Applications of liquid chromatography mass spectrometry in accelerating the pace of lead-drug candidate selection and pre-clinical development

This review discusses how the application and benefits of using LC/MS and LC/MS/MS assays in conjunction with *in vivo* and *in vitro* study designs can generate significant advantages in shortening timelines between discovery and pre-clinical development.

A successful lead-drug candidate development programme often requires considerations 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, 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 optimisation of drug candidates, applications of liquid chromatography mass spectrometry (LC/MS) and liquid chromatography, tandem mass spectrometry (LC/MS/MS) assays have become an extremely useful analytical chemistry tool to accelerate the transition from dis-

covery to the stages of pre-clinical 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 in shortening timelines and reducing cost during the preparation of an Investigational New Drug (IND) application.

The following discussion will review 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 optimisation of drug candidates. In addition, following the nomination of a lead drug candidate for pre-clinical development, integration of HPLC, LC/MS and LC/MS/MS assays during Chemistry, Manufacturing and Control (CMC) development of the drug substance, formulation of a pilot clinical

By Dr David Kwok

Table 1: Illustration of ADMET components and the associated examples of relevant *in vitro* versus pilot *in vivo* screening assays

ADMET Components	Examples of 'Drug-Like' Parameters	*In-vitro* Screening Assays	*In-vivo* Pilot Animal Studies
Absorption	Gut Epithelial Transport	MDCK/CaCo Cells Gut-Loop Models	Comparative IV, PO and IP Single Dose Administration
	Topical Skin Penetration	Dissolution Surrogate Model	Skin Stripping and Plasma Assay
Distribution	Blood Brain Barrier	Artificial Membrane / MDCK / CaCo	*In-vivo* CNS / Plasma Drug Ratio
	Serum Protein Binding	Equilibrium Dialysis / Ultrafiltration Assay	*In-vivo* Plasma Free Drug Fraction
	Tissue / Target Organ Distribution		Tissue Distribution (Cold / 14C-labeled) Study
Metabolism	Metabolic Stability	Human Primary Heptocytes and/or Animal / Human Liver Microsome	Single Oral Dose Metabolic Cage Study
	Metabolite Profile & Characterization		
	CYP450 Inhibition / Induction		
	Hepatic 1st-Pass		Hepatic Portal Vein Cannulation
	Enterohepatic Recycling		Bile Duct Cannulation
Excretion	Terminal Elimination Half-Life	Hepatocyte Time-Course Study	Single and Repeated Dose PK Study in Metabolic Cage
Toxicology	Safety Pharmacology	hERG Channel (Cardiovascular)	Mouse Micronucleus Assay, Tissue Pathology and Clinical Signs Following Single Acute / Subacute Dosing, Escalating Dosing or Repeated Dosing
	Target Organ Toxicity	Hepatocyte Apoptosis (Heptotoxicity)	
	Genotoxicology	Ames, Chromosomal Aberration, Mouse Lymphoma Assays	
Chemistry	Stability and Solubility		

drug product and QC of the clinical product as well as integration of bioanalytical assay requirements in support of toxicology and safety studies will be briefly reviewed with respect to assay validation strategies in context of Good Laboratory Practice (GLP).

In vitro and *in vivo* assays in ADMET optimisation

Following the screening and identification of compounds with desirable activity, the screening of drug candidates for desirable ADMET properties should follow an integrated lead optimisation and selection programme. The design of such a programme often requires considerations of several areas, including the number of molecular 'hit' candidates required to be optimised in the library. Secondly, the primary therapeutic indication, as well as the therapeutic advantages over existing marketed therapeutic products is also important. Experience drawn from historical pre-clinical 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 are useful to note.

Until recently, it has been common to encounter lead optimisation programmes working with combinatorial structural libraries of hundreds or thousands of compounds. Now it is more common to encounter the optimisation of a handful to a few

hundred molecular hits in a given primary therapeutic area. This trend of working with a small molecular 'hit' library is likely the result of awareness among scientists that the use of a mechanistic, knowledge-based approach of integrating biology and chemistry has generated higher quality 'hits' in a more cost-effective manner, compared to earlier approaches in the use of combinatorial chemistry and high-throughput screening approach. A number of recent literature reviews[1,2] have elaborated on the popular trend of working with a limited number of high quality molecular 'hits' during lead optimisation and selection.

