

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A REVISED TG 417

Toxicokinetics

INTRODUCTION

1. Studies examining the toxicokinetics (TK) of a chemical substance are conducted to obtain adequate information on its absorption, distribution, biotransformation (i.e. metabolism) and excretion, to aid in relating concentration or dose to the observed toxicity, and to aid in understanding its mechanism of toxicity. Basic TK parameters determined from these studies will also provide information on the potential for accumulation of the test substance in tissues and/or organs and the potential for induction of biotransformation as a result of exposure to the test substance.

2. TK data can contribute to the assessment of the adequacy and relevance of animal toxicity data (particularly chronic toxicity and/or carcinogenicity data) for extrapolation to human hazard and/or risk assessment. Additionally, toxicokinetic studies may provide useful information for determining dose levels for toxicity studies (linear vs. non-linear kinetics), route of administration effects, bioavailability, and issues related to study design. Certain types of TK data can be used in physiologically based toxicokinetic (PBTK) model development.

3. There are important uses for metabolite/TK data such as suggesting possible toxicities and modes of action and their relation to dose level and route of exposure. In addition, metabolism data can provide information useful for assessing the toxicological significance of exposures to exogenously produced metabolites of the test substance.

4. Adequate toxicokinetic data will be helpful to support the further acceptability and applicability of quantitative structure-activity relationships, read-across or grouping approaches in the safety evaluation of substances. Kinetics data may also be used to evaluate the toxicological relevance of other studies (e.g. *in vivo/in vitro*).

5. Unless another route of administration is mentioned, this Test Guideline is applicable to oral administration of the test substance.

DEFINITIONS

6. Definitions used for the purpose of this Test Guideline are provided in Annex.

INITIAL CONSIDERATIONS

7. Competent authorities have different requirements and needs regarding the measurement of endpoints and parameters related to toxicokinetics for different classes of chemicals (e.g. pesticides, biocides, industrial chemicals). Unlike most Test Guidelines, this Test Guideline describes toxicokinetics testing, which involves multiple measurements and endpoints. In the future, several new Test Guidelines

may be developed to describe each endpoint separately and in more detail. In the case of this Test Guideline, which test methods or assessments are conducted is specified by the requirements and/or needs of each competent authority.

8. There are numerous studies that might be performed to evaluate the TK behaviour of a chemical for regulatory purposes. However, depending on particular regulatory needs or situations, not all of these possible studies may be necessary for the evaluation of a chemical. Flexibility, taking into consideration the characteristics of the substance being investigated, is needed in the design of toxicokinetic studies. In some cases, only a certain set of questions may need to be explored in order to address chemical-associated hazard and risk concerns. In some situations, TK data can be collected as part of the evaluation in other toxicology studies. For other situations, additional and/or more extensive TK studies may be necessary, depending on regulatory needs and/or if new questions arise as part of chemical evaluation.

9. All available information on the test substance and relevant metabolites and analogs should be considered by the testing laboratory prior to conducting the study in order to enhance study quality and minimize animal usage. This could include data from other relevant test methods (*in vivo* studies, *in vitro* studies, and/or *in silico* evaluations). Physicochemical properties, such as octanol-water partition coefficient (expressed as log P_{OW}), pKa, water solubility, vapor pressure, and molecular weight of a chemical may be useful for study planning and interpretation of results. They can be determined using appropriate methods as described in the relevant OECD Test Guidelines. .

LIMITATIONS

10. This Test Guideline is not designed to address special circumstances, such as the pregnant or lactating animal and offspring, or to evaluate potential residues in exposed food-producing animals. However, the data obtained from a TG 417 study can provide background information to guide the design of specific studies for these investigations. This Test Guideline is not intended for the testing of nanomaterials.

ANIMAL WELFARE CONSIDERATIONS

11. Guidance on humane treatment of animals is available in OECD Guidance Document (GD) 19 (1). It is recommended that OECD GD 19 be consulted for all *in vivo* and *in vitro* studies described in this Test Guideline.

DESCRIPTION OF THE METHODS

Pilot Studies

12. The use of pilot studies is recommended and encouraged for the selection of experimental parameters for the toxicokinetics studies (e.g. metabolism, mass balance, analytical procedures, dose-finding, exhalation of CO₂, etc.). Characterization of some of these parameters may not necessitate the use of radiolabelled substances.

Animal Selection

Species

13. The animal species (and strain) used for TK testing should preferably be the same as that used in other toxicological studies performed with test substance of interest. Normally, the rat should be used as it has been used extensively for toxicological studies. The use of other or additional species may be warranted if critical toxicology studies demonstrate evidence of significant toxicity in these species or if

their toxicity/toxicokinetics is shown to be more relevant to humans. Justification should be provided for the selection of the animal species.

14. Unless mentioned otherwise, this Test Guideline refers to the rat as the test species. Certain aspects of the guideline might have to be modified for the use of other test species.

Age and Strain

15. Young healthy adult animals (normally 6-12 weeks at the time of dosing) should be used (see also paragraph 13). Justification should be provided for the use of strains and animals that are not young adults. All animals should be of similar age at the outset of the study. The weight variation should not exceed $\pm 20\%$ of the mean weight of the test group. Ideally, the strain used should be the same as that used in deriving the toxicological database for the chemical substance.

Number and Sex of Animals

16. A minimum of four animals of one sex should be used for each dose tested. Justification should be provided for the sex of the animals used. The use of both sexes (four males and four females) should be considered if there is evidence to support significant sex-related differences in toxicity.

Housing and feeding conditions

17. Animals should generally be housed individually during the testing period. Group housing might be justified in special circumstances. Lighting should be artificial, the sequence being 12 h light/12 h dark. The temperature of the experimental animal room should be 22°C ($\pm 3^{\circ}\text{C}$) and the relative humidity 30-70 %. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water.

