HPLC-API/MS/MS: a powerful tool for integrating drug metabolism into the drug discovery process

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HPLC combined with atmospheric pressure ionization (API) mass spectrometry (MS) has become a very useful tool in the pharmaceutical industry. The technique of HPLC-API/MS/MS is becoming very important for both drug discovery and drug development programs. In the drug discovery area, it has three major uses: (1) rapid, quantitative method development, (2) metabolite identification, and (3) multi-drug analysis. The sensitivity of the API source and the selectivity provided by tandem mass spectrometry (MS/MS) enable rapid, quantitative method development for drugs in plasma. Early information on the metabolism of candidate drugs can guide structural modifications, thereby improving the activity and/or bioavailability.

The use of mass spectrometry (MS) to support metabolism studies of drugs in development is well documented[1–6]. In the past, various 'off-line' MS methods were employed for metabolite identification; these procedures were typically reserved for candidate drugs in the development stage. In addition, GC-MS was employed in selected cases for quantitative analysis. More recently, with the introduction of commercially available HPLC combined with atmospheric pressure ionization/tandem mass spectrometry (HPLC-API/MS/MS), both metabolite identification and quantitative analyses are being performed routinely using this new technique.

Until recently, most analytical methods for the determination of a candidate drug were based on either GC or HPLC methods. The introduction of HPLC-API/MS/MS systems has provided new opportunities for rapid method development and metabolite identification, and this has helped to integrate drug metabolism into the drug discovery process.

Recent articles have described HPLC/API/MS/MS technology and provided some examples of its utility for specific assays[1–11]. In this report, the utility of HPLC-API/MS/MS systems for integrating drug metabolism into the drug discovery process will be discussed. A comparison will be made with GC and HPLC methodologies.

Rapid method development

Currently, various techniques are available to generate large numbers of compounds for biological screening, such as combinatorial synthesis and isolation from natural sources. The active compounds identified in these screens often require pharmacokinetic and *in vitro* metabolic evaluation. Over 100 compounds may have to be screened for their

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in vitro stability or *in vivo* pharmacokinetic parameters before a lead compound can be chosen. These *in vivo* and *in vitro* studies require large numbers of samples in various biological matrices that need to be subjected to quantitative analysis; rapid pharmacokinetic evaluation is therefore required.

In the past, standard methods used various sample clean-up methods (for example liquid/liquid phase extraction and solid-phase extraction) and GC or HPLC techniques for separation and detection of compounds[1,2]. As shown in Figure 1, these sample preparation methods typically required a sample volume of 200–1000 µl of plasma or serum. The separation method depended on the nature of the compound, and a significant amount of time was required to obtain the proper analytical conditions necessary to resolve the various components in the matrix. Often, a derivatization step was necessary to obtain enhanced sensitivity. This multi-step procedure could take 1–3 weeks to develop.

However, rapid method development (Figure 1) is possible when samples are analyzed by HPLC-API/MS/MS.

