A GUIDE TO EFFECTIVE METHOD DEVELOPMENT IN BIOANALYSIS
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DEVELOP MS/MS METHOD

Evaluate MS response and develop a multiple reaction monitoring methodology that provides the optimum MS conditions for detection of the compounds of interest.

DEVELOP LC METHOD

Robust chromatography will ensure the analyte(s) of interest separate from both endogenous interferences and drug metabolites.

DEVELOP SAMPLE PREPARATION METHOD

Preparing the sample for analysis is critical to ensuring that the assay conforms to recognized standards of compliance and performance. Analytical performance – whether sensitivity, reproducibility, or throughput – depends on a clean sample.

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Developments in bioanalytical technology and the application of pharmacokinetic (PK) principles have created a synergistic partnership that plays a vital, influential role in the discovery and development of new medicines. The origins of this partnership can be traced back some 30 years, and an understanding of the way in which the application of these two scientific disciplines has evolved is helpful in ensuring we make optimal use of current analytical technology.

Whilst the principles of pharmacokinetics (PK: the study of the change of drug concentrations with time) were first defined in the 1960s, the application of PK in optimizing drug therapy and evaluating bioavailability was truly made possible by the skills of the analytical chemists who pioneered the development of HPLC in the 1970s.

During the 1980s, HPLC-UV based assays routinely provided the plasma concentration data that were used to define drug exposure in test animals and in human subjects; the two main parameters of drug exposure being area under the plasma concentration-time curve (AUC) and maximum plasma concentration ($C_{\text{max}}$).

At this time PK was applied mainly as a descriptive science, essentially defining what happened to the test drug when it was administered to an animal in toxicology or to a healthy human subject in Phase I clinical pharmacology. Drug safety and tolerability, plus the determination of PK parameters like plasma clearance, volumes of distribution, elimination half-life, and bioavailability were and still are the objectives of Phase I studies.
The relationship between AUC and $C_{\text{max}}$ and key PK parameters such as plasma clearance, bioavailability, and bioequivalence are shown in Figure 1 in order to illustrate how PK principles are applied at various stages of the drug development process.


In Phase I, a poor PK profile would usually be characterized by low and/or highly variable oral bioavailability or a short elimination half-life. During later stages of clinical development, when the drug is evaluated in the intended patient population (Phases II and III of drug development), the incidence of adverse side effects attributable to drug metabolism or drug-drug interactions also led to termination of potential new medicines.

In all of these situations, an understanding of the plasma clearance mechanisms of the test drug would have provided an early warning of potential problems with the compounds. Furthermore, such an understanding could potentially have helped to avoid the progression of a poor drug candidate into clinical development studies and helped to select an alternative compound with a superior PK profile.

Thus demand arose to expand the application of PK and quantitative bioanalysis into lead-candidate optimization during drug discovery.

Progression to increased potency in pharmacology in the late 1980s was already placing extra demands on HPLC-UV based techniques. Whilst the use of fluorescence detection for LC – and on rare occasions GC/MS – could be used to improve assay sensitivity, neither approach was widely applicable for routine drug analysis. The desire to apply PK principles to drug discovery would not only require an improvement in assay sensitivity, significant increases in analytical capacity and faster sample turnaround would also be needed.

The analytical technology of the early 1990s appeared to have reached its limits, but the commercial development of atmospheric pressure ionization as an interface for LC heralded a revolution in bioanalysis. Within a few years, LC/MS moved from being the method of last resort for quantitative analysis to being the method of choice; the hope that pharmacokinetic principles could be applied extensively as a tool in the lead-optimization and candidate selection stages of drug discovery could, at last, be realized.

With the acceptance of LC/MS/MS as the gold-standard technology to support PK and drug metabolism studies comes significant improvements in assay sensitivity and specificity, plus the potential to reduce assay time per sample and consequently have a massive increase in the demand for quantitative drug analysis.

Generating vast quantities of analytical data is much easier and faster than ever, consequently a basic grounding in pharmacokinetic principles is helpful in appreciating how bioanalytical data are to be used in drug discovery and development. Here we will provide some examples of possible analytical strategies and an overview of the context for applying these in selected phases of drug discovery and development.
The technology available for bioanalysis has evolved over the last 30 years, with an explosion in progress over the last 10 years due to the practical application of MS, the evolution of column packings, and novel solid-phase extraction (SPE) techniques. Most recently the application of a revolutionary new technique, Waters® UPLC® Technology, has allowed the industry to further capitalize on the utility of MS and drive the limits of sensitivity and speed of analysis to new levels.

Figure 2. The changing landscape of chromatography technology in bioanalysis.
BIOANALYSIS IN THE DRUG DISCOVERY AND DEVELOPMENT LIFECYCLE

The bioanalytical process

The lead optimization/selection, confirmation, and testing process for new drug candidates is well-defined as a series of activities. Broadly, these can be split into discovery, lead optimization, and preclinical development, through to clinical evaluation (Phases I to IV).

Each stage places different requirements on the bioanalytical assay used to provide information. The use of LC/MS/MS assays provides the specificity, flexibility, and sensitivity to enable fast and effective decision-making at each stage.

PK and bioanalysis in drug discovery

Full PK characterization in the drug discovery phase is not required; however, in conjunction with in vitro techniques, the ability to quickly assess the bioavailability of a compound through bioanalysis gives a good indication of suitability for advancement to development.

Some analytical priorities are:
- Fast pass/fail determination of PK parameters
- Medium-sensitivity assay
- Minimum assay development
- High specificity for the compounds of interest

PK and bioanalysis in efficacy and safety studies

Phase I: First time into humans
The key requirements for this stage are that the assay must fully characterize the absorption and elimination phases of the plasma concentration-time curve. All metabolites must be fully resolved, identified, and quantified.

Adverse effects of a drug (toxicokinetics, TK) are investigated and require accurate measurement of AUC and Cmax after single and multiple doses. In this way the “no-toxic-effect dose level” can be established, a key parameter when dosing in first-time-into-human and further trials.

The demands placed on the bioanalytical assay are for:
- High sensitivity to ensure that the lowest effective doses can be identified
- High specificity to identify and quantitate metabolites
- Moderate throughput; sample groups are small
- Full validation is required

Phase IIa: Proof of concept
The drug compound is tested in small groups of patients to assess efficacy in treating the disease state. PK analysis is employed to assess the dose/exposure response (PK/PD). This is another key stage in deciding whether the drug should progress further through clinical trials and therefore incur the investment required.

Phase IIb
Dose ranging studies are carried out on patients to establish effective doses for Phase II trials.

Analytical priorities include:
- High-sensitivity assays
- High-specificity assays for drug compound and metabolites
- Fast turnaround of samples

Phase III: Long-term studies
Large numbers of patients take part in Phase III clinical trials with the objective of showing efficacy across a wide range of populations. Vast numbers of samples must be handled and analyzed with a bioanalytical assay that is specific, robust, and fast.
- Assay specific to very few analytes
- Robust to variations in matrix
- Ability to process very large volumes of data
Product life-stage management and bioequivalence studies

For marketed drugs, the application of PK and bioanalysis is predominantly focused on the evaluation of line-extension products, such as controlled-released formulations, and on the evaluation of generic formulations.

Relative bioavailability and bioequivalence determinations are both reliant on quality plasma-concentration/time-curve data: AUC, C_{max}, and occasionally T_{max} are the decision-making parameters for the test product.

In a bioequivalence study, the aim is to demonstrate that two formulations of an entity have the same bioavailability, to within certain limits. Regulatory requirements (e.g. U.S. FDA, EMEA guidelines) for such studies are explicit; thus the FDA guidance for generic products states that the “rate and extent of absorption are not statistically different” for test and reference formulations of the same molar dose.

In experimental pharmacokinetics, the term “absorption,” as used by the FDA, means systemic bioavailability (AUC_{0-∞}) and the statistical requirement is that the ratio of the two bioavailabilities must lie within the limits of 0.80% and 1.25% for test versus reference formulation. For a full explanation of the requirements, refer to the FDA guidelines.

For the bioanalyst, time spent on optimizing recovery of analyte from SPE sample preparation and adopting strategies for eliminating matrix effects for LC/MS/MS will be rewarded by improved assay performance. A small CV on the assay data will reduce the number of samples that need to be assayed for each study, as the need to have large numbers of subjects in studies to get over the problem of high variability can be avoided.

Assay sensitivity is also important in bioequivalence studies as this helps ensure that the elimination phase of the plasma-concentration/time-curve is defined fully.

If the lower limit of quantification (LOQ) is high, this can cause problems when estimating the elimination rate constant. This in turn can cause a problem when calculating the AUC_{0-∞} as this is derived using the elimination rate constant to estimate the part of the concentration curve from the time of the last quantifiable concentration to infinity (∞). A small CV on assay data will improve accuracy in determination of the elimination rate constant and elimination half-life.

It is important to acknowledge that many factors contribute to variability in the conduct of bioequivalence studies. Inter-subject variability is an ever-present factor in pharmacokinetics; some of the variability can be explained by differences in expression of drug metabolizing enzymes in the liver and gastrointestinal tract from one subject, and there can also be genetic and ethnic influences with some drugs. Thoughtful study design is vital in controlling variability and, as we indicate above, the efforts of the bioanalyst can have a tangible and beneficial impact.
BIOANALYSIS: FROM VIAL TO FILE

OVERVIEW

Pharmaceutical companies – small and large, generic and innovators, plus the companies that serve them – face endless challenges.

