Drug discovery is the process of generating compounds and evaluating all their properties to determine the feasibility of selecting one new chemical entity (NCE) to become a safe and effective drug. Pharmaceutical companies are under ever-increasing pressure from both their business competition and the patient population to increase the rate of drug discovery. The rapid increase in the rate at which NCEs can be synthesized by chemists and tested has meant that thousands of compounds per year undergo the lead selection and optimization process at major pharmaceutical companies. Although medicinal chemists will continue to search for programs and techniques to predict animal and human pharmacokinetics (PKs), the need to obtain experimental PK data from laboratory animals early in the discovery paradigm is still paramount (1, 2). PK properties are important decision-making criteria for selecting...
drug candidates in the initial phases of drug discovery. A key parameter in drug metabolism and PKs is the plasma concentration of the new drug after it is administered to laboratory animals.

For scientists involved in the bioanalytical component of drug discovery, the primary ongoing challenges are the speed and effectiveness of assay methods that are used in screening drug candidates. A fast turnaround time from sample receipt to the PK report provides one important set of information about a potential lead drug; the report requires minimal resources and often gives “go/no go” feedback to chemists (2–4). On the other hand, assay effectiveness is critical for analytical work as well. Assay performance is typically judged by precision, accuracy, and sensitivity. Because of its inherent selectivity and sensitivity, LC/MS/MS has proven to be both fast and accurate and is now the bioanalytical tool of choice for most pharmaceutical companies (1–6).

In many cases, an LC/MS/MS method suitable for a drug discovery PK assay can be developed in only a few hours if the compound and the chromatography are well behaved. Although the method would not be good enough to meet Good Laboratory Practices, it would be sufficient for use as a new drug-discovery PK assay (1). The bigger challenge occurs when a difficulty is encountered in the development of the assay. Often, the solutions presented in the literature only focus on one step in the method development process. We believe that it is important to consider the whole bioanalytical method development process from start to finish. What is needed is a systematic procedure for quickly developing a useful assay even when problems are encountered. Figure 1 shows our six-step paradigm for developing a fast and reliable assay for drug discovery.

**Tandem MS selected reaction monitoring**

Atmospheric-pressure ionization (API) interfaces, which consist of ESI and atmospheric pressure chemical ionization (APCI), are ideally suited for interfacing with tandem MS. These two techniques typically produce protonated molecules ([MH]+) in the positive mode with very little, if any, fragmentation. Thus, all of the current from the analyte is directed into only one ion—an ideal situation for tandem MS experiments. The rationale for choosing one interface over the other is most often based on the solution-phase proton affinity of the analyte: ESI normally requires preprotonated ions in solution and thus works best with fairly basic or acidic compounds, whereas APCI is well suited for the analysis of more neutral compounds (7).

The first step is to develop a selected reaction monitoring (SRM) method for the target compound. Typically, the SRM transition with optimized collision energy can be found by infusing the analyte stock solution with a generic tune-and-calibration file on a triple quadrupole mass spectrometer. For example, in the positive ionization mode, the [MH]+ is selected with the first quadrupole (Q1) and is then focused into the collision cell (Q2), where it is fragmented by collision-induced dissociation into various product ions. One product ion is selected with the third quadrupole (Q3), and only ions of that particular m/z are sent to the detector.

**FIGURE 1.** Fast method-development paradigm in drug discovery.
The highly specific nature of the SRM experiment with the triple quadrupole mass spectrometer was first described by Brotherton and Yost about 20 years ago (8). Their basic principle was that the multiple stages of selection in the MS/MS system reduced the noise more quickly than the signal, thereby creating a net improvement in S/N. Therefore, the SRM method typically provides very high selectivity for bioanalytical quantitation.

Because the analytical method will be used with multiple species, it should have high selectivity, ideally only responding to the compound of interest. This high degree of selectivity means that the sample analysis time can be very short (1–3 min); thus, the selectivity of the method directly impacts not only the speed of analysis but also the time required to develop the method.

Sample preparation
Sample preparation is still a topic of high importance when an LC/MS/MS method is developed to assay biological samples. Because of the large amount of proteins in plasma samples, conventional HPLC columns will not tolerate the direct introduction of plasma; therefore, most bioanalytical assays have a sample preparation step to remove >90% of the proteins from the sample (6, 9). Other important reasons to have a sample preparation step include reducing matrix components in the samples and eliminating ion suppression (also called matrix effects) in the MS response (10). In addition, the method’s performance should remain reasonably consistent over time. The results should be relatively free from systematic errors; any relative errors should be characterized and consistent and meet acceptability guidelines for the method (1). Therefore, sample preparation is used to ensure that a method maintains certain basic elements of ruggedness and consistency that are expected in any bioanalytical assay.

