Trap for MABs: Characterization of Intact Monoclonal Antibodies Using Reversed-Phase HPLC On-Line with Ion-Trap Mass Spectrometry

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For the first time, the characterization of intact 150-kDa monoclonal antibodies (MABs) using a commercially available three-dimensional ion-trap mass spectrometer (IT-MS) is reported. The IT-MS analysis was performed on-line with reversed-phase high performance liquid chromatography (RP-HPLC) on a POROS column using a nontraditional solvent system of acetonitrile, isopropanol, ethanol, and water in formic acid. The operating parameters of the IT-MS were optimized by extending the mass range to m/z 4000 and elevating the tube lens offset voltage value to around -100 V. Mass accuracy better than 300 ppm (± 40 Da) has been routinely achieved for these macromolecules. Multiple peaks 162 Da apart due to the hexose variants of the monoclonal IgG antibodies were partially resolved in mass spectra. Several commercial and chimeric antibodies have been investigated in this study. (J Am Soc Mass Spectrom 2005, 16, 307–311) © 2004 American Society for Mass Spectrometry

The human monoclonal immunoglobulin γ (IgG) molecule is the most popular modality in the modern protein pharmaceuticals because of its controlled functions, predictable physiochemical properties, and long life in circulation [1]. Monoclonal antibodies (MABs) have been successfully commercialized in the biotechnology field for the past ten years. These products have emerged as primary protein therapeutic research and development platforms. Although antibodies have structural similarities, their post-translational modifications on heavy and light chains require constant monitoring from cell culture to final formulated products. Thus, it is obvious that the analysis of intact therapeutic monoclonal antibodies by reversed-phase (RP) high-performance liquid chromatography (HPLC) online with mass spectrometry (LC/MS) would dramatically reduce sample preparation and data interpretation and also minimizes putative modifications that may occur during sample preparation, such as during peptide mapping experiments after enzymatic digestion [2].

In recent years, electrospray mass spectrometry (ESI-MS) [3], techniques to obtain maximum sample transfer efficiency [4, 5] and combination of ionization modes and mass analyzers [6] have enhanced the ability to characterize small to medium sized proteins (<100

kDa) by mass spectrometry. However, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) [7, 8], ESI orthogonal-TOF [9, 10] and ESI quadrupole [2, 11–13] mass spectrometers have been the instruments of choice for MABs and macromolecules, largely due to the large m/z range of the TOF and high ion transmission efficiency of the quadrupole mass analyzers. Unfortunately, on-line RP-HPLC has not accompanied these previous studies of intact monoclonal antibodies.

Reversed-phase liquid chromatography is an important component of intact protein analysis because it has the ability to separate species based on minor structural heterogeneity and, coupled to standard HPLC detectors, provides quantitative information about the main component, variants, and cleavage products. Because of the large size and hydrophobic nature of antibodies, employment of reversed phase liquid chromatography on-line with mass spectrometry for the analysis of intact antibodies has recently been developed in our laboratories [14–16]. Until recently, perfusion chromatography utilizing highly cross linked polystyrene-divinylbenzene (POROS) resins has often been used for separation and purification of antibodies [17, 18]. This technique suffers from limited separation efficiency attributable to high flow rates of 0.5–3.0 mL/min required for separation and signal suppression due to trifluoroacetic acid (TFA)-containing mobile phases (acetonitrile/water/TFA) which is not the most popular solvent for electrospray ionization of proteins [19]. Thus, an alternate combination of mobile phase consist-

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ing of ethanol/propanol/formic acid was employed as Solvent B for RP LC/MS analysis of glycosylated peptides [20].