Test Substance

18. A radiolabelled test substance using ^{14}C should be used for all mass balance and metabolite identification aspects of the study; however, if it can be demonstrated that:

- the mass balance and metabolite identification requirements can be met using the unlabelled test substance,
- the analytical specificity and sensitivity of the method used with non-radioactive test substance is equal to or greater than that which could be obtained with the radiolabelled test substance,

then, the radiolabelled compound does not need to be used. Other radioactive and stable isotopes may be used, particularly if the element is responsible for or is a part of the toxic portion of the compound. If possible, the radiolabel should be located in a core portion of the molecule which is metabolically stable (it is not exchangeable, is not removed metabolically as CO_2 , and does not become part of the one-carbon pool of the organism). Labelling of multiple sites or specific regions of the molecule may be necessary to follow the metabolic fate of the compound.

19. The radiolabelled and non-radiolabelled test substances should be analyzed using appropriate methods to establish purity and identity. The radiopurity of the radioactive test substance should be the highest attainable for a particular test substance (ideally it should be greater than 95 %) and reasonable effort should be made to identify impurities present at or above 2 %. The purity, along with the identity and proportion of any impurities which have been identified, should be reported. Individual regulatory programs may choose to provide additional guidance to assist in the definition and specifications of test substances composed of mixtures and methods for determination of purity.

Dose Selection

Pilot Study

20. Usually a single oral dose is sufficient for the pilot study. The dose should be non-toxic, but high enough to allow for metabolite identification in excreta (and plasma, if appropriate) as well as to meet the stated purpose of the pilot study as noted in paragraph 12 of this Test Guideline.

Main Studies

21. For the main studies, a minimum of two doses, typically by the oral route, is preferred since information gathered from at least two dose groups may aid in dose setting in other toxicity studies, and help in the dose-response assessment of already available toxicity tests.

22. Where two doses are administered, both doses should be high enough to allow for metabolite identification in excreta (and plasma, if appropriate). Information from available toxicity data should be considered for dose selection. If other information is not available (e.g., on repeated dose toxicity) a value for the higher dose that is below the LD50 estimate or below the lower value of the acute toxicity range estimate may be considered. The lower dose should be some fraction of the higher dose.

23. If only one dose level is investigated, ideally the dose should be high enough to allow for metabolite identification in excreta (and plasma, if appropriate), while not producing apparent toxicity. A rationale should be provided as to why no second dose level has been included.

24. If the effect of dose on kinetic processes needs to be established, two doses may not be sufficient and at least one dose should be high enough so as to saturate these processes. If the area under the plasma concentration-time curve (AUC) is not linear between two dose levels used in the main study, this is a strong indication of saturation of one or more of the kinetic process starting to occur somewhere between the two dose levels.

25. For test substances of low toxicity, a maximum dose of 1,000 mg/kg body weight should be used. Chemical-specific considerations may necessitate a higher dose depending on regulatory needs. Dose selection should always be justified.

26. Single dose toxicokinetic and tissue distribution data may be adequate to determine the potential for accumulation and/or persistence. However in some circumstances repeated dose administration may be needed i) to address more fully the potential for accumulation and/or persistence or changes in TK (i.e., for instance, enzyme induction and inhibition), or ii) as required by a competent authority. In studies involving repeated dosing, while repeated low dose administration is usually sufficient, under certain circumstances repeated high dose administration may also be necessary (see also paragraph 57).

Administration of Test Substance

27. The test substance should be dissolved or suspended homogeneously in the same vehicle employed for the other oral gavage toxicity studies performed with the test substance, if such vehicle information is available. Rationale for the choice of vehicle should be provided. The choice of the vehicle and the volume of dosing should be considered in the design of the study. The customary method of administration will be by gavage; however, administration by gelatin capsule or as a dietary mixture may be advantageous in specific situations (in this case, justification should be given). Verification of the actual dose administered to each animal should be provided.

28. The maximum volume of liquid to be administered by oral gavage at one time depends on the size of the test animals, the type of dose vehicle, and whether or not feed is withheld prior to administration of the test material. The rationale for administering or restricting food prior to dosing should be provided. Normally the volume should be kept as low as practical for either aqueous or non-aqueous vehicles. It should not normally exceed 10 mL/kg body weight for rodents. Volumes of vehicles used for more lipophilic test substances might start at 4 mL/kg body weight. For repeated dosing, when daily fasting would be contraindicated, lower dose volumes (e.g., 2-4 mL/kg body weight) should be considered.

29. Intravenous (IV) administration of the test substance and measurement of the test substance in blood and/or excreta may be used to establish bioavailability or relative oral absorption. For the IV study, a single dose (usually equivalent to but not to exceed the lower oral dose – see dose selection) of test substance is administered using an appropriate vehicle. This material should be administered in a suitable volume (e.g. 1 mL/kg bw) at the chosen site of administration to at least four animals of the appropriate sex (both sexes might be used, if warranted, see paragraph 16). A fully dissolved or suspended dose preparation is necessary for IV administration of the test substance. The vehicle for IV administration should not interfere with blood integrity or blood flow. Anesthesia should be used if one cannulates the jugular vein (for administration of chemical and/or collection of blood) or if one uses the femoral artery for administration. Due consideration should be given to the type of anaesthesia as it may have effects on toxicokinetics. Animals should be allowed to recover adequately before administration of the test material.

30. Other routes of administration, such as dermal and inhalation, (see paragraphs 74-78) may be applicable for certain chemicals, considering the expected human use or exposure.

Measurements

Mass Balance

31. Mass balance is determined by summation of the percent of the administered (radioactive) dose excreted in urine, faeces, and expired air, and that present in tissues and residual carcass.

