The use of High Performance Liquid Chromatography (HPLC) columns in Biomolecule analysis

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Abstract: Biomolecules are a topic of interest to many individuals and organisations including;

- Medicinal research groups looking at patient treatments focused on rare or underfunded diseases
- Pharmaceutical companies looking to target particular diseases with smaller toxicological side effects.
- Governments and authorities aiming to improve healthcare and reduce associated costs
- Environmental groups hoping to reduce emissions of chemical pharmaceuticals and by products into the environment.

Since this is a rapid growth area, we look at some of the chromatographic separation challenges faced and the ability of recent HPLC column technology to impact on the biomolecule separations required.

Introduction

Since the first approved biomolecule, synthetic Insulin in 1982, biomolecular analysis has become of great interest in many pharmaceutical and bio startup companies due to the rapid expansion of their therapeutic use, high specificity and also the sales

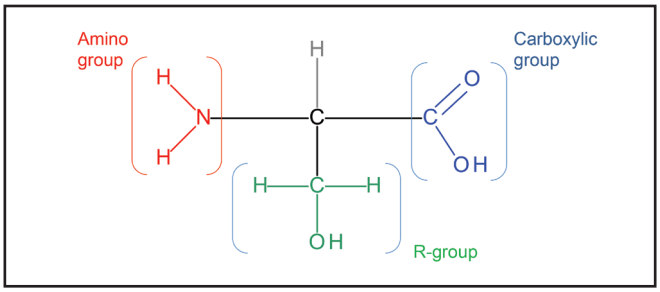

Figure 1. Generic structure of an amino acid

revenue that can be achieved. In 2015 six of the top 10 selling drugs were biomolecules [1], however 5 of these will lose patent protection in the next 3 years, leading to a whole host of potential biosimilar challengers. So the test will be to fill the revenue void with new molecules/biomolecules whilst balancing the contest afforded by biosimilars which will be queuing up to create revenue of their own.

The focus of drug development for pharmaceutical companies has traditionally been small molecules, which have a limited number of impurities present in the final product. The change to developing biologics over the past thirty years is now seen by many as the future of modern drug design. The number of potential impurities that are generated during the biomolecular manufacturing process can be many hundreds of times larger than that present with small molecules. As a result, analytical techniques and in particular LC-MS chromatographic techniques need to be able to separate highly complex sample mixtures that are often only available with low abundance impurities. The challenge of sensitivity has been ongoing in small molecule analysis for many years and is well understood with solutions to these challenges having been developed. However, for these new larger biomolecules, the specific challenges being faced are different and compounded by the diversity in molecules.

The range of biomolecules includes but is not limited to amino acids, peptides, proteins, monoclonal antibodies (mAbs),

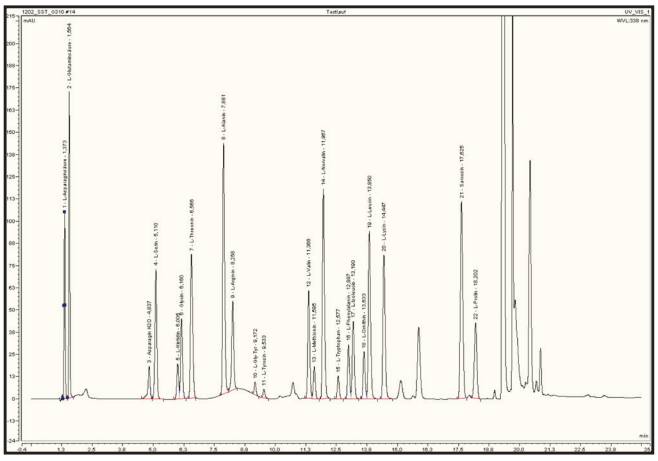

Figure 2. Separation of 22 Amino acids on 1.7um Fortis H2o (polar embedded) 150x2.1mm 0.45ml/min A: 10mM Sodium acetate + TEA pH 7.3 buffer B: 20:80:80 10mM Sodium Acetate (pH 7.3): MeOH : ACN

OPA/FMOC derivatisation 0-1min 2% B 1-17min 60% B 18min 100%B Hold to 25mins.