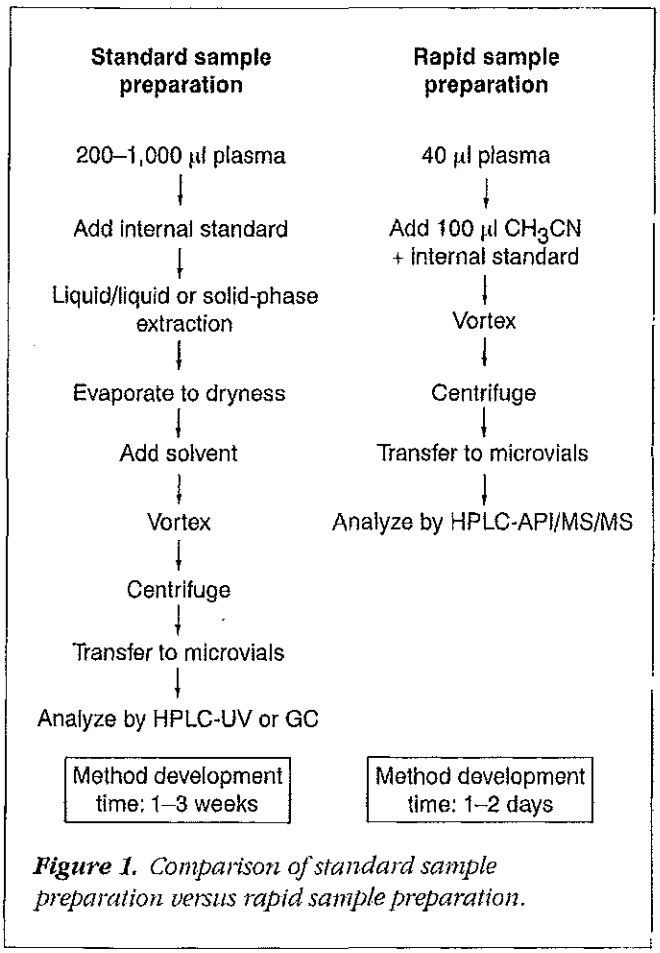

Figure 1. *Comparison of standard sample preparation versus rapid sample preparation.*

Sample clean-up involves protein precipitation. This only requires 20–40 µl of plasma or serum. Current API source designs are well suited to accept hundreds of injections of this type of sample, with minimal source cleaning needed between sample sets. In addition, HPLC method development is less demanding because of the added selectivity provided by the mass spectrometer. Compounds with different molecular weights do not need to be resolved. Typically, 1 or 2 days is sufficient to develop a method for a compound that is part of an ongoing series of compounds being screened for their pharmacokinetic parameters.

By using one HPLC-API/MS/MS system, one operator can typically develop methods for two compounds and the methods used to analyze one set of samples for each compound per week. This can result in a rapid turnaround of samples so that the required pharmacokinetic information can be provided to the drug discovery team in a timely manner. Evolving strategies (see below) may help to improve this throughput in the near future.

The two main reasons why HPLC-API/MS/MS allows rapid method development are the inherent selectivity and sensitivity of the instrumentation. The selectivity of HPLC-API/MS/MS is on account of the specificity provided by tandem mass spectrometry (MS/MS)[3]. When used for quantitative analysis, tandem mass spectrometry with a triple stage quadrupole mass spectrometer is used as follows. A specific ion is selected in the first quadrupole, typically the protonated molecule (MH^+) for the compound of interest. The selected ion then enters the collision cell (the second quadrupole) of the instrument and fragments into one or more smaller (product) ions. These fragment ions are analyzed in the third quadrupole and detected. Because of the mass selectivity provided by a tandem mass spectrometer, the high chromatographic resolution typically required for UV or fluorescence detection is not necessary. Usually, extensive sample clean-up procedures are not required. The sensitivity of the HPLC-API/MS/MS system is provided in part by the API source. For typical pharmaceutical molecules, assays can be readily developed for concentrations in the range of 10–25 ng/ml using only 40 µl of plasma. Thus, preconcentration or derivatization techniques are generally not required.

Serial bleeding in rats

Previously, one rat per time point was the standard procedure for pharmacokinetic studies in the rat. Currently, using HPLC-API/MS/MS, methods can be developed based

on the analysis of 40 µl of plasma or serum (Figure 1)[12], or even on 10–20 µl of plasma or serum if lower sample volumes are necessary[13]. Because of these small sample volume requirements, it is possible to analyze samples from rats that are subjected to serial bleeding. In serial bleeding, a single rat is dosed with the test compound and sampled at multiple time points (typically five to eight). At each time point a small volume of blood is removed (typically 200 µl). For mean data, three rats can be dosed. The advantages of serial bleeding in rats are:

- Fewer rats are needed to obtain pharmacokinetic data on a compound.
- Smaller amounts of the test compound are needed for dosing.
- There is less variability in the data.
- The entire set of pharmacokinetic parameters (C_{max}, area under curve, $t_{1/2}$) are available for each animal.

Figure 2 shows data obtained from (a) a serial bleeding study and (b) a multi-rat study for the same compound dosed orally at 10 mg/kg. It can be seen that there is a better correlation between the individual data and the mean data for the serial bleeding study than for the multi-rat study.

