Toxicokinetics in preclinical evaluation

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Toxicokinetic evaluation is both a regulatory and scientific requirement in the drug development process. However, as a fairly new term, its use within the industry is still confused. Toxicokinetics is the generation of kinetic data to assess systemic exposure, either as an integral component of preclinical toxicity studies, or in specially designed supportive studies. These data help to understand the relationship between observed toxicity and administered dose. They also play a role in the clinical setting, assisting in the setting of plasma limits for early human exposure and in the calculation of safety margins.

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The word toxicokinetics is a relatively new one. Its use has changed over the past 25 years to something quite separate from what is known as drug absorption, distribution, metabolism and excretion (ADME), namely, the study of pharmacokinetics. In essence, it is a comparatively small but important component of the complete kinetics programme used in drug development. The International Conference on Harmonization (ICH) has issued a global definition of toxicokinetics, describing it as '...the generation of pharmacokinetic data, either as an integral component in the conduct of non-clinical toxicity studies or in specially designed supportive studies, to assess systemic exposure' [1]. However, despite such a definition, confusion still exists regarding the exact nature of toxicokinetics; this is reflected in another recent definition of toxicokinetics as '...the modelling and mathematical description of the time-course of disposition (absorption, distribution, biotransformation and excretion) of xenobiotics in the whole organism'. [2]. Overall, it is safe to say that pharmacokinetics is used as an in-depth characterization of the properties of a drug, while toxicokinetics is used in the safety assessment of a drug, usually at doses associated with undesired toxic effects.

Several regulatory guidelines recommend toxicokinetic measurement. First, there is a need to describe the systemic exposure achieved in animals, and its relationship to dose level and the time-course of the toxicity study [1]. Second, exposure data in animals should be evaluated before human clinical trials [3]. Third, information on systemic exposure of animals during repeated-dose toxicity studies is essential for the interpretation of study results, to the design of subsequent studies and to the human safety assessment [4]. Perhaps more importantly, toxicokinetic evaluation makes for good science. Exposure in the animal species needs to be related to administered dose and any observed toxicity. In addition, species, sex, and inter-animal variability also need to be compared. The resulting data are used both to set plasma limits for clinical exposure (based on potential toxicity), and to calculate safety margins. Many drugs show species difference; for example, clearance of nicardipine in the plasma of rats is high compared with other species, including humans [5]. Proxicromil causes liver toxicity (with elevated plasma levels) in the dog but not in the rat or monkey owing to their inability to metabolize the drug through hepatobiliary saturation [6].

Gender also affects the kinetics of drug metabolism. For example, with drugs, such as pentobarbital, morphine and methadone, male rats have a much higher liver metabolism than females, resulting in lower plasma levels [7]. A recent survey by the Japanese Pharmaceutical Manufacturers Association compared the results from 102 repeat-dose toxicity studies (ranging from one to 12 months) in mouse, rat, dog and monkey [8]. Sex differences were observed in 41 out of 92 of the studies, primarily consisting of higher exposure in female rats. Exposure as observed from plasma levels increased in 35 out of 96 studies and decreased in 14 out of 96 studies.

Measuring toxicokinetics

There is a regulatory expectation to ensure that analytical methods used to determine plasma concentrations of pharmaceuticals are

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of adequate sensitivity and precision [1,9]. Such regulations require that the evaluation uses validated analytical methods and conforms to Good Laboratory Practice (GLP). In addition, the number of sample time-points must be to be frequent enough to estimate exposure [1]. Generally, toxicity studies use a range of time-points and replicates to provide toxicokinetic data, although staggered and sparse sampling (to reduce animal numbers) has been reported to give accurate results [10–13]. Analytical methods used in such studies include gas chromatography (although this is rarely used), HPLC (UV or fluorescence), LC, LC–MS, LC-MS-MS, and capillary electrophoresis (again, rarely used, and more for proteins). Results are then analysed using a set curve-prediction package (e.g. WinNonLin from Pharsight; http://www.pharsight.com). For replicate designs, toxicokinetic measurements are taken at similar pre-set time-points and the mean of the measured values is then taken to provide an estimate of drug exposure. Typically, three-or-more animals are used to provide data for each time-point [4–8]. For staggered designs, more sampling times are scheduled [10–12] and individual animal data are used for the toxicokinetic calculations. Although staggered designs are less sensitive, they are still used by various pharmaceutical companies for rodent and/or primate studies. The following parameters are usually measured:

