Advances in Bioseparations

The topic of Bioseparations is gaining importance in the separations science community with the increased appearance on the market of pharmaceuticals derived from biotech origins. The Chrom Soc had not addressed the topic in a dedicated meeting for over 6 years hence this meeting at GSK, Ware organised extremely competently by Greg Jonas and Ajit Shah from the society.

> ***"Moving away from the issues of speed and resolution and towards separation techniques saw Andy West from GSK present on the topic of 'Nano LC – Is it worth the effort?"***

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Richard Houghton from Quotient Bioresearch (formerly HFL Newmarket) made the first presentation and posed the question 'UHPLC - Just another technique for High Speed Bioanalysis?' His presentation comprised of an examination of the use of small particle sized stationary phases as used for enhancing the speed of analysis for both small and large molecules. Examples of both small and large biomarker molecules using a rather unique approach involving strategies developed when working in the field of proteomics to identify targeted proteins which are viewed to be indicative of doping with protein based therapeutics or gene transfer technologies on thoroughbred racehorses were shown. The method of analysis was LC/MS/MS with MRM and the outline methodology is shown in *Figure 1*.

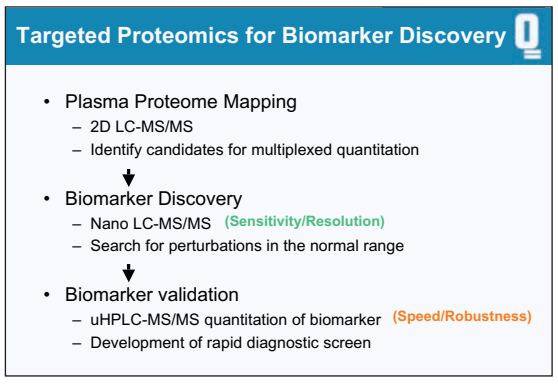


Figure 1. Workflow for biomarker discovery.

A summary of the different chromatographic techniques is shown in *Figure 2* below and from this the following conclusions were drawn;

Runtime - decreases as one progresses from nanospray to UHPLC.

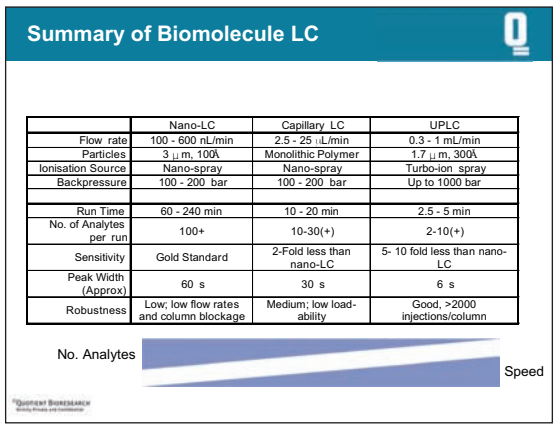
The number of analytes - that can be monitored in a single run goes from the 100`s in nanospray to 10 with UHPLC.

Peak Width – due to the resolving power of the nanospray but also peak widths. Narrow peaks in UHPLC means there is a need to limit the MRMs monitored to ensure the 15 data points across a peak for good P&A data.

Sensitivity – currently the best detection limits for protein quantification reported in the literature are 5 pg/ml.

Robustness – Nanospray was found to be 'fiddly' through to UHPLC which is very robust.