For drug discovery, protein precipitation (PP) is the most common sample preparation procedure; liquid–liquid extraction (LLE) and solid-phase extraction (SPE) have also been reported as preparation techniques for plasma samples (1, 3, 11–20). PP is the simplest approach because it requires no method development, and it removes the majority of the protein from the sample. SPE is a very effective method for removing matrix components and often provides the cleanest extract. However, this performance comes at a price: SPE method development is generally the most complex and time-consuming, and it typically involves multiple method optimization steps. LLE has also been used extensively to remove unwanted matrix components; it is based on the principles of differential solubility and partitioning equilibrium of drug molecules between aqueous (sample) and organic phases. It offers reasonably easy method development and is an effective sample cleanup process. Although LLE is generally easier to implement than SPE, it is still more labor-intensive than PP.

Direct sample injection techniques (e.g., restricted-access media, on-line SPE, and turbulent-flow chromatography) have been developed as an alternative to off-line sample preparation to meet fast-turnaround requirements; however, they typically need complex analytical configurations, and this limits their usage (6, 21–23). PP is still the most popular sample preparation technique for biological matrices, such as plasma (1–3, 5). PP works largely because of the high selectivity of LC/MS/MS in SRM mode; typically, there is little or no response from any compound other than the analyte of interest. In essence, the mass spectrometer is used for the bulk of the “separation” process, and the LC serves in no small part as an automated sample introduction technique. Thus, sample cleanup time is minimized by using PP. Method development time is also reduced from weeks to 1 day.

A standard PP procedure using acetonitrile that contains the internal standard, with a 1:3 plasma:solvent ratio, can be used for a plasma test sample in a 96-well plate. After the acetonitrile solution is added, PP is facilitated by vortexing and centrifuging the plate (24). A 10-μL aliquot of the supernatant is then injected directly into an LC/MS/MS system that uses a generic HPLC gradient and the targeted SRM transition setting for the analyte.

Checkpoint #1: Matrix-effect test
In the SRM mode, monitoring the specific precursor mass of the drug ion and its transition to a characteristic product ion can, in most cases, eliminate interference from other components within the sample. However, some challenges still remain, such as matrix-effect problems, which can result in significant quantitation errors (1, 10, 25–27). Undetected matrix-effect problems could lead to major mistakes in calculating PK parameters.

In the U.S. Food and Drug Administration guidelines for bioanalytical validation, matrix effects are defined as “interference from matrix components that are unrelated to the analyte” (28). This broad definition includes both ion enhancement and ion suppression, which can be caused by ionization competition among co-eluting components within the API source (10, 25). Compared with ion enhancement, ion suppression is more problematic in that it will reduce the sensitivity of the assay. It is also a more common problem. The possible mechanisms for ion suppression are competition for limited surface excess charge, incomplete evaporation, ion pairing, and competition for protons in the gas phase (25).
Because of the importance of the matrix-effect potential in an LC/MS/MS assay, an evaluation of this effect is the first checkpoint in our flowchart. Several approaches, such as postcolumn infusion (10, 25, 26, 29, 30), direct comparison, pre- and postspiking comparisons, and the standard addition method, have been developed to evaluate the matrix effects with different experimental techniques. Each has advantages and disadvantages (25). In our laboratory, postcolumn infusion is the primary approach for detecting matrix effects. Control sample extracts are injected onto the HPLC column under generic gradient conditions while a constant amount of analyte is infused into the HPLC eluent before it enters the mass spectrometer. Any ion suppression is observed as the variation of MS response of the infused analyte, as compared with the response from the injection of blank mobile phase.

If no matrix effect occurs (i.e., no effect during the elution window for the analytes and the internal standard), proceed to checkpoint #2. However, if any significant matrix-effect problems arise, then nonroutine assay options should be implemented. For example, different chromatography can be used to separate the target compound from the matrix-effect region of the chromatogram, or LLE or SPE can be used to obtain cleaner samples and remove the matrix-effect problem.

