Technical Report

No 46

# EC 7th Amendment: Role of Mammalian Toxicokinetic and Metabolic Studies in the Toxicological Assessment of Industrial Chemicals

May 1992

ISSN-0773-8072-46

TOXKIN-7

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**SUMMARY**

Experience obtained with pharmaceuticals and crop protection chemicals suggests that information on the absorption, distribution, metabolism and elimination of chemicals in mammals can assist in the interpretation of toxicity studies. The fate of a chemical as described by toxicokinetics and metabolism is closely related to the toxic effects. This awareness has led the EC to include toxicokinetic and metabolic information in its legislation governing the toxicity testing of industrial chemicals. Information requested for new chemicals is dependent on the amount of substance to be placed on the market. Progressing through notification Levels 1 and 2, an increasing amount of toxicokinetic and metabolic data is required. The concept of compound-tailored approaches is allowed and rigid procedures have not been defined in the regulatory requirements. The resulting requirements at Level 1 ("basic toxicokinetic information") and at Level 2 ("additional toxicokinetic studies which cover biotransformation, pharmacokinetics") have to be interpreted.

The principles and methods used in the conduct of toxicokinetic and metabolic studies are reviewed to provide background and some key reference material is given. Much of this science has been developed from research on pharmaceuticals, veterinary medicines and crop protection chemicals. Each of these classes is treated differently in legislation and there are good reasons to conclude that industrial chemicals have to be considered as a separate class. Toxicokinetic and metabolic studies are lengthy and sophisticated and because of the varied nature and use of industrial chemicals, non-routine approaches will be necessary.

Various factors affecting toxicokinetics and metabolism are reviewed. One of the most significant of these, species differences, leads on to a

consideration of the extrapolation of the experimental data to man. The use of toxicokinetic and metabolic studies in toxicology, including the interpretation of results and their impact on hazard assessment is discussed.

The basic toxicokinetic and metabolic information required at notification Level 1 is interpreted by ECETOC to mean an assessment of absorption and excretion of the test substance. Physico-chemical properties and analogies based on structure-activity relationships can be of predictive value. Results of toxicity testing may also offer evidence for absorption and elimination. If such approaches provide no information, absorption-excretion studies based on elements of the EC testing guideline are proposed. If there is evidence of non-absorption then no further testing is needed.

"Additional toxicokinetic studies which cover biotransformation, pharmacokinetics", as required at notification Level 2, is interpreted by ECETOC to mean that quantitative data should be obtained on absorption, excretion, and possibly information on distribution and metabolism. The acquisition of information required at this Level is best approached in two stages. Stage 1 is the conduct of a single oral dose mass-balance study using quantitative analytical methods. If non-absorption can be demonstrated and no toxicity has been observed at this stage in the general testing and if no structure-activity considerations give cause for concern, it may be that no further work need be carried out. If these conditions are not fulfilled, or if there is a specific toxicological problem to address, Stage 2 testing will be necessary. This may involve tissue distribution, blood kinetics or biotransformation.

Testing may be impossible under certain circumstances due to the physical and chemical properties of the test substance. Many industrial chemicals are either insoluble, polymeric, highly reactive, or ill-defined. It is recommended that, particularly at Level 2, an extensive evaluation be conducted between the Manufacturer and the Authorities, with a view to generating an agreed approach.

## 1. INTRODUCTION

Protection of man against potential risks from chemicals requires that appropriate precautionary measures for their safe handling, use and disposal are observed. The nature and extent of the measures to be taken for a given chemical depend on its inherent toxicological properties and the potential exposure.

National Authorities and International Organisations have provided guidance on how to determine and evaluate these two factors of risk assessment for different categories of chemicals. Registration schemes have been enforced for pharmaceuticals and agrochemicals in many countries; these specify in detail the design, performance, interpretation and reporting for the required safety investigations.

Legislation exists for industrial chemicals. Within Europe production volume is the main criterion for the initiation of a process with steps of increasing toxicity information. This approach is governed by EC Council Directive 67/548/EEC, the 7th Amendment of which is presently in preparation (EEC, 1990).

