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Current and future trends in the application of HPLC-MS to metabolite-identification studies

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Metabolic determinations are an integral part of every drug-discovery and drug-development program. Recent emphasis has been to increase sample throughput while, at the same time, increase information content within assays. To this end, screening for potential drug–drug interactions, overall metabolic stability and metabolite profiles are used early in discovery to select compounds for development. The throttle on the metabolism discovery engine is limited by the time required for data processing and reporting of the information-rich assays used in discovery-stage metabolism studies. In this article I examine how to increase throughput screening in drug discovery using novel liquid chromatography and mass spectrometry as the preferred analytical tool, and potential solutions to maximize output.

Drug metabolism and the pharmaceutical industry

The pharmaceutical industry is undergoing major changes, driven mainly by changes in the competitive and technological landscapes. Ever-decreasing cycle times and cost-cutting provide the impetus for innovative R&D partnerships and outsourcing that is reshaping the business strategies of many pharmaceutical and biotechnology companies. Today, the challenge is to find new ways to increase productivity, decrease costs and develop new therapies that will enhance human health and shareholder satisfaction. Questions remain about which strategies are effective in drug-discovery laboratories at reducing the time required to develop and market new drugs. Finding answers is crucial because research costs have spiraled upwards and increased scrutiny by the regulatory agencies lengthens the time required for a pharmaceutical company to get new drugs to the market.

A crucial part of the decision process is how and when a particular drug candidate is eliminated from the development phase. Historically, few new ideas in discovery are converted into products. In the context of the type of assays and the kind of information that is needed to make decisions earlier, the debate is whether to apply the, so-called, 'fail early, fail cheap' paradigm or the 'maximizing the shots on goal' paradigm. It is important to recognize that most development candidates fail as a result of either preclinical toxicity or lack of therapeutic efficacy [1]. Ten years ago, the main reason for failure of compounds was poor pharmacokinetics. The advent of high performance liquid chromatography (HPLC), coupled with atmospheric pressure ionization mass spectrometry in the early 1990s, enabled discovery-stage pharmacokinetic studies that have reduced attrition drastically. Now, liquid chromatography coupled to mass spectrometry is being adopted across all areas of drug discovery to provide early data that might help in understanding the metabolic fate/liabilities of a drug candidate and, thus, saving time and reducing costs in the long-term.

Studies of drug metabolism have a vital role in the pharmaceutical industry. The identification of *in vitro* and *in vivo* drug metabolites is part of the discovery and development programs of all pharmaceutical and biotechnology companies. During early absorption, distribution, metabolism and excretion (ADME) studies [2–4], scientists develop and utilize *in vitro* models and rapid methods to evaluate (bio)pharmaceutical properties and ADME parameters to support lead optimization and drug-formulation development. The ultimate goal is to develop fast, accurate and relevant higher-throughput models that correlate *in vitro* parameters with *in vivo* pharmacokinetics (*in vitro–in vivo* correlation), which requires in-depth, mechanistic understanding of the processes involved. Typically, the study of drug metabolites focuses on toxicological responses in selected animal species and how to balance the presence of metabolites in one species but not another. In other words, the metabolites that are generated are compared to

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those that are either expected or found in humans. Generally, metabolism leads to the formation of inactive compounds that are excreted. However, some metabolites lead to toxicity [5–7] and, potentially, to either the termination of the program or the re-optimization of that particular drug.

Drug-metabolism studies have key roles in medicinal chemistry for lead optimization, detection of potentially toxic metabolites, and identifying the route and rate of drug clearance from the body. Because of the sensitivity, speed of analysis and ease of use, the preferred analytical strategy for metabolite identification and quantitative studies is liquid chromatography coupled with mass spectrometry [8–16]. In particular, electrospray is a soft ionization technique that is amenable to the ionization of predominantly polar pharmaceutical drugs and their metabolites, which includes many classes of compound. It is generally accepted that ~90% of compounds are ionizable by electrospray and the remainder are ionized by other common techniques such as atmospheric pressure chemical ionization, atmospheric pressure photoionization, chemical ionization and electron impact ionization. Several mass analyzers have been used for this type of analysis, including tandem quadrupoles, linear and three-dimensional ion traps, hybrid quadrupole orthogonal time-of-flight (TOF) mass spectrometers, Orbitrap and Fourier Transform Mass Spectrometry (FTMS) [17–21]. The information from each of these mass analyzers is somewhat different. Some approaches alone reduce the amount of time needed to decipher a biotransformation pathway for a particular xenobiotic. For example, the data obtained with quadrupoles and ion traps is low resolution, whereas that from TOF, Orbitrap and FTMS is high resolution and provides accurate mass measurements that help with data interpretation.