**Checkpoint #2: Interference test**

Another challenge is background interferences, which are becoming more common as researchers strive to reach lower limits of quantitation (LOQ). Recent technological advances provide a unique enhanced mass resolution capability for a triple quadrupole mass spectrometer. It is important to remember that normally the higher the mass resolution, the less signal will be obtained on a triple quadrupole MS system. For a triple quadrupoleMS instrument, unit mass resolution is normally defined as 0.7 Da fwhm (31). To achieve the best balance of sensitivity and selectivity on the TSQ Quantum MS system in enhanced mass resolution mode, settings of Q1 = 0.2 Da fwhm and Q3 = 0.7 Da fwhm are recommended (31). Chromatographic peak areas typically decrease by 30–60% when mass resolution is increased from 0.7 to 0.2 Da fwhm (31, 32).

Figure 2 shows representative mass chromatograms of a proprietary drug candidate in rat plasma. In this case, significant background interference of the signal of this compound was observed at the unit mass resolution setting (Q1 = 0.7 Da fwhm; Figure 2a). An S/N of 8 was calculated. Hence, the LOQ for this compound at unit mass resolution under these conditions was ~250 ng/mL. When the same sample was assayed at an enhanced mass resolution (Q1 = 0.2 Da fwhm), a much cleaner SRM peak was observed (Figure 2b). The enhanced mass resolution allowed the removal of a considerable amount of isobaric chemical interference through improved mass selectivity. In addition to a more uniform SRM peak, an improved S/N = 31 was obtained. The dramatic decrease in background noise at the enhanced mass resolution was responsible for this significant improvement in S/N, despite the loss of a factor of 3–4 in peak area for the analyte. Because the S/N of 31 at 250 ng/mL was above that required for the LOQ, the assay LOQ could be set to 25 ng/mL under the enhanced mass resolution condition.

The background interference can be a more common problem as a very low LOQ is sought (31–33). Xu et al. reported a twofold sensitivity enhancement in both plasma and brain samples when they used the enhanced mass resolution instead of the unit mass resolution (32). It is also important to realize that setting up enhanced mass resolution methods requires more attention to detail than unit mass resolution methods. For example, the mass setting for the precursor ion selection is more critical in an enhanced mass resolution assay because the analyte mass peak is narrower (31). Therefore, it is important to use the appropriate mass setting for the Q1 precursor ion (set to the nearest 0.1 Da, not to the nominal mass) to avoid missing the top of the mass peak; that would lead to the selection of ions from either the ascending or descending side of the normal distribution of the mass peak for the precursor ion.

Furthermore, as discussed by Jemal and Ouyang, the precursor ion (Q1) mass values appear to change slightly as the mass resolution is changed (33). Typically, a change of 0.1 Da is observed when the Q1 mass setting is changed from 0.7 to 0.2 Da fwhm (33). Therefore, for routine use of enhanced mass resolution, precursor ion scanning immediately before the start of the analysis is recommended to update the exact precursor ion mass in the SRM table. It is normally not as important to recheck the product ion (Q3) mass setting because the mass resolution for the product ion in the SRM method is typically set at 0.7 Da fwhm.

These examples have shown the strength of using the additional resolving power of the quadrupole mass analyzer (at enhanced mass resolution) to eliminate background interferences without going through a more complex sample preparation procedure or revising the chromatographic conditions. This option can significantly reduce method development time. If the interference problem cannot be solved by enhanced mass resolution, then nonroutine assay options, for example, different chromatography, LLE, or SPE, can be used to eliminate the problem.
Checkpoint #3: Standard curve linearity

A standard curve from low to high concentration (generally 1–5000 ng/mL) can be obtained by spiking the blank sample matrix with stock solutions. Typically, a triple quadrupole mass spectrometer will give a linear response with 3–4 orders of magnitude in the dynamic range of the drug concentration. The regression weighting parameter is often $1/x$ or $1/x^2$ ($x$ is the drug concentration), which is needed to make the low end of the standard curve fit correctly. At higher concentrations, the standard curve may deviate from a linear response because of ionization factors or instrumental detection limits (9). This nonlinearity can often be compensated for by the use of either a power curve or quadratic fitting in the regression step (1).

Another important aspect is the reproducibility of the standard curve. Typically, two standard curves are obtained, one at the beginning of the assay and one at the end. After the assay, the proper standard curve range, which must include at least five consecutive concentrations, is selected (1). At least 75% of the assayed standards in the range must be included in the regression analysis and must be back-calculated to within 27.5% of their nominal values, and the regression $r^2$ must be $\geq 0.94$ (1).

