The Changing Face of Bioanalysis

by Howard Hill

This is a pharmaceutical industry perspective of the way bioanalytical technologies are changing in response to the changing types of molecules under development and the regulatory drivers required to "prove" effectiveness.

In this context bioanalysis is used to mean the measurement of drugs in biological fluids not the technologies used to elucidate and characterize the structure of biologicals.

1. Introduction

What are the drivers for change? Are they the same now as in past? What has changed now? Perhaps one of the earliest drivers for change was pharmacokinetics, a science whose origins can be traced back to 1930s, [1] concerned with the mathematics of drug absorption, distribution and excretion. It was not until the 1950/60s that it became widely used by drug developers to modify and monitor the development of their formulations. An important aspect of drug development was the need to relate the pharmacokinetics and metabolic profile of the drug in animal safety testing with those in Human. Nevertheless drug plasma levels still lacked a "direct" relationship to the pharmacological response. "We don't make drugs (in this case a diuretic) for their plasma levels, we make them for their effects" so said a Big Pharma Research Director when told in the 1970s that the techniques used to measure the plasma levels were too insensitive, although the physiological effects were obviously quantifiable.

2. Broadening the Scope of Bioanalysis

The development of pharmacokinetic / pharmacodynamic (PK/PD) models [2] in the 1970s` led to the rise of measuring pharmacological responses and effects. While short term effects were measurable e.g. antihypertensives reduce blood pressure, the question remained and still does do they extend the length of life or indeed the quality of life - major criteria in determining the value of a drug. This led to the wider use of markers of physiological response i.e. biomarkers.

While responses measured correlate closely with the plasma levels of the drug and help in understanding and evaluating the pharmacology of the drug, they do not necessarily predict the long term outcomes of therapy. Biomarkers which predict the ultimate response in life expectancy are few and far between and have become known as surrogate markers / endpoints. Nevertheless the development of the application of biomarkers, more recently driven by the FDA's Critical Path Initiative [3] has seen a dramatic rise in the quantitation of the type and number of biomarkers endogenous to plasma.

The bioanalysts role has therefore broadened into measuring both drug and biomarkers concentrations in biological fluids. The two are now irrevocably intertwined. Indeed there is a strong case to be made for seeing drug levels and pharmacokinetics as just another biomarker. Many of the earliest drug concentration assays were biomarkers. Following dosing of antibiotics such as the sulfonamides and later the penicillins, plasma levels were measured by the extent of inhibition of bacterial growth on agar plates, in reality one of the first in vitro biomarker assays.

The advent of gas liquid chromatography in the 1950s preceded by paper chromatography, thin layer chromatography, and not forgetting Tswett's contribution to liquid chromatography some 50 years earlier, are pivotal events in the analyst's ability to measure drug levels in biological fluids. Each one improving the specificity and sensitivity of the measurements achievable.

The development of new technologies was in many cases driven by the ability to meet an unmet need. Necessity is the mother of invention. It is, and always has been important to discriminate between a technology fulfilling a need and the use of technology just because it exists. This latter philosophy has led to development of laboratory cupboards full of interesting equipment; whose existence to management is kept a guarded secret. In earlier times when profit margins and growth in the pharma industry seemed to have no bounds this may have been forgivable. Today when the cost of instrument development is high and is matched only by the diversity of innovation in an ever expanding and technologically driven world, containing costs is a major driver. Manufacturers must cover their development costs (and more) – therefore the development of niches which may be scientifically clever and add interest for the bench scientist but raise the cost of the drug development programme may be difficult to justify.

So what do analysts now have in their toolbox, and what is likely to change?

3. Liquid Chromatography – Mass Spectrometry (LC-MS) the mainstay of Bioanalysis

LC-MS has been the major bioanalytical technique for almost 20 years, it replaced both LC-UV and ancillary detectors such as fluorescence, electrochemical detectors, as they developed after the introduction of HPLC in the early seventies. HPLC provided the perfect foil to Gas Chromatography which was originally developed to measure volatiles in the

oil and related industries, although it was rapidly adopted by the pharmaceutical Industry. Most drugs are polar in nature and not amenable to GC. However by judicious derivitisation techniques they could be made volatile and sensitive to detectors such as the electron capture detector, originally developed to quantify chlorinated pesticides while nitrogen phosphorus detectors were ideal for measuring organophosphorus pesticides and related environmental contaminants [4].

