flow was combined with a make-up flow in the coaxial CE–MS interface. Accurate mass measurements on the sample mixture were made in the profile mode of the data system, under the same sample and make-up flow conditions as used for source and interface tuning.

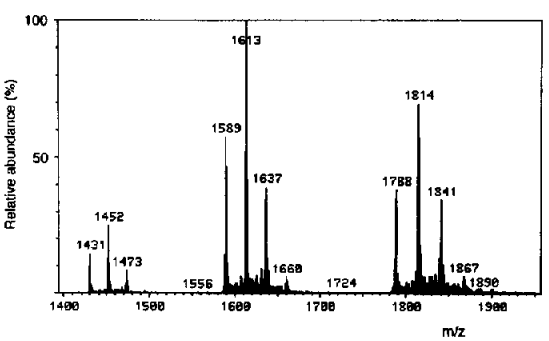

Fig. 2. Ionspray mass spectrum obtained by continuous pressure feed via a CE capillary of the reaction mixture obtained from the synthesis of conjugates of naproxen (M_r 230) with lysozyme (M_r 14 305).

RESULTS AND DISCUSSION

Fig. 2 shows the ionspray spectrum obtained by continuous pressure feed of the sample solution (in buffer of pH 4.6) via the CE capillary, combined with a make-up flow of water–methanol–acetic acid (40:60:1, v/v/v). The spectrum shows ions of charge state 8, 9 and 10 at m/z 1786–1890, 1587–1662 and 1429–1496, respectively. The ion series at m/z 1431, 1589 and 1788 corresponds to lysozyme carrying ten, nine and eight protons, respectively. The reaction products give rise to the series m/z 1452, 1613, 1814, to the series 1473, 1637, 1841 and to the series 1494, 1660 and 1867. Accurate m/z values were measured separately in the profile mode. The molecular masses can be determined from the measured m/z values using a simple algorithm presented previously [14]. The determined average molecular masses are 14 303.7, 14 516.0, 14 728.7 and 14 938.9. These values correspond to native lysozyme and 1:1, 2:1 and 3:1 naproxen–lysozyme conjugates, for which the calculated average molecular masses are 14 305.2, 14 517.4, 14 729.6, 14 941.8, respectively. The determined values are slightly below the calculated values, but the error is only 1 in 10^4 and the results confirm the presence of the naproxen conjugates in the sample. The calculated increment for the covalent bond between naproxen and one of the lysines in lysozyme through formation of an amide is 212 u. If complexation had taken place via non-covalent bonds, the observed mass increment for a naproxen molecule bound to lysozyme would have been 230 u, which is not observed and clearly not the case in the reaction product.

Next, the conjugates were separated and identified by on-line CE–MS. A commercially available deactivated column was used to minimize the adsorption of lysozyme and its conjugates. Basic proteins such as lysozyme have been reported to adsorb on the walls of undeactivated bare fused-silica CE capillaries [2,15]. Figs. 3 and 4 show the separation of lysozyme and its three naproxen conjugates (1:1, 2:1 and 3:1) at pH 4.6 and 5.6, respectively. Lysozyme and its conjugates can just be separated at pH 4.6 (Fig. 3). The spectra recorded for each compound

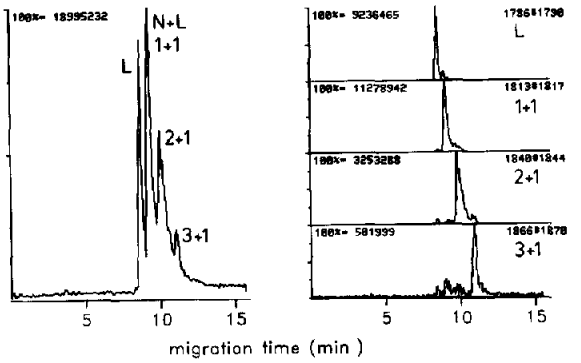

Fig. 3. Total ion current trace and ion current traces of individual components obtained by CE–MS at pH 4.6.

confirm the presence of native lysozyme and 1:1, 2:1 and 3:1 naproxen–lysozyme conjugates. The ion current profiles of the individual components with eight charges show some residual tailing due to sample adsorption. At pH 5.6 (Fig. 4) the separation is seemingly improved in the total ion current trace. However, the ion current profiles of the individual components show that lysozyme is apparently adsorbed on the column, but displaced and released when the 1:1 conjugate elutes. At pH 4.6 the averaged mass spectrum of the 1:1 conjugate recorded in scans 253–264 (Fig. 5, top) and the averaged mass spectrum of the 2:1 conjugate recorded in scans 277–279 (Fig. 5, bottom) contain minor contributions of earlier eluting components at m/z 1788 (top) and at m/z 1816 (bottom). The peak at m/z 1788, due to native lysozyme (indicated with an arrow) in the mass spectrum of the 1:1 conjugate re-

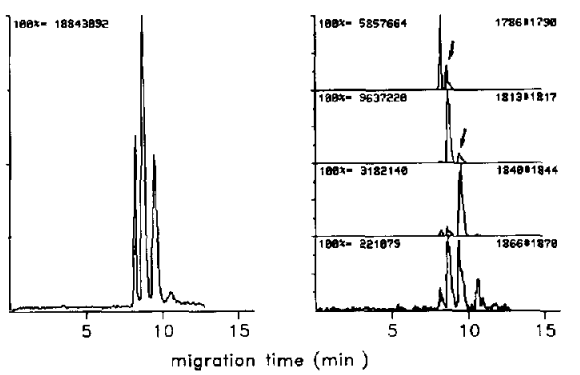

Fig. 4. Total ion current trace and ion current traces of individual components obtained by CE–MS at pH 5.6.