- maximum plasma concentration (C_{max});
- area under curve (AUC) of plasma concentration (exposure) versus time;
- time to reach maximum plasma concentration (T_{max}); and
- time to 50% plasma level ($t_{1/2}$; often, this parameter is not measured in routine toxicity studies owing to limited datasets).

Although some kinetic data might be generated in animal models during early drug efficacy studies, initial toxicokinetic data are generally collected before Phase I investigations during toxicology studies. Further toxicokinetic data are collected in longer-term toxicology and related studies as drug development proceeds. Toxicokinetic data are also likely to be needed for a change in clinical route; for example, AUC and/or C_{max} values might be altered or the metabolic route might change. For older compounds resurrected for development, toxicology regimes might not have included toxicokinetic measurements and might not have been performed to GLP. Thus, toxicokinetic assessment in bridging toxicology studies is needed. Marketed drugs used in combination might require further assessment as the kinetics of a drug can change. For example, a twofold increase in AUC for one drug was observed when combined with another drug, resulting in increased toxicity [14].

Box 1. Measuring toxicokinetics: factors to consider

- Plasma (common) versus whole-blood or serum (less common) measurements. Whole blood is the matrix of choice for drugs with variable kinetics (e.g. cyclosporin) as it gives a better indication of cellular accumulation.
- Unbound drug versus bound drug. Unbound drug in plasma is the most relevant indirect measure of tissue concentration. It has been reported that similar exposure of a drug was noted at a low toxic level in rat, dog and monkey compared to humans, using area under the curve (AUC) measurements of total drug (i.e. with no safety margin). However, unbound drug exposure was >20-fold the safety margin [5].
- Exposure based on active entity and not salt (assume dissociation to the active form occurs in blood).
- Racemate versus enantiomer analyte. A method for chiral conversion might be needed early in development.
- Non-linear dose kinetics; for example, increased exposure owing to saturation of a clearance process or a long plasma half-life, or decreased exposure owing to auto-induction of metabolizing enzymes.
- Parent (always) versus metabolite(s) (rarer) analysis.
- Pro-drugs, where the metabolite is the active material (e.g. cyclosporin, enalapril, levodopa and cyclophosphamide).
- Drug is metabolized to pharmacological or toxicological metabolites that contribute to the overall response.
- When extensive metabolism occurs and the measurement of a major metabolite is the only measure for estimating exposure.
- Human metabolites not found in animal studies.

Several factors need to be considered when measuring toxicokinetics (Box 1) and in interpreting the results. The severity of a toxic response might become more marked with increasing drug dose without an apparent increase in the AUC value as a result of saturation kinetics (e.g. protein binding and metabolism). This can lead to an underestimation of effective systemic exposure; for example, unbound drug might increase with dose with no apparent change in AUC, or increased absorption might be counteracted by enhanced clearance. By contrast, there might be no observed toxicity and no increase in exposure with increasing dose, owing to a non-toxic drug, lack of metabolism to an active form, or saturation of absorption. In cases such as these, toxicokinetic data provide a means of validating the duration and extent of exposure. If good exposure margins are found, the highest dose producing no increase in AUC is probably acceptable for further drug

development, although there is the option of using a different dose route, formulation or species. Finally, exposure might be limited owing to marked toxicity at higher dose levels – the choice of study dose levels would therefore be based on toxicity measurements only.