Oligonucleotides, DNA and RNA. Each gives a specific challenge based on polarity, size, conformation, resolution of 'critical pairs' or retention mechanisms. Many chromatographic methods can be used, with size exclusion (SEC), ion-exchange (IE), reversed phase (RP), ion-pair and hydrophilic interaction liquid chromatography (HILIC) being the most popular current choices. Some of the challenges involved in the analysis of these biomolecules will be examined, with a consideration of the evolving particle technology and variation of method parameters in the development of a separation. All the aforementioned are issues that the analyst has to take into account thus ensuring an accurate qualitative and quantitative method is produced for the separation of these complex proteins and peptides.

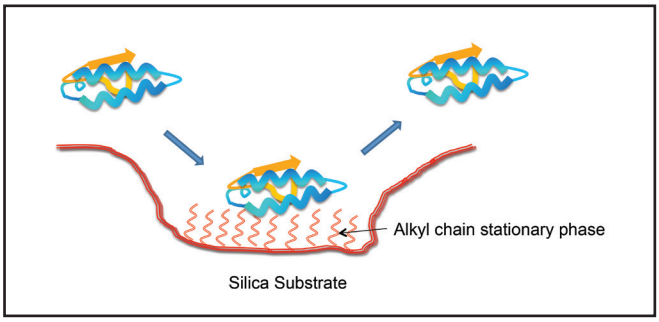

Figure 3. Retention by adsorption of a protein, by the 'hydrophobic foot'

Building Blocks

Amino acids are the initial building blocks for proteins, and form a primary structure. There are 22 naturally occurring amino acids, comprising generically of a carboxylic group and an amino group bound to a central carbon atom (Figure 1). A hydrogen atom occupies the third bonding site on the carbon and variable 'R' group occupy the fourth.

This causes numerous challenges in obtaining a chromatographic separation, due to the considerable diversity of the various analytes involved including but not limited to;

- a variety of hydrophobicities
- different functionalities,
- o Mixtures of basic, acidic and neutral.

The amine and carboxylic functional groups present allow the amino acid to have amphiprotic or zwitterionic properties, with either the carboxylic acid or the amino group typically being in a charged state at any given time.

At this stage the amino acid molecules are not yet large enough to warrant larger (>200 Å) pore size silica, so a more common 100 Å silica template can still be employed. The real challenge here being based around the correct choice of buffer, pH, derivatisation reagent and stationary phase to encompass the varied hydrophobicity across the spectrum of 22 molecules. A combination of sodium acetate and triethylamine (TEA) along with a polar-endcapped stationary phase provides a suitable separation in one 25 minute run (Figure 2).

Protein Separations

Coupling of several amino acids will increase the size of the molecule, and will eventually result in the formation of larger peptides and proteins. The larger molecules will present differing problems to that of the 'simple' amino acids, such as complexity in size and conformation, and greater sensitivities to environmental factors which can alter the properties of the compound. These larger molecules provide new challenges including a differing mechanism of retention and therefore separation. Larger biomolecules adsorb to the hydrophobic alkyl chain surface of the stationary phase by a 'hydrophobic foot' (Figure 3) and are eluted once a specific concentration of organic modifier is reached necessary to cause desorption, as discussed in the use of Geng and Regniers 'Z number' [2]. Access to the pore structure and therefore the surface area and stationary phase plays a more critical role with these large molecules, what is not wanted is an exclusion effect that sweeps the large proteins through the column with little or no retention. Analysts will typically move to a 300-400Å pore size silica in order to account for this. Larger pore size silica's (upto 1000Å) are available for Size Exclusion Chromatography (SEC) but these can also lead to issues with retention since pore size and surface area are intrinsically linked, hence bigger pore size leads to smaller surface area.

