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Multiple heart-cutting two dimensional liquid chromatography mass spectrometry: Towards real time determination of related impurities of bio-pharmaceuticals in salt based separation methods

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ABSTRACT

Many of the chromatographic methods used in industry to determine related impurities in bio pharmaceuticals employ salt containing mobile phases. "Salty" mobile phases often provide superior chromatographic performance but are not compatible with mass spectrometry (MS) detection. Peak tracking necessary for method development is therefore often based on peak areas and the chemist's experience/intuition. In addition, MS characterization of impurities usually is done by offline fraction collection, which apart from being time consuming often suffers from poor recovery or the degradation of impurities collected. The recent development of multiple heart-cutting (MHC) two-dimensional liquid chromatography (2D-LC) provides a way to address these problems. This study shows how MHC 2D-LC–MS can be used to obtain almost real time MS data for bovine insulin related impurities present at low level (<<0.03%). High quality MS spectra were obtained even for a first dimension using a mobile phase containing high concentrations of sodium, sulphate and phosphate. Thereby MHC 2D-LC–MS offers a possibility to eliminate the guesswork currently associated with peak tracking during method development. Furthermore, in contrast to current characterization methods involving fraction collection, solvent reduction/exchange etc., MS determination is done directly, which markedly shortens the workflow (from days to hours) and reduces the risk for poor recovery and degradation.

1. Introduction

Within the pharmaceutical industry in general and the bio-pharmaceutical industry in particular chromatographic purity methods often utilize mobile phases containing a high concentration of non-volatile salt. For small molecules and peptides reversed phase chromatography (RPC) mobile phases often are based on phosphate salts. For RPC of proteins trifluoroacetic acid (TFA) is the dominant mobile phase additive. Characterization of proteins does, however, also require size exclusion chromatography (SEC), ion exchange chromatography (IEC) and hydrophobic interaction chromatography (HIC). These chromatographic modes involve the use of very high concentrations of sodium chloride or other salts.

In order to identify or track impurities it is necessary to use mass spectrometry (MS). Unfortunately, direct coupling of MS detection to LC is incompatible with mobile phases containing high concentrations of non-volatile salt. Inorganic ions such as sodium, sulphate, and phosphate result in noisy MS spectra with high levels of adduction and salt clusters covering a large part of the mass spectral range. Such ions also suppress the ionization of target compounds in the electrospray process, resulting in a significantly reduction of the MS signal. In addition, an increasing deposit of salt in the ion source is formed that also reduces the signal and eventually blocks the entrance to the low pressure part of the MS. It is possible to obtain MS data with TFA present at low concentration in the mobile phase, but typically this also results in marked signal suppression. Both TFA and chloride under acidic condition cause corrosion. Due to this salt incompatibility it has not been realistic to utilize MS for peak tracking during method development when working with salt based mobile phases. Instead, peak tracking has historically been based upon peak areas and the chemist's experience/intuition. For the same reasons characterization of impurities by MS has required time consuming fraction collection followed by subsequent analysis of the fractions by LC–MS with volatile mobile phase additives. This process of fraction collection and re-analysis often suffers from poor analyte recovery and/or degradation of collected impurities.

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Single heart-cut two-dimensional liquid chromatography (2D-LC) has been applied for peak purity analysis as well as identification of impurities in pharmaceuticals [1–3]. However, as the name indicates, it only allows capture and analysis of one cut per run or at least a very limited number of cuts depending on the second dimension (^{2}D) cycle time. Only when the ^{2}D cycle has finished can the next cut be sampled. When an impurity elutes from the first dimension (^{1}D) column during the ^{2}D separation of the preceding cut, this impurity cannot be analyzed by the ^{2}D separation [4]. This is a serious drawback since it becomes very tedious to identify the 5 to 15 impurities that typically are present in a degraded drug product a few at a time in several analyses where the cuts are spaced to be compatible with the timing of ^{2}D analyses. This is especially problematic if the impurities elute in close proximity in the ^{1}D separation such that only one can be handled in each of several 2D-LC analyses. If retention times are shifting somewhat as they often are, several attempts are needed in order to ensure that each impurity has been analyzed.

Comprehensive 2D-LC, where the effluent from the entire ^{1}D separation is analyzed in the ^{2}D column, has also been applied for determination of impurities in small molecule pharmaceuticals [5,6] as well as for peptide mapping [7], however, this requires a very slow ^{1}D separation along with an extremely fast ^{2}D separation (10–30 s) in order to avoid compromising the resolution obtained by the ^{1}D separation in the process of collecting fractions from the first dimension [8–10]. In addition, the high ^{2}D flow rates needed for such fast separations typically must be split to allow coupling to MS detection.

Multiple heart-cutting (MHC) 2D-LC [4,10–12] provides a way to address the limitation of the single heart-cut and comprehensive modes of 2D-LC. A multitude of heart-cuts from the ^{1}D separation are sampled into loops or trap cartridges [13], where they remain parked until their analysis with the ^{2}D separation. The parking functionality disconnects ^{1}D and ^{2}D timescales, thus the conditions used for the two separations can be optimized independently in order to maximize resolution and/or desalt cuts prior to MS analysis. Since a multitude of cuts can be taken from the ^{1}D, the time needed for comprehensive characterization of multiple impurities in a sample is significantly reduced compared to single heart-cut 2D-LC.