While a range of cumbersome interfaces for LC-MS were developed e.g. the moving belt, it was not until MS detector coupled with HPLC through the development of the Atmospheric Pressure Interface (API), that chromatographic detection and drug analysis was revolutionized, with increased sensitivity, increased selectivity, increased throughput and more rapid method development. The classical Sciex III was originally styled a biomolecular analyzer used in the elucidation of protein structure. Bioanalysts were now able do things they could not previously do (sensitive assays for polar compounds were significant triumphs), so much so the gas chromatography rapidly declined followed by HPLC. These techniques have not been totally displaced, and in the hands of niche providers with expertise they continue to thrive e.g. steroidal hormones, lipidic molecules which are largely insensitive to many of these detectors including MS, unless derivatised [5].

LC-MS was not without its limitations. Largely the dreaded matrix effect [6] where coeluting compounds modified the ionisation process and response of the analyte. While phospholipids are seen as major contributors to this effect, the need for the separative power of the column made a comeback and remains a powerful parameter in LC-MS. Indeed UPLC (ultrahigh pressure liquid chromatography), has breathed new life into LCMS improving separation while still maintaining short assay times.

UPLC (together with sub 2 micron particles based columns) has resulted in increased throughput and the possibility of increased sensitivity. In HPLC-UV this technology reigns supreme. Its uptake as the front end of LC-MS-MS has been slower, as many LC-MS methods in bioanalysis already have short run times – but with UPLC even shorter times with better resolution should be possible.

4. Nanotechnologies

The development of small scale techniques driven by developments in nanotechnology has resulted in new instrumentation, not necessarily designed for the bioanalyst but no doubt likely to impact on it in the future. The need for micro instrument e.g. mass spectrometers in space exploration are likely to lead to smaller lab based instruments [7]. In addition small sample volume, high throughput and robust instrumentation needs are also driving the process of miniaturization i.e. chip technology.

UPLC may have elements of miniaturization i.e. particle size of column packing but the rest of the infrastructure is significantly large albeit with a "small" foot print. Miniaturization not only accelerates assay times but significantly reduces the space needed to accommodate the equipment or a per sample calculation. This is not just a current trend driven by new technologies, the original magnetic sector machines e.g. LK 9000 coupled to a GC of the early 1970s was enormous when compared with today's ubiquitous quadrupole mass spectrometers. The use of chip based technologies has produced CE on a chip (attached to an MS detector)! We await chip based MS detectors or detectors which may be analyte specific but highly sensitive and commensurate with the size of the CE chips[8].

Many advances have been through the ability to hyphenate techniques such as the GC to the MS in the 1960s and LC to MS using the API interface in the late 1980s along with linked columns in heart cutting, multi dimensional chromatography and other variants on a theme, some varying in name only others with major technological differences. Indeed the success of bioanalysis has been the story of hyphenation; analysts have hyphenated to improve specificity and sensitivity and beyond, and they continue to do so eg LC – MS, LC – UV – MS, LC – UV; LC – (reactor, Chemical, Photolytic) – UV, LC – ESI – TOF, GC – MS, GC-MS-TOF etc. etc. all have a role in bioanalysis. While not all techniques have routine application in bioanalysis their problem solving capabilities in niche areas is unparalleled.

5. The Rise of Biologics

So is LC-MS here to stay? It has been unchallenged over 20 years albeit with many tweaks and variants. Is the future MS based or is there something else waiting in the wings? Supercritical Fluid Chromatography (SFC) [9] and its alter ego steam chromatography have been in existence for decades and about to become the next new revolution every few years. Also NMR, perhaps the ideal technology - minimal sample preparation, no chromatography, good specificity but… sensitivity? What are the limits of LC-MS what will the future look like, what are the drivers? Analysis of the problems facing the industry may give an indication of where the future lies.