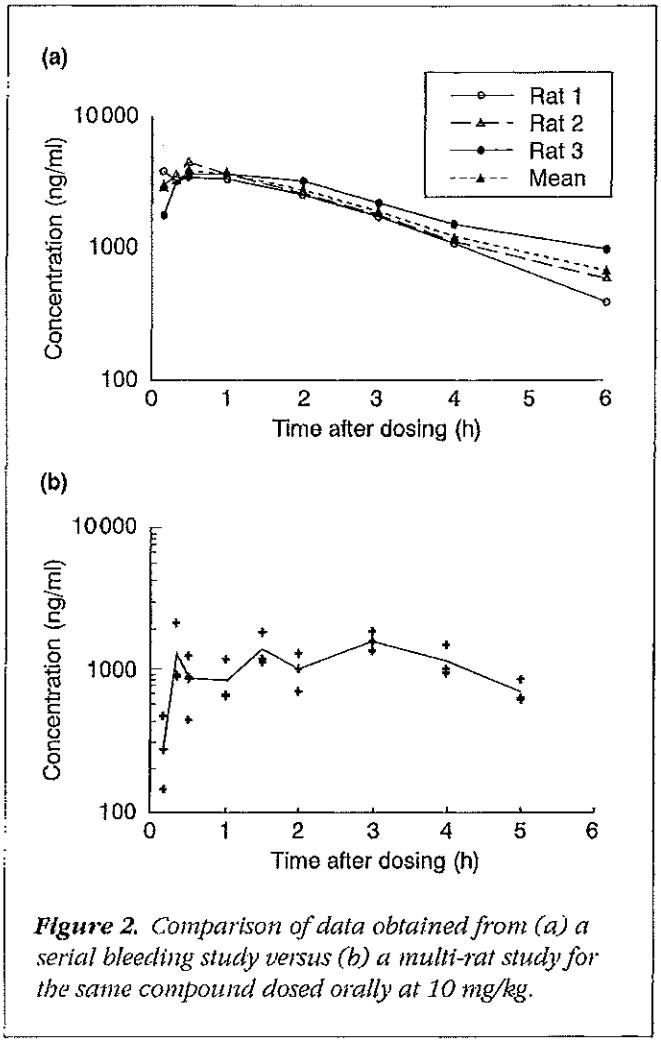

Figure 2. *Comparison of data obtained from (a) a serial bleeding study versus (b) a multi-rat study for the same compound dosed orally at 10 mg/kg.*

Metabolite identification

Previously, metabolite identification was reserved for compounds in the development phase. One reason for this was that the standard method for metabolite identification relied on radiolabeled drug. As shown in Figure 3, the process of synthesizing the drug and collecting, purifying, and analyzing the metabolites typically takes 2–4 months. This time frame is not acceptable by current drug discovery standards. In the drug discovery phase, HPLC-API/MS/MS can be used for metabolite identification. As shown in Figure 3, extensive metabolite identification can be completed in 1 week. In many cases, 1 day is sufficient to obtain useful information on the plasma metabolites of a drug dosed in an experimental animal. A more extensive look at other fluids (urine, bile) or other tissues (e.g. brain, heart) could take 1 or 2 weeks. In either case, significant amounts of information on the metabolism of a compound can be obtained in a relatively short period of time.

Metabolite identification in drug discovery provides early information that can lead to structural changes in the current lead compound, improving such pharmacokinetic parameters as oral bioavailability, half-life ($t_{1/2}$), or C_{max}. Often these parameters can be changed by improving the metabolic stability of the compound. In order to improve metabolic stability, it is very important to know how a compound is metabolized. The goal of drug discovery is to progress a lead compound into a final candidate drug that can be placed in the development stage. The traditional role of drug metabolism in drug discovery was often limited and, in the past, consisted mainly of producing a pharmacokinetic profile of lead compounds. Early metabolite identification can provide information on how to improve the metabolic stability of the lead structure. In this way, future lead compounds might be a metabolite identified from the previous lead drug or an analog of the previous drug designed to block the major route of metabolism. In either case, metabolite information in early drug discovery may lead to a much faster progression from the early lead drug to the final candidate drug.

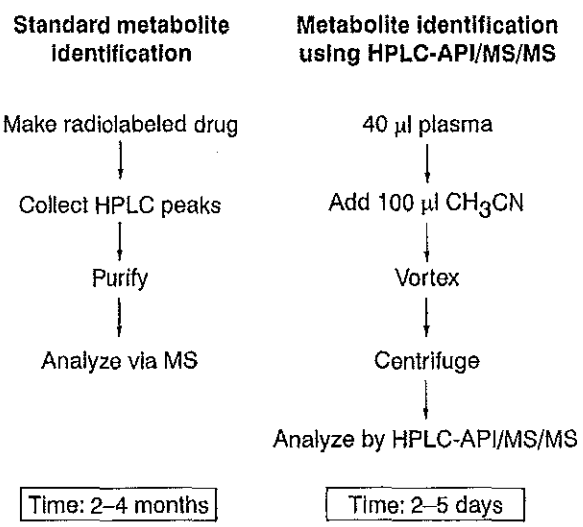

Figure 3. *Comparison of standard metabolite identification procedures versus metabolite detection and identification using HPLC-API/MS/MS.*