MHC 2D-LC has successfully been applied for the analysis of traditional small molecule pharmaceuticals [12] as well as bio-pharmaceuticals [13–16]. Masuda *et al.* [13] described a MHC 2D-LC system capable of trapping six cuts on individual trapping columns prior to desalting and subsequent MS or LC–MS analysis. The system was evaluated for IEC-RPC-MS analysis of tryptic protein digests. Zhang *et al.* [12] presented a similar but loop-based system. RPC-RPC applications were shown both for peak purity analysis and for MS determination of small molecule impurities in a salt containing ^{1}D separation. Schneider *et al.* [14] described the application of a commercial 12 loop MHC 2D-LC system for N-glycan analysis of a bio-pharmaceutical protein by HILIC-IEC (hydrophilic interaction chromatography – IEC). Stoll *et al.* [16] used the same type of system for analysis of monoclonal antibody charge variants using IEC-RPC. In this application the ^{2}D was mainly used for desalting prior to MS. A 12 loop MHC 2D-LC–MS was also used for HIC-RPC by Staples [15] for determination of oxidized antibody species. Also for this application the ^{2}D was mainly used for desalting prior to MS. Recent reviews by Stoll [8,17] give a good overview of the application of 2D-LC for analysis of bio-pharmaceuticals.

The current study investigated the use of state of the art MHC 2D-LC–MS for tracking and characterizing bio-pharmaceutical peptides at levels typically found for impurities during method development when employing reversed phase chromatography in conjunction with salt based mobile phases. Bovine insulin and related impurities were selected as model substances. Both chromatographic and MS performance aspects were investigated including peak shape, detection limits, selectivity, number of cuts and presence of salt related adducts and clusters in MS spectra.

2. Material and methods

The samples used in the study were generated by exposing 1 mg/mL of bovine insulin (Sigma-Aldrich, St Louis, MO) in 25 mM of sodium phosphate pH 7 at 50 °C for 3 days.

Data were acquired using an Agilent 1290 Infinity 2D-LC system (Waldbronn, Germany) with Multiple Heart-Cutting operated through Agilent OpenLAB CDS ChemStation Edition, version C.01.07. MS data were obtained using an Agilent 6550 iFunnel QTOF LC/MS System operated with MassHunter Workstation software (B06.01). Qualitative evaluation of MS data was performed using MassHunter Bioconfirm (B07.00).

The first dimension included a binary pump, auto-sampler, column thermostat, diode array detector (DAD) equipped with a 10 μL flow cell. Sampling rate 20 Hz. An Agilent Poroshell HPH C18 150 × 2.1 mm, 2.7 μm column was used at 40 °C. Solvent A was 40 mM $(NH_4)_2HPO_4$ and 60 mM Na_2SO_4 titrated with 85% H_3PO_4 to give pH 2.2 and solvent B was acetonitrile (MeCN)/water 80:20 v/v. Gradients were from 29 to 43%B in 28 min then in 1 min to 90%B, which was held for 1 min before a reduction back to 29%B at 31 min. Flow rate 300 μL/min.

The second dimension used binary pump, column thermostat, and DAD with a 10 μL flow cell acquiring data at a 40 Hz rate. An Agilent Poroshell HPH C18 50 × 2.1 mm, 2.7 μm column was used at 40 °C. Solvent A was 0.1% formic acid (FA) in water and solvent B was 0.09% FA in MeCN/water 80:20 v/v. Initial ^{2}D-gradient: 6.25%B at 0 min, (to 23% B at 0.1 min, 32%B at 2.1 min), 90%B at 2.2 min, held until 2.3 min, 6.25%B at 2.4 min. A gradient shift [9] was programmed changing the part of the ^{2}D-gradient in brackets in a linear fashion reaching (to 25% B at 0.1 min, 38%B at 2.1 min) at 15.2 min 2D-LC analysis time and finally (to 27% B at 0.1 min, 40%B at 2.1 min) from 15.3 min onwards. Flow rate 400 μL/min. ^{2}D-gradient stop time = 2.5 min ^{2}D-cylce time = 4 min. Analysis stop time was set to 38 min in the ^{2}D pump. HPH columns were originally selected to provide the flexibility to utilize high pH if needed. The pore size of this column is sufficiently large (100 Å) to accommodate insulin and degradation products. Since the study was conducted at low pH other C18 columns commonly used for peptide mapping could probably have been used.

The MHC 2D-LC interface is shown in Fig. 1 [18]. Two 6-pos/14-port selector valves are connected to a 2-position/8-port modulator valve. Each selector bears a cluster of six 40 μL sampling loops. This provides two parking decks (A and B) with 12 loop positions. Switching the modulator valve places a deck in sampling or alternatively in ^{2}D-analysis position. The switch of the selector valves provides access to the discrete loop positions.

During the split-flow focusing experiment a bypass capillary was connected to ports 6 and 7 in the modulator valve (Fig. 1). The principle of this approach is described in Results and Discussion.

Q-TOF MS data were acquired in positive mode between m/z 100 and 3200 at a rate of 2 Hz. The instrument was equipped with the Dual AJS electrospray ionization source operated at the following gas flows and temperatures. Gas flows: Nebulization gas = 35 psi, drying gas = 15 L/min, sheath gas = 12 L/min. Temperatures: Sheath gas = 375◦C, drying gas = 200◦C. The electrospray capillary voltage was 2500 V and nozzle set to 300 V. A m/z 121.0508 and 922.0098 was used for internal mass calibration.

3. Result and discussion

Bovine insulin and related impurities were selected as model substances for the current study. In order to generate degradation