Since the invention [21] and pioneering works of Wolfgang Paul on the three-dimensional quadrupole ion-trap (QIT) [22], numerous landmark research papers have described interesting modes of operation and demonstrated a variety of specific applications [23–26] that illustrate the usefulness of this nascent type of mass spectrometry, especially for small molecules and peptides. Unfortunately, without RP-HPLC, the analysis of intact antibody [27] has required a tedious combination of analytical techniques and extensive modifications of the ion trap analyzer. The main reasons for the lack of published material is thought to be attributable first, to the well recognized limitations of the ion-trap mass spectrometers to detect highly charged ions of MABs due to the space charge issues [28], and second, several suitable solvent systems and optimal ionization voltages must be determined to deliver intact antibodies and to initiate the ionization process in the ion source region (spray needle [“nozzle”]/skimmer) of an ion-trap mass spectrometer, respectively. This is the first report about analysis of intact monoclonal antibodies by electrospray ionization on a commercial three-dimensional quadrupole ion trap mass spectrometer coupled with RP-HPLC (IT-LC/MS). This innovative technique was achieved by interfacing a POROS column that is operated in reversed-phase mode using nontraditional solvents of acetonitrile, isopropanol, ethanol and water in formic acid (FA). The RP-HPLC system first allowed us to obtain acceptable chromatographic resolution of intact antibodies and their degradation products or clip forms. More importantly, molecular masses of the intact antibodies were obtained with unprecedented accuracy by optimizing the tube lens offset voltages and automatic gain control (AGC) to obtain maximum sample transfer efficiency while perhaps minimizing space charging effects.

Experimental

A commercial IgG1 antibody Herceptin (calculated MW = 148,058 Da, Genentech, San Francisco, CA) [29] and a chimeric IgG2 antibody produced at Amgen (calculated MW = 147,353 Da, Amgen Inc., Thousand Oaks, CA) were used in this study. The IgG2 antibody was subjected to an accelerated stability study under elevated temperatures, which caused degradations of the molecule. The HPLC system consisted of a HP-1100 (Agilent, Palo Alto, CA) equipped with a heated 75 °C POROS R1/10 1-1014-24 (Applied Biosystems, Framingham, MASS) analytical column (4.6 mm × 50 mm) and operated at a flow rate of 450 µL/min. The RP-HPLC system was connected on-line to a Thermo Finnigan LCQ DECA ion trap spectrometer equipped with an off-axis electrospray ionization (ESI) source. Ten µg aliquots of the antibodies were injected per analysis. The IgG1 antibody, Herceptin, was evaluated by IT-

LC/MS utilizing an elution composition of 0.08% aqueous FA (Solvent A) with a linear gradient of acetonitrile/ethanol (8:2 vol/vol) in 0.08% FA (Solvent B). After a 3 min isocratic hold, a linear gradient started at 20% B and increased to 55% B in 12 min (2.92% B/min) followed by an isocratic hold for 3 min. The column was then further washed to 98% B in an additional 12 min (3.58% B/min) of a 30 min gradient. This solvent/gradient system is referred here as ACN/ETOH containing. It is noticed that IgG1 fractionate differently than IgG2 antibody with this solvent/gradient system.

To effect acceptable resolution and separation of IgG2 antibody preparation, our laboratory evaluated many mobile phase solvent systems. One of the better mobile phase solvent systems for an IgG2 chimeric antibody was ACN/IPA/ETOH/WA, which was composed of the following: 0.08% aqueous FA (Solvent A) and acetonitrile/isopropanol/ethanol/water (6.0:1.5:1.5:1.0 vol/vol/vol/vol) in 0.08% FA (Solvent B). Following an isocratic post-injection hold, 5 min, the linear gradient started at 25% B was increased to 60% B within 15 min and held for 5 min. The column was then washed to 98% B (2.53% B/min) for the next 15 min of a 40 min gradient. Depending upon the types of antibodies and their specific formulation buffers, the column wash cycle could take 30 min.

