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BY DAVID KWOK, PhD

LIQUID Chromatography MASS Spectrometry

ACCELERATING THE PACE OF LEAD DRUG CANDIDATE SELECTION AND PRECLINICAL DEVELOPMENT

Introduction

A successful lead drug candidate development program often requires consideration of chemical and biochemical *in vitro* assays in the identification of active "hit" compounds. This early hit compound identification stage is commonly followed by *in vitro* and *in vivo* studies, conducted in parallel, to permit further selection of lead candidates with desirable absorption, distribution, metabolism, excretion and toxicology (ADMET) properties, as well as efficacy, to assist in the prediction of clinical "drug-like" properties.

During these early stages of discovery involving identification, selection and optimization of drug candidates, applications of liquid chromatography mass spectrometry (LC/MS) and liquid chromatography tandem mass spectrometry (LC/MS/MS) assays have become extremely useful analytical chemistry tools to accelerate the transition from discovery to preclinical development. The use of appropriate LC/MS and LC/MS/MS assays, in conjunction with proper *in vitro* and *in vivo* study designs, can generate significant benefits by shortening timelines and reducing cost during the preparation of an Investigational New Drug (IND) application.

The following discussion reviews selected examples of LC/MS and LC/MS/MS assays useful in support of *in vitro* and *in vivo* study designs, to accelerate the evaluation of drug-like properties during identification, selection and optimization of drug candidates. In addition, a brief review is given on integration of bioanalytical assay requirements in support of toxicology and safety studies, with respect to assay validation strategies in context of Good Laboratory Practice (GLP).

In Vitro and *In Vivo* Assays in ADMET Optimization

Following the screening and identification of compounds with desirable activity, the screening of drug candidates for desirable ADMET properties should follow an integrated lead optimization and selection program. The design of such a program often requires considerations of several areas, including the number of molecular hit candidates required to be optimized in the library. The primary therapeutic indication, as well as the therapeutic advantages over existing marketed therapeutic products, is also important. It is useful to note experience drawn from historical preclinical failures — including poor aqueous solubility, poor gut absorption, short half-life, mutagenicity, toxicity in target organ, hepatotoxicity, lack of animal and human ADMET correlation and signification CYP450 drug-drug interaction.

Given the task to focus on a limited number of high-quality molecular hits, an effective ADMET lead optimization and selection strategy should be planned with screening assays that can provide the most relevant prediction of drug-like properties in the clinic. As an example, the screening of CYP450 induction or inhibition, particularly 3A4 isozyme using human primary hepatocytes, has become a very important selection assay to predict clinical drug-drug interaction. Similarly, CNS drugs candidates with desirable drug-like properties often exhibit efficient blood-brain barrier transport and low serum protein binding. Although high throughput MDCK/CACO assays or artificial membrane assays are used for blood-brain barrier screening, they suffer from low predictability for specifically transported substances (transporters, P-glycoprotein, brain-specific receptors) due to differences between peripheral epithelial cells and brain endothelial cells. Alternatively, *in vivo* animal experiments using radiolabelled or non-radiolabelled compounds have the highest biological relevancy, but incur the highest costs.

In addition to the need for relevant ADMET properties, physiochemical properties of a drug candidate substance are also important. The crystal form of the drug substance during drug development, with respect to crystalline/amphorous, polymorphs, solvates, hydrates and salts selection, are also very important chemical parameters — all of which affect the intellectual property, chemical stability and bioavailability of the drug substance.[1] From this brief discussion, it can be recognized that an effective ADMET lead optimization and selection strategy must be customized for each of the approximately 500 known therapeutic targets specifically for a clinical market. The new ADMET optimization paradigm often involves a parallel approach of evaluating solubility, absorption, metabolism, formulation, pharmacokinetics, toxicity and efficacy, all of which are equally important interrelated parameters in early compound optimization.[2,3,4]

LC/MS and LC/MS/MS in ADMET *In Vitro* and *In Vivo* Studies

Prioritization for the evaluation of specific ADMET components and decisions on the use of selected *in vitro* and/or *in vivo* assays is often shaped by considerations — including the number of compounds being investigated, costs, timelines and prior experience on the safety and ADMET issues of related compounds. Regardless of *in vitro* or *in vivo* assays, the use of an LC/MS or LC/MS/MS assay method in support of ADMET investigations can generate significant benefit by reducing turnaround time and assay costs.