At a corporate level, this may be to ensure that they are first to market with a new drug and enjoy a level of appropriate patent protection.

For companies like CROs, servicing the needs of clients quickly and profitably are key factors to success.

One of the ways to ensure a competitive position and success in the highly-competitive life sciences marketplace is to apply the advantages of innovative laboratory technologies to foster achievement and exceed business goals.

Liquid Chromatography (LC) is a technique commonly used throughout the pharmaceutical industry since it lends itself very well to the analysis of drug compounds across the whole of the research and development and quality control process. The flexibility in today’s number and types of hyphenated detection techniques, such as mass spectrometry (MS), allows quicker and more accurate quantification and identification, enabling critical analysis and decision-making events to be made earlier in the drug development process.

Analytical challenges in bioanalysis

The use of LC/MS/MS for bioanalysis is accepted as the analytical tool of choice for both selective and sensitive detection of compounds of interest in difficult matrices.

For laboratory analysts, developing an LC/MS/MS method that conforms to all the requirements laid out for a regulated environment has its own layers of challenges – in addition to ensuring that this method achieves business goals of producing results efficiently and effectively.

Waters has a history of technical innovation and applications experience designed to address these challenges. Our unique understanding of laboratory needs can help the laboratory scientist successfully streamline the analysis of bioanalytical assays.

Steps to successful method development

In this applications notebook, we address the challenges faced when developing an LC/MS/MS method for bioanalysis.

A well-thought-out, simple approach is often the best way to start and will be applicable to the larger proportion of compounds that will be encountered. Following the outlined approaches will allow the development of a method without having to consult a more experienced analyst or bring in consultants.

This approach, coupled with innovative advances in LC/MS/MS technology from Waters, will improve the speed of bioanalytical method development, provide excellent results, help ensure compliance with regulatory standards, and increase the speed of analysis.
INTRODUCTION

The development of an LC/MS/MS method requires three separate methodologies to be developed:

- Sample preparation
- Chromatography
- Multiple reaction monitoring (MRM) mass spectrometry

An optimized LC/MS/MS method encompassing these three techniques may require an iterative approach since changes in one can affect another.

- An LC method is required to determine the efficiency and cleanliness of the solid-phase extraction process.
- An MRM MS method is required in the optimization of the LC method, to confirm resolution of the compounds of interest from endogenous metabolites or other interferences.
- Logically, the first method developed is the MRM MS method. This allows the scientist to confirm that the compounds of interest are suitable for this detection technique, and it also provides information about other potential ions in the sample.
- Furthermore, the MRM method can be optimized to reduce the chances of spurious transitions that can compromise the integrity of the technique in the context of the fully developed method.

Thus the process of method development starts with a process of several iterations, and is followed by testing the method and making further adjustments to obtain the final method. Figure 1 demonstrates this end-to-end method development workflow.

Figure 1. Method development workflow for bioanalysis or bioequivalence by LC/MS/MS.
Understanding the challenges of method development

Critical to obtaining an appropriate method is a full appreciation of all the key factors involved with bioanalytical studies. For example:

- **The matrix** – Plasma, serum, urine, and tissue are common matrices and have differing implications for clean-up and accounting for interferences in the sample.
- **The number of samples** – And, consequently, the number of analyses. In a high-throughput environment, the suitability of a method for automation can prove critical.
- **Urgency** – To meet a required short turnaround time, analytical speed is balanced against other considerations. Automated processes can play a role.
- **The number of analytes to be quantified** – If there are many analytes, this will determine the complexity of the separation and the scope of the method development process.
- **Required level of sensitivity** – Arguably, the primary consideration should be that detection and quantitation of the lower levels of analyte is possible.
- **Pharmacokinetic profile of the analyte** – An understanding of the PK profile will allow the scientist to determine the lower levels of quantitation required.

Another important consideration is from where in the drug discovery and development process the samples are derived.

For example, if the samples are from first-time-into-human studies, resolution is more important than throughput, since the number of samples is low but the fate of the compound of interest is unknown. Of key importance is the resolution of the peak of interest from all drug-related metabolites and other endogenous compounds.

In the later Phase II/III and IV clinical studies, the human metabolism of the compound would have been completely characterized and the number of samples increases to the thousands. High throughput may not be the best choice as compared to improving analytical efficiency. Advantages can be found in automating processes as well as fast, sensitive, and reproducible chromatography with detection and data-capturing capabilities that can deal with the amount of data being generated.

The process of method development

The method development process can be broken down into components with a logical progression. In practice, the process is more a series of iterative and interlinked steps that may often need to be revisited and adjusted to finally arrive at the best method.

Fully understanding these interdependencies and the requirements placed on the assay by these challenges leads to an assay that is fit for purpose.

Since the method will ultimately be carried out using a real sample, it is good scientific practice to obtain some representative samples for the method development process. For example, if the method is to be used to treat patients who are diabetic, obtaining plasma from these type of patients may be critical to successfully developing the assay.

Using a representative sample often leads to the need for redevelopment of the LC, MS, or sample preparation step due to issues detected in one of the other processes. For example, a spurious MS transition may require the MS/MS method to be redeveloped to eliminate this effect, or the LC method to be modified to eliminate the coelution of a thermally-labile conjugate metabolite. Utilizing a logical, well-thought-out process can make adjustments easier to track, trace, and confirm.

We will now provide an outline and rationale for each step discussed so far that will also shed light on the iterative processes and how they can be managed.
DISCUSSION

MS/MS method development

The first step in the method development process is to evaluate the MS response and develop a multiple reaction monitoring (MRM) methodology that provides the optimum MS conditions for detection of the compounds of interest. As part of the MS evaluation, it is necessary to determine:

- Whether the compound can be detected by mass spectrometry without the need for chemical modification
- The mode of ionization best suited for detection, such as electrospray (ESI) or atmospheric pressure chemical (APCI)
- The polarity that provides the best response

The process of developing an MRM method can be laborious and, once again, involves a series of iterations to achieve the optimum set of conditions.

The Waters Xevo™ TQ Mass Spectrometer provides robust quantitative capabilities for demanding bioanalytical applications and complex sample matrices. The Xevo TQ is an advanced tandem quadrupole mass spectrometer that provides uncomplicated access to superior HPLC and UPLC®/MS/MS performance, delivering the highest levels of sensitivity, selectivity, robustness, speed, and accuracy.

Two proven and user-friendly features in Waters mass spectrometers are ESCi® Technology and IntelliStart™ Software. ESCi allows fast switching between ionization modes without having to physically change the source, as well as the ability to quickly switch from positive to negative modes. ESCi enables you to quickly determine the most sensitive mode of ionization, which also reveals the most sensitive MRM path for detection and quantitation. Using the information gained from this process allows IntelliStart Software to help you quickly and automatically optimize the mass spectrometer’s parameters for MRM MS analysis.

It is good practice to check whether this initial MRM method provides sufficient sensitivity for an aqueous sample before further work is carried out. If the sensitivity is not sufficient for the requirements of the assay, then it may be necessary to introduce a concentration step in the sample preparation process, or to use a higher-sensitivity form of chromatography such as microbore or capillary LC.

Liquid chromatography

Developing robust chromatography is critical to the success of a bioanalysis method. The goal of the chromatographic process is to separate the analyte(s) of interest from both endogenous interferences and drug metabolites. Coelution of endogenous metabolites or other endogenous or exogenous species may cause ion suppression or ion enhancement, both of which are detrimental to the development of a quantitative bioanalytical assay.

The Waters ACQUITY UPLC® System enables you to achieve higher chromatographic resolution and sensitivity and greater throughput using sub-2 μm particle technology. The proven design of the ACQUITY UPLC System, used in laboratories for business-critical
applications worldwide, ensures that you will simultaneously gain higher quality information from your samples and optimize laboratory productivity. This highly robust, dependable, and reproducible system eliminates significant time and cost per sample from analytical processes while improving the quality of results. By outperforming traditional or optimized HPLC, the system allows chromatographers to work at higher efficiencies with a much wider range of linear velocities, flow rates, and backpressures.

The first step when developing the LC section of an LC/MS/MS method is to determine the retention characteristics of the compound(s) of interest. This is easily achieved by screening using a simple reversed-phase gradient and a high retention column such as C₁₈ at both low and high pH. This allows selection of the most promising conditions from which to continue the development of the LC methodology.

Good practice here is to refer to knowledge gained from previous experiences, such as animal studies or in vitro experiments. If samples from these studies are available, they should be injected onto the chromatography system to gain an understanding of where these compounds elute. Resolution of the analyte of interest from any conjugate metabolite, such as a glucuronide or sulphate (which may be thermally labile and decompose in the source to form the aglycone) is essential to prevent an overestimation of the concentration of the parent (dosed) compound and hence an incorrect determination of the pharmacokinetic profile of the compound and the exposure to the drug (AUC).

In general, it is best to modify the chromatography to move retention of the analyte of interest towards the end of the chromatographic analysis since, with the exception of N-oxides, most metabolism reactions result in a product that is more polar than the compound of interest.

After determining the pH that produces the greatest retention of the analyte of interest, the chromatographic process can be adjusted by altering the slope of the gradient to ensure sufficient resolution is obtained between the peak of interest and other drug-related metabolites.

The next step is to evaluate the retention profile of the endogenous metabolites. An effective tactic is to monitor either the full-scan MS profile of the endogenous metabolites or a specific known transition, such as precursors of m/z (positive ion) 184 for phospholipids. Using this information, the chromatography can be adjusted to affect how the analyte resolves from the endogenous compounds in the sample.