As a final thought the presenter offered the opinion that



	Nano-LC	Capillary LC	UPLC
Flow rate	100 - 600 nL/min	2.5 - 25 µL/min	0.3 - 1 mL/min
Particles	3 µm, 100Å	Monolithic Polymer	1.7 µm, 300Å
Ionisation Source	Nano-spray	Nano-spray	Turbo-ion spray
Backpressure	100 - 200 bar	100 - 200 bar	Up to 1000 bar
Run Time	60 - 240 min	10 - 20 min	2.5 - 5 min
No. of Analytes per run	100+	10-30(+)	2-10(+)
Sensitivity	Gold Standard	2-Fold less than nano-LC	5- 10 fold less than nano-LC
Peak Width (Approx)	60 s	30 s	6 s
Robustness	Low; low flow rates and column blockage	Medium; low load-ability	Good, >2000 injections/column

Figure 2. Biomolecule LC Summary.

monoliths were the best option in terms of column technology options since they offer opportunities to optimise speed (short columns) or resolution (long columns).

The second presentation was from Daniela Fraier from Bioanalysis PDM at Pfizer UK, who spoke on 'Solving bioanalytical challenges in an evolving discovery environment'. The type of work that defines bioanalysis at Pfizer was outlined in that it involves supplying bioanalytical solutions to bring compounds through from candidate nomination and clinical phase. It also demands rational approaches are developed to deal with the physiochemical diversity of a large number of compounds and corresponding large number of biological matrices. Furthermore there is a need to harness the synergies between general bioanalysis expertise and specialist biomarker knowledge in order to solve quantitative issues associated with challenging compound Chemistries. There is a final requirement that involves the implementation of novel analytical technologies and methodologies.

Involved in developing a bioanalytical strategy to meet the requirements necessitates analysing and overcoming the limits imposed on the various steps in the analytical process, viz;

- Optimising Method Development – this was helped by using a Decision making tool designed for method development and ACD/Chromgenius & ChemAxon/Marvin software used to predict a suitable extraction method.
- Reducing matrix effects – Defined in FDA Guidelines: "Interference from matrix components that are unrelated to the analyte" and broadly covers Ion Suppression & Enhancement along with Co-elution of components during the ionisation that affects signal intensity & therefore quantitation accuracy.
- Increasing speed and sensitivity – looking at UHPLC and Fused core technology options.
- Looking to both short and long term future technology shifts which may improve the efficiency of the Bioanalytical section – summarised in *Figure 3* below

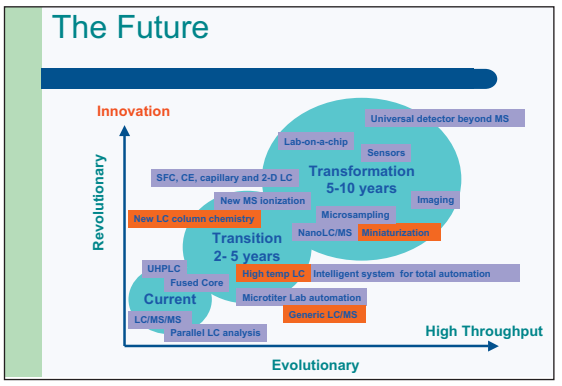


Figure 3. Future for bioseparation techniques.

Moving away from the issues of speed and resolution and towards separation techniques saw Andy West from GSK present on the topic of 'Nano LC – Is it worth the effort?'

The department in which the presenter works is the Analytical Biochemistry and Biophysics group and the responsibilities include Protein characterisation, Protein-ligand interactions, Post translational modifications, Cellular Pathways and protein analysis and MALDI imaging. A range of top end Mass Spectrometers from Thermo Fisher Scientific, Waters/Micromass, ABI/Sciex and Brucker and Nano LC Systems from wares (Nano Acquity), Agilent (Nano and Cap HPLC) and KYA Corp (DiNa direct nano flow systems) were used. However different ionisation sources and interfaces operate at different optimum flow rates eg Waters/Sciex prefer ca. 300nl/min but Thermo prefers 500nl/min. Also Sciex works better with higher organic content than the others, thus requiring an additional make up flow. A variety of application examples ranging from analysis of Endogenous Proteins, Chemical Proteomics and Phosphorylated proteins using "Phos Tag" technology (*Figure 4*) were conducted.