The main requirements for metabolite identification are good chromatographic separations, full-scan sensitivity and exact mass in full scan mode and MS/MS. Typically, the samples to be analyzed vary greatly. They might be either *in vitro* or *in vivo* samples. *In vitro* samples tend to produce less complex results than *in vivo* samples and, therefore, are easier to analyze. In most cases, when analyzing *in vitro* samples, only the major metabolites are reported. At this stage of the drug-screening process, it is important to have evidence about the major metabolic route of the drug of interest. By contrast, *in vivo* samples are more complex because they contain many endogenous compounds, and the xenobiotics tend to be present at much lower concentrations than in the corresponding experiments *in vitro*. Typically, the metabolites are not visible clearly in the total ion current chromatogram and, therefore, their detection is difficult. This is especially true for first-in-human experiments when knowing the circulating metabolites might provide valuable information to refine the strategy for clinical development. The use of radio-labeled compounds makes this process easier, but by the time the labeled compound is available, resources might have been wasted.

Chromatographic separation of metabolites from endogenous matrix peaks also has a vital role in metabolite identification. During the past 30 years, HPLC has become widely accepted and employed in pharmaceutical laboratories worldwide. HPLC technology has not evolved a great deal during this time in terms of hardware performance, but it has become more reliable and easier to use. Although there have been significant advances in column technology, including particle size, porosity, chemical stability and bonded ligands (for example chiral), until recently, chromatographic performance has not increased dramatically. The development and commercial availability of porous, sub-2 μm material has enabled better chromatographic separations with increased sensitivity and resolution in a much faster timeframe than previously [22–26]. Smaller particle sizes enable the speed of separation and peak capacity to be extended to new limits. The combination of these sub-2 μm materials with dedicated, purpose-built instrumentation [e.g. UltraPerformance LC® (UPLC) from Waters and Accela™ (Thermo)] will allow faster separations with excellent peak capacities for complicated matrices such as plasma, bile, urine and feces. In addition, the use of porous, sub-2 μm particles in liquid chromatography allows a wider 'sweet spot' in the van Deemter curve for the increased flow rates without the loss of chromatographic resolution, and smaller particles increase the efficiency of separation because efficiency (N) is inversely proportional to particle size (dp). The linear velocity of the mobile phase (flow rate for a fixed column ID) at which the maximum efficiency occurs increases as dp reduces. Furthermore, with sub-2 μm particles, the flow-rate region at which the optimal efficiency is obtained is much wider. The overall result is higher efficiency at high flow rates, resulting in faster analyses and better sensitivity.

UPLC™ technology is illustrated in Figure 1, which compares the separation of *in vivo* metabolites of verapamil in rat urine using HPLC and UPLC. From this it is clear that more information is obtained when a better chromatographic separation is achieved because more metabolites are detected in less time.

Because of the need to decrease analysis time, scientists often used to sacrifice resolution for speed. UPLC technology overcomes such problems, as illustrated in Figure 2, which shows a separation of six hydroxylated metabolites of buspirone [27] in <1 min without loss of chromatographic resolution. With this approach, typical peak widths at the base of the chromatographic peaks are 1–3 sec. Therefore, to keep up with the pace of data acquisition the mass spectrometer must acquire data quickly enough to match the chromatographic output. Mass spectrometers such as FTMS or Orbitrap can not keep up with this type of analytical strategy because they need a longer scan time to acquire high-resolution data, typically 1 sec to achieve a resolution of 60 000 FWHM. As a result, few data points will be collected, which might result in the loss of information. By contrast, mass analyzers, such as TOF mass spectrometers, do not suffer from this effect as the measurement of mass spectra occurs in very short intervals. Typically, 0.1 second is all that is needed to scan from, for example, 1–1000 mass units.

For illustration purposes, a TOF analyzer can be compared with a photographic camera taking snapshots of the m/z values of an assembly (beam) of ions; the faster the repetition rate at which the camera shutter is clicked, the more mass spectra can be taken in a very short time. For TOF analysers, it is not uncommon to measure several thousand mass spectra in less than one second. All such spectra can be added to each other digitally, a process that leads to improvements in the signal:noise ratio in the final accumulated total.

For some analyses, however, speed is of secondary importance, and peak capacity and resolution are the priority. For example, *in vivo* metabolism studies present a large number of small, but