Samples such as serum, plasma, urine, tissue or *in vitro* assay samples are commonly presented to the analytical laboratory in 50 µl to 100 µl aliquots. Additionally, an analytical method must be able to detect drug concentrations at low nanogram levels. A common LC/MS/MS approach involves chromatography of an extracted sample, followed by electrospray ionization of a drug molecule as a $[M+H]^+$ protonated molecular ion, under solution conditions suitable for chromatography and ionization

Figure #1 LC/RM/MS chromatogram showing both MS and radioactivity detection of the ^{14}C-labelled drug at 1 ng/ml (A), which becomes undetected by MS at 100 pg/ml.

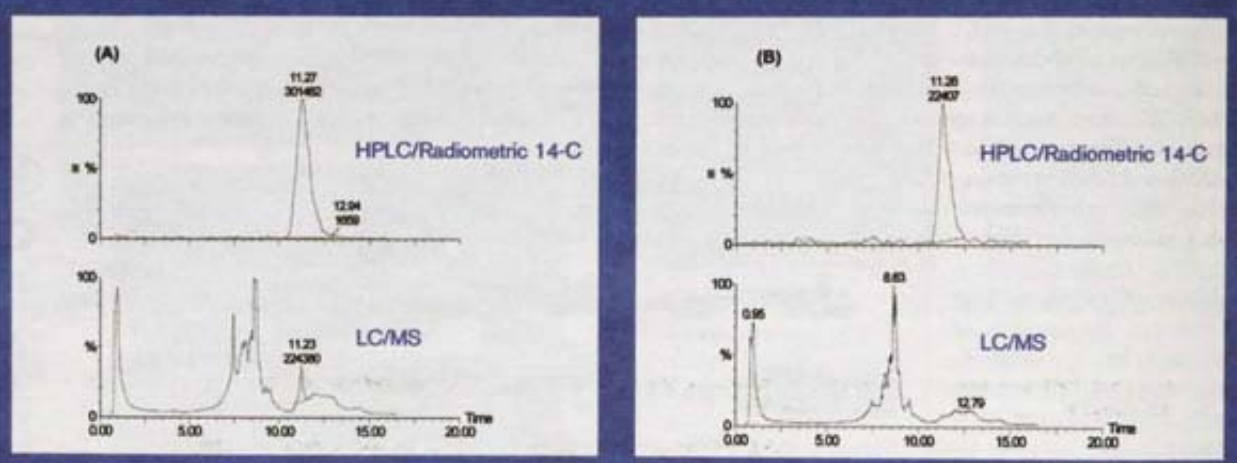

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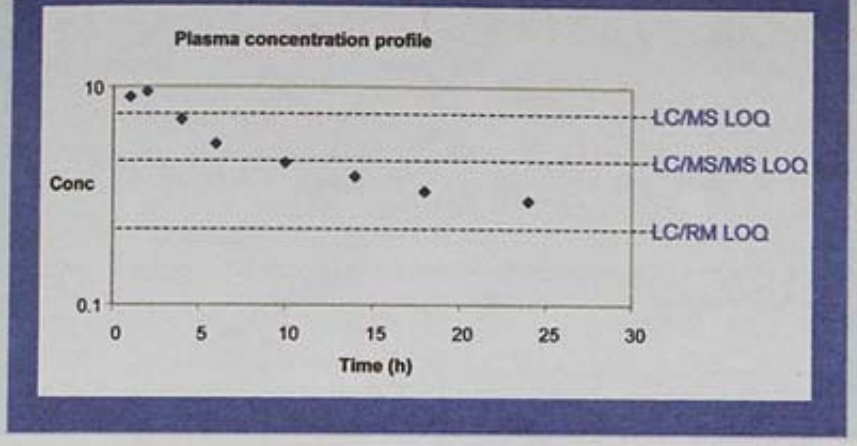
Figure #2 PK study of a drug candidate requiring low picogram plasma concentrations not adequately assayed by LC/MS and LC/MS/MS approach.



of the drug molecule. The molecular parent ion is initially detected by the first of two mass spectrometers operating in tandem. These are separated by a collision cell, where the molecular ion is induced to produce daughter ion fragments, which in turn are detected by the second mass spectrometer. This parent-ion/daughter-ion (MS/MS) detection approach provides a very specific measurement of ionized drug substances at low nanogram levels and is less reliant on chromatography to resolve co-eluting sample matrix components. In contrast, an LC/MS assay approach often relies on chromatographic separation of a drug molecule from sample matrix components, followed by detection of the protonated molecular ion.