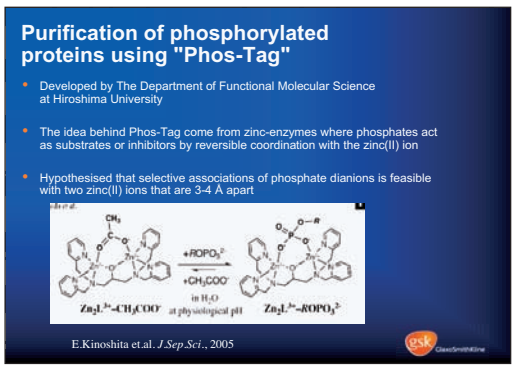


Figure 4. Phos-Tag modus operandii.

Summarising, the presenter concluded that Nano LC Provides sensitivity, resolution and is 'fairly easy' to use. The challenges remaining are to keep the system at optimum performance and the careful preparation of samples. Mass Spectrometry continues to improve and one of the challenges continues to be that of getting the sample into a suitable form to deliver it to the ion source.

The podium was then given over to Chris Smith from Astra Zeneca who spoke on the subject of ' Hot water chromatography for metabolite quantification using ICP-MS.' After outlining the theoretical reasons for using 'hot' (> 60C) water to decrease the viscosity of the mobile phase and hence allowing higher flow rates and lower pressure drops to be obtained an example of how the van Deemter curve is affected at different temperatures was shown (*Figure 5*) to illustrate that mass transfer effects are changed allowing more efficient separations at higher temperatures.

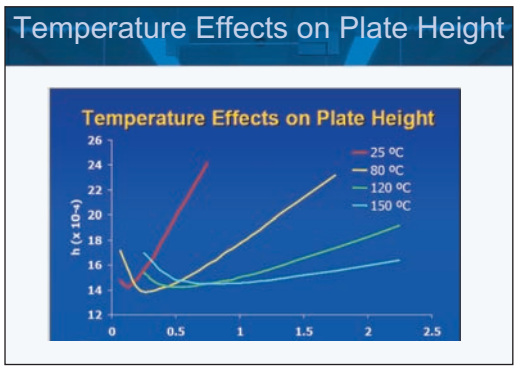


Figure 5. Plate height as a function of temperature.

Bearing this advantage in mind, coupling 'Hot water' with an analyser such as ICP-MS (Inductively Coupled Plasma –MS), which allows both qualitative and quantitative determination of, in this case, elements present in a sample, enhances both HPLC and MS detection. So why does ICP-MS have a presence in a DMPK laboratory? It is an element specific mass spectrometer that can be used to measure Halogens, Sulphur, Phosphorous and metals such as Pt and Fe already present (unlabelled). Results obtained are independent of chemical structure and suffer very little from sample matrix effects. This makes it ideal when one wishes to obtain impurity and metabolite profiling as well as excretion balances. It is similar to radio labelling without the need to radiolabel!!

A typical mechanism of profiling is shown in *Figure 6* below.

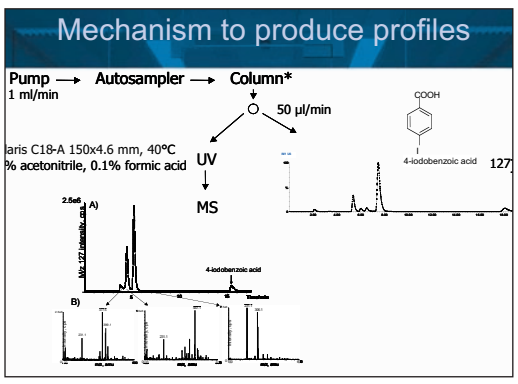


Figure 6. Profile mechanisms.