Given the task to focus on a limited number of high quality molecular 'hits', an effective ADMET lead optimisation and selection strategy should be planned with screening assays that can provide the most relevant prediction of drug-like properties in the clinics. 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. As an alternative, *in vivo* animal experiments with radiolabelled or non-radiolabelled compounds have the highest biological relevancy but are associated with the highest costs.

In terms of ADMET lead optimisation and selection strategy to produce drug candidates that are commercially competitive compared against existing marketed products, screening assays can be designed towards the selection of candidates which improve potency, increase selectivity, increase chemical stability, lower toxicity or deliver higher bioavailability. These improvements in drug-like properties have been identified by a recent literature review of among 29 recently launched drugs[1].

In addition to the need for relevant ADMET properties, physiochemical properties of a drug candidate substance are also important 'drug-like'

attributes. 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 affecting the intellectual property, chemical stability and bioavailability of the drug substance[3]. From this brief discussion, it can be recognised that an effective ADMET lead optimisation and selection strategy must be customised specifically for each of the approximate 500 known therapeutic targets specifically for a clinical market. The new ADMET optimisation paradigm often involves a parallel approach of evaluating solubility, absorption, metabolism, formulation, pharmacokinetics, toxicity and efficacy which are equally important inter-related parameters in early compound optimisation[4-6]. An illustration of ADMET components and the associated examples of relevant *in vitro* versus pilot *in vivo* screening assays are summarised in **Table 1**.
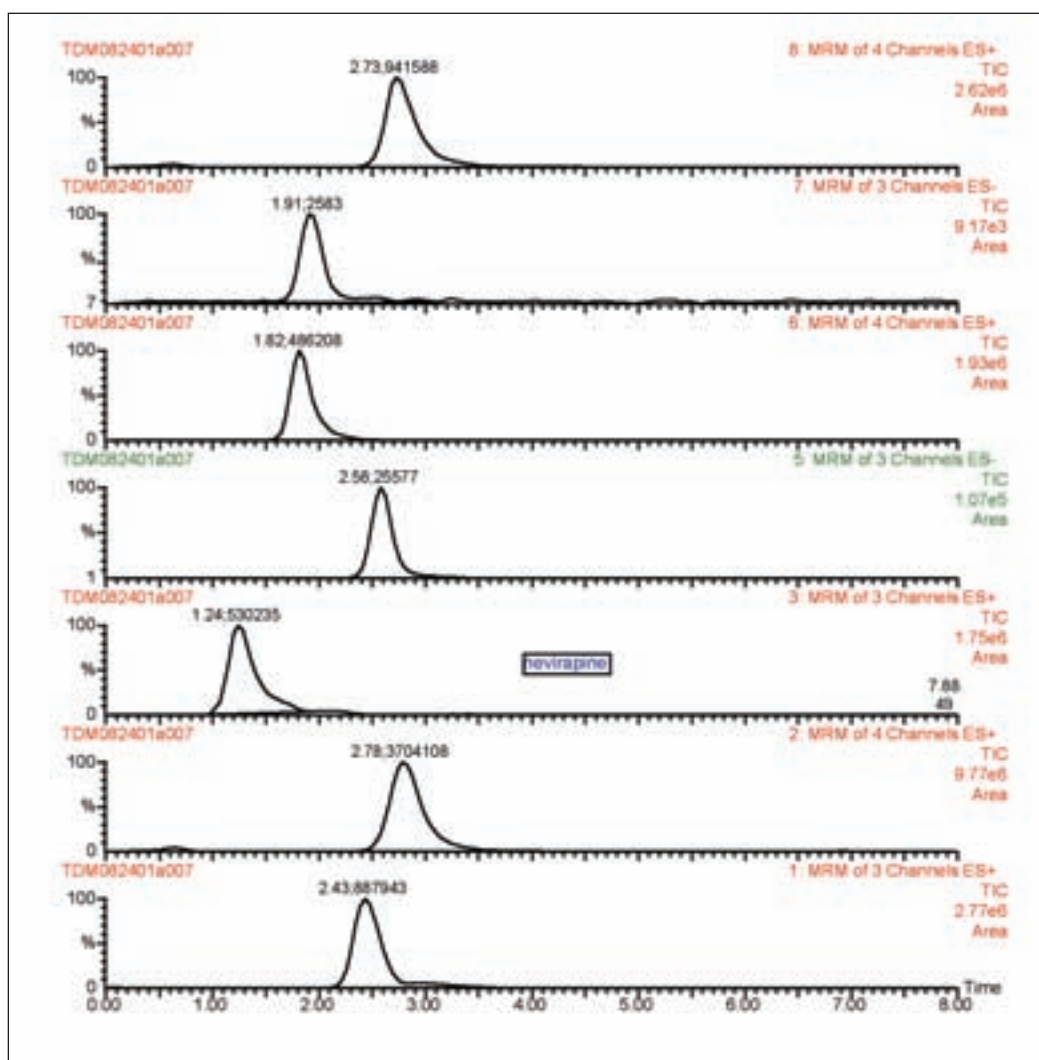