It is important here to evaluate some typical patient plasma to ascertain the profile of real patients, rather than that of control plasma/serum/urine. Using this information, more robustness can be built into the methodology, or more realistic levels of detection can be determined.

Iteration back to the solid-phase or MS/MS methodology may be required to help obtain the assay performance required.

**Sample preparation**

Selection of the most appropriate sample preparation step depends on the assay sensitivity required, duration of use of the assay, and the complexity of the task.

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**HOT TOPICS IN BIOANALYSIS: INCURRED SAMPLE REANALYSIS**

Although the U.S. FDA guidelines for Incurred Sample Reanalysis (ISR) have not yet been established, the likely impact on laboratories is that additional samples will need to be analyzed.

- **Dealing with an increase in input** can be easily dealt with by replacing traditional HPLC system with the Waters ACQUITY UPLC System. The system decreases run times while maintaining data quality, which can lead to greater productivity in the laboratory.

- **The use of Waters Oasis SPE** can eliminate the potential sources of ISR failure, resulting in more robust and highly selective assays.
If the assay lifetime is short, then it may not be appropriate to develop a highly sophisticated method; on the other hand, if the methodology is going to last for an extended period of time or will be applied to a high throughput scenario, then it makes good economic sense to expend the time to develop a robust, reliable sample clean-up step. Furthermore, reducing sample complexity will allow the LC cycle time to be significantly reduced without affecting the assay performance, and hence increase productivity.

The two most common biological samples that require analysis are:
- Plasma/serum, to determine the pharmacokinetic profile of the analyte and thus the drug clearance, half-life, and bioavailability
- Urine, to determine the renal elimination profile of the compound

The sample preparation process fulfills three major roles:
- Removal of protein-related materials that may contaminate the chromatography column
- Elimination of endogenous compounds, such as phospholipids that are the major cause of ion suppression/enhancement in LC/MS
- Concentration to increase assay sensitivity

Common techniques for sample preparation are protein precipitation with acid or organic solvent, solid-phase extraction, and liquid/liquid extraction.

Protein precipitation only addresses the removal of proteins. Phospholipids and other contaminants that remain in the matrix may cause ion suppression or enhancement, which will lead to inconsistencies or inaccuracies in detection and quantitation of the compound of interest, as well as a reduction in sensitivity. Contaminants will also foul the column, reduce column lifetime, and reduce linearity and run-to-run reproducibility sooner than if the sample was cleaner.

If the sample contains only a very small amount of the compound of interest and the analyst is working at the limits of sensitivity for the assay, there is no scope for inclusion of a concentration step within this method to increase sensitivity. Nevertheless, protein precipitation remains a popular method for sample clean-up because it is relatively fast and cheap.

Liquid/liquid extraction of a sample has advantages over protein precipitation in that the sample can be concentrated. However, selecting the right solvents for sample partitioning makes developing this extraction method laborious. Solvent cost and disposal are also factors that must be considered, as well as the lack of potential for automation. In a high-throughput scenario, LLE becomes highly labor intensive.

HOT TOPICS IN BIOANALYSIS:
DETECTION AND CHARACTERIZATION OF METABOLITES

Current U.S. FDA guidance documents recommend the characterization of metabolites that are formed with an exposure of greater than 10% of that of the parent drug compound.

- The resolving power of the Waters ACQUITY UPLC System allows separation of the drug compound from its metabolites and other potential interferences without sacrificing speed or sensitivity.
- The ability of Waters mass spectrometers to switch ionization modes quickly during fast UPLC peaks (1-2 seconds), and the ability to detect all compounds under optimum conditions, leads to greater productivity and maximum sensitivity.
- Confirmation of metabolite identity is possible using Waters Xevo TQ MS’s ScanWave™ collision cell technology for full-scan spectral data acquisition during the mass spectrometer’s MRM response.

>> Refer to FDA guidelines on www.fda.gov for details.
Solid-phase extraction (SPE) offers many supports and phases for sample clean-up. Waters Oasis® SPE sorbents can be utilized in a very simple and effective methodology; selection of the correct sorbent for removal of exogeneous components results in a very clean sample for subsequent analysis. Two protocols, combined with four sorbents, provide the flexibility to extract acids, bases, and neutrals with high analyte recovery and the cleanest extracts.

Use of Oasis µElution Plate formats results in increased sensitivity through not only the cleanliness of the sample — the high capacity of the sorbent also lends itself well to a concentration step (up to 15X) with no evaporation or reconstitution, a potentially critical factor in low-dose scenarios.

By using either high or low pH with the Oasis sorbent, selectivity for the analytes of interest increases. Furthermore, for high throughput, Oasis sorbent lends itself well to automated sample clean-up, providing excellent recovery and linearity across a wide range of analytes.

Cleanliness of the extraction is evaluated by LC analysis. Once the best sorbent has been selected the methodology can be optimized for cleanliness by evaluating the effect of changing the organic solvent wash composition.

**Method validation**

Once the assay has been optimized for either sensitivity or throughput, the final assay can be validated using the criteria outlined by the U.S. FDA in their guidelines documentation.

FDA guidelines require that bioanalytical assays conform to specific criteria. A full treatment of the rules and regulations can be found on the FDA website, but some of the requirements as they pertain to LC/MS/MS assays in regulated bioanalysis are outlined here:

- **Accuracy and precision**
  - Use QC samples to determine accuracy and precision
  - QC samples must be prepared at three concentrations — low, medium, and high
  - Review inter-day and intra-day variation
  - ± 15% variation
  - 2/3 of QC samples must pass

- **Standard curve linearity**
  - Simplest fit possible

- **Carryover must be measured**

- **Matrix effects**
  - < 15% variability in absolute matrix effects across six lots of samples in the matrix

- **LLOQ and limits of detection (LOD) must be measured**
  - LLOQ: > 5% level in blank matrix
  - LOD: > 3% level in blank matrix

A well-validated method will encompass these major criteria and also define other aspects of the assay.

IntelliStart System Suitability Software, in conjunction with Waters MassLynx™ Software’s QCMonitor™ for quantitative data quality monitoring, will help monitor the status of the assay, allowing the analyst to quickly determine assay failure and take remedial action. All changes to the assay are filed and stored as part of the method in order to remain compliant.

**CONCLUSION**

Developing a robust and compliant LC/MS/MS assay has traditionally been the domain of very experienced analysts. Waters’ advanced and user-friendly instruments and software offer superior capabilities for the bioanalytical laboratory, providing elegant, robust tools and techniques that allow less-experienced analysts to quickly and successfully develop fit-for-purpose LC/MS/MS assays.
**INTRODUCTION**

Liquid Chromatography (LC) coupled with tandem quadrupole mass spectrometry (LC/MS/MS) and operated in multiple reaction monitoring (MRM) mode is the analytical method of choice for the determination and quantification of drugs and their metabolites in biofluids and tissues.

This is due to the strong specificity and therefore high levels of sensitivity that LC/MS/MS is capable of achieving. However, utilization of this technique often requires companies to hire specialists or to train current personnel, resulting in a significant investment in time and resources.

An LC/MS/MS method requires a robust MRM method. When developing an MRM method, the analyst must first determine whether the compound of interest will ionize, and if it does, understand the best way to ionize and also obtain the best m/z value (parent or daughter ion) that will offer the best sensitivity and selectivity.

The parameters that must be determined for successful MS detection and quantitation of a chosen compound are:

- Ionization mode
- MRM transition ions
- Capillary voltage
- Cone voltage
- Desolvation gas flow
- Source temperature
- Collision energy

Optimization of these parameters may require a number of iterations before the best MRM method is created for the assay.

Choosing the right mode for ionization is an important factor and is usually the first condition to be determined. Occasionally an experienced scientist will be able to make an educated and informed guess by looking at the structure of the compound, but otherwise the mode of ionization is selected by trying each of the modes of ionization, both positive and negative. This often means that the source must be exchanged in between modes.

The other parameters (cone voltage, capillary voltage, desolvation gas flow, source temperature, and collision energy) that need to be selected for optimal operation can also sometimes be determined through experience, but trial and error are common techniques.

For non-experts, developing the right MRM method can be time consuming and daunting.

**Innovations in MS hardware and software**

The proliferation of LC/MS/MS in the laboratory has resulted in the need to make this technique more accessible to any analyst as it becomes part of the routine analytical landscape. Often there may be several MS systems in the lab – but not necessarily several analysts with that expertise.

Waters’ development of user-friendly and intuitive tools that can assist or completely automate the operation of mass spectrometers provides an essential benefit in numerous ways:

- Savings in time that allows experienced analysts to direct their time towards tasks that require a higher level of expertise
- Non-expert analysts will be able to develop optimal MRM methods with confidence and also very quickly, speeding up the process
- Providing consistency in the way instrumentation is utilized and information is transferred into a GLP-compliant software package reduces the potential for errors
Waters has developed proven hardware and software technologies for our tandem quadrupole mass spectrometers that simplify development of an MRM method. These tools allow even the most inexperienced user to develop a MRM method – making this advanced MS technique highly accessible and valuable for any bioanalytical laboratory. Two such enabling technologies are ESCi® and IntelliStart.

- ESCi is a technique from Waters that allows the user to automate testing of the four modes of ionization without changing the source. Not only can the user readily establish the best mode of ionization that will yield the most sensitivity, ESCi also clarifies the best m/z ion to monitor in the assay.