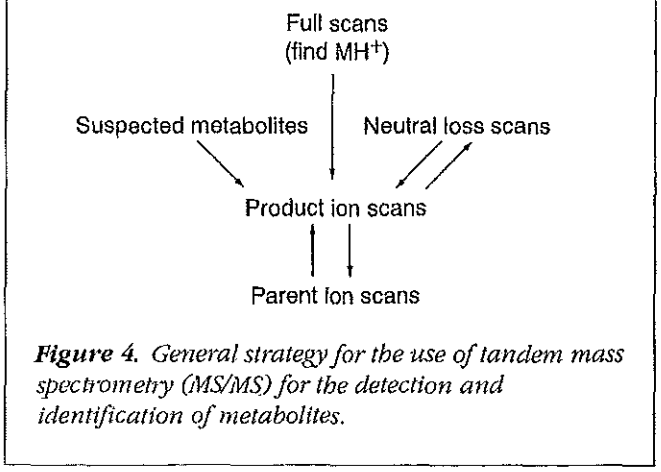

Figure 4. *General strategy for the use of tandem mass spectrometry (MS/MS) for the detection and identification of metabolites.*

The methodology for using tandem mass spectrometry (MS/MS) to identify metabolites is well documented[2–5,9,11]; however, the application of this methodology to drug discovery is a relatively recent development. Figure 4 shows a general strategy for the use of MS/MS for the detection and identification of metabolites without the need for a radiolabeled drug. A full-scan mass spectrum displays all masses detected over the desired mass range. This provides no structural information on the compounds detected, so there could be substantial contributions from unrelated compounds, solvent ions or chemical noise. Structural information can be obtained on masses of interest by subjecting them to MS/MS. The most common type of MS/MS experiment is a product ion scan. In this case, ions of interest are isolated and fragmented into several product ions. These product ions provide characteristic structural information about the original ion. For example, a suspected metabolite ion would fragment into product ions that either resemble the original parent drug or differ from the product ion masses by the mass of the metabolite modification. A second MS/MS mode is a precursor ion scan. Here, a characteristic product ion is specified and all ions that fragment to form this product are monitored. In this manner, a characteristic fragment of the parent drug can be selected to screen for possible metabolites that might produce the same fragment. The third MS/MS mode is a neutral loss scan. This is similar to a precursor ion scan except that the characteristic fragment selected is a neutral species rather than an ionic species. Conjugated species such as glutathione or sulfate give characteristic neutral losses when fragmented in a mass spectrometer; therefore, these metabolites can often be detected with a neutral loss scan.

In addition to the identification of metabolites of lead compounds, it is very important to give some estimate of the levels of these metabolites compared with the dosed drug. If a metabolite is minor (1% or less of the dosed drug), it may not be important to block its route of metabolism. If the metabolite is major (>20% of the dosed drug), it may become the new lead drug, or modification of the site of metabolism may lead to a better compound. The best way to quantitate a metabolite is to compare its response with an authentic standard of the metabolite (as is done with the dosed drug). However, a metabolite standard is not usually available, and the only estimate that can be made is to use the calibration curve for the dosed drug to estimate the level of the metabolite. It is known that MS responses for various compounds can be quite different; but, in many cases, the response of the metabolite will be within a factor of two to three of the dosed drug. Thus, while this method of estimating the level of one or more metabolites is not ideal, it can provide useful information.

Figure 5 shows a mass chromatogram of drug B dosed at 10 mg/kg orally in the rat. The top trace shows the internal standard, which is a structurally similar analog that is used for quantitation. The second trace shows the peak for drug B. The third trace shows three M+16 metabolites that were monitored for this compound. The bottom trace is the reconstructed ion chromatogram which is the sum of the top