Figure 1: A representative electrospray LC/MS/MS chromatogram from the simultaneous assay of seven drug compounds
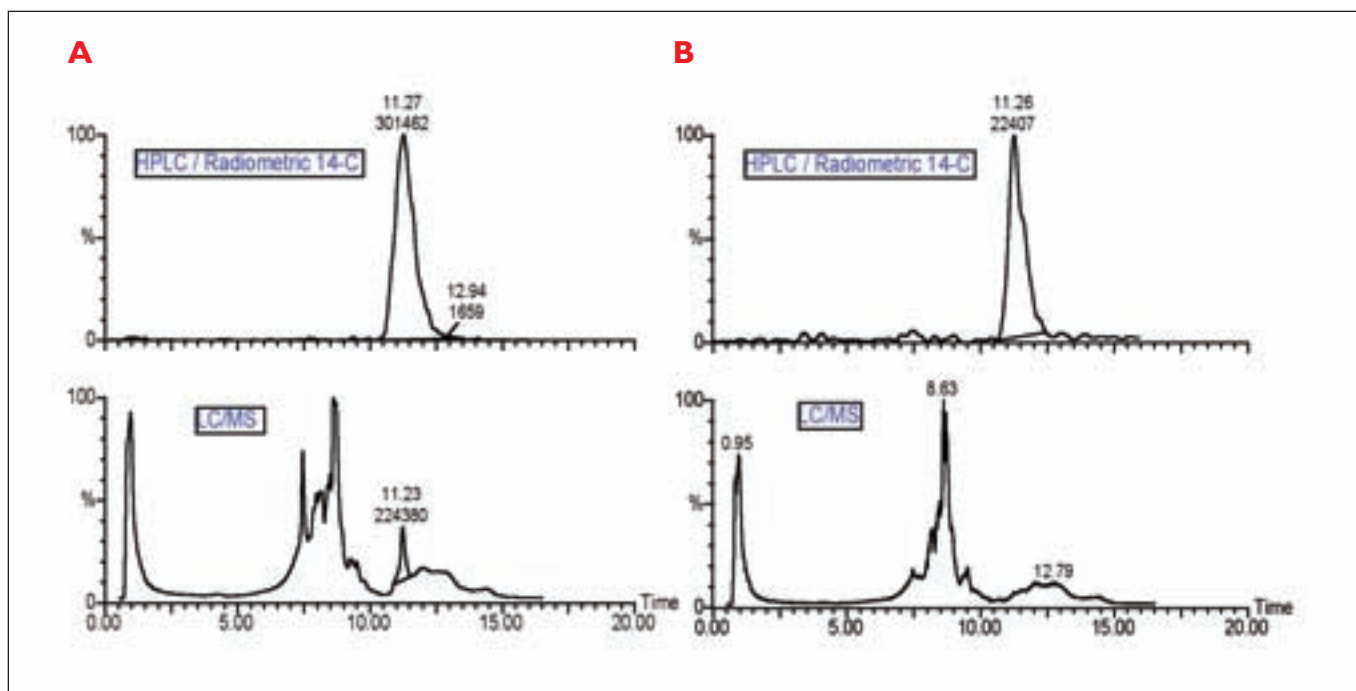
LC/MS and LC/MS/MS applications in ADMET *in vitro* and *in vivo* studies