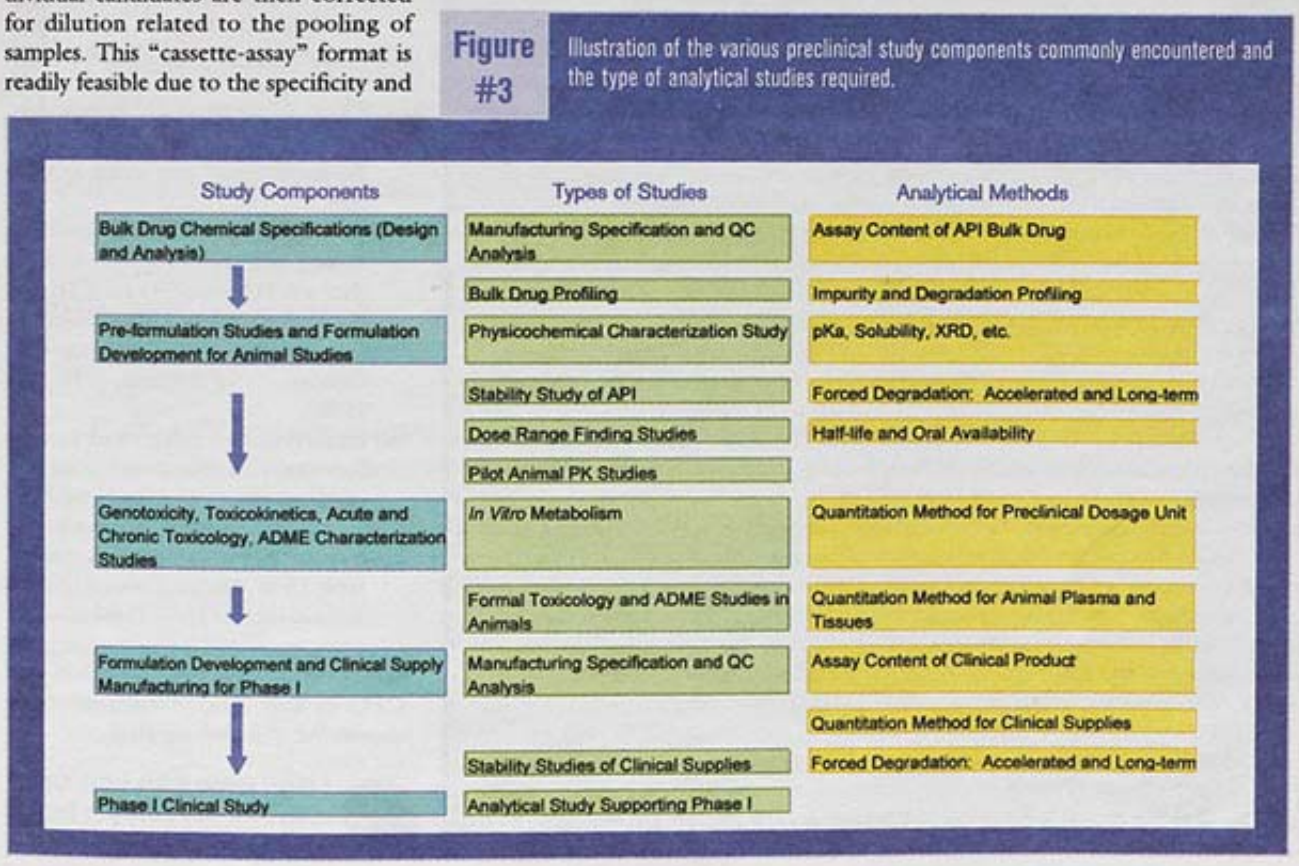
To reduce the analytical data turnaround time and the additive costs of assaying multiple samples, samples collected from *in vitro* assays of five or six individual drug candidates can often be pooled and assayed by a single LC/MS/MS assay. Assay results for individual candidates are then corrected for dilution related to the pooling of samples. This "cassette-assay" format is readily feasible due to the specificity and sensitivity of an LC/MS/MS assay approach, though an LC/MS approach may often prove to be suitable if adequate chromatographic separation amongst the drug candidates is achieved. This cassette-assay approach can effectively reduce the number of *in vitro* CYP450 metabolic screening samples or other *in vitro* assays, while avoiding potential drug-drug interaction of cassette-incubation of multiple drug candidates.

