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# Analytical challenges for conducting rapid metabolism characterization for QIVIVE

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ABSTRACT

For quantitative *in vitro–in vivo* extrapolation (QIVIVE) of metabolism for the purposes of toxicokinetics prediction, a precise and robust analytical technique for identifying and measuring a chemical and its metabolites is an absolute prerequisite. Currently, high-resolution mass spectrometry (HR-MS) is a tool of choice for a majority of organic relatively lipophilic molecules, linked with a LC separation tool and simultaneous UV-detection. However, additional techniques such as gas chromatography, radiometric measurements and NMR, are required to cover the whole spectrum of chemical structures. To accumulate enough reliable and robust data for the validation of QIVIVE, there are some partially opposing needs: Detailed delineation of the *in vitro* test system to produce a reliable toxicokinetic measure for a studied chemical, and a throughput capacity of the *in vitro* set-up and the analytical tool as high as possible. We discuss current analytical challenges for the identification and quantification of chemicals and their metabolites, both stable and reactive, focusing especially on LC–MS techniques, but simultaneously attempting to pinpoint factors associated with sample preparation, testing conditions and strengths and weaknesses of a particular technique available for a particular task.



## 1. Introduction

In toxicology and pharmacology, identification and quantification of the substance under study has been the first prerequisite since the times of Paracelsus, even if analytical technologies in the past were quite insufficient from the modern perspective. In current pharmacology, measurements of the parent drug and its metabolites span from early discovery phase to pharmacovigilance studies, for the simple reason of elucidating dose-concentration-response relationships of active or toxic entities towards multiple targets in the body. Quantitative *in vitro–in vivo* extrapolation (QIVIVE), *i.e.* prediction of *in vivo* parameters on the basis of *in vitro* (and *in silico*) studies has become a paradigm in drug development (see Pelkonen et al., 2011). QIVIVE is even more important in the proactive elucidation of chemical risks, because in most cases it is not possible to expose human beings deliberately to chemicals used as, *e.g.*, pesticides, household chemicals, cosmetic ingredients, industrial chemicals etc (Basketter et al., 2012; Hartung et al., 2013). In such a situation, the risk assessment, *i.e.* building a plausible and reliable sequence of events from exposure to toxicokinetics to toxicodynamics is almost wholly dependent on the measurement of a chemical and its metabolites in *in vitro* test systems and in experimental animals. Because of ethical, scientific and legislative reasons have made animal experimentation increasingly less justifiable, the prediction of toxicokinetic behaviour of a chemical on the basis of QIVIVE is becoming an essential prerequisite for the proper risk assessment (Adler et al., 2011; Coecke et al., 2012). Another point of importance is the increasing reliance on the use of human tissues and enzymes in *in vitro* testing systems because of known species differences in drug and other xenobiotic metabolism.

## 2. Challenges for analytics

In the current toxicology research, there are several challenges: A large number of chemicals to be analyzed (*e.g.* inadequately studied chemicals under REACH programme), an enormous and variable chemical space of existing and future chemicals, a multitude of possible metabolites and other transformation products, a need of high throughput screening and high content screening, a multitude of biological matrices in which chemicals should be measured, a need of (toxicologically relevant) quantification (*e.g.* unbound, free,

*Abbreviations:* HR-MS, high-resolution mass spectrometry; LC/MS, liquid chromatography–mass spectrometry; LPME, liquid phase microextraction; NMR, nuclear magnetic resonance (spectroscopy); TOF, time-of-flight (MS); UHPLC, ultra-high performance liquid chromatography.

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concentrations), limitations of various techniques and so on. Below we discuss many of the above challenges from the analytical perspective, focusing especially upon the early phases of research aimed at producing reliable data for QIVIVE. We do not deal with *in silico* approaches except as tools for helping the interpretation of data produced by analytical techniques. In any case, *in silico* approaches, including quantitative structure-activity (property) relationships and various molecular modelling techniques to predict toxicokinetic processes, need *in vitro* and *in vivo* data for the validation (Pelkonen et al., 2011).

## 3. Conditions for rapid metabolism characterization for QIVIVE

There exists a common consensus on the general scheme for producing metabolism data by *in vitro* testing systems: An experimental design and platform to obtain metabolism data; a proper comprehensive enzyme source (*e.g.* liver microsomes, or a metabolically competent cell line such as human hepatocytes), suitable sample preparation technique, and a reliable and robust 'fit-for-purpose' analytical technique. By suitable modifications of this general scheme, a number of important measures of metabolism and ensuing toxicokinetics should be obtained:

(1) Disappearance rate, clearance, enzyme kinetics
(2) Metabolite identification, profile, quantification
(3) Reactive metabolites
(4) Enzyme identification

On the basis of the primary measures, it is possible to obtain information on inhibition and induction potential, *i.e.* pharmacokinetic interactions, on interindividual variability, and on species differences. Obviously the above data are of importance for clinical use, on the one hand, and for the extrapolation of animal toxicity tests into human situation, on the other.