This Council Directive requires information on the toxicokinetics of industrial chemicals at Levels 1 and 2 of notification. As the wording of the 7th amendment is somewhat vague, guidance regarding a rational approach for this category of chemicals is required. To meet this aim ECETOC established a Task Force with the following Terms of Reference:

- review the regulatory requirements for mammalian toxicokinetic and metabolism studies with respect to industrial chemicals,

- review the contribution of toxicokinetic and metabolic information to the design and interpretation of toxicity studies,

- discuss the relevance of toxicokinetics and metabolism in the assessment of hazard from industrial chemicals for man,

- propose a suitable range and appropriate sequence of studies which provide the toxicokinetic and metabolic information required for industrial chemicals.

This report describes the regulatory requirements (Section 2), gives the scientific background pertaining to toxicokinetics and metabolism (Sections 3 - 5) and makes recommendations on a stepwise approach for the evaluation of new chemicals (Section 6).

# 2. REGULATIONS FOR INDUSTRIAL CHEMICALS REGARDING TOXICOKINETICS AND METABOLISM

## 2.1. Legislative Position: Europe (EC)

### 2.1.1. Regulatory Requirements

Legislation is in a transitional phase, currently in annex VII of Council Directive 79/831/EEC relating to the classification, packaging and labelling of dangerous substances (6th amendment) applies (EEC, 1979). At notification Level 2 toxicokinetic studies are required for new substances which are placed on the EC market in quantities reaching 1,000 tonnes/year or exceeding a total of 5,000 tonnes irrespective of time (Level 2). The type of studies to be carried out are selected by the notifier in consultation with the competent authority.

In the proposal for the 7th Amendment (EEC, 1990) "basic toxicokinetic information" is required at Level 1 of notification (100 tonnes/year or when the total market exceeds 500 tonnes irrespective of time). In the legislation "basic toxicokinetic information" is not explained. At Level 2 (5,000 tonnes) additional "toxicokinetic studies which cover biotransformation, pharmacokinetics" are required. The interpretation of these terms is given in Section 3.1. and Section 6.

### 2.1.2. Test Guidelines

Examples of toxicokinetic tests are outlined in Commission Directive 88/302/EEC (EEC, 1988) [included as Appendix 1] which is an addition to the annex V of the 6th amendment and can be summarised as follows (the terms used are those quoted in the legislation):

Absorption studies,

- the determination of test substance and/or its metabolites in excreta, exhaled air and carcass (i.e. radioactivity balance);
- the comparison of biological response between test and reference groups (eg. oral vs iv);

- the determination of plasma levels of test substance and/or its metabolites.

Distribution studies,

- whole body autoradiography;
- the determination of test substance and/or its metabolites in organs/tissues at various times after administration.

Excretion studies,

- the determination of test substance and/or its metabolites in excreta and exhaled air;
- the determination of test substance and/or its metabolites in milk.

Biotransformation studies,

- the determination of metabolites in excreta in vivo;
- the determination of metabolites in vitro;
- biochemical studies.

This guideline is very similar to OECD Guideline No 417 (OECD, 1984).

2.2. Legislative Position: United States (US)

The EPA is open to toxicokinetic and metabolic studies in the testing of industrial chemicals. Normally no such studies are required by the EPA for Pre-Manufacturing Notification (PMN) of new chemicals.

The EPA is currently proposing study guidelines (EPA, 1991) for testing required under TSCA and FIFRA. This proposed rule is a joint guideline to harmonise the "pharmacokinetics" testing guidelines of the Office of Toxic Substances (OTS) and the Office of Pesticide Programmes (OPP). The main features of the US guidelines are presented below:

- absorption by relevant routes of exposure;
- biological half-life of parent compound and its metabolites and of their accumulation;
- tissue distribution of parent compound and its metabolites;

- metabolism pathways;
- routes and rates of elimination of parent compound and its metabolites.

The preferred species is the rat. The number of animals required is 5/sex/dose for a total of at least 10 animals. At least 2 dose levels should be used. The low dose should correspond with a no-effect-level whereas the higher dose should produce toxicity. The routes of administration are the oral, the dermal, the inhalation and the i.v. route.