Absorption

32. An initial estimation of absorption can be achieved by excluding the percentage of dose in the gastro-intestinal (GI) tract and/or faeces from the mass balance determination. For the calculation of percent absorption, see paragraphs 33 and 34. For investigation of excreta, see paragraphs 44–49. If the exact extent of absorption following oral dosing cannot be established from mass balance studies (e.g., where greater than 20 % of the administered dose is present in faeces), further investigations might be necessary. These studies could comprise either 1) oral administration of test substance and measurement of test substance in bile or 2) oral and IV administration of test substance and measurement of net test substance present in urine plus expired air plus carcass by the two routes. In either study design, measurement of radioactivity is conducted as a surrogate method for chemical-specific analysis of test substance plus metabolites.

33. If a biliary excretion study is undertaken, the oral route of administration is typically used. In this study, the bile ducts of at least four animals of the appropriate sex (or of both sexes, if warranted) should be cannulated and a single dose of the test substance should be administered. Following administration of the test substance, excretion of radioactivity/test substance in bile should be monitored as long as necessary to estimate the percentage of the administered dose that is excreted via this route, which can be used to directly calculate the extent of oral absorption, as follows:

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Percent absorption = (amount in bile + urine + expired air + carcass without GI tract contents) / amount administered x 100

34. For the IV study, the disposition of the test substance is compared with the results from dosing via the oral route to determine the relative absorption of the oral vs. IV doses. Note that since IV dose absorption is 100 % by default, the absolute fraction of oral absorption is obtained with this method. The equation below also accounts for both biliary elimination and enterohepatic recirculation reabsorption by both routes. In this study, at least four animals (of the same sex and strain used in the oral route experiment) should be given the test substance, via IV administration in a suitable vehicle (see paragraph 29). The percentage of administered dose absorbed for the oral route is calculated as follows:

Percent absorption = [recovery_{oral} / recovery_{IV}] x [dose_{IV} / dose_{oral}]

where recovery = amount of administered dose recovered in urine + expired air + carcass without GI tract contents

This comparative recovery method is based on comparable fractions of biliary elimination and enterohepatic recirculation reabsorption via the oral and IV routes. If the test substance is unmetabolized, this percent absorption calculation can also be considered a surrogate for chemical-specific determination of bioavailability (see paragraph 35).

Bioavailability

35. Bioavailability can be determined from plasma/blood kinetics of the oral and IV groups, as described in paragraphs 50-52, by specific chemical analysis of the test substance and/or relevant metabolite, therefore not requiring radiolabeled test substance. The calculation of bioavailability (F) of the test substance or relevant metabolite(s) can then be made as follows:

$F = (AUC_{exp} / AUC_{IV}) \times (Dose_{IV} / Dose_{exp})$

where AUC is the area under the plasma concentration-time curve, and exp is the experimental route (oral, dermal or via inhalation).

36. For use in risk assessment of systemic effects, bioavailability of the toxic component is in general preferred over the percent absorption when comparing systemic concentrations from animal studies with analogous biomonitoring data from worker exposure studies. The situation may become more complex if doses are in the non-linear range so it is important that toxicokinetic screening determines doses in the linear range.

Tissue Distribution

37. The percent of the total (radioactive) dose in tissues as well as residual carcass should at the minimum be measured at the termination of the excretion experiment and at earlier time points if necessary (see paragraph 38) to determine tissue distribution of the test substance and to aid in establishing mass balance. Tissues that should be collected include liver, fat, GI tract, kidney, spleen, whole blood, residual carcass, target organ tissues and any other tissues (e.g. thyroid, erythrocytes, reproductive organs, skin, eye (particularly in pigmented animals)) of potential significance in the toxicological evaluation of the test substance. Analysis of additional tissues at the same time should be considered to maximize utilization of animals and in the event that target organ toxicity is observed in sub-chronic or chronic toxicity studies. The (radioactive) residue concentration and tissue-to-plasma (blood) ratios should also be reported.

38. The evaluation of tissue distribution at additional time points such as the time of peak plasma\blood concentration (e.g. T_{max}) or the peak rate of urinary excretion, obtained from the respective plasma\blood kinetic or excretion experiments may also be needed or required by a competent authority. This information can be useful for understanding toxicity and the potential for test substance and metabolite accumulation and persistence. Justification for sample selection should be provided; samples for analysis generally should be the same as those above (see paragraph 37).

39. Quantification of ^{14}C residues for tissue distribution studies can be performed using organ dissection, homogenization, combustion and/or solubilisation, followed by liquid scintillation counting (LSC) of trapped residues. Certain techniques, currently at various stages of development, e.g., Quantitative whole-body autoradiography and receptor microscopic autoradiography, may prove useful in determining the distribution of a test substance in organs and/or tissues (2) (3).

40. For routes of exposure other than oral, specific tissues should be collected and analyzed, such as lungs in inhalation studies and skin in dermal studies. See discussion below regarding Alternate Routes of Exposure.

Metabolism

41. Excreta (and plasma, if appropriate) should be collected for identification and quantitation of unchanged test substance and metabolites as described under paragraphs 44-49. Pooling of excreta to facilitate metabolite identification within a given dose group is acceptable. Profiling of metabolites from each time period is recommended. However, if lack of sample and/or radioactivity precludes this, pooling of urine and faeces across several time points is acceptable but pooling across sexes is not acceptable. Appropriate qualitative and quantitative methods should be used to assay urine, faeces, expired radioactivity from treated animals, and bile if appropriate.