Diffusion of large molecules is also affected by the type of silica particle morphology used, Gritti showed how the use of core-shell particles could lead to a reduction in the C term [3] in the van-Deemter equation when compared with fully porous particles as the large molecules show a slow diffusion. Efficiency in large molecule analysis is a combination of the exclusion and the diffusion of the molecule from and across the porous structure. To obtain the highest efficiencies and therefore resolution of large molecules

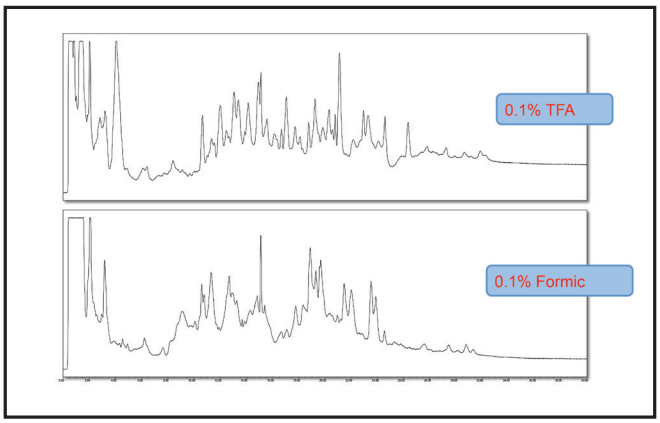

Figure 4. Separation effect of differing buffer, TFA vs Formic acid

requires large core-to-particle ratio and a 200-300Å pore size. Reducing the c-term with a core-shell particle due to this smaller pore/core ratio is therefore positive in reducing band broadening. Investigation into commercialisation of fully non-porous materials is also underway to ascertain whether a non-porous particle will bring even more beneficial characteristics to the separation process.

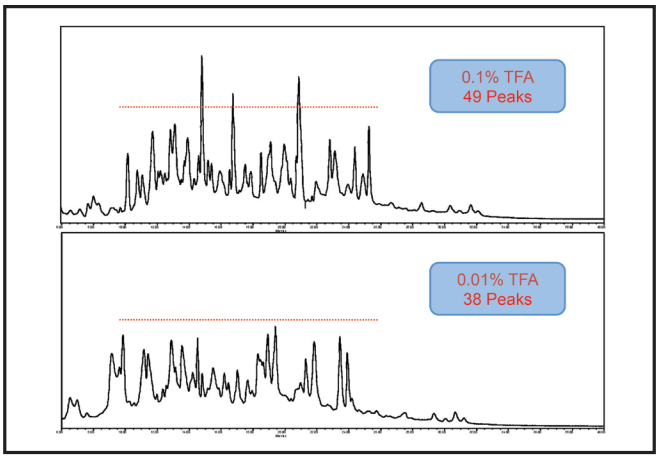

Figure 5. Effect of buffer concentration on resolution of peaks

Chromatographic Requirements

The choice of buffer and mobile phase in biomolecule analysis is quite limited from a traditional LC perspective. Trifluoroacetic acid (TFA) is the most common mobile phase modifier primarily used as a pH control and an ion-pair reagent, although formic acid has found use also. It can be seen in (Figure 4) using a UHPLC column (1.7um FortisBIO) how the selectivity in 0.1% TFA vs 0.1% formic acid is very different, chiefly due to the ion-pair ability of TFA, much greater resolution being achieved in TFA. Concentration of buffer (Figure 5) can also play a significant role in the number of peaks detected, and this is an area to be optimised for each set of molecules.

Whilst methanol could be used, acetonitrile is the most common organic solvent. Acetonitrile has become the standard due its greater ability to operate at low <210 nm [4] wavelength. As discussed previously the adsorption of the proteins and peptides is very sensitive to organic modifier, which makes isocratic separations something of a rarity. Gradient elution is most common, typically with very shallow gradients being successful at separating structurally similar proteins.

Chromatographic Considerations

Due to the low abundance of many of these new compounds of interest, the need for high sensitivity is a major requirement; hence the use of mass spectral detection as a sensitive, selective detector has become more and more prevalent. The use of electrospray ionisation (ESI) revolutionised the analysis of proteins. In combination with time-of-flight (TOF), Orbitrap™ and Fourier transform ion cyclotron resonance (FTICR) it allows for huge amounts of structural resolution data to be obtained [5].

