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The emergence of new protein and peptide-based drugs makes necessary the development of rapid and sensitive methods to check consistency between and within batches of biotechnology pharmaceuticals to insure product quality. The types of analytical methods that are typically used for conventional drugs may not be appropriate for biotechnology pharmaceuticals, which are often of greater molecular size and complexity. Some of these tests are microbiological and animal assays in addition to conventional analyses such as HPLC. Analytical approaches that are capable of distinguishing differences (or sameness) of biotechnology products may be of significant benefit.

We chose insulin as a model therapeutic protein for the development and evaluation of a method, based on hydrogen/deuterium exchange and electrospray ionization (ESI) mass spectrometry. The method was evaluated for distinguishing among different types of insulin (r-Human, isomeric LysPro, porcine and bovine) for confirming the identification of the type of insulin present.

ESI mass spectra were collected by using a prototype VG ZAB-T four-sector tandem mass spectrometer equipped with a VG electrospray source. A syringe pump was used to infuse a solution of 50/49/1 water/acetonitrile/formic acid to the spray needle at a rate of 10 L/min. Samples incubated for various time periods were introduced into the solution via a 20-L loop Rheodyne 7125 valve. The spray needle was maintained at 8000 V, and the counter electrode (pepper pot) potential was 5000 V. The sampling cone, skimmer lens, skimmer, hexapole and ring electrode were 4196, 4159, 4156, 4154, and 4117 V, respectively. Nitrogen was used separately as both bath and nebulizer gas with flow rates of 400 and 12 L/h, respectively. The bath-gas temperature was maintained at 50 °C. The mass spectrometer was calibrated from m/z 600 to 2600 by using a solution of CsI. All experiments made use of only the first two sectors. Ten scans were signal averaged and processed by using the VG Opus operating system and a DEC-alpha work station. The raw ESI spectra were transformed by using a Maximum Entropy algorithm (MaxEnt) obtained from Micromass.

The sample (1 mg) was dissolved in 500 L of H₂O, and several 20-L aliquots were removed to determine the extent of H/D exchange at different time points in the rate study. The exchange was initiated by mixing a 20-L aliquot with 180 L of D₂O. After an exchange period, which varied from 30 s
to 24 h, the exchange was quenched by adding 500 L of ice-cold, 50/49/1 water/acetonitrile/formic acid solution. A 20-L aliquot with a final concentration of 10 pmol/L was loop-injected immediately following the quench for ESI-MS analysis.

When we analyzed samples that were quenched after different times of H/D exchange (up to 24 h), we obtained a time-dependent increase in the mass of insulin (Figure 1). The results show that one can determine reliably whether an insulin sample is r-Human or isomeric LysPro by measuring the extent of deuterium incorporation after the exchange had become relatively constant (e.g., after 15 minutes). Although one can easily recognize bovine and porcine insulins because they have different molecular masses than that of human insulin, one can find additional confidence that the sample has not been subjected to denaturing conditions by the unique extents of H/D exchange.

To verify that the H/D exchange method described is sensitive to insulins that are subjected to denaturing conditions, bovine insulin was overtly denatured by heating a solution to 80°C for 15 min. The denatured bovine insulin exchanged 33 protons after 30 minutes of incubation, as shown in Figure 2, whereas the native bovine insulin exchanged only 23 protons. After 12 hours of incubation, the denatured bovine insulin exchanged 15 protons more than the native bovine insulin.

We propose the following protocol as a method for screening protein analogues and for determining the consistency among batches of protein preparations. The protein (in the present case, insulin) is submitted to H/D exchange for a time period at which exchange has become relatively constant (for insulin, > 15 min). The exchange is quenched, and the extent of exchange analyzed by ESI-MS. In the case of insulins, the isomeric LysPro and r-Human insulins after 30 minutes of incubation exchanged 35 and 28 protons, respectively, while porcine and bovine insulins exchanged 25 and 23 protons, respectively (Figure 3).
This assay method was tested by analyzing ten insulin samples of different types that were submitted as unknowns. Assignments based on deuterium incorporation after 30 minutes were correct in all cases, suggesting that ESI-MS in combination with H/D exchange is a reproducible and reliable method for distinguishing r-Human insulin from LysPro insulin and for evaluating the consistency of different batches of different insulin types.