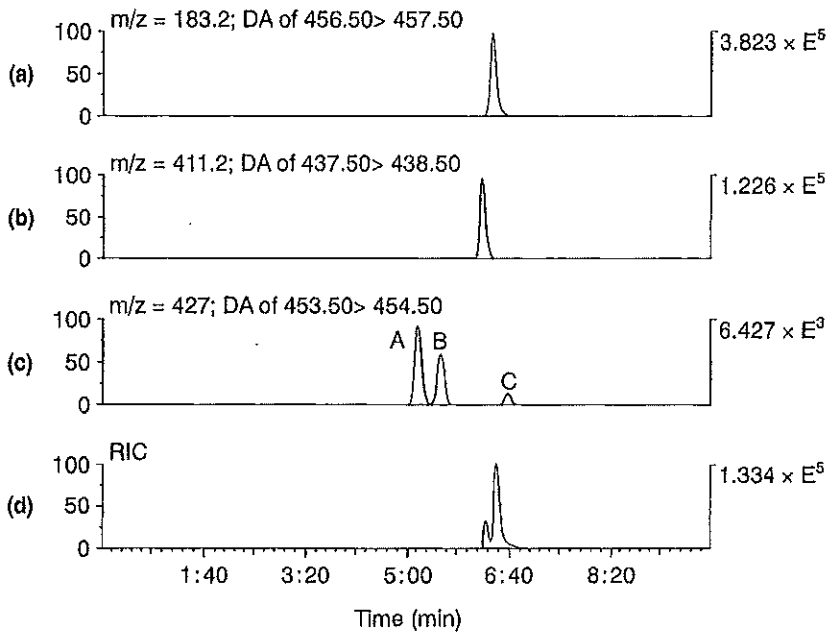

Figure 5. *Mass chromatograms from the HPLC-API/MS/MS analysis of an extract of a rat plasma sample from a 10 mg/kg oral dosing study: (a) internal standard, (b) dosed drug, (c) peaks (designated A,B and C) from three M+16 metabolites, (d) reconstructed ion chromatogram (RIC); DA = daughter ion.*

three traces. Each mass chromatographic trace is normalized to the highest peak. This figure shows the excellent selectivity of the HPLC-API/MS/MS technique; this is typical of the type of data that can be obtained by this procedure.

Figure 6 shows an example of a discovery drug that was extensively metabolized. The orally dosed drug (parent) reached a maximum level of less than 100 ng/ml. Three of its metabolites reached levels that were estimated to be significantly higher than the parent drug. A fourth metabolite showed very low levels. This information provided important clues concerning what part of the molecule needed be modified in order to improve the metabolic stability of the compound.

Multiple analysis

Another advantage of the HPLC-API/MS/MS technology is that it can be used for multiple analyses, which can be defined as the determination of more than one compound in one chromatographic assay. Use of this capability is a recent development for HPLC-API/MS/MS. This feature can be utilized in at least two different ways:

- analysis of multiple studies and
- multi-drug study analysis.

In either case, the analytical challenge is the same.

In multiple analysis, spiked plasma (or serum, etc.) calibration samples are prepared with more than one analyte. The simplest example is two analytes, although the analysis of samples containing as many as 10 or 20 analytes has been reported[15]. The basic principle is the same regardless of the number. Figure 7 shows the results of the analysis of four drugs plus one internal standard in a single HPLC-API/MS/MS assay, in which each analyte has a unique MS/MS 'analytical window'. The four drugs are compounds with similar structures that are spiked at 50 ng/ml each into rat plasma, resulting in four calibration curves (one for each of the four drugs) for the single analytical run. Thus, in the same analytical run, samples from one or more single

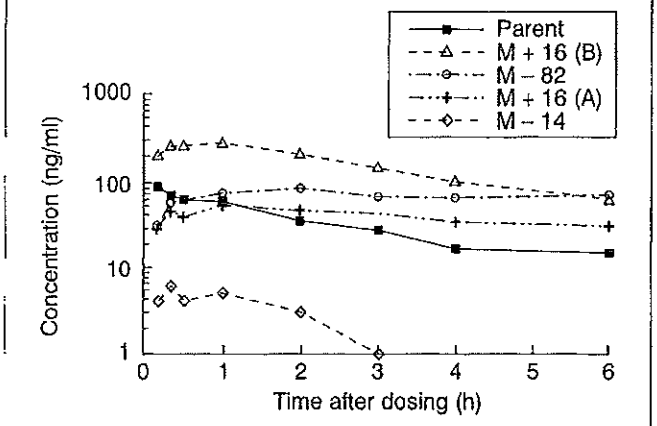

Figure 6. *Results from the analysis of samples obtained from a discovery drug that was dosed orally at 10 mg/kg; the dosed compound levels are compared with estimated metabolite levels. The metabolites are identified by their mass difference from the drug. The two M+16 metabolites are distinguished as A and B.*

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