The ion trap LCQ Deca mass spectrometer was operated under Xcalibur 1.2 software (Thermo Finnigan, San Jose, CA). The mass range of 1200–3800 *m/z* was calibrated in the positive ion mode with multiple-ion monitoring of four ions signals of $[M + 7H]^{7+} = m/z$ 1730.0, $[M + 6H]^{6+} = m/z$ 2018.5, $[M + 5H]^{5+} = m/z$ 2421.8, $[M + 4H]^{4+} = m/z$ 3026.0 of a truncated form (MW: 12,103 Da) of a sTNF-R1 protein [30] for this study instead of polypropylene glycol (PPG). Mass spectra of both IgG1- and IgG2 antibody analyses were acquired over a range from *m/z* 2100–3800. The mass spectrometric acquisition required the tube lens offset voltage was set at –100 V (±25 V). The voltage of the ion-spray needle was 5000 V (±200 V) and the temperature of the heated capillary was 280 (±20 °C). The AGC setting of full mass was occasionally changed to 5 × E8 from conventional setting of 5 × E7. Other targets and duration of micro-scan were approximately at their default values.

Results and Discussion

Since the amino acid sequences of antibodies used in this report are known, the purpose of this study was to confirm unambiguously the molecular weight of these antibodies by IT-LC/MS. Figure 1a shows an HPLC-UV chromatogram of the IgG1 (Herceptin) antibody after separation on POROS column. Figure 1b is a positive ion ESI mass spectrum of the main chromatographic peak acquired in the range from *m/z* 2400–3500 and featuring multiply charged ions from 44+ to 60+. Figure 1c shows the convoluted and deconvoluted ESI mass spectrum of IgG1 antibody with resolving masses 148,098 and 148,260 Da. The difference in mass of ~162

Figure 1. (a) A RP-HPLC chromatogram of an IgG1 antibody used in this study with UV detection at 215 nm. Inset shows a generic schematic of IgG1 antibody. (b) An expanded positive ESI mass spectrum of intact IgG1 antibody Herceptin from m/z 2400–3500 with charged ions ranging from 43^+ to 61^+ . The spectrum was reconstructed by removing excessive noise signals and artifacts caused by the application software. (c) The corresponding convoluted and deconvoluted mass spectra (reconstructed) of Herceptin antibody and its associated structural micro-heterogeneity: 148,098 Da and 148,260 Da, respectively. The observed mass difference of 162 Da is correlated with a loss of a hexose residue (Hex, 162 Da), which is a typical heterogeneity of glycosylation observed in IgG antibodies produced in mammalian cells.

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[13]. When the same ACN/ethanol containing mobile phase was used for IgG2 antibody, chromatographic resolution efficiency was significantly reduced (data are not shown). To improve elutropic properties of the mobile phase and obtain better separation of the degradation products of IgG2 antibody, iso-propanol was added to Solvent B. Thus, depending upon the subclass of antibody being examined, IgG1 or IgG2, it would be important to optimize the chromatographic mobile phase to effect maximal resolution. Figures 1a and 2a illustrated the need to have a different mobile phase system to obtain better chromatographic resolution for IgG2 antibodies compared to an IgG1.

Figure 2a shows an HPLC UV chromatogram of the IgG2 antibody. On the chromatogram, the low MW cleavage products of 12 to 24 kDa (clips) eluting from 9.5 to 9.8 min were chromatographically separated from the high MW components of the degraded IgG2 sample. Because of the limited resolution of the chromatographic peaks eluting from 10.3 to 11.5 min, we were not able to accurately measure the masses of degrada-

Figure 2. (a) A RP-HPLC chromatogram of an IgG2 antibody used in this study after separation on POROS column with UV detection at 215 nm. The observed multiple degradation products were induced by storage at elevated temperatures over an extended period of time. (b) The corresponding positive ESI mass spectrum of the chromatographic peaks eluting from 11.3 to 13.5 min in (a) featuring multiply charged ions from 44^+ to 63^+ . The deconvoluted mass spectrum of the observed mass of 147,348 Da of this IgG2 antibody is closely matched its theoretical mass of 147,353 Da.