Toxicokinetic evaluation in preclinical studies

Safety pharmacology studies

Core studies in safety pharmacology comprise *in vivo* CNS, cardiovascular and respiratory assessments. Although toxicokinetic assessment is not specifically mentioned in the guidelines [15], it enables researchers to correlate any observed effects with systemic level of the drug. However, it is possible to cross-reference dose level with exposure in toxicity studies.

Single-dose and rising-dose toxicity studies

Single-dose studies are usually performed in rodents. Although toxicokinetic evaluation is not routinely included in such studies, plasma samples can be taken and stored for later analysis. However, toxicokinetics can be assessed for some drug classes, or in screening studies (e.g. in a series of candidates or when choosing a suitable formulation). Rising-dose studies are performed in non-rodent models. Here, toxicokinetic evaluation takes place at various time-points for each new dose level. Such an evaluation is especially useful if higher-dose emesis occurs as it can reveal whether exposure to the drug still occurred.

Repeat-dose toxicity studies

Repeat-dose studies generally use both rodents and non-rodents and are usually of four-week duration to support the Phase I clinical study. No rigid detailed procedures for the application of toxicokinetics are recommended in regulatory guidance documentation [1]. Assessment usually occurs in both sexes on Day 1 and during Week 4 for all drug dose levels and/or controls (the latter is performed to enable similar treatment of all groups where satellite animals are not used and/or to demonstrate a lack of drug contamination). Often, satellite animals are used in rodent studies owing to the small dose-volume. There are also strict restrictions on blood volume available (no more than 10% of circulating volume can be taken). Sample size is typically 0.25–0.50 ml day^{-1} in rodents and up to 1 ml day^{-1} in non-rodents. Sampling times vary based on the presence (or lack) of pharmacokinetic data, but are often taken 0.5, 1.0, 2.0, 4.0, 8.0, 12.0 and 24.0 h post-dose, with only the parent drug generally being measured. Toxicokinetic values are normally calculated as mean SD; statistical evaluation is not usually performed owing to large intra- and inter-individual variation and the small number of samples or animals used. The results give information on exposure, dose proportionality, sex- and species-difference, and potential accumulation and inhibition, and help to support dose-selection for subsequent studies.

Further repeat-dose toxicity studies

Further repeat-dose studies are usually performed in rodents and non-rodents for up to six and 12 months, respectively, to enable longer clinical exposure. Assessment often occurs in a similar way to shorter-term toxicity studies, although it is possible to reduce sampling times because the drug profile is known from earlier studies. Both parent drug and metabolites can be assessed.

Reproduction toxicity studies

Reproduction toxicity measurements are taken in studies of fertility (rat), embryo-foetal development (rat and rabbit) and peri- or post-natal development (rat). There is a regulatory expectation for toxicokinetic data in pregnant animals, although no specific guidance is given [1,16]. Data from non-pregnant animals is useful to set dose levels, and the limitation of exposure is usually governed by maternal toxicity. The point at which toxicokinetic evaluation is performed varies among pharmaceutical companies but often takes place in embryo-foetal studies at the beginning and end of gestation in the main study animals themselves. However, it can also occur in preliminary studies or in main studies with satellite animals.

Genotoxicity studies

Drug development usually needs to be supported by two *in vitro* studies and one *in vivo* study [17]. *In vivo* investigations usually use a rodent micronucleus (bone marrow or peripheral erythrocytes) test or chromosome aberration (bone marrow cells) test. There is a regulatory expectation to demonstrate exposure to the drug either with toxicity or toxicokinetic data [1,18]. In rodents, specific toxicokinetic evaluation might not be necessary as it is possible to cross-reference with toxicity studies.