The following dosing schedules may be envisaged:

- single i.v.-dose at an appropriate dose level;
- single oral dose at a low dose and a high dose level;
- single 6 hour inhalation exposure at a low and at a high concentration level;
- repeated dosing studies by the oral, the dermal and the inhalation route.

In all studies, except the repeated dose studies, the animals should be kept in individual metabolism cages for seven days or until 90% of the administered dose is excreted.

2.3. Comment

Test guidelines for toxicokinetic and metabolic studies exist only in EC and US legislation. The EC-guideline is almost an identical copy of OECD Guideline No 417 (OECD, 1984). The general concept of this legislation is that data should be obtained on absorption, distribution, excretion and metabolism in order to assist in the evaluation of test results from toxicology studies and in the extrapolation of data from animals to man. The requirements of the EC and the US are kept as broad as possible to allow for a compound-tailored study design.

# 3. PRINCIPLES AND METHODS USED IN TOXICOKINETIC AND METABOLIC STUDIES

## 3.1. General

### 3.1.1. Definitions

The terms used to address kinetic studies have been the subject of extensive discussion and considerable confusion over their meaning. Therefore some explanation and definition is essential. Kinetics, in the context of this report, describes the extent and the time course of adsorption, distribution and excretion of a foreign compound in the body following intentional or incidental exposure. Within this context the objective of "pharmacokinetic studies" is the optimisation of the dosage of medicines, whereas the objective of "toxicokinetics" is the assessment of systemic exposure to chemicals. Thus "pharamacokinetic studies" are an integral part of the pre-clinical evaluation of drugs whilst exposure to industrial chemicals is incidental to occupation (ie. multiple low exposures) or accidental (ie. single high exposure).

While kinetics describes the extent and time course of phenomena, the term metabolism or synonymously biotransformation describes the chemical modification of a foreign compound following exposure. The parent compound is usually converted into another compound often with completely different chemical, physico-chemical or biological properties. Although such reactions have kinetic aspects they are not normally included within toxicokinetics.

It should be noted that in the 7th Amendment (EEC, 1990) toxicokinetics is defined as the combination of pharmacokinetics (sic) and metabolism.

### 3.1.2. Prerequisites

Experimental approaches to the study of metabolism are different from those used for kinetic studies and require non-routine research effort in contrast to other studies in experimental toxicology.

The most relevant routes of exposure for industrial chemicals are the dermal and the inhalation routes. This is in contrast to the routine toxicity testing, for which initial information on toxicokinetics and metabolism is obtained generally from oral tests. It is only after having established the toxicokinetic parameters and metabolism of the chemical in oral tests that additional tests can be designed to measure absorption via the dermal and the inhalation route.

### 3.1.3. Methods

Toxicokinetic and metabolism studies can be carried out using non-labelled compounds, stable-isotope-labelled compounds, radioactively labelled compounds or using dual (stable and radio-) labelling. The labels should be placed in metabolically stable positions, the placing labels such as $^{14}C$ in positions from which they can enter the carbon pool of the test animal should be avoided. The radiolabelled compound must be of high radiochemical purity and of adequate specific activity to ensure sufficient sensitivity in radioassay methods. Separation techniques are used in metabolism studies to purify and to separate the several radioactive fractions in biota such as urine, plasma and bile. These techniques range from relatively simple approaches such as liquid-liquid extraction and column chromatography to more sophisticated techniques such as high pressure liquid chromatography. These methods also allow for the establishment of a metabolite profile when radiolabelled material is used.

Quantitative analytical methods are required to follow concentrations of parent compound and metabolites in the body as a function of time. The most common techniques used are gas chromatography coupled to sensitive and specific detectors such as electron capture, Fourier transform infra-red and mass detectors and high performance liquid chromatography with UV detection. The cost of radiosynthesis is often seen as an impediment to the use of radiochemicals. Often it will be cheaper and faster than approaches which require the development of other analytical methods for parent compound and major metabolite(s). It is important to note that kinetic parameters cannot be calculated from measurement of

total radioactivity. An analytical step is required to define the radioactivity as chemical species. This is also usually faster than cold analytical techniques. "Dual" labelling (eg. $^{13}C$ and $^{14}C/^{12}C$) is the method of choice for structural elucidation of metabolites (by MS and NMR spectroscopy). A cold analytical techniques which incorporates stable isotope labelling (for GC/MS or LC/MS analysis) is a useful combination. Unless this latter method has already been developed for the test compound in various matrices (urine, faeces, blood, fat, liver, kidney, etc), the use of radiolabelled compound will be less costly than other methods. The decision must be made on a case-by-case basis but it will usually be the case that where minimal toxicokinetic information is sought, the method development costs (radiosynthesis or cold GC/LC methods) will outweigh the cost of the dosing/analysis phase of the study.