In a similar manner, cassette-dosing study design involving co-administration of a mixture of five to six test compounds can also be considered in support of *in vivo* pilot PK and tissue distribution studies. Following administration of a drug candidate mixture, plasma and/or tissue samples are collected for simultaneous assay of the drug candidates typically supported by a LC/MS/MS assay approach. This cassette-dosing approach can also be performed with comparative intravenous, oral and intraperitoneal routes of administration of a drug mixture to three parallel groups of animals. While cassette-dosing data may be confounded with *in vivo* drug-drug interaction, use of a cassette-assay approach will provide economy in reducing the number of samples requiring assay measurement.

Another important application of LC/MS or LC/MS/MS assays, using simultaneous online radiometric (RM) ($^{14}C/^{3}H$) measurement is increasingly useful in support of *in vivo* metabolism and tissue distribution studies, particularly for drugs candidates exhibiting low plasma concentrations. Online radiometric/mass spectrometry detection involves splitting the HPLC column effluent for simultaneous online detection of a $^{14}C/^{3}H$-labelled drug using a flow-through liquid scintillation detector, operating in parallel with a mass spectrometer. The use of radiometric detection provides assay sensitivity, often in the low picogram range, surpassing LC/MS and LC/MS/MS detection. This LC/RM/MS/MS approach is particularly advantageous for detection of compounds with a neutral charge structure, inherently exhibiting low electrospray sensitivity. The ability of a LC/RM/MS assay to provide quantitation of a radiolabelled molecule at 100 pg/ml, compared with the lack of detection by MS at the same concentration, is illustrated in Figure 1. This relatively low limit of quantitation has permitted the *in vivo* study of drug candidates at reduced doses. As shown in Figure 2, the PK study of a drug candidate is feasible at low picogram plasma concentrations not adequately assayed by LC/MS and LC/MS/MS assays.

Following method development, method validation and cross-validation studies for plasma matrices from different animal species are required to demonstrate reliability of drug quantitation in various plasma and tissue sample matrices. Integration of the requirements for method cross-validation study in human plasma and other human biological sample matrices is advisable, to permit application of a comparable bioanalytical method in support of preclinical and clinical Phase I studies.

Figure #3 Illustration of the various preclinical study components commonly encountered and the type of analytical studies required.



LC/MS and LC/MS/MS Applications in Preclinical Development

During preclinical development of a new drug candidate, studies relating to chemistry, formulation, animal pharmacology, safety/toxicology, manufacturing and clinical supplies studies are required to meet regulatory requirements for an IND submission.[5,6] At the onset of a preclinical program, careful planning of an integrated strategy for all analytical chemistry requirements (Fig. 3) is paramount, and will result in significant time and cost savings.

Analysis of Drug Substance Bulk Material

Manufacturing release requirements for bulk material as an active pharmaceutical ingredient (API) requires that QC assays are validated, and that purity of the API is documented in a Certificate of Analysis, along with other relevant official chemical and safety compendium specifications. For a synthetic small molecule new chemical entity (NCE) drug candidate, or for a semi-synthetic molecule, HPLC/UV detection methods are often easily developed and implemented in most research laboratories for QC of bulk materials. HPLC/UV methods are often robust when validated over concentrations in the µg/ml to mg/ml range. For an isolated natural product drug candidate, complementary LC/MS and/or GC/MS methods are often required to characterize the chemical profile and to determine the relative composition of major and minor components in the bulk material. Chemistry information and chemical specification data generated for the API bulk material will form an important component of the CMC section of an IND application. Information on the

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impurity and degradation profile of synthetic or isolated API will also be a necessary component of the Drug Master File.

While an HPLC/UV method is commonly applicable and desirable as a QC method — due to its relatively low capital cost and its availability in most laboratories — the design of an HPLC method should ideally be capable of measuring impurities and degradation products as a "stability indicating" method. Impurities or degradation products above a relative amount of 0.05% or 0.1%, depending on the proposed maximum daily dose, must be characterized and quantitated.[7]

To meet this requirement, an HPLC/UV method should be validated using representative batches of materials, derived from scale-up synthesis or isolated materials, to demonstrate sufficient sensitivity and specificity for impurities or degradation products. Also often considered are forced-degradation stability studies under prescribed thermal, chemical and photolytic conditions, as well as shelf-life storage stability studies of the API under accelerated and long-term conditions.[8] The presence of potential impurities or degradation products insensitive to UV detection is an important consideration, and can be verified using an online LC/UV/MS approach as one of the validation components.