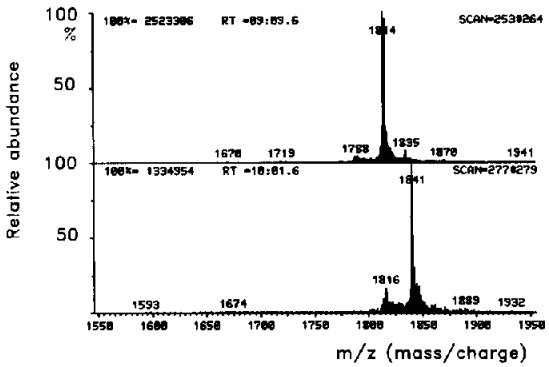

Fig. 5. Ionspray mass spectra of the 1:1 and 2:1 naproxen–lysozyme conjugates obtained by CE–MS at pH 4.6.

corded in scans 243–246 (Fig. 6, top) and the peak at m/z 1816 due to the 1:1 conjugate in the mass spectrum of the 2:1 conjugate recorded in scans 263–272 (Fig. 6, bottom) demonstrate that interference from earlier eluting components is stronger at pH 5.6. Explaining the inference noted above by adsorption and displacement may seem speculative. A more thorough discussion would require knowledge of the modification of the capillary wall. Unfortunately, such information is not available as the CE capillary is deactivated by a proprietary process. Mass spectrometric fragmentation of sample ions with loss of naproxen under the conditions chosen in this study can be ruled out. First, the ion source and ion transport conditions were mild enough to prevent fragmentation. Second, the interference problem observed is clearly pH dependent. Fragmentation by collision-induced dissociation inside the mass spectrometer would be entirely dependent on the voltage settings of ion optics elements, which were kept the same in all experiments.

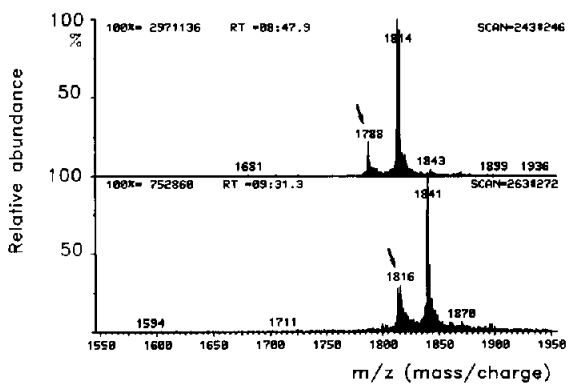

Fig. 6. Ionspray mass spectra of the (top) 1:1 and (bottom) 2:1 naproxen–lysozyme conjugates obtained by CE–MS at pH 5.6. The arrows point to co-eluting native lysozyme (m/z 1788) and 1:1 naproxen–lysozyme (m/z 1816).

The 3:1 conjugate, which is a minor component in the mixture, was split into four peaks, in particular at pH 5.6 as shown in Fig. 4. Protein adsorption alone cannot account for the observation made here. We assume that relatively large amounts of lysozyme and its 1:1 and 2:1 conjugates apparently carry small amounts of the 3:1 conjugate in their zones. The problems observed in our experiments cannot be detected by non-specific detectors such as a UV detector. This highlights one of the advantages of the use of a mass spectrometer as a detector in CE.

The peak areas of the individual compounds in the ion current profiles in Fig. 3 show that the relative amounts of the native lysozyme and the 1:1, 2:1 and 3:1 conjugates are about 70, 100, 40 and 5%, respectively. These relative amounts correspond well to the relative abundances of the native lysozyme and its conjugates in the spectrum recorded without separation by continuous pressure feed of the sample solution (Fig. 2).

The spectra recorded by continuous pressure feed showed ions of charge states 10, 9 and 8. In contrast, the spectra recorded with CE–MS showed only ions of charge state 8. As the mass range of our instrument is limited to m/z 2000, we cannot observe the expected ions of charge states 7 and 6. The shift to lower charge states also takes place when the high voltage (30 kV) is applied to the front end of the CE capillary during continuous pressure feed of the sample. These results show that the electrophoretic transport of ions in the CE capillary influences the electrospray ionization process. For unknown reasons, lysozyme and its conjugates appear to collect fewer protons when +30 kV is applied to the front end of the CE capillary. This phenomenon will be studied further in the future.

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