Prioritisation on the evaluation of specific ADMET components and decisions on the use of selected *in vitro* and/or *in vivo* assays are often shaped by considerations such as 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 LC/MS or LC/MS/MS assay method in support of ADMET investigations can generate significant benefit in 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 50µl to 100µl aliquots and 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 ionisation of a drug molecule as a [M+H]$^+$ protonated molecular ion under solution conditions suitable for chromatography and ionisation of the drug molecule. The molecular parent ion is initially detected by the first of two mass spectrometers operating in tandem 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

ionised drug substances at low nanogram and is less reliant on chromatography to resolve co-eluting sample matrix components. In contrast, a 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 which permit simultaneous measurement of a mixture of five or six compounds. 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 the LC/MS/MS assay approach, though the LC/MS approach may often prove to be suitable if adequate chromatographic separation among the drug candidates is achieved. As an illustration, a representative LC/MS/MS chromatogram of seven protease inhibitors is presented in **Figure 1**, showing simultaneous assay of seven drug compounds. This 'cassette-assay' approach can effectively reduce the number of *in vitro* CYP450 metabolic screening samples or other *in vitro* assays and yet avoid potential drug-drug interaction of cassette-incubation of multiple drug candidates.

In a similar manner, in support of *in vivo* pilot PK and tissue distribution studies, a cassette-dosing

study design involving co-administration of a mixture of five to six test compounds can also be considered. 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. Comparison of AUC_{IV} to AUC_{ORAL} can provide timely and valuable data on elimination of half-life and oral bioavailability, while a comparison of AUC_{ORAL} to AUC_{IP} can provide of mechanistic information regarding the presence of gut transport barrier or first-pass metabolism. While 'cassette-dosing' data may be confounded with *in vivo* drug-drug interaction, the use of the '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 on-line radiometric ($^{14}C/^3H$) measurement is increasingly useful in support of *in vivo* metabolism and tissue distribution studies, particularly for drugs candidates exhibiting low plasma concentrations. On-line radiometric/mass spectrometry detection involves the use of splitting the HPLC column effluent for simultaneous on-line detection of a $^{14}C/^3H$-labelled drug using a flow-through liquid scintillation detector operating in parallel to 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 a particular advantage for detection of compounds with a neutral charge structure, inher-

ently exhibiting low electrospray sensitivity. The ability of a LC/RM/MS assay to provide quantitation of a radio-labelled molecule at 100pg/ml is illustrated in **Figure 2**, compared with the lack of detection by MS at the same concentration. This relatively low limit of quantitation offered by LC/RM/MS/MS assays have permitted the *in vivo* study of drug candidates at reduced doses. As illustrated in **Figure 3**, 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.

In addition to the advantage of LC/RM/MS/MS assays operating at low picogram sensitivity over LC/MS or LC/MS/MS assays, the use of radiolabelled drug candidates in *in vivo* metabolism studies can also generate timely identification of metabolites across animal species. As illustrated in **Figure 4**, radiometric measurements were performed for plasma samples collected from rat, mouse and dog studies. From the same assay, detection of the parent drug along with multiple metabolites by MS was conveniently carried out based on the availability of mass specific information.

