Annex H. Biokinetics and xenobiotic bioavailability

Since the techniques used for assessing biokinetics and xenobiotic bioavailability are complex, cost-intensive and time-consuming, routine use in the laboratory may not always be feasible, and therefore may prove more useful in troubleshooting the *in vitro* method.

Different processes result in a freely dissolved concentration that is not the same as the nominal concentration, (i.e., the added concentration). These processes are described in e.g., (Heringa et al., 2006[1]) and (Groothuis et al., 2015[2]), and were one of the main topic investigated by the FP7 EU Project Predict-IV, aimed to improve the predictivity of *in vitro* methods for unwanted effects of drugs after repeated dosing integrating biokinetics and biodynamic data. As one of the project outputs, a step-wise strategy was applied to measure and model cell exposure levels over time of a selected number of drugs in the developed *in vitro* assays. The strategy and the major obtained results are described in (Kramer et al., 2015[3]).

Evaporation / plastic and glass binding / sorption

In vitro systems are often open, with a small gap between the well plate and the lid, to allow air circulation for provision of oxygen for the cells and removal of excess CO_2. This air circulation allows volatile substances to evaporate into the air of the incubator. This may decrease the concentration in the medium in the test system, but can also contaminate medium in e.g., blank wells, as the substance can dissolve from the air into the medium of other wells present in the well plate or even the incubator. An example showing the effect of evaporation on test results can be found in Tanneberger (Tanneberger et al., 2010[4]). It may also be advisable to use tape/**foil** to cover culture plates in order to avoid evaporation of volatile substances and cross-contamination between wells **(e.g., OECD TG 442D).**

Lipophilic substances tend to bind to the plastic the cell culture plates are made of, although differences exist among the types of plastic used. The adsorption to polycarbonate is limited, but in organ-on-a-chip devices made of Polydimethylsiloxane (PDMS), there will be partitioning between the PDMS and the medium. PDMS is even used as an extraction material for Solid Phase Microextraction (SPME) (Heringa and Hermens, 2003[5]) and is therefore not suitable for *in vitro* test devices for testing of chemical substances. Examples where considerable binding to plastic was measured can be found in Kramer *et al.* (Kramer et al., 2012[6]) who also discuss how the addition of serum to medium decreases the binding to plastic. Other examples are reviewed in (Kramer et al., 2015[3]), reporting results of the Predict-IV project on cyclosporine A, amiodarone and chlorpromazine. The addition of serum to medium decreases the binding to plastic, but likely also the uptake into the cells (Pomponio et al., 2015[7]). Glass is a better material to avoid binding but very lipophilic substances are known also to bind to glass. Silanised glass can decrease this binding even further.

Sorption of the test item to cell-attachment matrices (e.g., collagen or matrigel layer used with hepatocytes in culture) is a specific aspect of interaction with the test device, although the relationship between a test item's lipophilicity and binding to is not as clear cut as it is for binding to plastic laboratory ware. The possible physical sequestration of test items, can lead to overestimating intracellular concentrations (Kramer et al., 2015[3]). Adsorption by coating material on plastics and feeder cells should also be considered.

Chemical degradation by hydrolyses and phototoxicity

The aqueous environment of the medium in an *in vitro* test enables spontaneous hydrolysis (i.e., without the aid of an enzyme) of substances with structures sensitive to this chemical reaction. During the time the test system, e.g., the well plate, is outside of the incubator, light will reach the medium and photolysis can take place for light sensitive substances. Therefore, information on hydrolysis and photolysis sensitivity is necessary before a substance is tested in an *in vitro* method (Section 6.2). More generally, each test facility should have adequate test item characterisation procedures in place to identify if the test item characteristics are compatible with the *in vitro* method.

Metabolism/metabolic stability

Some cell types have metabolic capacity, meaning that they contain significant levels of enzymes that convert the test substance to another substance. Especially cells originating from liver, intestine and lung are known to possess metabolic capacity, in decreasing order. In test systems with such cells, especially from these tissues, the concentration of the test item may decrease because of this metabolism, and the concentration of metabolites will increase. When a positive hazard response is obtained in such a cell system, it may thus either be caused by the test item itself, or its metabolite(s). Where there is a lag time in the response (compared to the positive control or other reference items), it could be that metabolite(s) are responsible (Pomponio et al., 2015[7]).