42. Reasonable efforts should be made to identify all metabolites present at 5 % or greater of the administered dose and to provide a metabolic scheme for the test substance. Compounds which have been characterized in excreta as comprising 5 % or greater of the administered dose should be identified. Identification refers to the exact structural determination of components. Typically, identification is accomplished either by co-chromatography of the metabolite with known standards using two dissimilar systems or by techniques capable of positive structural identification such as MS, NMR, etc. In the case of co-chromatography, chromatographic techniques utilizing the same stationary phase with two different solvent systems are not considered to be an adequate two-method verification of metabolite identity, since the methods are not independent. Identification by co-chromatography should be obtained using two dissimilar, analytically independent systems such as reverse and normal phase thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). Provided that the chromatographic separation is of suitable quality, then additional confirmation by spectroscopic means is not necessary. Alternatively, unambiguous identification can also be obtained using methods providing structural information such as: liquid chromatography/mass spectrometry (LC-MS), or liquid chromatography/tandem mass spectrometry (LC-MS/MS), gas chromatography/mass spectrometry (GC-MS), and NMR spectrometry.

43. If identification of metabolites at 5 % or greater of the administered dose is not possible, a justification/explanation should be provided in the final report. It might be appropriate to identify metabolites representing less than 5 % of the administered dose to gain a better understanding of the metabolic pathway for hazard and/or risk assessment of the test substance. Structural confirmation should be provided whenever possible. This may include profiling in plasma or blood or other tissues.

Excretion

44. The rate and extent of excretion of the administered dose should be determined by measuring the percent recovered (radioactive) dose from urine, faeces and expired air. These data will also assist in establishing mass balance. Generally, total recoveries of administered test substance (radioactivity) in the order of > 90 % are considered to be adequate. The quantities of test substance (radioactivity) eliminated in the urine, faeces, and expired air should be determined at appropriate time intervals (see paragraphs 47-49). Repeated dose experiments should be properly designed to allow for collection of excretion data to meet the objectives described in the paragraph 26. This will allow for comparison to single dose experiments.

45. If a pilot study has shown that no significant amount of test substance (radioactivity) (according to paragraph 49) is excreted in expired air, then expired air does not need to be collected in the definitive study.

46. Each animal is to be placed in a separate metabolic unit for collection of excreta (urine, faeces and expired air). At the end of each collection period (see paragraph 47-49), the metabolic units should be rinsed with appropriate solvent (this is known as the “cage wash”) to ensure maximum recovery of the test substance (radioactivity). Collection of excreta should be terminated at 7 days, or after at least 90 % of the administered dose has been recovered, whichever occurs first.

47. The total quantities of substance (radioactivity) in urine are to be determined for at least two time points on day 1 of collection, one of which should be at 24 h post dosing, and daily thereafter until study termination, unless pilot studies suggest alternate or additional time points for collection.

48. The total quantities of test substance (radioactivity) in faeces should be determined on a daily basis beginning at 24 h post-dosing until study termination, unless pilot studies suggest alternate or additional time points for collection. A rationale should be provided for alternative collection schedules.

49. The collection of expired CO₂ and other volatile materials may be discontinued in a given study experiment when less than 1 % of the administered dose is found in the exhaled air during a 24-h collection period.

*Time Course Studies**Plasma/Blood Kinetics*

50. The purpose of these studies is to obtain estimates of basic TK parameters [e.g. C_{max}, T_{max} half-life (t_{1/2}), AUC] for the test substance. These studies may be conducted at one dose or, more likely, at two or more doses. Dose setting should be determined by the nature of the experiment and/or the issue being addressed. Kinetic data may be needed to resolve issues such as substance bioavailability and/or to clarify the effect of dose on clearance (e.g. to clarify whether clearance is saturated in a dose-dependent fashion).

51. For these studies a minimum of four animals of one sex per dose group should be used. Justification should be provided for the sex of the animals used. The use of both sexes (four males and four females) should be considered if there is evidence to support significant sex-related differences in toxicity.

52. Following administration of the test substance (radiolabelled), blood samples should be obtained from each animal at suitable time points using appropriate sampling methodology. The volume and number of blood samples which can be obtained per animal might be limited by potential effects of repeated sampling on animal health/physiology and/or the sensitivity of the analytical method. In some instances, it might be necessary to pool sample from more than one animal. If a radiolabelled substance is used, analysis of total radioactivity present might be adequate. If so, total radioactivity should be analyzed in

whole blood and plasma or plasma and red blood cells to allow calculation of the blood/plasma ratio. In other circumstances, more specific investigations requiring the identification of parent compound and/or metabolites, or to assess protein binding might be necessary.

Other Tissue Kinetics

53. The purpose of these studies is to obtain time course information to address questions related to issues such as toxic mode of action, bioaccumulation and biopersistence via determination of levels of test substance in various tissues. The selection of tissues and the number of time points evaluated will depend on the issue to be addressed and the toxicological database for the chemical substance. The design of these additional tissue kinetics studies should take into account information gathered as described in paragraphs 37-40. These studies might involve single or repeated dosing. A detailed rationale for the approach used should be provided.

54. Reasons for performing other tissue kinetic studies might include:

- Evidence of extended blood half-life, suggesting possible accumulation of test substance in various tissues or
- interest in seeing if a steady state level has been achieved in specific tissues (e.g. in repeated dosing studies, even though an apparent blood steady state level of test substance may have been achieved, there may be interest in ascertaining that a steady state level has also been attained in target tissues);

55. For these types of time-course studies, an appropriate oral dose of test substance should be administered to a minimum of four animals per dose per time point and the time course of distribution monitored in selected tissues. Only one sex may be required, unless gender specific toxicity is observed. Whether total radioactivity or parent substance and/or metabolites are analyzed will also depend on the issue being addressed. Assessment of tissue distribution should be made using appropriate techniques.