**Mystery problem**

If the standard curve is not linear or reproducible, more investigation is needed. A proprietary discovery drug was administered to dogs via intravenous (IV) and oral (PO) routes. Dog plasma samples were collected between 7 min and 48 h afterward. The accuracy of the data was critical to calculating key PK parameters. The standard curve fit in neither a linear regression nor a common quadratic regression; this indicated a problem with the assay (Figure 3). One possible explanation was that some nonspecific binding occurred during the preparation of the spiked standards. When the stock solutions were tested under the same LC/MS/MS conditions, a perfect linear response of 0–1000 ng/mL was obtained ($r^2 = 0.9993$); this indicated that the problem was not the instrumentation and was most probably caused by the sample preparation step. To test reproducibility, the stock solution standard curve was reassayed with eight replicates. Reproducibility was very good, which indicated that the compound’s stability in organic solvent was not the issue.

It is well known that certain drugs bind nonspecifically to some 96-well plates (34). Because all the stock solution standard curve samples were prepared in glass vials, the same brand of glass vials was used for plasma sample preparation; the typical 96-well plate was not used (Figure 4). The first standard curve (series 1) showed very good linearity with $r^2 = 0.9993$. However, in the rest of the curves, the peak area ratio kept decreasing over time, and the curve shape started to become similar to the shape

![Figure 3](image-url)

**Figure 3.** Standard curve in dog plasma was assayed by LC/MS/MS with replicates in a 96-well plate.

![Figure 4](image-url)

**Figure 4.** Reproducibility of standard curves in dog plasma prepared in glass containers. The protein precipitation supernatants from a set of standard curves (0–5000 ng/mL) were continuously injected 9 times.
of the initial plasma standard curve prepared in the 96-well plate (Figure 3). These data clearly indicated that the assay problem came from the dog plasma matrix rather than from the sample container types.

To investigate this hypothesis, we first prepared the dog plasma PP supernatant matrix and then spiked the stock solutions into it for a set of concentrations of 0–1000 ng/mL. Under the same LC/MS/MS conditions, the dog plasma supernatant standard curves were assayed with eight replicates. The results showed a similar trend to that seen in the previous experiments (Figures 3 and 4). Therefore, these data indicated that some component in the dog PP supernatant matrix caused this unusual phenomenon, so the need for another sample preparation procedure was indicated.

At this point, we chose LLE rather than SPE to achieve a cleaner sample primarily because it was easy to implement LLE. The compound had good solubility in methylene chloride, so it was tested as the extraction solvent. After the LLE sample preparation procedure, the standard curves assayed by LC/MS/MS fit an excellent linear regression with \( r^2 = 0.9991 \). The reproducibility of the eight replicates was also very good. Although the exact cause of the unusual nonlinear behavior was still not clear, the final method that was developed was acceptable. The IV/PO dog PK study samples were assayed, and the PK data were reported (the last step!) in a timely manner to the drug discovery team. A popular PK calculation tool that can be used for preclinical studies is Watson LIMS (1).

Future perspectives

Revolutionary changes during the past decade, particularly the introduction of the robust, broadly applicable techniques of ESI and APCI, have made MS an essential tool in the optimization of the in vivo PK parameters of lead molecules in the drug discovery stage. As both MS and sample robotic instrumentation improve, opportunities to increase throughput for PK studies will continue to exist. Although not widely practical at this time, microfluidics and miniaturization hold promise in terms of throughput advantages. HPLC-on-a-chip devices are being developed and will someday be interfaced to an MS system. Combining microchip-based separation devices with ESI MS could provide high-throughput sample assays on the nanoliter scale, with hundreds of channels being processed in parallel and total assay time shrinking from hours to minutes or seconds. The capability to work at the nanoliter scale can be an advantage in sample-limited situations, which are often encountered in the pharmaceutical drug discovery process. Although some successes have been achieved in these areas, enormous technical obstacles in separation and detection sensitivity need to be overcome before these approaches can be practical.

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Walter A. Korfmacher is the director of, Xiaoying Xu is a group leader of, and Jing Lan is an associate scientist in exploratory drug metabolism at the Schering-Plough Research Institute. The group’s interests are mainly in the use of HPLC, combined with API MS or tandem MS, for the quantitative assay of various pharmaceutical molecules in plasma at the nanogram-per-milliliter level. The group is also interested in developing strategies for applying new MS techniques to drug metabolism and drug discovery. Address correspondence about this article to Xu at Schering-Plough Research Institute, 2015 Galloping Hill Rd., Kenilworth, NJ 07033 (xiaoying.xu@sphcorp.com).

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