For the reliable QIVIVE, experimental details affecting the final outcome, a toxicologically relevant concentration of a chemical and its metabolism, should be rigorously studied and controlled. However, this discussion is beyond the scope of this review article and we refer to recent review articles which deal with the details of '*in vitro* kinetics' or 'biokinetics' (Pelkonen et al., 2008, 2009a; Blaauboer, 2010). Also, we do not describe various schemes for QIVIVE, which are dealt in other articles of this special theme issue.

## 4. Analytical techniques vis-a-vis chemical space

In this review we describe principally techniques employing mass spectrometer as a detector, connected with liquid chromatography as a separation technique. Here it is sufficient to note that there are important classes of chemicals that usually need other analytical techniques, *e.g.* metals and metalloids, many nanoparticles, polymers etc. Many of these substances are not metabolized to a significant extent, so the analytical method for the parent(s) should be available. However, a large majority of substances and their metabolites currently of toxicological and pharmacological interest are organic molecules with a molecular weight about 150–1000 Da, which are usually well suitable for the LC/MS studies. Still within the domain of organic substances there is significant chemical variability which may affect the selection of analytical techniques. One such 'real-life' example of tentative selection of an analytical technique for a mixed group of chemicals is shown in Table 1.

Generally, LC/MS techniques offer the most effective technology to analyse the drug pharmacokinetics and clearance, and to screen and identify metabolites, in both *in vitro* and *in vivo* conditions (Lee and Kerns, 1999; Kostiainen et al., 2003; Tolonen et al., 2009). Ultra-high-performance chromatographic techniques (UHPLC, also known UPLC), together with high-resolution mass spectrometry (HR-MS) are techniques of huge developments in the recent years (Nováková and Vlckobvá, 2009; Zhu et al., 2011; Maurer, 2010; Josephs, 2012). The ability of these high-resolution MS-techniques to acquire data with a very high sensitivity and detection specificity for a high number of compounds at the same time, and, without the need for compound-specific detection parameters, has made them suitable tools to gather information about the parent compound and both expected and unexpected metabolites in a single LC/MS run. Often also high-resolution fragment ion data for elucidation of the metabolic biotransformation sites can be acquired at the same time. In addition to the advances with the high-resolution mass analyzers, the ion source technology has enabled the use of LC/MS with higher chromatographic flow rates, even several ml/min, with very high sensitivity, and applicability for various types of analytes, using various ionization techniques (Kostiainen and Kauppila, 2009). Comparison of various available MS equipments is summarized in Table 2.

## 5. Sample preparation and stability in biological matrices

The purpose of sample preparation is to clean the sample both physically and chemically, so that it can be injected to analytical system (LC/MS) without interferences. These interferences might arise *e.g.* from physical blocking of the HPLC column or from contaminating the mass spectrometer ion source, or they might be observed as data-related problems such as decreased selectivity of analysis or biased quantification of the experiment. In addition to the analytical parameters, such as sensitivity, selectivity, repeatability, matrix effects, robustness, sample preparation process affects also total time consumption and price of the analysis.

For *in vitro* metabolism studies, the most recommended methods of sample preparation are easy and generic methods that should have an equal recovery for both the parent drug and its metabolites, so that the sample preparation method does not bias the metabolite profile or even lead to false negatives for certain metabolites. The repertoire of sample preparation tools includes methods based *e.g.* on filtration, ultrafiltration, centrifugation, protein precipitation, liquid-solid extraction with sonication or microwaves, liquid–liquid extraction (LLE), liquid–liquid micro extraction (LLME), single drop micro extraction (SDME or LPME), solid phase extraction (SPE), ion-exchange, solid phase micro extraction (SPME), microdialysis, restricted access media (RAM), just to mention few of them. In some cases also derivatization methods are used, *e.g.* to increase retention in liquid chromatography or volatility in gas chromatography, to improve mass spectrometric ionization, to increase extraction yield, to stabilize the analyte, or to introduce fluorescent moiety to the structure, but these methods are quite rare in analysis of traditional small chemicals or their metabolites. Some excellent recent review papers cover this area very thoroughly (Chen et al., 2008; Nováková and Vlckobvá, 2009).

## 6. Screening parent chemicals and/or metabolites at the high throughput mode

The need for fast throughput for *in vitro* clearance and metabolite information on a large number of different compounds has clearly turned the drug industry-practice towards the use of novel liquid chromatography and mass spectrometry tools and the toxicology risk assessment community has followed the suite. The arrival of liquid chromatographic systems with sub 2 μm particle sizes, and instruments tolerating even 15,000 psi (1000 bar) backpressures, has improved chromatographic resolution and