Enzyme Induction/Inhibition

56. Studies addressing the possible effects of enzyme induction/inhibition or biotransformation of test substance under study may be needed under one or more of the following cases:

1. Available evidence indicates a relationship between biotransformation of test substance and enhanced toxicity;
2. The available toxicity data indicate a non-linear relationship between dose and metabolism;
3. The results of metabolite identification studies show identification of a potentially toxic metabolite that might have been produced by an enzyme pathway induced by the test substance;
4. In explaining effects which are postulated to be linked to enzyme induction phenomena;
5. If toxicologically significant alterations in the metabolic profile of the test substance are observed through either *in vitro* or *in vivo* experiments with different species or conditions, characterization of the enzyme(s) involved may be needed (e.g., Phase I enzymes such as isoenzymes of the Cytochrome P450-dependent mono-oxygenase system, Phase II enzymes such as isoenzymes of sulfotransferase or uridine diphosphate glucuronosyl transferase, or any other relevant enzymes). This information might be used to evaluate the pertinence of species to species extrapolations.

57. Appropriate study protocols to evaluate test substance related changes in TK, suitably validated and justified should be used. Example study designs consist of repeated dosing with unlabeled test substance, followed by a single radiolabelled dose on day 14, or repeated dosing with radiolabelled test substance and sampling at days 1, 7 and 14 for determination of metabolite profiles. Repeated dosing with radiolabelled test substance may also provide information on bioaccumulation (see paragraph 26).

SUPPLEMENTAL APPROACHES

58. Supplemental approaches beyond the *in vivo* experiments described in this Test Guideline may provide useful information on the absorption, distribution, metabolism or elimination of a chemical in certain species.

Use of in vitro information

59. Several questions concerning the metabolism of the substance may be addressed in *in vitro* studies using appropriate test systems. Freshly isolated or cultured hepatocytes and subcellular fractions (e.g. microsomes and cytosol or S9 fraction) from liver may be used to study possible metabolites. Local metabolism in the target organ, e.g. lung, may be of interest for risk assessment. For these purposes, microsomal fractions of target tissues may be useful. Studies with microsomes may be useful to address potential gender and life-stage differences and characterize enzyme parameters (K_m and V_{max}) which can aid in the assessment of dose dependency of metabolism in relation to exposure levels. In addition microsomes may be useful to identify the specific microsomal enzymes involved in the metabolism of the substance which can be relevant in species extrapolation (see also Paragraph 56). The potential for induction of biotransformation can also be examined by using liver subcellular fractions (e.g., microsomes and cytosol) of animals pretreated with the substance of interest, *in vitro* via hepatocyte induction studies or from specific cell lines expressing relevant enzymes. In certain circumstances and under appropriate conditions, subcellular fractions coming from human tissues might be considered for use in determining potential species differences in biotransformation. The results from *in vitro* investigations may also have utility in the development of PBTK models (4).

60. *In vitro* dermal absorption studies may provide supplemental information to characterize absorption (5).

61. Primary cell cultures from liver cells and fresh tissue slices may be used to address similar questions as with liver microsomes. In certain cases, it may be possible to answer specific questions using cell lines with defined expression of the relevant enzyme or engineered cell lines. In certain cases, it may be useful to study the inhibition and induction of specific cytochrome P450 isozymes (e.g., CYP1A2, 2A1, and others) and/or phase II enzymes by the parent compound using *in vitro* studies. Information obtained may have utility for similarly structured compounds.

Use of Toxicokinetic Data from Toxicity Studies as Complementary Information

62. Analysis of blood, tissue and/or excreta samples obtained during the conduct of any other toxicity studies can provide data on bioavailability, changes in plasma concentration in time (AUC, C_{max}), bioaccumulation potential, clearance rates, and gender or life-stage changes in metabolism and kinetics.

63. Consideration of the study design can be used to answer questions relating to: saturation of absorption, biotransformation or excretion pathways at higher dose levels; the operation of new metabolic pathways at higher doses and the limitation of toxic metabolites to higher doses.

64. Other hazard assessment considerations could include issues such as:

- Age-related sensitivity due to differences in the status of the blood-brain barrier, the kidney and/or detoxification capacities;
- Sub-population sensitivity due to differences in biotransformation capacities or other TK differences;
- Extent of exposure of the foetus by transplacental transfer of chemicals or of the newborn through lactation.

Use of Toxicokinetic Modeling

65. Toxicokinetic models may have utility for various aspects of hazard and risk assessment as for example in the prediction of systemic exposure and internal tissue dose. Furthermore specific questions on mode of action may be addressed, and these models can provide a basis for extrapolation across species, routes of exposure or dosing patterns. Data useful for developing PBTK models for a chemical in any given species include 1) partition coefficients, 2) biochemical constants and physiological parameters, 3) route-specific absorption parameters and 4) *in vivo* kinetic data for model evaluation (e.g. clearance parameters for relevant (> 10 %) excretion pathways, K_m and V_{max} for metabolism). The experimental data used in model development should be generated with scientifically sound methods and the model results validated. Chemical- and species-specific parameters such as absorption rates, blood-tissue partitioning and metabolic rate constants are often determined to facilitate development of non-compartmental or physiologically-based models (*ref to IPCS document if available*).

DATA AND REPORTING

66. It is recommended that the study report include a table of contents.

Body of the Report

67. The body of the report should include information covered by this Test Guideline organized into sections and paragraphs as follows:

Summary

68. This section of the study report should contain a summary and analysis of the test results and a statement of the conclusions drawn from the analysis. It should include a summary of the study design and a description of methods used. It should also highlight the key findings regarding mass balance, the nature and magnitude of metabolites, tissue residue, rate of clearance, bioaccumulation potential, sex differences, etc. The summary should be presented in sufficient detail to permit evaluation of the findings.

Introduction

69. This section of the report should include the study objectives, rationale and design, as well as, appropriate references and background history, if any.