Since the acceptance of LC-MS for the quantitation of small molecules, there has been the rise of biotechnology based products, alternatively known as biologicals. This chemical group is made up largely of macromolecules such as proteins (native and modified), monoclonal antibodies (also proteins), vaccines (protein and DNA based), oligonucleotides as well as a group of compounds/therapies classified as Advanced Therapies [10].

Many of these therapeutic molecules can be measured by what have become loosely known as ligand based assays. The American Association of Pharmaceutical Scientists (AAPS) recognised the "unique" features of these technologies by the development of a focus group, Bioanalytical Ligand based Assays [11]. Many of these techniques have been in existence for over 50 years in a wide variety of formats. While they have found use in measuring macromolecules and some small molecules in automated clinical systems, the application to measuring drugs in biological fluids has been limited usually to those drugs where achieving the required sensitivity by chromatographic techniques was not possible or in a limited number of cases where high sample throughput was pivotal to achieving timely regulatory submission). However, the development of LC-MS almost eliminated the use of this technology for small molecules.

The rise in biologicals, now projected to be 30-50% of the New Chemical Entities (NCEs) coming to the market in the next 5-10 years is driving development in ligand based assay techniques. Growth in small molecule NCEs has been less than 1% per annum over the last 10 years while the growth in large molecules has been about 25%. There may not be a fall in the absolute numbers of small molecule NCEs – but the future growth in NCEs, is biologicals [12].

Thus the small molecules NCEs market may be regarded as mature in terms of drugs to market but also the technologies used to quantify them in biological fluids. The major development in new technologies over the last five years has therefore been driven by this rise in the growth of biologics. Concomitantly there has been a rise in the role of biomarkers in drug development; many of these biomarkers are macromolecules and the development of biological drugs has driven the need to measure a wide variety of biomarkers produced in response to these therapies. Frequently biologics toxicity is an exaggerated pharmacology in a desired therapeutic effect, largely caused by getting the dose and ergo the plasma concentration wrong. The Tegenero (TG N142) episode is a typical biologic, which at excessive dose levels initiated the so called cytokine storm of biomarkers which are readily quantifiable in biological fluids. Allegedly over 100

inflammatory mediators such as TNF alpha and Interleukins e.g. IL1, 6, 10 as well as a range of other inflammatory modulators are released by the immune system are released during this storm [13].

Thus while biological drugs and biological biomarkers share many physico-chemical features the terminology should not be used interchangeably. Nevertheless together they have driven the need to measure more and more macromolecule analytes using ligand based assays. While the 1960s and 1970s saw the development of a "daunting" range of formats (Table 1), akin to the chromatographers ability to hyphenate, the 1980s saw the development of 96 well plates for immunoassay which increased samples analysis throughput. Although incubation times were not significantly reduced, it was largely the ability to automate the sample preparation process, reading the results in a compact format and saving space and materials which drove this development.

Table 1: Immunoassay Formats

Radioimmunoassay	RIA
Immunoradiometric Assay	IRMA
Enzyme Immunosorbent Assay linked	ELISA
Enzymatic Multiplied Immunoassay Technique	EMIT
Fluorescence Polarization Immunoassay	FPIA
Substrate Labelled Fluorescent Immunoassay	SLFIA
Anitgen Capture Enzyme Immunoassay	Ag CEIA
Antibody Capture Enzyme Immunoassay	Ab CEIA
Dissociated Enhanced Lanthanide Fluorescent Immunoassay	(DELFIA)

Recent developments like the Luminex® [14] and MesoScale Development (MSD)® electrochemical luminescence system [15] are examples of multi-analyte systems, the former using beads with a range of antibodies attached, while the MSD system uses a well system with multiple antibodies attached. Not all assays are mutually compatible in one "analysis". It is not possible to pick and mix the assays of choice in one system. Effectively the assay time is reduced by the ability to analyse multi analytes at one time, even though incubation times may not be much different from conventional 96 well plate systems.

Another major use of these technologies is to screen the body's response to neutralizing antibodies. As their name implies these antibodies bind with the drug in the body and neutralize the effectiveness of the drug, it is essential that the potential incidence for this in clinical trials is determined as this can have a major impact on the interpretation of PK data. While the format of assay changes, the technologies used to screen for the possible presence of neutralizing antibodies are the same as those for measuring plasma levels of the drug.