In addition to the high sensitivity and specificity of LC/MS/MS measurements, advances in LC/MS/MS techniques have also enabled reliable quantitation methods to be developed in a much shorter timeframe than normally required for development of a conventional HPLC method. 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 different plasma and tissue sample matrices. Integration of the requirements for
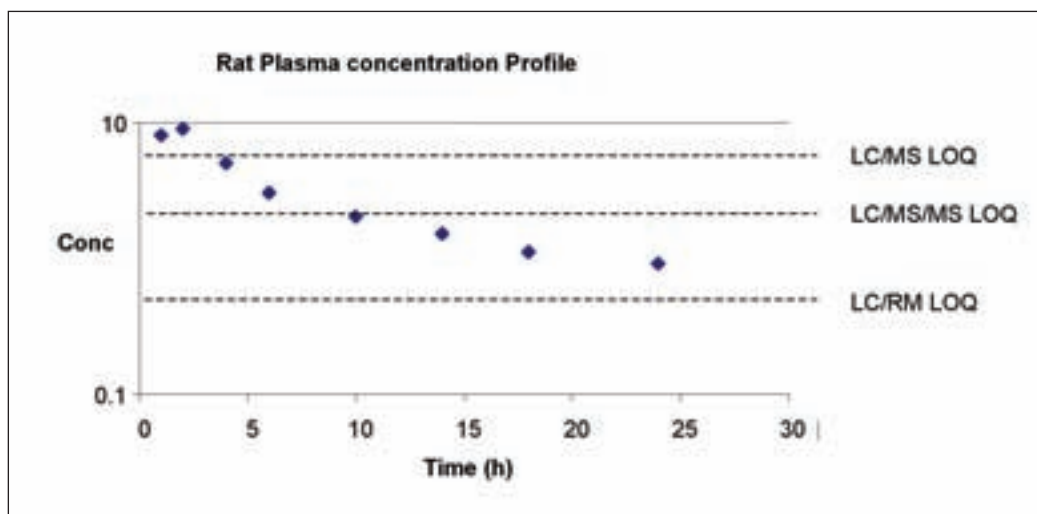

Figure 3: PK study of a drug candidate requiring low picogram plasma concentrations not adequately assayed by LC/MS and LC/MS/MS approach

Analytical Instrumentation

References

1 Proudfood, JR. Drugs, leads, and drug-likeness: an analysis of some recently launched drugs. Bioorg Med Chem Lett. 2002 Jun 17;12(12):1647-50.

2 Bleicher, KH, Bohm, HJ, Muller, K, Alanine, AI. Hit and lead generation: beyond high-throughput screening. Nat Rev Drug Discov. 2003 May;2(5):369-78. Review.

3 Clas, SD. The importance of characterizing the crystal form of the drug substance during drug development. Curr Opin Drug Discov Devel. 2003 Jul;6(4):550-60.

4 Caldwell, GW, Ritchie, DM, Masucci, JA, Hageman, W, Yan, Z. The new pre-clinical paradigm: compound optimization in early and late phase drug discovery. Curr Top Med Chem. 2001 Nov;1(5):353-66.

5 Guidance for industry. Drug metabolism/drug interaction studies in the drug development process: Studies in-vitro. Department of Health and Human Services, US Food and Drug Administration, Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research, April 1997.

6 Guidance document. Metabolic drug interactions: Studies in-vitro and in-vivo. Therapeutic Products Directorate, Health Canada, May 2000.

7 Investigational new drug (IND) application process. US Food and Drug Administration, Center for Drug Evaluation and Research, May 29, 2001.