The problems lie in the use of incompatible mobile phases with the mass spectroscopy (MS) spray. Whilst reversed phase (RP) chromatography mobile phases are volatile enough to be sprayed in ESI, the use of TFA previously discussed as a useful ion-pair reagent can act as a suppressor of the ESI spray and therefore have a major influence on the signal strength obtained and the ensuing sensitivity of the protein/peptide detection.

Sensitivity will also be hugely affected in modern chromatography by the choice of chromatographic particle. Over the past 30+ years particle size choice has changed significantly moving from irregular 30-50 µm silica, to 10µm spherical particles, then 5µm and then 3µm spherical particles. With decreasing particle size, we observe increasing detection sensitivity. Recently two different particles are becoming commonplace, namely 1.7µm particles used for Ultra High Pressure Liquid Chromatography (UHPLC) and core-shell (fused-core) particles for high efficiency, low backpressure separations. Both if used correctly can offer the analyst an increase in throughput, efficiency and resolution. Due to impact of pressure on the confirmation of proteins, very different separations can be achieved with the two techniques, inversion of the hydrophobic/hydrophilic ratio leads to weaker retention if hydrophobic nature is distorted between native and tertiary globular forms.

Broad peaks in biomolecule analysis are an issue caused by the slow radial diffusion of large molecules, which is a problem not seen when analysing small molecules which have a greater diffusion rate and so there are less equilibration effects observed due to the 'c' term of the van Deemter equation. If we wish to analyse complex samples such as peptide digests with high resolution, then peak shape will play a significant role. To achieve highest peak capacity on these separations we require sharp efficient peak shapes from the stationary phase. High surface coverage of the chosen ligand, with a pure silica inert substrate, typically C4 or C18, will help reduce secondary interactions, and reduce silanol effects that cause poor peak shapes. Bio stationary phases can be investigated in a similar fashion to small molecule columns using Tanaka, Synder, or Euerby et. al [6-8] to probe the silica surface for detrimental residual silanols. Although this is an area currently overlooked and is open to greater evaluation.

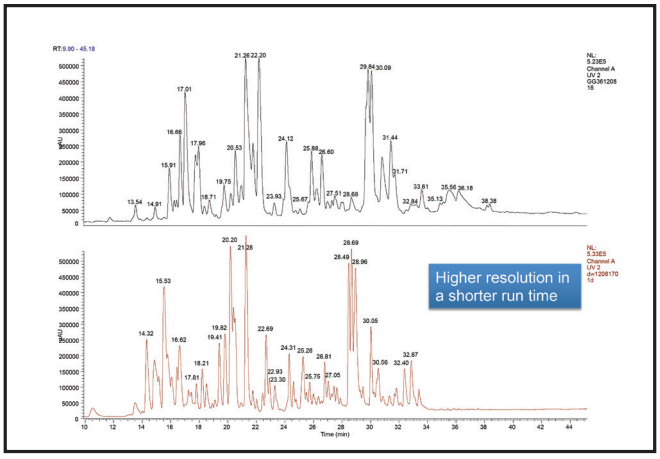

Figure 6. Improvement in sensitivity and resolution using UHPLC. A) 5um Vydac RP18 150x2.1mm b) 1.7um FortisBIO 100x2.1mm

Moving forward…

UHPLC offers high speed and high sensitivity separations, however the cost is the high backpressure generated and the requirement for specialist UHPLC instrumentation and the costs associated with acquisition. When comparing UHPLC with HPLC for bioseparations it can clearly be seen that UHPLC offers greater sensitivity and

resolution of the same sample even when using a shorter column length (Figure 6). The high efficiency of the separation is due to the small 1.7µm particle being used, since efficiency is inversely proportional to particle size.