tion products or isoforms of this antibody. Figure 2b is a positive ion ESI mass spectrum of the chromatographic peaks eluting from 10.3 to 13.5 min acquired in the range from m/z 2200–3400 and featuring multiply charged ions from 44^+ to 63^+ . Figure 2c shows the deconvoluted ESI mass spectrum of IgG2 antibody in the mass range of the intact molecule with measured mass of 147,348 Da. The glycosylation profile featuring different number of hexoses was not resolved in this case. The measured mass agreed well with the calculated $MW = 147,353$ Da for glycosylation structure without any terminal hexose residues [13]. These results have been confirmed by size exclusion chromatography and MALDI-TOF analyses in our laboratory (data are not shown). The control experiments confirmed that the degradation products of the IgG2 antibody were indeed caused by the storage conditions and not by the low pH, elevated temperature environment on the POROS column. It was also observed that the multiple chromatographic peaks eluting from 10.3 to 13.5 min were either obscured or totally hidden when using the mobile phase containing only ACN/ethanol/water/FA combination of solvents. The addition of iso-propanol to our perfusion chromatography system improved separation and elution properties similar to our laboratory experiments on silica based C8 stationary phases [15]. In this report, the mass accuracy was measured and averaged after three consecutive LC/MS runs for each antibody and it was determined within 300 ppm for IgG1 antibody and 100 ppm for IgG2 antibody.

Our experiments focused on optimization of the IT-LC/MS show that the ion transfer efficiency from atmosphere to the ion trap could be greatly improved by increasing amplitude of the tube lens offset voltages. The tube lens voltage provides the potential voltage drop to accelerate the highly charged ions during the ionization spray processes by confining and focusing them at the entrance of the ion-trap skimmer after they exist the heated capillary [31]. In this region, the electrospray particles move with the velocity of sound (~ 300 m/s) as a result of the free jet expansion into the vacuum. For the 150-kDa ions of antibody, this velocity corresponds to a relatively high kinetic energy of ~ 60 eV. It required a larger focusing potential (~ 100 V) provided by the tube lens to focus the ions into the orifice of the skimmer. In addition, we believe that another factor to consider is the dissociation of ion in the nozzle/skimmer region, as was pointed out by Rockwood and co-workers [32]. It is easier to dissociate highly charged ions by acceleration in the nozzle/skimmer because of at least two factors. First, because they are more highly charged, they acquire more kinetic energy by being accelerated. Second, highly charged ions tend to be inherently less stable because Coulombic repulsion destabilizes them, making it easier to break ions into fragments. Therefore, if the potential voltage drop is set too high, loss of ions will occur because they will dissociate, especially for highly charged ions. Thus, the voltage drop potential in the nozzle/skimmer needs

to be high enough to desolvate intact antibody ions, but not so high as to dissociate them.

Conclusions

We have demonstrated, for the first time, that intact 150-kDa monoclonal antibodies and their potential degradation products could be characterized by electrospray ionization using a standard commercial three-dimensional ion-trap mass spectrometer coupled online with RP-HPLC (IT-LC/MS). To the best of our knowledge, these 150-kDa antibodies are the largest proteins detected by an unmodified ion-trap mass spectrometer thus far. In this report, we focused our applications on a POROS analytical column to accomplish our objectives of determining molecular masses of antibodies. A combination of employing the nontraditional solvent systems and optimizing the transfer efficiency of the sample by lowering the tube lens offset voltages of the QIT led to the successful development of this "Trap for MABs" method. We anticipate that the chromatographic peak resolutions as well as the sensitivity of the mass spectrometer can be substantially improved with either narrow bore POROS columns or other commercially available columns or other solvent systems. The analysis of intact antibodies in protein pharmaceuticals opens a new field of applications for three dimensional ion trap mass spectrometry, which, until recently, has been considered as mainly a tool for peptide mapping of proteins after their enzymatic digestion.

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