8 Investigational new drug application. Therapeutic Products Directorate, Health Canada, September 2001.

9 ICH Q3A: Impurities in New Drug Substances (International Conference on Harmonization of Technical Requirements for the Registration of Drugs for Human Use, Geneva, Switzerland, January 1996).
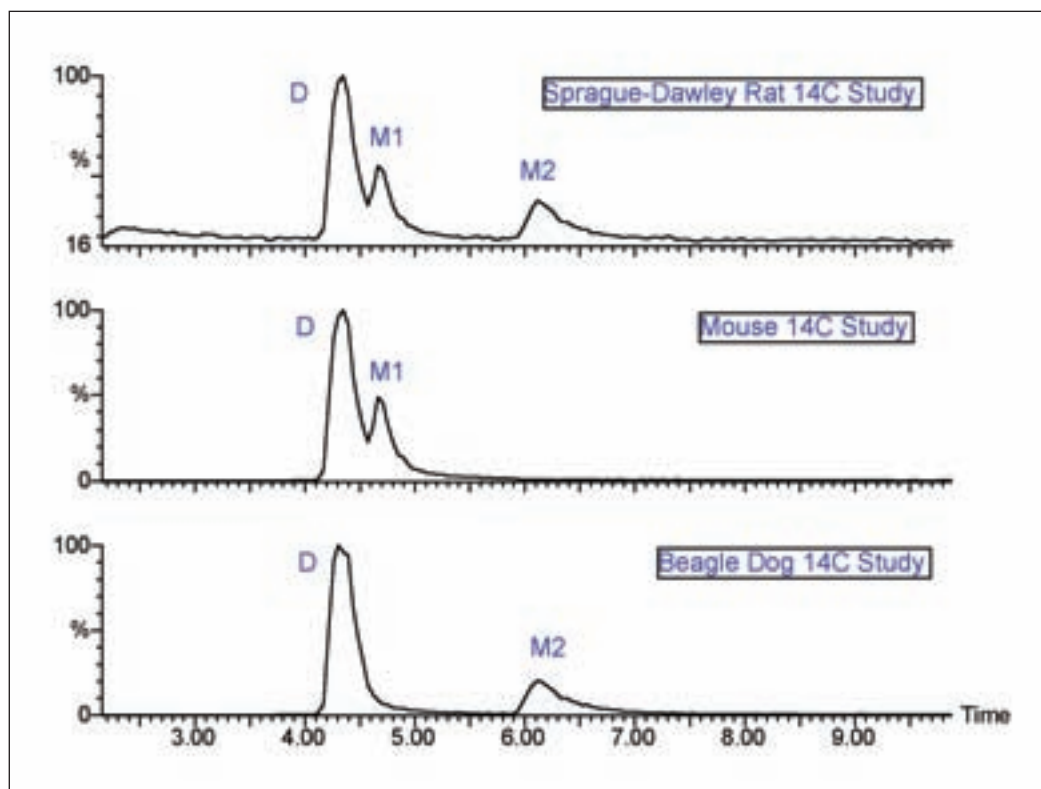
Figure 4: LC/RM/MS radiometric assay of plasma samples collected from rat, mouse and dog studies, showing detection of the ^{14}C-labelled parent drug (**D**) followed by two chromatographically resolved ^{14}C-labelled metabolites (**M1** and **M2**). From the same assay, identification of the parent drug and characterisation of the metabolites by mass spectrometry was carried out conveniently on-line

method cross-validation study in human plasma and other human biological sample matrices are advisable to permit application of a comparable bioanalytical method in support of pre-clinical and clinical phase-I studies.

LC/MS and LC/MS/MS applications in pre-clinical development

During pre-clinical 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 Investigational New Drug (IND) submission[7,8]. At the onset of a pre-clinical programme, careful planning of an integrated strategy for all analytical chemistry requirements is paramount and will result in significant time and cost savings. To illustrate the various pre-clinical study components commonly encountered and the type of analytical studies required, a schematic summary of studies generally involved is presented in **Figure 5**. The analytical chemistry requirements and applications of LC/MS and LC/MS/MS assays in sup-

port of drug substance and clinical drug product development during pre-clinical studies are discussed below.

Analysis of drug substance bulk material

Manufacturing release requirements for bulk material as an active pharmaceutical ingredient (API) requires that quality control (QC) assays are validated and that purity of the API documented in a Certificate of Analysis along with other relevant chemical and safety official 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 of µg/ml to mg/ml range. For an isolated natural product drug candidate, complimentary LC/MS and/or GCMS methods are often required to characterise 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 Chemistry, Manufacturing and Control section of an IND application. Information on impurity and degradation profile of synthetic or isolated API will also be a necessary component of the Drug Master File.

While a HPLC/UV method is commonly applicable and desirable as a QC method due to the 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, are required to be characterised and quantitated[9].