Carcinogenicity studies

Lifetime studies in the rodent are needed to support the long-term clinical use of pharmaceuticals [19]. Dose selection is usually determined as the maximum tolerated dose (MTD), which is a 25-fold AUC ratio (rodent to human), or by dose-limiting pharmacodynamic effects, saturation of absorption, or a maximum feasible dose [9]. Selection based on AUC is less common as a 25-fold ratio is often not feasible. Indeed, at the highest dose level, most drugs do not yield AUC values of more than 5–10-fold the human

AUC [20]. There is a regulatory expectation for information on systemic exposure to the parent drug and metabolites [1,21]. Although no details are given as to when such measurements should be taken, it is recommended that monitoring should occur on a few occasions during the study, although it is not essential for monitoring to occur beyond six months [1]. However, pharmaceutical companies use various strategies for such monitoring times (e.g. Weeks 1, 13, 26 and 52, Weeks 1 and 26, or Weeks 26 and 52). It should be noted that, owing to high variability in plasma concentration, toxicokinetic data from aged rats (above one-year old) are not useful for estimating exposure. Sampling times depend on available kinetic data but can range from full profile (up to 24 h) to limited time-points.

Using toxicokinetics for clinical studies

Regulatory guidance outlining which toxicity studies are necessary to support human Phase I, II and III studies, and product license application is available [3]. The extent of the preclinical toxicokinetic evaluation for each clinical phase varies significantly among pharmaceutical companies. At the least, the company might only generate toxicokinetic data from the four-week repeat-dose toxicity studies for Phase I investigations. At the most, the company might undertake a full pharmacokinetic profile (including *in vitro* metabolism studies), and toxicokinetic measurements from four- and 13-week repeat-dose toxicity studies prior to Phase I. Toxicity assessments enable the No Observed Effect Level (NOEL) or No Observed Adverse Effect Level (NOAEL) to be established for a potential new drug, based on clinical observations, bodyweight, food consumption, clinical pathology, organ weights, necropsy examination, and histopathology. Toxicokinetic data from either NOEL or NOAEL [and subsequent toxic level(s)] can be used to give guidance to the clinical investigator by providing suitable safe starting and upper doses in the initial single-dose Phase I study. For further clinical studies using multiple dosing, toxicokinetic data from toxicity studies provide information on possible increases or decreases of drug in plasma. Cases where human plasma levels in a Phase I study are higher than in the animal study NOEL or NOAEL values need to consider the effects of different metabolism and plasma protein binding. This might result in the use of a different species in the toxicity study and/or a change of formulation to enable reassessment of safety margins.

Figure 1 shows an example of toxicokinetic data from toxicity studies for an analgesic drug. In another recent example, toxicokinetic evaluation in toxicity studies for a drug with anti-viral action showed that exposure on Day 1 was 2–3-fold (C_{max}) or 4–6-fold (AUC) higher in the rat than in the dog, at equivalent doses. The toxicities observed were in the liver and kidney. Interestingly, in the dog, although C_{max} and AUC values increased proportionally with dose on Day 1, values decreased between Day 1 and Week 4 at higher doses. It was initially thought that drug-metabolizing enzyme induction in the liver had occurred, as was consistent with increases in liver enzymes, liver weights, and single cell necrosis. However, an *in vitro* assay of terminal liver samples showed that this was not the case, and, on repeated administration, the reduction in plasma levels was related to either reduced absorption or increased clearance. Further evaluation at lower dose levels showed that C_{max} and AUC increased with dose on both Day 1 and Week 4 with similar values.