Materials and Methods

70. This section of the report should include detailed descriptions of all pertinent information including:

(a) Test Substance

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This subsection should include identification of the test substance: chemical name, molecular structure, qualitative and quantitative determination of its chemical composition, chemical purity and whenever possible, type and quantities of any impurities. It should also include information on physical/chemical properties including physical state, colour, gross solubility and/or partition coefficient, stability, and if appropriate, corrosivity. If the test substance is radiolabelled, information on the following should be included in this subsection: the type of radionuclide, position of label, specific activity, radiochemical purity, and if applicable, information of isomer.

The type or description of any vehicle, diluents, suspending agents, and emulsifiers or other materials used in administering the test substance should be stated.

(b) Test Animals

This subsection should include information on the test animals, including selection and justification for species, strain, and age at study initiation, sex as well as body weight, health status, and animal husbandry.

(c) Methods

This subsection should include details of the study design and methodology used. It should include a description of:

- (1) How the dosing solution was prepared and the type of solvent or vehicle, if any, used;
- (2) Number of treatment groups and number of animals per group;
- (3) Dosage levels and volume (and specific activity of the dose when radioactivity is used);
- (4) Route(s) and methods of administration;
- (5) Frequency of dosing;
- (6) Fasting period (if used);
- (7) Total radioactivity per animal;
- (8) Animal handling;
- (9) Sample collection and handling;
- (10) Analytical methods used for separation, quantitation and identification of metabolites;
- (11) Limit of detection for the employed methods;
- (12) Other experimental measurements and procedures employed (including validation of test methods for metabolite analysis).

(d) Statistical Analysis

If statistical analysis is used to analyze the study findings, then sufficient information on the method of analysis and the computer program employed should be included so that an independent reviewer/statistician can re-evaluate and reconstruct the analysis.

In the case of systems modelling studies such as PBTK, presentation of models should include a full description of the model to allow independent reconstruction and validation of the model (see paragraph 65 and Annex: Definitions).

Results

71. All data should be summarized and tabulated with appropriate statistical evaluation and described in the text of this section. Radioactivity counting data should be summarized and presented as appropriate for the study, typically as microgram or milligram equivalents per mass of sample, although other units may be used. This section should include graphic illustrations of the findings, reproduction of representative chromatographic and spectrometric data, metabolite identification/quantification and proposed metabolic pathways including molecular structure of metabolites. In addition the following information is to be included in this section, if applicable:

- (1) Justification for modification of exposure conditions, if applicable;
- (2) Justification for selection of dose levels;
- (3) Description of pilot studies used in the experimental design of the follow-up studies, if applicable. Pilot study supporting data should be submitted;
- (4) Quantity and percent recovery of radioactivity in urine, faeces, expired air, and urine and faeces cage wash, as appropriate. For dermal studies, include recovery data for treated skin, skin washes, and residual radioactivity in the covering apparatus and metabolic unit as well as results of the dermal washing study (see paragraph 75);
- (5) Tissue distribution reported as percent of administered dose and concentration (microgram equivalents per gram of tissue), and tissue-to-blood or tissue-to-plasma ratios;
- (6) Material balance developed from each study involving the assay of body tissues and excreta;
- (7) Plasma concentrations and toxicokinetic parameters (bioavailability, AUC, C_{max} , T_{max} , clearance, half-life) after administration by the relevant routes of exposure;
- (8) Rate and extent of absorption of the test substance after administration by the relevant routes of exposure;
- (9) Quantities of the test substance and metabolites (reported as percent of the administered dose) collected in excreta;
- (10) Reference to appendix data which contain individual animal data for all measurement endpoints (e.g., dose administration, percent recovery, concentrations, TK parameters, etc);
- (11) A figure with the proposed metabolic pathways and the molecular structures of the metabolites.

Discussion and Conclusions

72. In this section the author(s) should:

- (1) Provide a proposed metabolic pathway based on the results of the metabolism and disposition of the test substance;
- (2) Discuss any potential species and sex differences regarding the disposition and/or biotransformation of the test substance;
- (3) Tabulate and discuss the identification and magnitude of metabolites, rates of clearance, bioaccumulation potential, and level of tissue residues of parent, and/or metabolite(s), as well as possible dose-dependent changes in TK parameters, as appropriate;

- (4) Integrate into this section any relevant TK data obtained in the course of conducting toxicity studies;
- (5) Provide a concise conclusion that can be supported by the findings of the study;
- (6) Add Sections (as needed or appropriate)

73. Additional sections should be used to include supporting bibliographic information, tables, figures, appendices, etc.

ALTERNATIVE ROUTES OF EXPOSURE

Dermal

Dermal Treatment

74. Alternative routes only need to be considered if human exposure occurs in this way. Before starting dermal treatment, TG 427 should be consulted (6). Dermal doses could be selected based on expected human exposure and/or doses at which toxicity occurred in dermal toxicity study. One or more dose levels for the test substance should be used in the dermal treatment. The test substance (e.g., neat, diluted or formulated material containing the test chemical which is applied to the skin) should be the same (or a realistic surrogate) as that to which humans or other potential target species might be exposed. The dose level should be selected in accordance with paragraphs 20-26 of this Test Guideline. The dermal doses should be dissolved, if necessary, in a suitable vehicle and applied in a volume adequate to deliver the doses. Shortly before testing, fur should be clipped from the dorsal area of the trunk of the test animals. Shaving may be employed, but it should be carried out approximately 24 h before the test. When clipping or shaving the fur, care should be taken to avoid abrading the skin, which could alter its permeability. Approximately 10 % of the body surface should be cleared for application of the test substance. With highly toxic substances, the surface area covered may be less than approximately 10 %, but as much of the area as possible is to be covered with a thin and uniform film. The same treatment surface area should be used for all dermal test groups. The dosed areas are to be protected with a suitable covering which is secured in place. The animals should be housed separately.