- Optimization of the rest of the MS instrument’s conditions for the MRM method can also be carried out quickly and effectively using IntelliStart. IntelliStart is a software tool for Waters mass spectrometers that optimizes all the other parameters required for MS analysis. In a matter of just a few key strokes and in around four minutes, a method can be optimized and ready to be used in an LC/MS/MS assay.

The application for developing a MRM method for ibuprofen illustrates the application of ESCi and IntelliStart in optimizing the MS conditions.

**DISCUSSION**

**Development of an MRM method for ibuprofen**

A Waters ACQUITY UPLC®/MS/MS System was used to rapidly develop an MRM method for the non-steroidal anti-inflammatory drug (NSAID) ibuprofen with subsequent incorporation into an LC/MS/MS method for separation, detection, and quantitation.

**Materials and methods**

Development of the MRM part of the MS analysis for the drug was carried out using a Waters tandem quadrupole mass spectrometer as part of a full LC/MS/MS system utilizing UltraPerformance LC® (UPLC®) Technology.

Instruments were controlled with Waters software, which also acquired the data for subsequent processing. The operation of ESCi and IntelliStart are embedded within the software, facilitating full documentation of the processes and conditions and transfer into reports and records. Intuitive screens guided us quickly and easily through the process of developing and optimizing the MRM method for ibuprofen.

An overview of the workflow for the development of an MRM method in bioanalysis is illustrated in Figure 1. These are the steps that are generally taken to determine the correct parameters for running an MS method subsequent to UPLC separation and MS detection.

**Figure 1. General workflow for development of MRM method for bioanalysis.**

IntelliStart was used to automatically optimize the source conditions and reduce the time taken to develop the MRM method.
EXPERIMENTAL

Using IntelliStart and ESCi, the MRM method development process was greatly simplified. The steps taken to develop the method and complete the workflow in Figure 1 are detailed in Figure 2.

To begin the process, neat sample was introduced into the mass spectrometer by direct infusion. (Other common methods of introduction are via a fast gradient elution off a column or a direct loop injection.)

Using ESCi for steps 1 and 2 of the optimization process allowed us to determine which was the best ionization source for analysis of the compound – in less than one minute and three keystrokes.

The results from ESCi clearly illustrate that ibuprofen ionized in both APCI and ESI negative modes; ESI negative yielded the best sensitivity and was therefore chosen. This step in the optimization process also told us that the molecular ion observed with the best sensitivity was m/z 205, as shown in Figure 3.

Figure 3. ESCi of ibuprofen.

With the use of IntelliStart Software, the other parameters for optimization of the MS method could now be obtained.

The molecular ion mass was entered into IntelliStart Software (step 3) and the default ranges for cone voltage and collision energy were used to enable IntelliStart Software to automatically determine all other parameters, such as optimized voltages, desolvation temperatures, gas flows, and MRM transition – performing this step in less than 4 minutes.
A report was automatically generated specifying the optimized settings for the MRM method. The report also detailed the daughter ion spectra along with cone voltage and collision energy values (Figure 4).

Having successfully and quickly developed the MS method for detection of ibuprofen, the MRM method parameters determined by IntelliStart Software were automatically inserted into the instrument method (Figure 5) for subsequent use in the method for optimizing the chromatographic conditions for ibuprofen. Because the data was automatically transferred, transcription errors were avoided and time was saved.

A complete UPLC/MS/MS method for the determination of ibuprofen in biological fluids could now be developed with all the optimized parameters for detection and quantitation in place (Figure 6).

**LC conditions**

- **LC system:** Waters® ACQUITY UPLC® System
- **Column:** Waters ACQUITY UPLC BEH C₁₈ Column 2.1 x 50 mm, 1.7 µm
- **Column temp.:** 40 °C
- **Flow rate:** 50 µL/min
- **Mobile phase A:** 0.1 % Ammonium Hydroxide in H₂O
- **Mobile phase B:** MeOH
**CONCLUSION**

Use of intuitive tools like ESCi and IntelliStart technologies provide an efficient, rapid, and effective approach to developing MRM methods for assays.

Developing the MRM method for ibuprofen actually took an inexperienced user about 4 minutes. In this time, the best ion for monitoring was observed and MS parameters were set up and entered in the method for ibuprofen appropriate to a regulated environment.

In a setting where there are many LC/MS/MS systems, this is a clear advantage in terms of level of expertise required to operate the system and allowing more experienced personnel to direct their time towards tasks that demand a higher level of expertise.

**References**


Morphet J and Hancock P. Waters Application Note. 2007; 720002329en.

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**MS conditions**

- **MS system:** Waters tandem quadrupole mass spectrometer
- **Ionization mode:** ESI negative
- **Capillary voltage:** 3.8 KV
- **Cone voltage:** 15 V
- **Desolvation temp.:** 450 °C
- **Desolvation gas:** 900 L/Hr
- **Source temp.:** 150 °C
- **Acquisition mode:** MRM transition 205 > 161
- **Collision energy:** 10 eV

---

![Figure 6. Ibuprofen chromatogram.](image-url)
STRATEGIC LC METHOD DEVELOPMENT FOR BIOANALYTICAL ASSAYS

OVERVIEW

The process of bioanalysis in a regulated environment involves the development of a high sensitivity MS/MS assay for detection of an analyte in the low ng to pg/mL range. Critical to the success of the assay is the separation of the analyte of interest from the endogenous material in the sample, such as phospholipids and exogenous metabolites from the dosed compound.

Rigorous demands are placed on the development of an LC method for detection and quantitation of a specific compound and associated components in a biological matrix. Criteria that the method must meet include:

- Accuracy
- Precision
- Specificity
- Detection limit
- Quantitation limit
- Linearity
- Range
- Robustness

Developing an LC method that encompasses all these parameters requires a series of experiments to determine the best and most effective separation conditions. A method development strategy that is systematic in its approach can greatly simplify the process and allow the scientist to develop optimal separation conditions quickly and efficiently.

Before going down the path towards developing a method, you need to consider how several factors will impact your assay: column, solvent delivery, pH, and organic modifier.

Column choice

C₁₈ columns are generally the initial column of choice when developing an LC method due to their high retention and suitability for a wide range of applications. Using a short column allows a fast run time while providing sufficient capacity for resolution of most analytes and associated species. A column that can operate over a wide range of pH conditions (pH 1 to 10) is an important factor to consider as it will provide the flexibility to develop the most sensitive and robust method possible.

Solvent delivery and gradient chromatography

The use of gradient chromatography can greatly accelerate the screening process. A gradient can be optimized for a short run time and consequently enables a fast and effective assay. Simple modifications of a gradient can shorten a run time by half while still retaining resolution and sensitivity of the key analytes and freedom from interfering influences.

pH

Often the analyte of interest will be polar. Other compounds of interest in the matrix may differ in terms of their chemical properties. For these reasons it is important that the method is robust to extremes of pH. The ability to use pH as tool in the retention and resolution of component peaks in an assay can greatly enhance selectivity and sensitivity.

Organic modifier

A level of organic modifier in the mobile phase is essential in reversed-phase chromatography. Choosing which organic modifier to use also has an impact on the sensitivity and selectivity of an assay. Acetonitrile and methanol are the most commonly used modifiers. Chromatographic results can differ markedly depending on which solvent is used, adding an extra dimension of flexibility to the development of a separation.
When all these aspects have been considered and optimized for the analytes of interest, the assay can be checked to determine the lower limits of detection (LLOD) and quantitation (LLOQ). For most compounds, using a simple step-by-step strategy will ensure the development of an assay which conforms to the demands required for a regulated assay.

**Technology for bioanalytical methods**

Waters has outlined a simple yet effective and comprehensive strategy for optimizing a method for analysis of analytes in a complex matrix such as blood, plasma, urine, or tissue.

Use of the ACQUITY UPLC® System allows this process to take place quickly and effectively. Screening runs can take place in as little as two minutes. Automation for screening additional parameters greatly increases productivity and confidence in results.

In the less-frequent cases where a compound does not separate well and further chromatographic development is needed, an experienced analyst will be able to use the results of a method developed using this process to determine further steps for improvement.

---

**SCREEN FOR ANALYTE RETENTION**
Using a 5 cm x 2 mm C18 column, screen analyte retention employing a 5 to 95% organic gradient over 2 min at both low and high pH. Both methanol and acetonitrile should be screened as organic modifiers.

**SELECT pH**
Select the pH that gives the best retention.

**SELECT ORGANIC MODIFIER**
Select the pH and organic solvent that gives the best retention, sensitivity, and selectivity from metabolites.

**OPTIMIZE THE GRADIENT**
Optimize the separation by taking the measured organic elution composition, e.g. the middle of the gradient = 50%. From this electronic elution composition, create a focused gradient using a starting composition 15% lower than and a finishing composition 10% higher than the measured elution composition. The gradient should terminate with a step wash at 95% organic solvent.

**ASSESS INTERFERENCES**
Assess any interference from residual phospholipids by calculating the matrix effect. Monitor phospholipids at 184 m/z transition.

**TEST IN MATRIX**
Test the new gradient by monitoring the resolution of the analyte under test from known metabolites and endogenous material in the sample. Use these findings to modify the gradient conditions to achieve optimal separation and sensitivity.

**CHECK SENSITIVITY**
Check the sensitivity using neat standard. If the required sensitivity is not achieved to allow for the detection of the elimination phase of the PK curve, seek assistance from an experienced analyst.