To meet this requirement, a 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. Forced-degradation stability studies under prescribed thermal, chemical and photolyt-

ic conditions as well as shelf-life storage stability studies of the API under accelerated and long-term conditions are also often considered[10]. The presence of potential impurities or degradation products insensitive to UV detection is an important consideration and can be verified using an on-line LC/UV/MS approach as one of the validation components.

Analysis of formulation materials and clinical finished products

A series of physiochemical characterisation and formulation development studies of the drug substance is necessary in support of properly designed formulations for any route of administration. Oral solutions, suspensions, solid dosage units or parental formulations are commonly encountered and formulation data gathered during the pre-clinical studies are transferable to the design of a finished dosage product in support of clinical phase-I studies. Physiochemical characterisation studies including pKa, log P, solubility, polymorphism, particle size, hydration, material flow and other studies are often carefully planned in support of the manufacturing requirements of the clinical supplies.

Figure 5: Illustration of the various pre-clinical study components commonly encountered and the type of analytical studies required

Analytical Instrumentation
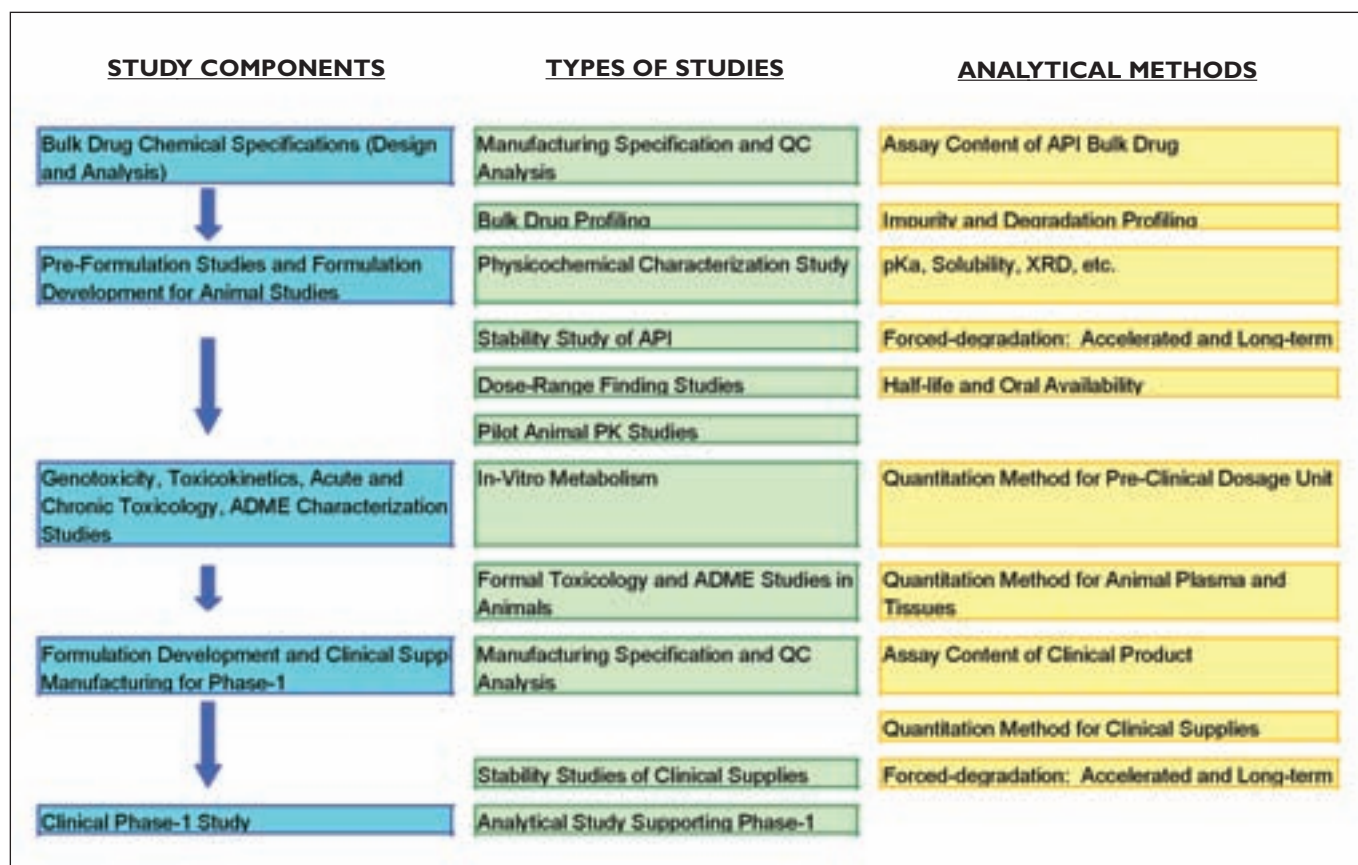
10 Stability testing of Drug Substances and Drug Products. Department of Health and Human Services, US Food and Drug Administration, Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research, June 1998.