Dermal Washing

75. A washing experiment should be conducted to assess the removal of the applied dose of the test substance by washing the treated skin area with a mild soap and water. A single dose should be applied to two animals in accordance with paragraph 20 of this Test Guideline. At the end of the experiment, the treated areas of the animals should be washed with a mild solution of soap and water (e.g. 2 to 5 min). The amounts of test substance recovered in the washes should be determined to assess the effectiveness of removal by washing.

76. Unless precluded by corrosiveness, the test substance should be applied and kept on the skin for a minimum of 6 h. At the time of removal of the covering, the treated area should be washed following the procedure as outlined in the dermal washing study (see paragraph 75). Both covering and the washes should be analyzed for residual test substance. At the termination of the studies, each animal should be humanely killed in accordance with (1), and the treated skin removed. An appropriate section of treated skin should be analyzed to determine residual substance (radioactivity).

77. For the assessment of pharmaceuticals, longer application times (including up to 24 h) may be appropriate and tested materials may not be washed off.

Inhalation

78. A single concentration (or more if needed) of test substance should be used. The concentration(s) should be selected in accordance with paragraphs 20-26 of this Test Guideline. Inhalation treatments are to be conducted using a “nose-cone” or “head-only” apparatus to prevent absorption by alternate routes of exposure (7). If other inhalation exposure conditions are used, justification for the modification should be documented. The duration of exposure by inhalation should be defined; a typical exposure is 4-6 h.

REFERENCES

- (1) *OECD (2000), Guidance Document on Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation; Environmental Health and Safety Publications, Series on Testing and Assessment N°19, ENV/JM/MONO(2000)7.*
- (2) *Solon E. G., Kraus L (2002) Quantitative whole-body autoradiography in the pharmaceutical industry; Survey results on study design, methods, and regulatory compliance, J Pharm and Tox Methods 46, 73-81.*
- (3) *Stumpf, W. E. (2005). Drug localization and targeting with receptor microscopic autoradiography. J. Pharmacological and Toxicological Methods, 51, 25-40.*
- (4) *Loizou G, Spendiff M, Barton HA, Bessems J, Bois FY, d'Yvoire MB, Buist H, Clewell HJ 3rd, Meek B, Gundert-Remy U, Goerlitz G, Schmitt W. (2008): Development of good modelling practice for physiologically based pharmacokinetic models for use in risk assessment: The first steps. Regulatory toxicology and pharmacology 50, 400 – 411*
- (5) *OECD Guideline for Testing of Chemicals: 428 “Skin Absorption: In Vitro Method” (Adopted 13 April 2004)*
- (6) *OECD Guideline for Testing of Chemicals: 427 “Skin Absorption: In Vivo Method” (Adopted 13 April 2004)*
- (7) *OECD (2009) Guidance Document on on Acute Inhalation Toxicity Testing. Environmental Health and Safety Monograph Series on Testing and Assessment No. 39*
- (8) *Barton, H.A., et al. (2006), The Acquisition and Application of Absorption, Distribution, Metabolism, and Excretion (ADME) Data in Agricultural Chemical Safety Assessments, Critical Reviews in Toxicology, 36: 9-35*
- (9) *Pharmacokinetics, 2nd edition, Milo gibaldi and Donald Perrier, 1982, Marcel Dekker, Inc., New York*

ANNEX: DEFINITIONS

Absorption: Process(es) of uptake of substances into or across tissues. Absorption refers to parent compound and all its metabolites. Not to be confused with “bioavailability”;

Accumulation (Bioaccumulation): Increase of the amount of a substance over time within tissues (usually fatty tissues, following repeated exposure); if the input of a substance into the body is greater than the rate at which it is eliminated, the organism accumulates the substance and toxic concentrations of a substance might be achieved;

ADME: Acronym for “Absorption, Distribution, Metabolism, and Excretion”;

AUC: (Area under the plasma concentration-time curve): Area under the curve in a plot of concentration of substance in plasma over time. It represents the total amount of substance absorbed by the body within a predetermined period of time. Under linear conditions, the AUC (from time zero to infinity) is proportional to the total amount of a substance absorbed by the body, irrespective of the rate of absorption;

Autoradiography: (Whole-body autoradiography): Used to determine qualitatively and/or quantitatively the tissue localization of a radioactive substance, this technique uses X-ray film or more recently digital phosphorimaging to visualize radioactively labeled molecules or fragments of molecules by recording the radiation emitted within the object under study. Quantitative whole-body autoradiography, compared to organ dissection, may have some advantages for the evaluation of test substance distribution and the assessment of overall recovery and resolution of radioactive material in tissues. One significant advantage, for example, is it can be used in a pigmented animal model to assess possible association of the test substance with melanin, which can bind certain molecules. However, while it may provide convenient whole body overviews of the high-capacity-low-affinity binding sites, this technique might be limited in recognizing specific target sites such as receptor-binding sites where relatively high-resolution and high-sensitivity are required for detection. When autoradiography is used, experiments intended to determine mass balance of administered compound should be conducted as a separate group or in a separate study from the tissue distribution experiment, where all excreta (which may also include expired air) and whole carcasses are homogenized and assayed by liquid scintillation counting;

Biliary excretion: Excretion via the bile ducts;

Bioaccumulation: See “Accumulation”;

Bioavailability: Fraction of an administered dose that reaches the systemic circulation or is made available at the site of physiological activity. Usually, bioavailability of a substance refers to the parent compound, but it could refer to its metabolite. It considers only one chemical form. Nota Bene: bioavailability and absorption are not the same. The difference between e.g. oral absorption (i.e. presence in gut wall and portal circulation) and bioavailability (i.e. presence in systemic blood and in tissues) can arise from chemical degradation due to gut wall metabolism or efflux transport back to the intestinal lumen or presystemic metabolism in the liver, among other factors (8). Bioavailability of the toxic component (parent compound or a metabolite) is a critical parameter in human risk assessment (high-to-low dose