Professor Dave Perrett from Bart's and the London School of Medicine & Dentistry gave an enthralling and entertaining talk on the topic of 'Biomarkers in a Biomedical environment – yesterday and today'. He quantified the role of urine profiling in that its purpose is to generate patterns in biofluids in order to understand metabolism, to identify differences in patterns to aid diagnosis and to generate quantitative and qualitative differences i.e. Biomarkers. After reviewing the last 40 years or so of metabolite profiling experiments and equipment used he postulated (with examples) that much of the 'omics' research quoted today is merely a re-hash of previously accepted scientific facts. Never the less the advent of more selective and sensitive instrumentation has been shown to help diagnose certain conditions. An example using Capillary electrophoresis and cyclodexdrin additives to the mobile phase was shown which helps to diagnose Downs syndrome presence in patients (*Figure 7*).

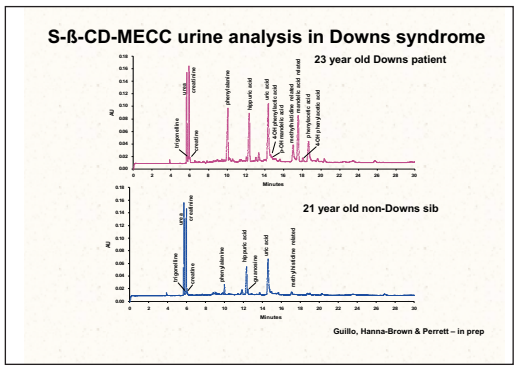


Figure 7. Urine analysis on Downs and non-Downs patients.

Following on from there the possibilities of using multidimensional techniques to further aid the identification of important metabolites within patient urine samples becomes realistic.

Such a generic 2D HPLC system was constructed using 2 independent high pressure binary gradients for both dimensions, two six port valves and a PDA UV detector for all the eluates. The 2D columns used were SCX/SAX Spherisorb and a C18 monolith .The scheme is outlined below in *Figure 8*.

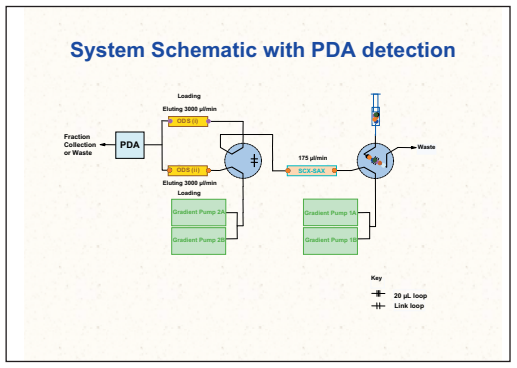
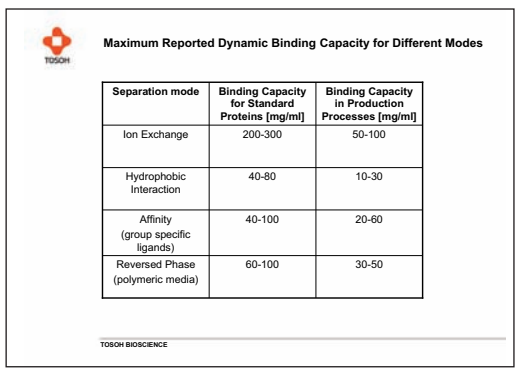


Figure 8. PDA Schematic.

The system was used to analyse samples obtained from patients suspected of having Adenose Deaminase Deficiency (ADA) or SLID sometimes known as 'Baby in a Bubble disease' and also Lesch-Nyhan Syndrome to aid diagnosis and treatment where possible.

Most of the presentations had centred on the use of silica based stationary phases but Egbert Muller from TOSOH BioSciences GmbH gave a presentation aimed at those present who worked with resin based supports. His talk on 'New IE & HIC Chromatography Resins for the Production Chromatography of Biomacromolecules (Properties and Optimisation Procedures)' was a review of the advances that had been made during the manufacture of resins by his company which had ultimately resulted in higher values of binding capacities, both static and Dynamic. Reported Values for different modes of chromatography are shown in *Figure 9* below.