Protein binding

Protein binding can not only affect the freely dissolved concentration of substances as Heringa *et al* demonstrated where moderate differences in protein concentration in the test system resulted in the EC_{50} for a substance shifting by two to three orders of magnitude based on nominal concentration; but it also it is a factor to consider when comparing the responses between different *in vitro* systems. In systems where protein concentrations are relatively high, it requires more test item to achieve the same freely dissolved concentration, and therefore bioavailable concentration, as in those assays with lower protein concentrations. Testing at concentrations approaching the solubility limit of the test item within the test system does two things: first it provides the best experimental design to compare effects across *in vitro* test systems of similar endpoint but different protein concentration, and it reduces the potential for false negative results by testing to optimise the freely dissolved test chemical concentration in the test system. When working near the solubility limit of chemicals in test solution it can be helpful to include a concentration where solubility appears to be exceeded in the test run to better be able to distinguish it from concentrations where solubility is not exceeded. The next lowest test concentration would then be the concentration used to determine if there was activity in the assay for that test chemical. This strategy is particularly advantageous when the results of *in vitro* assays are to be used to identify chemicals with any potential for

activity, especially if further testing hinges on the results from these assays. Such a strategy has been considered to be advantageous in minimizing the chance of false negative results (Schmieder et al., 2014[8]).

The effect of protein in test systems is not only important for cell-based systems, but is also relevant to cell-free systems. For example, a receptor binding assay conducted using a cytosolic or nuclear preparation from a tissue may have more total protein than a competitive binding system using recombinant-expressed receptor protein. In the assay with greater concentration of non-receptor protein, it would take a higher nominal concentration of test item to displace the endogenous ligand from the receptor, and therefore a higher apparent IC_{50} based on nominal concentration when compared to a competitive binding assay with less total protein, but same receptor protein.

Serum is often added to cell culture medium to supplement it with important factors required for cell proliferation and maintenance. Serum-free medium is available and used, but not all cell types thrive in such culture conditions. Serum contains proteins, including albumin, which has non-specific binding sites, to which most organic substances tend to bind. As proteins are large molecules that do not transfer across a membrane, the binding to a protein renders a test item unavailable for cellular uptake, thus unable to reach any target inside the cell. Thus increasing the protein content in a test system can decrease the freely dissolved test item available to reach the target by shifting the equilibrium between freely dissolved and protein bound test item. Examples of the effect of serum protein binding can be found in (Heringa et al., 2004[9]) and in (Pomponio et al., 2015[7]).

On the other hand, serum proteins can also make some test items more accessible or more stable, e.g., for medical devices a medium with serum is preferred for extraction because of its ability to support cellular growth as well as to extract both polar and non-polar substances. In addition, protein binding also occurs *in vivo*. Therefore an *in vitro-in vivo* extrapolation method was developed to extrapolate nominal effective *in vitro* concentrations to equivalent *in vivo* plasma concentrations by accounting for the differences in protein concentrations (Gülden and Seibert, 2003[10]).

Cell membrane absorption

Cell membranes are composed of fatty acids, thus providing a lipid environment in which lipophilic substances will like to absorb. These absorbed molecules are then also not available for a target inside the cell. Examples showing the effect of membrane sorption can be found in Gülden *et al.* (Gülden, Mörchel and Seibert, 2001[11]) and in Bellwon *et al.*, (Bellwon et al., 2015[12]).