In comparison the newer core-shell technologies offer high efficiency and potential for reduced analysis times without significant backpressure increases over traditional fully porous particles of

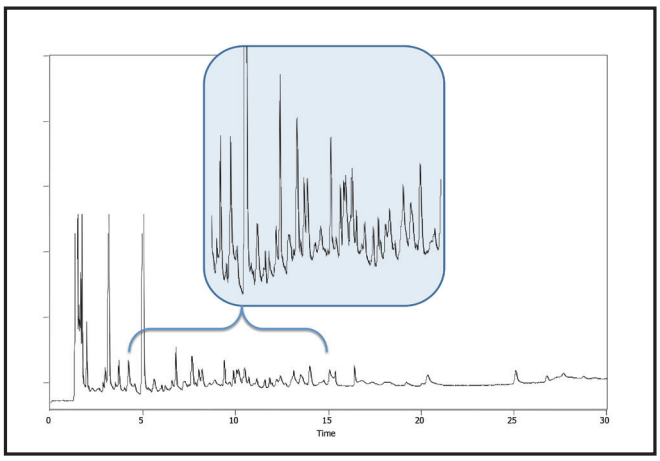

Figure 7. Use of a core-shell particle to achieve high resolution. 2.6um SpeedCore BIO C18 A: 0.1% TFA in Water B: 0.1% TFA in MeCN 10-90 % 60min gradient

equivalent size. This leads to the ability to operate standard 400bar LC systems with a variety of column geometries and flow rates. These particles become applicable to all pharmaceutical methods created and the necessary iterations such as impurity profiling, parent drug purity, preparative scale up and manufacture and QC. Transfer between global sites, transfer to CRO and other associated industry partners is also achievable due to the resilience, reproducibility and robustness of methods.

Core-shell particles can be used to increase the resolution of components in complex mixtures using shallow gradients. We highlight the use of an experimental core-shell column with large pore size diameter for molecular access, but a low pore volume to particle size ratio helping to reduce the c-term (Figure 7). Biomolecules prefer slower flow rates than small molecules so having a stationary phase/particle with low dispersion due to a smaller diffusion path is most advantageous.

One of the possible limitations of core-shell technology is the loading factor. In comparison to fully porous particles approximately 65% loading capacity is achieved [9] due to the reduced pore volume. The lower surface area of these materials has been shown to compromise the quantity of compound that can be injected on column. This may be less of a problem at the R&D stage of molecule design but could be a factor later at the production stage where both resolution and loading have a great impact on the recovery of pure drug that can be achieved.

Each technique has its advantages and drawbacks the analyst can select either particle technology and achieve a high efficiency, high speed separation, but should at the same time be aware of the limitations of each technique.

Conclusion

Our discussion has centred on the many challenges that we face in achieving acceptable separation, resolution and sensitivity in large molecule analysis. The sheer diversity of molecule size, conformation, charge and polarity will lead to varying interaction mechanisms being required. The number of fragments, charges and conformation multiply the complexity once the protein begins to denature or fragment. It was seen how the use of ion pair reagents is both an advantage and a disadvantage at the same time within potential chromatographic – MS methods, and this is just one of the compromises that must be overcome.

Accurate qualitative analysis of large molecules with low abundance will be aided by the use of advanced MS instrumentation and the use of chromatography columns containing small particles or core-shell particles, both of which offer the ability to obtain high efficiency, higher speed separations.

There are many challenges facing the biologics market, complexity of the samples, understanding the genetic make-up of therapeutic target areas, the manufacture costs, premium pricing, increased pressure from biosimilar approvals, quality standards and product innovation. Despite these hurdles the biologics market is forecast to be a high growth area in the foreseeable future.

Similar challenges in the pharmaceutical arena have been seen before with small molecules and generics in the last 20+ years. In the meantime, we in the chromatography industry must stay ahead of the game in order to serve the needs of the analyst in their drive for progress. This will not be easy as the samples are so complex and with some unique and challenging concepts, but as always we will strive to produce products that can provide the gains in sensitivity, throughput and resolution in order to meet the challenge.

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