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*Figure 1. The Waters bioanalysis method development workflow.*
APPLICATION EXAMPLE

An efficient LC method development approach in the analysis of risperidone

Risperidone is an antipsychotic drug marketed as Risperdal. It is rapidly metabolized in the liver to 9-hydroxyrisperidone, which is the primary circulating species. Since the 9-OH metabolite has the same activity as the parent, it must also be quantified. The following assay was carried out using clozapine as an internal standard.

Using the previously-described Waters bioanalytical LC method development workflow, we were quickly able to establish an effective method for the separation and quantitation of the three analytes in human plasma.

Key to a fast effective separation was the use of UPLC® Technology with the ACQUITY UPLC System, which allowed us to optimize speed, resolution, and sensitivity with no compromises in any of these key parameters for successful analysis. Furthermore, the use of MS/MS for highly sensitive and selective detection resulted in an assay that enabled us to detect and quantitate all three analytes at very low levels.

The role of UPLC in method scouting

Developing and optimizing a method is a necessary task that nonetheless detracts from the task of running analyses. Use of UPLC to carry out fast chromatography allows you to make quick decisions about methodology and therefore dramatically reduces the amount of time needed to develop and optimize a method.

Column choice

We selected the ACQUITY UPLC BEH Column for this analysis. Its unique column technology is a multifaceted tool that is robust to high and low pHs, making it suitable for a wide range of analyses. The column provides us with increased flexibility in effecting a separation and influencing selectivity and sensitivity.

The ACQUITY UPLC BEH Column’s small particle size (1.7 µm) in a narrow diameter column (2.1 mm) provides superior capacity, allowing complex mixtures to be separated quickly over a short column length (50 mm). In this case, run times of less than two minutes could be achieved resulting in fast and highly effective method development.

Both the parent and the metabolite in this case have a pKa above 7. In the case of basic compounds, chromatography is greatly improved by the use of high pH where basic compounds are in their neutral form, increasing their hydrophobicity. This typically results in higher MS sensitivity since the analytes elute in higher percentage organic, with a gain in efficiency in the MS source resulting in a higher analyte signal.

Solvent delivery and gradient formation

Capitalizing on the recent advances in column technology requires an equal level of innovation in the chromatographic system to deliver the mobile phase to the column and the mass detector to maximize the potential of these novel technologies.

The performance and unique design of the ACQUITY UPLC’s solvent management system provides a previously-unattainable level of precise, controlled solvent delivery for low-flow, micro, and narrow-bore chromatography. Without this attention to detail in instrument design, the advantages of innovative columns and detection systems cannot be realized.

pH considerations

The ability to manipulate pH to affect sensitivity and selectivity of a separation is a powerful tool in the development and optimization of LC methodologies. The ACQUITY UPLC BEH particle is robust to a wide range of pH values, from pH 1 to 10. UPLC’s speed and versatility for screening high and low pH when developing a method is essential to determining the best pH region for the separation.
**EXPERIMENTAL**

**Materials and methods**

- Initially we used the UPLC method scouting approach. We screened both low pH (formic acid) and high pH (ammonium acetate). Both these mobile phases are compatible with mass spectrometry.
- The organic modifiers used were acetonitrile and methanol.
- Use of the four solvent inlets on the ACQUITY UPLC Solvent Manager allowed us a high degree of automation in developing the method.
- Since there are only three compounds to detect, we used a fast gradient (2 to 98% over 2 minutes).
- Results of this scouting allowed us to pick the set of conditions that gives us the best starting point for peak shape, retention, and selectivity.

**Scouting method conditions**

<table>
<thead>
<tr>
<th>LC system:</th>
<th>Waters ACQUITY UPLC System</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column:</td>
<td>Waters ACQUITY UPLC BEH C18</td>
</tr>
<tr>
<td></td>
<td>2.1 x 50 mm, 1.7 µm</td>
</tr>
<tr>
<td>Mobile phase A1:</td>
<td>0.1% HCOOH in H2O (~pH 2.7)</td>
</tr>
<tr>
<td>Mobile phase A2:</td>
<td>10 mM NH₄COOH in H2O (pH 9)</td>
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<tr>
<td>Mobile phase B1:</td>
<td>MeOH</td>
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<tr>
<td>Mobile phase B2:</td>
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</tr>
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<td>Flow rate:</td>
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</tr>
<tr>
<td>Gradient:</td>
<td></td>
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<tr>
<td>Time (min) %A %B</td>
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<tr>
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</tr>
<tr>
<td>3.0</td>
<td>98 2</td>
</tr>
</tbody>
</table>

**Figure 2.** Evaluating peak shape, resolution, and sensitivity in the pH screening results.

Peak order:
1. 9-OH Risperidone metabolite
2. Risperidone
3. Clozapine (internal standard)
Injection volume: 5.0 µL
Column temp.: 50 °C
Sample temp.: 15 °C
Sample concentration: 100 ng/mL
Sample diluent: 50:50 H₂O/MeOH
Strong needle wash: 60:40 ACN/IPA 0.5% HCOOH
Weak needle wash: 95:5 H₂O/MeOH
Injection mode: Partial loop
MS system: Waters tandem quadrupole mass spectrometer
Ionization mode: ESI positive
Capillary: 3.0 kV
Source temp.: 120 °C
Desolvation temp.: 350 °C
Cone gas flow: 50 L/Hr
Desolvation gas flow: 700 L/Hr
Dwell time: 30 msec

Screening results

Figure 2 shows results of the initial screening run. The peaks are 9-hydroxyrisperidone metabolite, risperidone, and the internal standard, clozapine. All the results are plotted on the same intensity scale.

Select pH

From Figure 2, we could see enhanced sensitivity at high pH as well as improved resolution of the metabolite (peak 1) from risperidone. Consequently, we selected high pH. The next task was to select the organic solvent. Note that each screen took only 3 minutes.

Select organic modifier

Another look at Figure 2 shows us that methanol offers higher sensitivity at high pH than acetonitrile for the metabolite (peak 1). Also, at high pH with MeOH, the internal standard elution time is closer to the analytes, but not coeluting. We chose methanol to be our organic solvent for both retention and selectivity.

Optimize gradient

Further analysis of the chromatogram that we selected reveals that the analytes do not elute until after the first minute of the separation, and that the cycle time is three minutes including re-equilibration. The next step is to investigate the possibility of shortening the cycle (run-to-run) time.

Many parameters can be investigated for optimization: temperature, flow rate, column length, gradient slope, and profile. In this example, we will try to shorten the gradient by starting at higher organic since the analytes eluted at 70% organic and elute our compounds earlier while maintaining sensitivity and freedom from interferences.

Instead of starting the gradient at 2% organic, we elected to start at 50% organic. This shortened the gradient time (Figure 3) for a 50% reduction in cycle time.
Having developed a method that is optimal for speed, sensitivity, and resolution, we now need to check that in the real world of complex matrices, such as plasma, that this separation would be maintained in the presence of interferences.

Plasma contains a wide range of interferences that must be removed before analysis of the compounds of interest. In circumstances where the interferences cannot be removed through sample cleanup, it is important to check that the interferences (typically phospholipids) do not coelute with our desired chromatography.

For this part of the optimization process, we took matrix blanks and spiked them with low concentrations of the analytes. Figure 4 shows that there was significant separation of the analytes from the interferences. Calculated matrix effects were under 5% (see SPE section for calculation of matrix effects).

If desired, interferences due to endogenous phospholipids (identified as one of the major sources of matrix effects) can be monitored using the transition m/z 184 to 184. This confirms the presence and elution profile of the interfering component.

At the end of the process, we were satisfied that the separation was optimized for the three analytes of interest (Figure 4). The MRM transitions for the analytes confirmed their identity. The final step in the process of method development is to check the limits of quantitation and confirm that they meet requirements for the assay.

Checking assay sensitivity

The final stage of checking the sensitivity of the assay is to run neat standard. Here we chose to run each compound at 0.1 ng/mL, which is the desired LLOQ. Figure 5 demonstrates that this was easily achieved.
**CONCLUSION**

Use of a simple step-by-step process in combination with the analytical speed of the ACQUITY UPLC System allowed us to determine very quickly the optimum conditions for a separation. This process did not require an experienced analyst and thus provided laboratory savings in time as well as resources.

UPLC also facilitates faster analysis of samples, which results in higher levels of productivity and more efficient use of high-value capital equipment such as tandem quadrupole MS techniques.

**References**


Chambers E, Diehl D. Waters Application Note. 2007; 720002326en.

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**Figure 4.** Verifying the absence of matrix interferences.

**Figure 5.** The compound run at 0.1 ng/mL concentration.
INTRODUCTION

The rigorous criteria placed on a bioanalytical method, as outlined earlier in the LC Method Development section, can be seriously compromised by the cleanliness of the sample when it is introduced to the assay. Preparing the samples for eventual analysis is essential to ensuring that the assays conform to recognized standards of compliance and performance.

Often the factors that impact sensitivity, reproducibility, or throughput of the analytical system might not lie with the instrument. Optimal sample preparation techniques will improve analytical performance dramatically by removing interferences from the sample matrix and concentrating analytes of interest.

Common sample analysis challenges include:
- Ion suppression or ion enhancement
- Complex sample matrices
- Low recovery and inconsistent results
- Poor sensitivity
- Interfering peaks/cleanliness
- The need to concentrate or evaporate/reconstitute
- Limited sample volume
- Sample throughput

A major concern in LC/MS/MS techniques in regulated bioanalysis lies with the problem of ion suppression/enhancement through matrix effects, i.e. matrix interferences. Matrix effects can lead to inconsistencies in ruggedness as well as sensitivity of analyses.