11 ICH Q2A: Text on validation of Analytical Procedures (International Conference on Harmonization of Technical Requirements for the Registration of Drugs for Human Use, Geneva, Switzerland, March 1995).

12 ICH 2QB: Validation of Analytical Procedures: Methodology (International Conference on Harmonization of Technical Requirements for the Registration of Drugs for Human Use, Geneva, Switzerland, March 1997).

13 Analytical Procedures and Methods Validation – Chemistry, Manufacturing, and Controls Documentation. US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, August 2000.

14 'Section 21 Code of Federal Regulations, part 58' (US Government Printing Office, Washington, DC.

Analytical methods in support of formulation and physicochemical studies can be commonly adopted from validated chromatographic methods for the API, subject to modifications and cross-validation in conformance with cGMP requirements. New analytical methods may be required to be developed and validated for formulation excipients used in the finished product, such as antioxidant, adjuvant, preservative or any excipients important in affecting the quality and safety of the finished product.

Prior to the manufacturing of the clinical supplies, manufacturing-release and shelf-life stability, specifications are carefully designed based on quality and safety considerations. References should be made to official compendium and regulatory guidance with respect to analytical chemistry requirements for the finished dosage product developed[11]. Pilot-scale clinical supplies are often scheduled for accelerated stability studies and the use of validated 'stability-indicating' analytical methods for the API and excipients in the finished product are required.

In review of the number of analytical applications required in support of assaying of the API in various formulation materials and finished products, an early estimation of the amount for API requirements will ensure the availability of an adequate supply of API and avoid unexpected time delays. Cross-validation studies of each assay method can be planned and integrated with the applications in mind when demonstrating assay precision, accuracy, calibration linearity and specificity[12,13].

Working with CROs

Study Sponsors may often consider outsourcing specific study components to Contract Research Organisations (CROs) to permit the conduct of concurrent studies in order to meet time-limiting project milestones. While the selection of a CRO with broadly-based experience in various analytical applications is important, a Study Sponsor should also ensure that the CRO conforms with Good Laboratory Practices[14].

While the scientific and project performance standards are set high for both Sponsors and CRO scientists alike, a CRO is often expected to perform at a higher level of competency and efficiency as compared to the Sponsor's internal counterparts. To meet and to exceed this expectation, the personnel within a CRO must demonstrate a high level of dedication and commitment to his or her own success and the success of its Sponsors.

An important distinction an emerging biotech-nology research company should make when opting to outsource pre-clinical 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 biotechnology research based companies may prefer to outsource the entire pre-clinical programme 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 inter-related disciplines (chemistry, analytical, pharmacokinetics, biopharmaceutics and clinical) as the project advances through its development. This offers opportunities for time and cost-efficient project management in avoiding duplication in technology transfer between participating CROs and minimising the number of interactions between CROs.   DDW

Dr David Kwok graduated from his pharmacy training in 1984 and obtained his PhD in Medicinal Chemistry from the University of British Columbia, Canada in 1991. He served in a variety of management and scientific posts at Health Canada from 1991 to 1998. He is currently the founder and President/CEO of BRI Biopharmaceutical Research Inc, a bioanalytical chemistry contract research company.