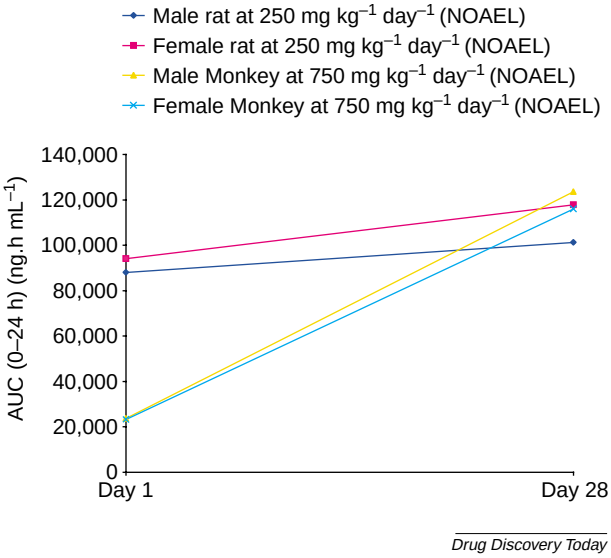

Figure 1. Plasma exposure [area under the curve (AUC)] on Day 1 and Day 28 from four-week toxicity studies in the rat and monkey. Data shown are for a drug with analgesic action, and were used to support early clinical trial work. The No Observed Adverse Effect Level (NOAEL) was established primarily on the basis of liver toxicity. Results showed that exposure increased proportionally with dose for both sexes but was much higher in the rat than in the monkey at the beginning of dosing. However, exposure increased with dosing in the monkey leading to similar plasma levels after four weeks. Early clinical work showed no evidence of accumulation.

Toxicokinetics considerations for different drug classes or dose routes

Anticancer drugs

Initial stages in the development of anticancer drugs are often supported by single- or limited-repeat-dose toxicity studies in the rodent and/or non-rodent, particularly for cytostatic drugs. There is a regulatory expectation for at least a limited toxicokinetic assessment (peak plasma levels

and AUC at the MTD) [22,23]. Such an assessment facilitates the choice of dose escalation during clinical studies. It is interesting to note that there have been examples where plasma levels (with toxicity) in animal studies have been lower than those found in human trials; however, the clinical benefit of using such drugs lacking a safety margin profile outweighs the risk of potential adverse findings.

Topical drugs

The extent of absorption and/or systemic exposure of topical drugs is usually determined from pharmacokinetic studies. However, systemic levels after topical administration must be measured in toxicity studies, with the sampling regimen depending on the extent of absorption.

Inhalation drugs

Toxicity studies of inhalation drugs routinely involve nose-only exposure chamber rodent studies, and dosing-mask non-rodent studies. The calculation of toxicokinetic values is problematic owing to the difficulty in taking blood samples during the exposure period and relating this to the actual and/or measured dose administered.

Biological drugs

The regulatory guidelines recognize the difficulties in measuring toxicokinetics in toxicity studies with biological drugs. However, systemic exposure should be monitored whenever possible [24]. A common problem is the presence of neutralizing antibodies (e.g. raised to a human protein), which affect kinetic profiles. For recombinant human proteins, endogenous (pre-dose) levels of the material must be measured as part of toxicity study design.

Continuous intravenous infusion

Drugs that are administered by continuous intravenous infusion are designed to provide constant systemic exposure without a (potentially) toxic peak (C_{max}), and can be used to overcome the short half-life of various drugs. Continuous-infusion toxicity studies can take place in rodent and non-rodent, and toxicokinetic assessments usually involve $C_{steady\ state}$ and $T_{steady\ state}$ measurements as well as AUC. Although the drug is constantly infused, blood samples need to be taken at the same time-points for each day of the study.

Dietary administration

Dietary administration enables drugs that are readily eliminated by the body to remain in the blood at steady-state levels. However, there is a high degree of inter- and intra-animal variation, especially in the rodent, owing to food consumption pattern. It should be noted that this slower rate of drug presentation (compared with oral administration) could decrease the bioavailability of a drug, owing to increased first-pass metabolism. For example, following gavage- versus dietary-administration of a lipid-lowering drug in the rat, effects on liver enzymes were reported at lower gavaged dose levels due to reduced bioavailability of the drug (AUC values were 2–8-fold lower by the dietary route) [6]. By contrast, even though bioavailability of an anti-psychotic drug was lower, liver toxicity (hyperlipidosis) was observed at lower dietary dose levels compared with gavage administration, owing to the formation of a higher proportional formation of a hydroxylated metabolite [6].