Measurement of free concentration/passive dosing

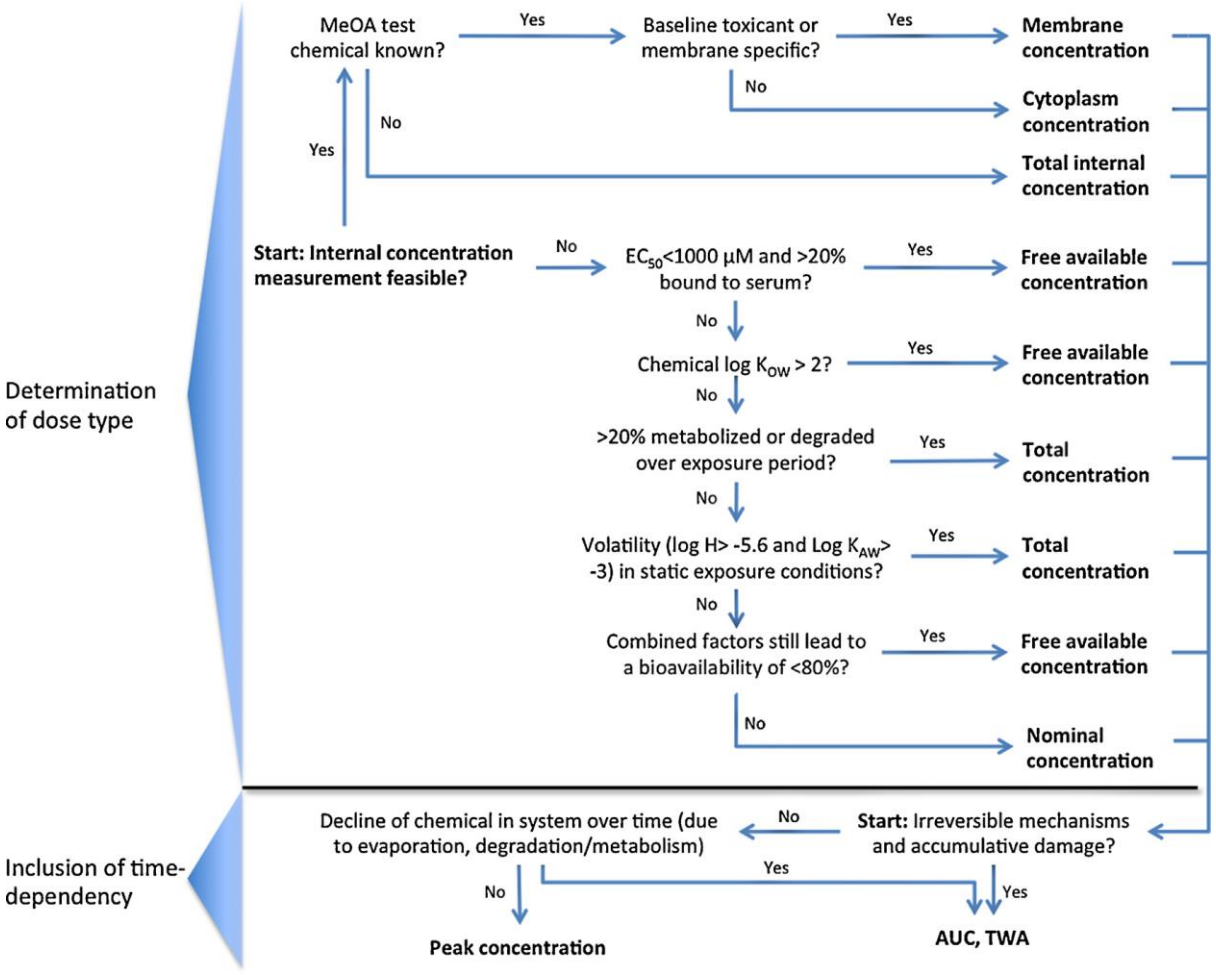
Clearly, several processes can influence how much of the test item will actually reach the target and is also related to its saturation concentration. If test results are based on the added, or nominal, concentrations, considerable variation between laboratories may be obtained. Furthermore, in vitro biokinetic processes are included in these test results (e.g., an EC50), rendering these unfit for extrapolation to in vivo (see In Vitro to In Vivo Extrapolation IVIVE below and (Kramer et al., 2015[3]). For example, if there is considerable evaporation, the EC50 in vitro will appear to be much higher than it will be in the same tissue in vivo. Thus, in order to obtain pure EC50 values, that relate target concentrations to responses, these target concentrations should be measured. As the precise concentration at the target site inside the cell is too difficult to measure, the best

approximation should be measured, i.e., the free concentration in the cell or in the medium. The free concentration in the cell is often still difficult to measure, therefore the free concentration in the medium (similar to the free concentration in the cell cytosol), or the total concentration in the cells (often for metals) are usually measured. Further information can then be added by calculations that take physicochemical and biochemical properties (e.g., transporter substrates) of the substances into account.