Working with CROs

An important distinction that an emerging biotechnology research company should make when opting to outsource preclinical studies is whether a CRO is being viewed as a strategic partner or strictly as a service provider. Due to the limited internal project management staff, emerging research-based biotechnology companies may prefer to outsource the entire preclinical program to a vertically integrated CRO capable of handling studies from discovery screening to IND filing. In doing so, a CRO is in a position to retain valuable "big-picture" knowledge between interrelated disciplines — chemistry, analytical, pharmacokinetics, biopharmaceutics and clinical — as the project advances through its development. This offers opportunities for time- and cost-efficient project management by avoiding duplication in technology transfer between participating CROs, and minimizing the number of interactions between CROs.

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A longer version of this article appears on our *Laboratory Focus* Web Portal at **www.biosciencesworld.ca**

TechDoctor

BY ANISSA MORAES

Dear Tech Doctor:
I'm curious about expression systems. I read your article on antibiotic use and it made me start thinking about some of the other things in cell culture that have to be meticulously monitored in order to keep the cells happy and growing. What are some of the more rugged strains and how would someone go about selecting an organism to use for expression? I know that some research has been done to deliver drugs in food plants, but what about run-of-the-mill expression?

D. Trapolsi, Philadelphia, PA

I am going to take a little licence with your question. I will go over expression system options and how to choose the right system for your needs. In this article, I will cover bacterial, *in vitro* and yeast expression and I will go over some examples of promising newer/developing microbial expression systems. In the next article, I will cover higher eukaryotic expression systems, the old standards and some of the up-and-coming systems (insect larvae, molecular farming, etc.).

Generally, there are a few things to keep in mind when you are choosing an expression system:
1) Cell growth rate/Speed of the expression system
2) Cost and complexity of the cell growth medium
3) Expected protein expression level
4) Ease of protein purification
5) Post-translational modifications/Protein folding achievable
6) Ability to scale up to large scale/Production-level expression

The fastest of all would have to be *in vitro* expression systems. Proteins can be expressed from a DNA template in less than two hours. The protein yield of an *in vitro* expression experiment can be as high as 1 mg of protein per millilitre of lysate. Often, proteins expressed *in vitro* are properly folded, post-translationally processed and functionally active. Provided that the chosen *in vitro* expression system has low or undetectable levels of the enzymatic activity of your protein of interest, it is possible to directly assay the translation reaction for activity. The ease of use and the speed with which you can express properly folded, functionally active proteins make it highly desirable. However, this is not an inexpensive system and large-scale expression can be cost prohibitive.

Speed, high protein-expression levels and the relatively low cost of growing bacteria are the key advantages of expressing proteins in bacterial cells and make this system an excellent choice for large- or production-scale protein expression. Bacteria cannot glycosylate proteins and they are very limited in their ability to post-translationally modify proteins. Bacteria are also a poor choice for expressing genes from eukaryotic genomic DNA sources, as they cannot process introns.

Then you have yeast: not quite as fast as bacteria, but then again, not too far off either. The doubling time of yeast is approximately 90 minutes, compared with an approximate 30-minute doubling time for bacteria. Like bacterial expression systems, there are several vector systems commercially available for yeast expression and there is a relatively small front-end preparation time commitment to use a yeast or bacterial expression system. Also like bacteria, growing yeast is not a complicated business. Yeast growth media is simple and inexpensive. The major advantage yeast have over bacteria is that they are eukaryotic cells. As eukaryotes, yeast will be better able to fold recombinant eukaryotic proteins, as well as phosphorylate and glycosylate (to an extent).

As a look to the future, work is being done to find new enzymes for laboratory use in thermophilic bacteria. Enzymes from thermophiles are not only more heat resistant than their standard counterparts, but also more chemically resistant and robust in general. The dream is to express your proteins in a soup of thermophilic enzymes and have the freedom to heat or add chemicals (i.e., oxidizing/reducing agents, etc.) almost at will. Some effort is also being put into using thermophiles as an expression system themselves. In other words, there could be competent strains of thermophiles using their big, strong enzymes to express your proteins. Not much chance of culture contamination at high temperature. Pretty exciting, as long as your protein isn't heat labile.

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