Maximum Reported Dynamic Binding Capacity for Different Modes

Separation mode	Binding Capacity for Standard Proteins [mg/ml]	Binding Capacity in Production Processes [mg/ml]
Ion Exchange	200-300	50-100
Hydrophobic Interaction	40-80	10-30
Affinity (group specific ligands)	40-100	20-60
Reversed Phase (polymeric media)	60-100	30-50

TOSOH BIOSCIENCE

Figure 9. Binding capacity values.

The conclusions were that a substantial increase in binding capacity can be achieved for IEX by new polymeric surface modifications, for HIC resins increases can be achieved by a pore size optimisation procedure and Optical binding conditions for MAb's (Mono Clonal Antibodies) can be predicted by Zeta Potential measurements.

An interesting end to the day was provided by Louise Royle, Ludger Ltd who spoke on the challenges involved in ' Glycan analysis for biopharmaceuticals'. Glycans are polymers of monosaccharide residues linked in branched or unbranched chains. Glycosylation is most commonly associated with either an N-linked glycan or O-linked versions.

An individual glycoprotein is a heterogeneous population of glycoforms that can be dependent upon growing conditions or cell type etc. The reason for the interest in Glycosylation is due to the fact that it is the most common post translational modification in biopharmaceuticals and as such has an impact upon Regulatory issues such as safety and efficacy and from a commercial viewpoint of patentability and consistency of products.

Effective Analysis and Monitoring of Glycosylation throughout the Drug Life Cycle not only allows optimisation of glycosylation but also can also increase product potency, decrease the risk of adverse reactions, reduce the risk of batch rejects, reduce manufacturing costs and reduce times for product development and regulatory approval.

The importance of improving the speed of profiling Glycans and the advances shown in that area are shown in *Figure 10* below where analysis times have been reduced from 180 minutes to 30 minutes by moving to a 3µm column.

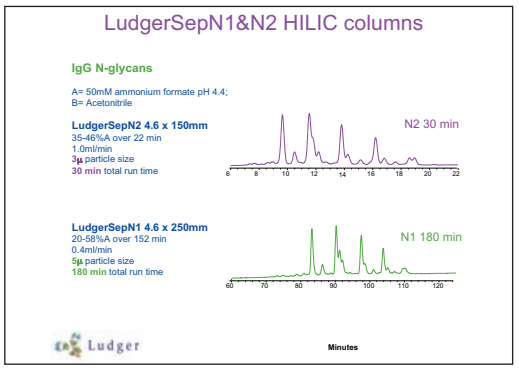


Figure 10. Improved separation times for IgG Glycans.

In terms of the contribution this makes to a full structural assignment of Glycans then this is outlined with complimentary techniques in *Figure 11*.

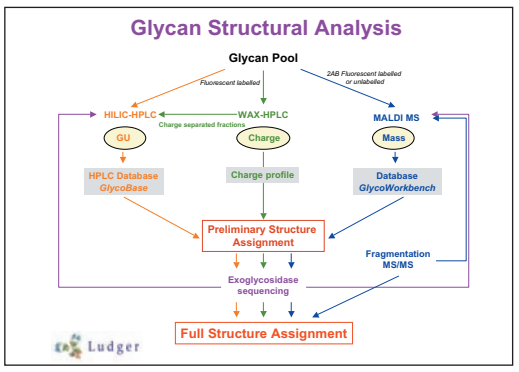


Figure 11. Pathway for full structure assignment.

SUMMARY

A different topic in many ways from the recent 'small molecule' centric meetings that have been organised by the Chrom Soc but nevertheless the topic of how to increase the speed of analysis even with Biomolecules still rears its head. The use of UHPLC appears to be creeping into the application area and the well-debated issues of small particles vs. monoliths vs. porous layers on solid centres will become more visible in the coming months. Also it is heartening to see that the resin manufacturers are advancing the properties of their offerings to the market place.