Drugs to be used in juveniles

Various drug classes have been tested for toxicity in juvenile animals to support paediatric clinical use (e.g. anti-asthma drugs). Recent changes in the regulatory environment have indicated a need for the testing of more classes of drugs in children to enable product license approval [25–27]. If juvenile animal studies are used to support paediatric clinical trials, toxicokinetic evaluation is essential, and pharmaceutical companies often use study designs that compare repeat-dose toxicity studies in adult animals. For example, a recent work with atomoxetine, a drug for attention-deficit–hyperactivity disorder to be used in the paediatric population, assessed the effects in juvenile rats and dogs [28,29]. Results were similar to those from adult animal studies, with the exception of rats, where exposure was initially higher but became similar with age. A repeat-dose toxicity study in juvenile dogs for cefmatilon hydrochloride hydrate (a cephalosporin antibiotic) also mirrored the adult study [30,31]. Plasma levels increased in a less-than-dose proportional manner in both studies.

Excipients

Novel excipients in drug formulations need to be considered as new chemical entities [4,32]. As with any new material for human use, toxicokinetic measurements of the excipients within separate toxicity studies might be needed.

Concluding remarks

Interpreting preclinical data from the range of studies performed during drug development requires a good understanding of the observed toxic response(s) versus drug exposure. This understanding is crucial for setting safe dose levels for clinical use of a potential new drug. Both species- and sex-differences exist in animal toxicity studies for toxicokinetic measurements, which obviously has relevance to the clinical evaluation. A lack of dose proportionality also has relevance to the clinical use of a drug and can be related to saturated mechanisms or auto-induction. These

mechanisms include intestinal-absorption saturation (e.g. with verapamil, cimetidine and salbutamol), enzyme-metabolizing system saturation (e.g. with salicylates, theophylline, paroxetine, phenytoin and acyclovir) and tubular re-absorption saturation (e.g. with L-carnitine).

Another important factor is the effect of higher plasma values observed later in a toxicity study, possibly caused by accumulation from a long half-life, reduced clearance, metabolizing-enzyme inhibition or enterohepatic re-circulation. Clearance can be affected either by capacity-limited elimination or impairment of hepatic function (e.g. with proxicromil). Drugs such as methadone have a long half-life and are subject to accumulation. Several examples of enzyme-inhibiting drugs can be found in the literature, including cimetidine, ciprofloxacin and ketoconazole. Enterohepatic cycling might prolong the action of some benzodiazepines and components of the contraceptive pill. In the dog, the ulcerogenic effects of indomethacin have been related to increased cycling compared with other species. Lower plasma values observed later in a toxicity study could be related to enzyme induction or first-pass metabolism. A range of enzyme-inducing agents are known to exist and include carbamazepine, phenobarbitone and phenytoin. The first pass of a drug (e.g. morphine) through the liver can reduce systemic bioavailability and can be considered a form of pre-systemic metabolism.

Robust toxicokinetic evaluation is an essential part of drug development. Assessment should comprise effective analytical methods (performed to GLP), adequate sampling (including controls where relevant), sufficient results (through the collection procedure), evaluation of metabolites, and measurements of human metabolite(s) in an animal species (where appropriate). In addition, it is important to perform toxicokinetic measurements in both safety pharmacology and *in vivo* genotoxicity studies (where cross-referencing with other studies is not possible) and in reproduction toxicity studies. Effective interpretation of toxicokinetic data in regulatory dossiers should also occur.

Future implications

This review has described how the activities associated with toxicokinetic evaluation have become more standardized and distinct from ADME over the years. However, the field is still changing, with more sensitive detection methods, reductions in sample volumes and the numbers of animals, and considerations for newer situations, such as the effects of biological drugs, and testing materials in juveniles or by continuous infusion. Finally, although toxicokinetic evaluation has a role largely associated with mainstream toxicology testing, it is increasingly overlapping with other areas of pharmacokinetics. For example, toxicokinetic assessments are now used to support the use of biomarkers, are used earlier in screening studies, provide data for allometric species scaling, and even play a role in measuring drug levels in non-plasma samples such as tissues, urine and bile. Although toxicokinetic evaluation is only a small part of the process of understanding the fate of a drug, it has a vital role to play in drug development – a role that continues to advance.

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