ENV

extrapolation, route-to-route extrapolation) for derivation of an internal value from the external NOAEL or BMD (applied dose). For liver effects upon oral administration, it is the oral absorption that suffices. However, for every effect other than at the portal of entry, it is the bioavailability that is in general a more reliable parameter for further use in risk assessment, not the absorption;

Biopersistence: See “Persistence”;

Biotransformation: (Usually enzymatic) chemical conversion of a substance of interest into a different chemical within the body. Synonymous with “metabolism”;

C_{max}: Either maximal (peak) concentration in blood (plasma/serum) after administration or maximal (peak) excretion (in urine or feces) after administration;

Clearance: Quantitative measure of the rate at which a substance is removed from the blood, plasma or a certain tissue per unit time;

Compartment: Structural or biochemical portion (or unit) of a body, tissue or cell, that is separate from the rest;

Detoxification pathways: Series of steps leading to the elimination of toxic substances from the body, either by metabolic change or excretion;

Distribution: Dispersal of a substance and its derivatives throughout an organism;

Enzymes/Isozymes: Proteins that catalyse chemical reactions. Isozymes are enzymes that catalyse the similar chemical reactions but differ in their amino acid sequence;

Enzymatic Parameters: K_m : Michaelis constant and V_{max} : maximum velocity;

Excretion: Process(es) by which an administered substance and/or its metabolites are removed from the body;

Exogenously: Introduced from or produced outside the organism or system;

Extrapolation: Inference of one or more unknown values on the basis of that which is known or has been observed;

Half-life ($t_{1/2}$): The elimination half-life is the time taken for the plasma concentration, as well as the amount of the test substance in the body, to fall by one-half;

Induction/Enzyme induction: Enzyme synthesis in response to an environmental stimulus or inducer molecule;

Linearity/linear kinetics: A process is linear in terms of kinetics when all transfer rates between compartments are proportional to the amounts or concentrations present, i.e. first order. Consequently, clearance and distribution volumes are constant, as well as half-lives. The concentrations achieved are proportional to the dosing rate (exposure), and accumulation is more easily predictable;

Linearity/Non-linearity can be assessed by comparing the relevant parameters, e.g. AUC, after different doses or after single and repeated exposure. Lack of dose dependency may be indicative of saturation of enzymes involved in the metabolism of the compound, an increase of AUC after repeated exposure as

compared to single exposure may be an indication for inhibition of metabolism and a decrease in AUC may be an indication for induction of metabolism [see also (9)];

Mass balance: Accounting of test substance entering and leaving the system;

Material balance: See mass balance;

Mechanism (Mode) of toxicity/Mechanism (Mode) of action: Mechanism of action refers to specific biochemical interactions through which a substance produces its effect. Mode of action refers to more general pathways leading to the toxicity of a substance;

Metabolism: Synonymous with “biotransformation”;

Metabolites: Products of metabolism or metabolic processes;

Oral Absorption: The percentage of the dose of test substance absorbed from the site of administration (ie: GI tract). This critical parameter can be used to understand the fraction of the administered test substance that reaches the portal vein, and subsequently the liver;

Partition coefficient: Also known as the distribution coefficient, it is a measure of the differential solubility of a substance in two solvents;

Peak blood (plasma / serum) levels: Maximal (peak) blood (plasma/serum) concentration after administration (see also “ C_{max} ”);

Persistence (biopersistence): Long-term presence of a substance (in a biological system) due to resistance to degradation/elimination;

Read-across: The endpoint information for one or more chemicals is used to make prediction of the endpoint for the target chemical;

Receptor Microscopic Autoradiography (or Receptor Microautoradiography): This technique may be used to probe xenobiotic interaction with specific tissue sites or cell populations as for instance in receptor binding or specific mode of action studies that may require high-resolution and high sensitivity which may not be feasible with other techniques such as whole-body autoradiography;

Route of administration (oral, IV, dermal, inhalation, etc.): Refers to the means by which substances are administered to the body (e.g., orally by gavage, orally by diet, dermal, by inhalation, intravenously, etc);

Saturation: State whereby the kinetic (e.g. absorption, metabolism or clearance) process cannot be exceeded

Sensitivity: Capability of a method or instrument to discriminate between measurement responses representing different levels of a variable of interest;

Steady-state blood (plasma) levels: Non-equilibrium state of an open system in which all forces acting on the system are exactly counter-balanced by opposing forces, in such a manner that all its components are stationary in concentration although matter is flowing through the system;

Systems Modeling (Pharmacokinetic-based, Physiologically-based Pharmacokinetic, Biologically-based, etc.): Abstract model that uses mathematical language to describe the behaviour of a system;

ENV

Target tissue: Tissue in which the principal adverse effect of a toxicant is manifested;

Tissue distribution: Reversible movement of a substance from one location in the body to another. Tissue distribution can be studied by organ dissection, homogenization, combustion and liquid scintillation counting or by qualitative and/or quantitative whole body autoradiography. The former is useful to obtain concentration and percent of recovery from tissues and remaining carcass of the same animals, but may lack resolution for all tissues and may have less than ideal overall recovery (<90%). See definition for the latter above;

T_{max}: Time to reach C_{max};

Toxicokinetics (Pharmacokinetics): Study of the absorption, distribution, excretion, and metabolism of substances over time;

Validation of models: Process of assessing the adequacy of a model to consistently describe the available toxicokinetic data. Models may be evaluated via statistical and visual comparison of model predictions with experimental values against a common independent variable (e.g. time). The extent of evaluation should be justified in relation to the intended use of the model.