The appropriate sample preparation technique can significantly enhance analyte signal and reduce unwanted noise, as well as significantly reduce or eliminate matrix effects.

Consequently, a highly selective sample preparation method is an important step in a bioanalytical assay to minimize matrix effects and sample variability and ensure compliance with the FDA guidance for bioanalytical method validation.

Matrix effects

LC/MS/MS is a powerful analytical tool utilized in quantitative bioanalysis because of its high sensitivity and selectivity; however it is susceptible to matrix effects.

In practice, matrix effects result from coeluting matrix components that impact the ionization of the target analyte, resulting either in ion suppression or ion enhancement.

Largely, the interference is due to endogenous phospholipids that are present in high concentrations in plasma samples, but other components such as dosing media, formulation agents, and even mobile phase modifiers can also contribute.

Due to the strong non-polar nature of phospholipids, they will elute late from a reversed-phase column and have the potential for interfering with non-polar analytes.

A further complication is the variation in endogenous interferences in plasma even within a species. This will lead to further imprecision that cannot be accounted for consistently across a series of samples. As a result, even manipulation of the chromatography to avoid interferences may not be fully mitigated. Consequently, the importance of eliminating these effects becomes more critical for a well-validated and compliant assay.

Assessment of matrix effects

Matrix effects can be described as the difference between the mass spectrometric response for an analyte in standard solution and the response for the same analyte in a biological matrix, such as plasma, and should be assessed experimentally.

A common method for assessment of matrix effects is through use of the post-extraction spike method. This is carried out by comparing the response of the analyte in neat solution to the response of the analyte spiked in to a blank matrix sample that has been carried through the sample preparation process, as shown in Figure 1.
Using the post-extraction spike methodology in Figure 1, quantitative effects on ion suppression or enhancement experienced by all analytes in the sample can be measured each time a change is made to the analytical method.

To illustrate this, the procedure was carried on a sample containing 0.5 ng/mL of terfenadine, an antihistamine marketed under various brand names. In this case, to demonstrate the methodology two spiked samples were cleaned using different methods:

A. Protein precipitation (PPT)
B. Mixed-mode solid-phase extraction (SPE)
C. Standard in solution

Then matrix effects were measured (Figure 2). The post-extraction spiked method showed very clearly the extent of matrix effects in each sample.

The post-extraction spike technique is used in the following proof of concept for the Waters Oasis® SPE 2x4 method to assess matrix effects after cleanup of a complex sample.

A simplified and streamlined approach to sample preparation

Clearly, matrix effects are wholly undesirable in a bioanalytical assay that is based on LC/MS/MS technology.

Matrix effects can be measured and factored into results, but the innate unreliability of the extent of the effect and the variation from sample to sample make it highly desirable to eliminate matrix effects completely.

Sample preparation using SPE technology has been utilized successfully, resulting in very clean samples and low-to-negligible matrix effects. Traditionally, developing a method for cleanup of sample by SPE has meant a long and laborious process fraught with many choices and development steps: the effort expended has not always justified the end result.

Here, we demonstrate a strategy for developing an SPE method that is simple, quick, applicable to a wide range of compounds, and results in a rugged method for sample cleanup on a manual or automated scale.
APPLICATION EXAMPLE I

Oasis 2x4 SPE method development

A novel strategy for the development of rugged SPE protocols, the Oasis® SPE 2x4 method is a simple, logical approach to the selection of an SPE sorbent and protocol for sample preparation. This method will result in high recoveries for most analytes on the first attempt.

The Oasis SPE family of products has characteristics that greatly enhance performance and ease of use, including excellent water wettability (no drying out), no silanol interactions, stability across a wide pH range, excellent recoveries for polar and non-polar analytes, and all have the familiar hydrophilic-lipophilic balanced (HLB) co-polymer backbone.

Taking full advantage of these characteristics, the 2x4 method is based on two protocols and four sorbents that provide the flexibility to extract acids, bases, and neutrals with high recoveries while removing matrix components that may interfere with analysis.

The flow chart in Figure 3 illustrates the required steps to develop an SPE method for sample preparation using Oasis sorbents.

Application of the 2x4 method quite simply involves:
- Characterization of the analyte (neutral, acid or base, pKa)
- Selection one of the four Oasis sorbents
- Application of protocol 1 or 2
- Determination of the SPE recoveries by LC analysis

Figure 3. Oasis 2x4 method for sorbent and protocol selection. The Elute 1 step recovers neutral analytes.

Proof of concept

To illustrate in practical terms how the method and protocols are applied, samples spiked with five different drugs were characterized for the proof of concept of the Oasis 2x4 method:
- Imipramine: pKa 9.4 (base)
- Ibuprofen: pKa 5.2 (acid)
- Valethamate: pKa > 12 (quaternary amine)
- Nonafluoropentanoic Acid: pKa < 0.5 (strong acid)
- Prednisone: neutral

These drug compounds were chosen because they represent a wide spread across the pKa range and represent a challenge for any sample preparation technique.
EXPERIMENTAL

Each of the above compounds was spiked into rat plasma. The concentrations and structures are shown in Figure 4.

Each plasma sample was diluted (1:1 v/v) and acidified with phosphoric acid (4% in water). Aliquots were taken and processed using the 2x4 method in the following way:
- Load prepared sample onto each sorbent
- Use the designated protocol for each sorbent
- Collect elution 1 and 2
- MD tip: collect and save all load and wash steps (to be analyzed only if analytes are not in the elution fractions)

LC/MS/MS was used to determine SPE recoveries. Figure 5 illustrates how the recovery of the analytes was determined and calculated.

RESULTS AND DISCUSSION

Excellent recovery and cleanup efficiency were achieved for each of the test analytes (Figure 6) when the recommended Oasis 2x4 method protocol was used. The neutral analyte had acceptable recoveries on all four sorbents and was recovered in the 100% organic elution step.
The matrix effect was also calculated for each analyte, Figure 7.

Figure 7 shows that the matrix effects were very low when the Oasis methodology was used for preparation of the samples.

**CONCLUSION**

Using the Oasis 2x4, method development for sample preparation and clean-up is greatly simplified and streamlined to quickly yield a method which is robust and covers a wide range of analytes with excellent recovery and low matrix effects.

**APPLICATION EXAMPLE II**

Elimination of matrix effects:
A comparison of sample preparation techniques

As discussed, elimination of matrix effects in an LC/MS/MS-based assay is highly desirable both in terms of confidence in results and compliance at all levels.

Sample clean-up can be approached in a number of ways, but the most common methods are by PPT, SPE, or liquid/liquid extraction (LLE).

PPT is only effective for removal of proteins. Plasma contains many endogenous compounds that can interfere with the analysis, compromising all the parameters essential for a well-validated assay. Other factors, such as contamination of the column due to contaminants in the sample, reduce the cost-effectiveness of PPT as a method for sample preparation. The impact on sensitivity can be manifold due to interfering species in the assay, through matrix effects, or simply due to the inability to include a concentration step in the sample preparation process.

LLE has the potential for concentration of the sample to enhance sensitivity of the assay. This method for sample preparation can also be highly selective. However, method development requires selecting the right solvents for partitioning the sample. If high throughput is required, the technique does not lend itself well to automation.

SPE is a highly-selective method for sample preparation that combines with the potential for sample concentration, ease of automation, and, as previously demonstrated, simple and highly effective method development.
**EXPERIMENTAL**

A study was carried out to compare matrix effects after the three techniques were used to clean plasma spiked with basic drug compounds.

The following protocols were employed to prepare the samples:

**Oasis MCX SPE (strong cation exchanger)**
- Dilute 0.25 mL plasma with 0.25 mL 4% $\text{H}_3\text{PO}_4$
- Condition/equilbrate cartridge
- Load diluted plasma
- Wash with 0.5 mL 2% HCOOH in $\text{H}_2\text{O}$
- Elute with 0.5 mL 4% NH$_4$OH in 100% MeOH
- Evaporate to dryness and reconstituted in 0.25 mL 50:50 MeOH/$\text{H}_2\text{O}$

**PPT**
- 0.75 mL ACN added to 0.25 mL plasma (3:1 ratio is an accepted standard)
- Vortex and centrifuge at 13,000 rpm for 5 min
- Remove supernatant and evaporate to dryness
- Reconstitute in 0.25 mL 50:50 MeOH/$\text{H}_2\text{O}$

**LLE**
- 75 mL 5% NH$_4$OH in MTBE added to 0.25 mL plasma
- Vortex and centrifuge at 13,000 rpm for 5 min
- Remove supernatant and evaporate to dryness
- Reconstitute in 0.25 mL 50:50 MeOH/$\text{H}_2\text{O}$

The prepared samples were then analyzed using LC/MS/MS and matrix effects were calculated using the post extraction spike method.

**RESULTS AND DISCUSSION**

The samples prepared using PPT yielded a high recovery for all the analytes (Figure 8), but Figure 2 shows 70% or greater suppression of all the analytes due to interferences in the matrix.

The samples prepared using LLE varied in recovery (Figure 8), but the extracts were quite clean as demonstrated in Figure 9.

The Oasis MCX SPE preparation yielded excellent and consistent recoveries across the analytes and also were very clean as evidenced by the negligible matrix effects.
CONCLUSION

Both liquid/liquid extraction and protein precipitation offer some form of cleanup, but with each the analyst must make compromises in recovery or cleanliness.