Methods with which the free concentration can be measured have been reviewed in Heringa *et al.* 2003 (Heringa and Hermens, 2003[5]). This review also describes how negligible depletion-solid phase extraction (nd-SPME) should be applied to measure free concentrations. This method is very suitable for *in vitro* tests, as it is suitable for small volumes. Examples of its application in *in vitro* tests are (Heringa et al., 2004[9]), (Broeders, Blaauboer and Hermens, 2011[13]) and (Kramer et al., 2012[6]).

Measuring the free concentrations does require extra effort and resources in the conduct of the *in vitro* test, as e.g., a chemical analysis method is necessary. This effort can be saved in some instances, depending on the properties of the test item: in case of very hydrophilic, non-volatile substances that hardly bind to serum proteins, there will hardly be any losses and the nominal concentration will be very similar to the free concentration. Figure A H.1 provides a decision scheme on which concentration should/can be used as dose metric (Groothuis et al., 2015[2]).

Figure A H.1. Flow chart to aid in choosing an appropriate dose metric for a specific *in vitro* toxicity test



Notes: First, a choice should be made for dose type based on the characteristics of the chemical and available knowledge. Then, the metric can be integrated or averaged in case of time-dependent exposure and irreversible mechanisms, or steady reduction over time. Peak concentration is defined here as the maximum concentration reached during the exposure period. Biokinetic/Toxicodynamic (BK/TD) may be applied to model partitioning and assess concentration changes over time.
Sources: The chart has been compiled by (Groothuis et al., 2015[2]) using literature data (Austin et al., 2002[14]); (Gülden et al., 2010[15]); (Gülden and Seibert, 2003[10]); (Knöbel et al., 2012[16]); (OECD, 2011[17]); (OECD, 2006[18]); (OECD, 2006[19]); (Reinert, Giddings and Judd, 2002[20]) and (Riedl and Altenburger, 2007[21]).

To avoid the effort of measuring free concentrations in every sample, passive dosing can be applied. In this method, a disk or ring of absorbent material, which is loaded with the test substance, is added to the sample. After a time of equilibration, the free concentration will have become proportionate to the concentration in the disk or ring, governed by the partition coefficient between water and the disk or ring material. If this partition coefficient has been predetermined, and if the amount of substance in the ring or disk by far exceeds the amount to be dissolved in the medium, then the free concentration in each sample can be easily calculated, and does not need to be measured. A more detailed description of the method (Smith, Oostingh and Mayer, 2010[22]) as well as a later study

(Smith et al., 2013[23]) provide examples of how passive dosing can be applied to *in vitro* tests.

In vitro to *in vivo* extrapolation (IVIVE) refers to the qualitative or quantitative transposition of experimental results or observations made *in vitro* to predict phenomena *in vivo*, on full living organisms. When the response of the *in vitro* test is plotted against the free concentration (or the nominal concentration only in case it can be demonstrated/estimated this approximates the free concentration), toxicity parameters such as the EC_{50} or a Benchmark Concentration (BMC) can be derived from the obtained curve. This *in vitro* toxicity parameter can be used as point of departure (PoD) for *in vitro* test circumstances and directly applicable to *in vivo* extrapolations (Blaauboer et al., 2012[24]); (Leist et al., 2014[25]) The corresponding *in vitro* concentrations can be converted into relevant plasma concentrations by taking the protein and lipid concentrations in plasma and cell culture medium into account (Bosgra and Westerhout, 2015[26]); (Zimmer et al., 2014[27]). In a final step, this concentration can be used as input for physiologically based pharmacokinetic (PBPK) models to estimate the dose that would result in the respective plasma concentration in man. This way an external Benchmark Dose (BMD) can be obtained. PBPK models describe the kinetic processes *in vivo*, relating external doses to tissue concentrations in time. For these models, some physical-chemical properties of the test substance need to be known, as well as some kinetic parameters such as the fraction absorbed, rate of metabolism, tissue partition coefficients, protein binding coefficients and urinary excretion rate (Louisse et al., 2010[28]). Good modelling practices for Physiologically Based PharmacoKinetic (PBPK) models have been described by Loizou *et al.* (Loizou et al., 2008[29]). The recommendations from a joint EPAA - EURL ECVAM on how Physiologically Based ToxicoKinetic (PBTK) modelling platforms and parameter estimation tools could enable animal-free risk assessment are reported in Bessems *et al.*, (Bessems et al., 2014[30]).

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