While sample preparation and plate reading technologies have no doubt accelerated assay throughput, in the past few years the acceptance of the Giros® technology [16], which uses microflow driven by centrifugal forces on a compact disk format has led to a dramatic reduction in assay times. The use of nanotechnologies / microfluids / miniaturization has led to increased reaction times, such that incubation times of less than four hours are possible for a single run. The throughput can therefore be increased to several thousand samples per week. While these technologies are readily usable for analytes for which reagents are available the ease of application to drug specific assays for PK is still evolving. Indeed a major limitation of immunoassays is the time consuming and sometimes serendipitous approach to achieving good sensitivity and specificity through the raising of appropriate antibodies [17]. So what about alternatives using chromatographic technologies?

Could LC-MS replace immunoassay as a technology for the quantitation of macromolecules? While the ability of LC-ESI ToF and SELDI-ToF based machines are able to discriminate between molecules differing in only a few Daltons, is this really an advantage? It is likely that immunoassay selectivity is not specific to small changes in the overall structure of the molecule. Indeed as long as there are no changes in the epitope, changes in the rest of the molecule eg. loss of a few methyls here a few hydroxy groups there, may not influence potency or the ability to bind with the epitope. In short ToF based assays could be too specific. On the other hand, the ability of LC-MS to accurately quantify large peptides following hydrolysis of the protein is well established. It may be possible therefore to identify a peptide, post hydrolysis, specific to the molecule of interest which can be accurately monitored and quantified which reflects the potency of the drug but not ephemeral changes in the drug structure.

So what of the holy grail of bioanalysis - you place a sample into the "sampler" and you just wait for the numbers (analogous to the clinical chemistry analyzers like the Hitachi 7600 Clinical Analyser). While this may be possible for established biomarker molecules, for drugs and biomarkers requiring highly sensitive optimised detection systems this may not be possible - but it should be possible to get close. On line sample preparation coupled with short selective chromatography and miniaturised MS detectors interfaced to an intelligent data system i.e. one that can review chromatographic quality, monitor parameters such as retention time drift, peak shape etc in real time, review QC parameters and automatically stop batches of samples if out of specification may be one path forward. It is possible that this could become a routine technology and if, when LCMS replaces immunoassay, will it be an automated protein hydrolysate of a plasma sample, followed by isolation and quantification of a peptide specific to the protein of interest. If so LCMS will be safe for another twenty years or more [18].

6. Summary

Historically there have been different drivers as to why we need drug / plasma levels in biological fluids. In addition to well established need for pharmacokinetic data the other current major driver from a regulatory perspective is the FDA's Critical Path Initiative relating to the role of biomarkers.

In many cases the technologies used by bioanalysts have not been developed for measuring drugs / biomarkers in biological fluids. They are technologies which have been adapted, modified, improved and hyphenated in order to make them adaptable for bioanalysts.

While current "regulatory" guidances on Methods Validation sets the boundaries for analytical methods they are not nor should they be the drivers for new technologies.

The growth in biological drugs has already accelerated the growth and diversification in ligand based assay technologies, which are not chromatographically related – yet; when will chromatographic techniques become a major competitor to ligand based assays in quantification of macromolecules in plasma?

The drive for miniaturization, accelerates the need for smaller (cheaper) versions of established technologies, producing shorter assay times through more rapid reactions and the use of smaller volumes i.e. increases productivity and reduces space and analyst time per assay.

So the future is smaller, faster more specific and as always for the "routine" mass market technologies must be robust. However there will always be a need for the smarter multi hyphenated technologies that are pivotal problem solvers. But whatever, chromatography is here to stay and LC-MS its flagship.

6. Glossary of Abbreviations

LC	Liquid Chromatography (used as an abbreviation for HPLC)
GC	Gas Chromatography
MS	Mass Spectrometry
HPLC	High pressure (performance) liquid chromatography
CE	Capillary Electrophoresis
ESI	Electro Spray Ionisation / Interface
ToF	Time of Flight
SELDI	Surface Enhanced Laser Desorption Ionisation

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