The samples prepared using Oasis MCX for solid-phase extraction yielded excellent recovery and cleanliness with no compromises. Thus matrix effects can be eliminated using Oasis MCX for a robust reliable method that is inherently consistent.

References
INFORMATICS IN BIOANALYTICAL LABORATORIES

INTRODUCTION

The pharmaceutical industry in any of its forms is heavily regulated. The implications of regulations are far-reaching, from qualification and monitoring of system performance to acquisition, storage, and retrieval of raw data, to documentation requirements whether for regulatory authorities or for a client.

The discovery process in any pharmaceutical company generates a diverse and large quantity of data. Some of the information in the early stages of discovery, i.e., ADME and lead optimization, is more qualitative. In the latter stages of clinical trials, information becomes quantitative.

The priorities of each stage differ also as do the regulatory demands, yet the information that each stage generates is critical to the decision-making process in the selection and development of a new drug candidate.

Handling such diverse requirements for a high volume of data can be time consuming and frustrating especially when attempting to mine accumulated data for the right information in the right format.

Waters’ suite of informatics products addresses the needs of not only the laboratory, but the workflow process throughout a company, to ease and facilitate the flow of scientific information. Here we will look at the types of products from Waters that carry out these functions and the ways in which they interface to yield an efficient, enhanced laboratory and business environment.

MassLynx Software

Waters MassLynx™ Software is a powerful package that balances simplicity and sophistication to make MS/MS techniques available to users of any skill level, from technicians to experienced mass spectrometrists.

MassLynx provides complete control of an LC/MS/MS system from its intuitive interface, which includes an integrated fluidics management tool with diagnostics software that facilitates automatic instrument tuning and full performance checks on the whole system, including auxiliary detectors. Combined with fully-compliant, secure software and a full audit trail feature, MassLynx provides total information management control of LC/MS/MS data for regulated laboratories.

Get the most from your instrumentation with MassLynx Software's seamless solutions for advanced applications:

- Streamline data acquisition and processing
- Automatically switch from MS to MS/MS acquisition when a compound of interest is detected
- IntelliStart™ Software allows even the most inexperienced user to develop an MRM method
- Security Manager allows configuration of access privileges for each user
- Secure file encryption and checksum technologies prevent accidental or deliberate tampering with files
- An audit log records all events that relate to file generation or manipulation, with a log file that can be easily filed and researched
- Application Manager packages offer specialized functionalities for specific processing challenges and requirements, for example:
  - QuanOptimize™ for optimization of MS and MS/MS method development
  - TargetLynx™ for automatic QC checks, including flagging out-of-range data
  - ProfileLynx™ for physicochemical property analysis
  - Additional software packages streamline identification and characterization processes

MassLynx Software has full compatibility with the Waters NuGenesis® Scientific Data Management System (SDMS) informatics platform, which allows data to be automatically captured and processed into relevant, compliant information files that can be managed and shared both locally and globally.
**NuGenesis SDMS**

The volumes of data produced by one laboratory or a number of laboratories can range from raw data to printed reports and all stages in between. Accessing and mining this data can be an all-consuming project, often requiring many man hours, resources, and frustration.

Within any laboratory there are many tools and techniques in use, very frequently with disparate methods for analyzing and storing the data and information gained. Nevertheless data and information must be integrated into more full documentation as a complete analysis of a sample or set of samples.

Collating and rationalizing the differing data is laborious. If a decision rests on the outcome of the collated information, then tools that can manage the data and information will provide valuable time savings.

A system that will serve as a repository for storage and management of all scientific information and data has the potential for coordinating the movement of all laboratory information, delivering results faster and allowing instant access to legacy data and information.

NuGenesis SDMS is an automated electronic repository that stores and manages all scientific information. Data from files and printed reports are automatically cataloged, are instantly retrievable, and are reusable through unique built-in tools that allow the user to publish and report results.

Essential for bioanalytical and bioequivalence laboratories needing rapid review, informed decisions, and efficient results summaries, SDMS helps with managing, finding, repurposing, and sharing information – increasing collaboration throughout a company and with other interested parties or clients.

Some of the tasks that SDMS carries out include:

- Capture of printed reports and key data, dramatically reducing use of paper forms
- Easier re-use of data and information, automatically cutting and pasting to assemble refined study reports, FDA reports, or client summaries
- Fully customizable to final purpose
- Absolute traceability – if results are queried and need to be backed up, they can be found instantly

- Automatically catalogs data for ease of search and traceability
- Totally GLP compliant with automatic audit trails
- Compatibility with popular statistical packages such as WinNonLin
- Seamless integration with Waters software, as well as software from other vendors

**Integrated authoring and reporting**

SDMS Vision Publisher™ is an effective documentation solution that consistently records and easily locates key experimental data. Helping to eliminate manual processes and transcription errors from the workflow, it is essential for the regulated bioanalysis/bioanalytical laboratory.

Seamless integration between SDMS and Waters software enhances experimental documentation, information tracking, and intellectual property management, leaving more time to carry out essential tasks in the lab and manage the flow of information. SDMS Vision Publisher can be fully deployed in a GLP-compliant laboratory, further ensuring compliance at all levels.

**Management of workflow**

Figure 1 represents a typical workflow in a laboratory. Information about the samples must be gathered, analytical measurements are performed, and the related raw data are created. The raw data are then evaluated and interpreted according to the technique and specific software used to generate the information. Data and information are then assimilated into a report for review and sign-off.

Each technique will produce a set of data that now must be archived in a structured way so that it can be accessed again easily for reuse and further documentation. Furthermore, all the additional evaluations such as statistical analyses and reports can be stored.

All the data and information can now be reused for evaluation and creating reports that are best suited for final purpose. Compatibility with other commercially-available packages ensures control over the final output.

Access to any of the data and information is quick, efficient and can be shared within a lab, across several labs, within a company, globally, or with a client. Total control and accessibility ensures full compliance and speeds up decisions and other processes.
APPLICATION EXAMPLE

Challenge

A clinical research company in the UK is challenged with the preparation, analysis, data management, and evaluation of 60,000 clinical samples per year.

Solution

NuGenesis SDMS print-captures all analytical data, extracts results, and exports these into a statistical bioanalysis package. Once the biostatistical report is generated and reviewed, this is then printed back into SDMS to complete the information trail.

Impact

NuGenesis SDMS drives the throughput and speed of analytical turnaround, and improves workflow by automating common and repetitive data input, resulting in the improvement of biostatistical analysis, reporting and decision making, all in a compliant environment.
CONCLUSION

Improving the flow of data and information through a laboratory can positively impact a company by enabling faster realization of the knowledge and understanding required to make informed decisions, and to communicate with interested and vested parties for better business practices.

Waters’ informatics solutions for the laboratory provide a total solution for information flow in the bioanalytical laboratory. Developed by scientists, for scientists, Waters understands the information management needs and business demands placed on a bioanalytical laboratory as well as the specific needs of the analytical scientist.
THE WATERS ACQUITY UPLC SYSTEM

Increasing productivity and profitability in quantitative bioanalyses

Client: A Contract Research Organization

BACKGROUND

As part of the drug development process, the safety and efficacy of drugs and their metabolites must be tested and demonstrated. The primary role of a contract research organization (CRO) is to quantify drug and metabolite concentrations in biological samples received from preclinical and clinical studies.

There are four main areas within which CROs receive samples:

1. Preclinical: Multiple animal species studies (i.e. primate, dog, rodent, etc.), with a large number of studies, but small sample numbers; moderate throughput analysis required

2. Clinical Phase I: First in-human study, with volumes of approximately 1,000 samples; high throughput analysis required

3. Clinical Phase II: Dose range and efficacy study, with thousands of samples; moderate throughput analysis required

4. Clinical Phase III: Wider patient studies, with tens of thousands of samples, but over an extended period of time; moderate throughput analysis required

CROs typically use HPLC/MS/MS quantitative analysis for these samples due to its sensitivity and selectivity.

CHALLENGE

Contract research laboratory revenues depend upon the number of samples that they can run. They have streamlined workflows in place to run samples in a highly efficient process, similar to a production line. The main issue CROs face is obtaining the maximum throughput and quality possible using their LC/MS/MS resources.

Pharmaceutical companies often require CROs to develop and validate their analytical methodology prior to sample analysis. Method development can be a time-consuming process, as several experiments have to be carried out prior to starting method validation. Such experiments include: sensitivity and linearity tests over different ranges, sample preparation experiments, qualitative matrix effects, etc. All of these experiments require instrument time that does not generate revenue and can take weeks to complete if the assay is problematic.

Preclinical sample analysis can also be challenging and normally involves a large number of studies spread out over an extended period of time, but with limited sample numbers. Results from these studies are required quickly.

The CRO laboratory’s ACQUITY UPLC System enables them to optimize their throughput for the most efficient use of their mass spectrometry systems. The speed and throughput of all aspects of their bioanalytical program has increased, from method development to clinical sample analysis, and with this comes an associated increase in revenue and productivity.
Due to lengthy HPLC run times, the CRO often has to analyze small batches of samples (i.e. 30 rat plasma samples) overnight, preventing the lab from performing higher revenue-generating studies.

Clinical sample analysis generates its own challenges. There are large sample numbers involved with the potential to generate large amounts of profit for the CRO, but there are several bottlenecks in this task, including sample preparation, LC/MS analysis, and results processing.

Both method development and preclinical are areas that require large amounts of instrument time with little or no scope to generate large returns. And although there is revenue to be realized in clinical sample analysis, it is most significantly limited by the speed of current HPLC/MS/MS technologies.

THE SOLUTION

The Waters® ACQUITY UltraPerformance LC® (UPLC®) System allows for rapid method development, with typical UPLC methods being three times faster than conventional HPLC. In addition, this increase in throughput is usually accompanied by improvements in sensitivity and chromatographic resolution, resulting in lower limits of quantification and reduced matrix effects.

For example, a method for the analysis of Risperidone and 9-Hydroxyrisperidone has recently been developed using HPLC and UPLC. The HPLC method’s run time is 5.5 minutes, while the UPLC run time is 1.5 minutes, a three-fold increase in throughput.

Additionally, the high capacity ACQUITY UPLC System sample manager allows for unattended operation, reducing the need for employees to work overtime to feed conventional HPLC autosamplers.

One particular CRO’s decision to convert from HPLC to UPLC has allowed them to benefit in all areas of bioanalysis.

Method development can be carried out faster. Instrument time is required during method development, during which the system is not generating revenue. Method development time can be broken down into several key tasks:

- **Linearity Test**: Analyses have to be performed to determine that the method is linear over the required concentration range. This is achieved by analyzing 12 standards in solution.
  - Time taken by HPLC = more than 1 hour
  - Time taken by UPLC = 20 minutes

- **SPE Method Development Plate**: A SPE method development plate may be used to determine the best sorbent and to get a good starting point for the SPE method. The development plate requires the analysis of 96 extracts. With UPLC, the method development plate can be extracted, the samples analyzed, and the data reviewed within a day. It would not be possible to review the data from HPLC until the following day.
  - Time taken by HPLC = 9 hours
  - Time taken by UPLC = 2.5 hours

- **SPE Method Development and Optimization**: The SPE method needs to be optimized and three sample batches (96 samples per batch) must be analyzed to prove that the method is suitable.
  - Time taken by HPLC = more than 3 days
  - Time taken by UPLC = 1 day
Taking these steps into account, a method that takes one week to develop by HPLC/MS/MS can be ready within two days using UPLC/MS/MS, therefore freeing up the instrument to run revenue-generating samples.

Small preclinical studies can also be analyzed in a shorter period of time. For example, a toxicology study could contain 30 rat and mouse plasma samples (50 samples in total when calibration and QC samples are included). These batches could be extracted and analyzed by UPLC in the same working day, allowing more samples or a different method to be run overnight. With HPLC, these batches would have run overnight without opportunity for additional batches to be analyzed.

- Time taken by HPLC = more than 10 hours
- Time taken by UPLC = less than 3 hours

Phase I fast turnaround samples can be analyzed in less time, making it easier to meet the tight deadlines required by customers.

As well, Phase II and III sample throughput significantly increases with UPLC.

### BUSINESS BENEFIT

In a typical CRO, the sample batches would be extracted during the working day and the LC/MS/MS performed during the night. When conventional HPLC systems are used, an average of two batches of samples are analyzed per day. With UPLC this increases to six batches per day, resulting in a 200 percent increase in sample throughput and revenue.

The difference between HPLC and UPLC consumable costs is negligible when compared to the revenue potential.

<table>
<thead>
<tr>
<th></th>
<th>HPLC</th>
<th>UPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Per Day</strong></td>
<td>$12,000</td>
<td>$36,000</td>
</tr>
<tr>
<td><strong>Per Week</strong></td>
<td>$60,000</td>
<td>$180,000</td>
</tr>
<tr>
<td><strong>Per Year</strong></td>
<td>$3,120,000</td>
<td>$9,360,000</td>
</tr>
</tbody>
</table>

Assuming a typical 96-sample batch generates $6,000.

Additionally, the high capacity ACQUITY UPLC System sample manager allows for unattended operation, reducing the need for employees to work overtime to feed conventional HPLC autosamplers.

The CRO laboratory’s ACQUITY UPLC System enables them to optimize their throughput for the most efficient use of their mass spectrometry systems. The speed and throughput of all aspects of their bioanalytical program has increased, from method development to clinical sample analysis, and with this comes an associated increase in revenue and productivity.

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THE WATERS ACQUITY UPLC/MS/MS SYSTEM

Increasing efficiency, productivity, and profitability for bioequivalence laboratories
Client: A Contract Research Organization in Latin America

BACKGROUND

Bioequivalence testing involves confirmation of the profiles of active pharmaceutical ingredient concentrations in human blood that has been sampled at various times following the administration of a referenced drug product and the generic test product.

The bioequivalence test, subject to U.S. FDA clinical and statistical standard procedures, provides the basis for expecting that the drug products are, while not identically formulated or manufactured, clinically comparable in safety and effectiveness in comparison to the referenced drug. Because typical bioequivalence trials employ numerous subjects, each studied on multiple occasions over a period of weeks per drug formulation, the costs are substantial to those companies charged with adequately demonstrating safety and effectiveness.

CHALLENGES

To meet FDA requirements and specifications, manufacturers and contract research organizations (CROs) must undertake rigorous, time-consuming, and extensive chemical analyses of the active and inactive ingredients in the drug product using analytical methods such as chromatography and mass spectrometry. Methods need to be acceptable in terms of linearity, sensitivity, selectivity, accuracy and precision, stability, and carryover.

Maintaining profitability depends largely on the number of studies an organization can complete while meeting FDA criteria. And because each study can involve thousands of samples across a wide range of complex molecules, achieving maximum laboratory operational efficiency is critical to remaining competitive.

One Latin American CRO laboratory was routinely employing HPLC with UV detection and electrochemical detection. The lab often had issues with sensitivity, which meant that defining pharmacokinetic profiles accurately could prove difficult—especially at low dosages. Subsequent sample concentration and matrix issues led to frequent sample re-analysis and a general lack of reproducibility. Thus, the time of analysis per sample was too long in many cases, affecting the completion of the study, as well as bottom-line costs and profitability.

THE SOLUTION

Implementing the Waters® ACQUITY UltraPerformance LC® (UPLC®) System with a Waters tandem quadrupole mass spectrometer made a substantial and immediate difference in the efficiency of this CRO laboratory. The ACQUITY UPLC System reduced the time of analysis 50 to 70% depending on the product, with improved resolution and reproducibility. With this powerful UPLC/MS/MS system, the laboratory’s problem of lack of sensitivity was overcome with a selective and repeatable assay.
The laboratory also realized a significant increase in the efficiency of their method development—even for the most complex molecules—so they were able to save time on analysis for a number of different analytical tasks.

In one example, a combination of two compounds, one a cholesterol-lowering compound and the other a non-steroidal anti-inflammatory, required low-level analysis. The compounds were very difficult to detect by conventional HPLC detection because of the lack of UV chromophore. By using the ACQUITY UPLC/MS/MS System with ESI, and taking advantage of the MS instrument’s unique positive/negative ionization mode switching feature, the laboratory achieved the necessary selectivity and sensitivity for the analysis of two molecules in the same run. The method was linear, reproducible, specific—and fast, taking only about four minutes per run.

In another example, the CRO laboratory performed analysis of anti-biotics with very low UV absorbance that were previously analyzed by HPLC with electrochemical detection, requiring long run times and system re-equilibration. With UPLC/MS/MS, the run time was reduced to four minutes, which in turn allowed the company to analyze more patient samples per day, with fewer repetitions per sample, shorter time of method development and validation, and less overall sample and solvent usage.

With the dramatic increase in throughput, the company was able to realize a 7.5-fold increase in productivity, accelerate their clinical study, and ultimately deliver a better pharmacokinetic profile of the different products under test.

**BUSINESS BENEFIT**

With the implementation of their first ACQUITY UPLC System for UPLC/MS/MS analysis, the CRO laboratory doubled the number of bioequivalence studies they were able to process per month. Realizing the scalable benefits, they purchased two additional systems over a period of two-and-a-half years to further optimize their efficiencies and increase their capacity.

Due to the fact that they could then have a quantifiable differentiation over competitive CRO operations utilizing HPLC, the company promoted rapid, sensitive assays with high quality and fast turnaround. Additionally, they were able to maximize their margins to become more competitive, capturing additional customers and gaining substantial market share, even against some of their most formidable overseas competitors.
**KEY WATERS TECHNOLOGIES FOR BIOANALYSIS**

**ACQUITY UPLC® System:** The ACQUITY UltraPerformance LC® (UPLC®) separation system lets you achieve higher chromatographic resolution and sensitivity and greater throughput using sub-2 µm particle technology.

**ACQUITY UPLC Columns:** The most technologically-advanced LC columns ever created. Designed, tested, and guaranteed for use in applications up to 15,000 psi (1000 bar), they provide unsurpassed efficiency, ruggedness and throughput. Combine faster separations with higher resolution by harnessing the full potential of small particles.

**MassLynx™ Mass Spectrometry Software:** Software for MS and MS/MS analysis. Easy-to-use instrument controls and software features simplify interaction with your MS system and improve laboratory productivity.

**Oasis® SPE:** Achieve robust, selective, and sensitive solid-phase extraction (SPE) methods without worrying about low recoveries caused by breakthrough, sorbent drying, pH limitations, and undesirable silanol activity.

**NuGenesis® SDMS Information Platform:** Automatically capture, secure, access, and disseminate information from any analytical technology.

**Xevo™ TQ Mass Spectrometer:** An advanced tandem quadrupole mass spectrometer with uncomplicated access to superior UPLC/MS/MS performance.

Learn more at [www.waters.com/bioanalysis](http://www.waters.com/bioanalysis)