It is important therefore, to attempt a preliminary assessment of absorption, elimination and metabolism without specific toxicokinetic and metabolic testing. This can be done by using information which is already available:

**Molecular weight:** small molecules are more likely to be absorbed than large molecules (e.g. >1000 daltons).

**Structure:** a consideration of the fate of structural analogues is often useful. Possible routes of metabolism can be readily predicted from structure but it is not possible to predict which of these many options will occur for a specific compound.

**Stability:** if low, then exposure to decomposition products may be important and absorption of the given compound may be limited.

**Ionisation:** passive diffusion across membranes occurs only for unionised forms, therefore pH may well affect absorption (and elimination).

**Water solubility:** water soluble compounds are often rapidly

absorbed and eliminated (via the kidneys in urine). Compounds of low solubility may be deposited at the dosage site and be poorly absorbed.

**Fat solubility** (or octanol/water partition coefficient): if high, ready passage across membranes will occur; accumulation in fat may occur (if biotransformation is slow); binding to plasma proteins and lipoproteins is likely.

**Vapour pressure**; if high, inhalation exposure is likely, as is elimination via the lungs in expired air.

**Toxicity:** When a clear dose-effect relationship can be established in toxicity studies this presents a clear indication that the substance is absorbed. Difference in response in a mutagenicity test with and without S9 indicates that the substance is metabolised and that the metabolite(s) formed are biologically active.

## 3.2. Absorption

### 3.2.1. Principles

The process by which foreign compounds cross body membranes and enter the bloodstream is referred to as absorption. In most cases, this consists of passive transport down a concentration gradient. Small hydrophilic molecules may pass through aqueous channels or pores in the membranes. For some chemicals, absorption via facilitated diffusion, active transport or pinocytosis has been reported.

The most important sites of absorption are gastrointestinal tract, skin and lungs [fig.1]. In the case of absorption from the gastrointestinal tract, the compound reaches the systemic circulation usually after passage through the liver via the hepatic portal vascular system. As the liver possesses considerable metabolic capacity, the chemical may not reach the circulation intact. Some metabolic activity also exists in the

lung and skin, but in most cases compounds reach the bloodstream unchanged after passage of the skin or lung membranes [fig.1].

Absorption of chemicals via the skin is hindered by its low permeability. Specifically the non-vascularised outer layer of the skin functions as a barrier for passive transport, so that entry is only possible by preferential absorption of lipid soluble compounds via the more permeable sweat and sebaceous glands, or by the hair follicles. Damage to the skin and irritant effects facilitate diffusion of foreign compounds. Factors effecting percutaneous absorption have been reviewed (ECETOC, 1992).

Absorption via the lungs is an important route for toxic gases, vapours of volatile liquids, aerosols and in some cases airborne particles. The lung, and specifically the alveoli, has a large surface area with highly permeable membranes. Therefore, rapid absorption of foreign compounds can occur. The deposition of aerosols in the lung is very dependent on the particles sizes, as only those with small diameter (<5 μm) reach the alveoli when inhaled. The absorption of gases is affected by their solubility and their partial pressure in the inspired air. Particulate matter can be absorbed in the lungs by macrophages.

Absorption from the gastrointestinal tract may occur along the whole length of the tract. The site of gastrointestinal absorption also depends on ionisation of the chemical at given pH. The pH can vary from acidic (pH 2) in the stomach to pH 6 in the intestine. Most compounds are absorbed from the gastrointestinal tract by passive diffusion of their non-ionised forms. Nevertheless as the tract has specialised transport systems for the absorption of nutrients and electrolytes some foreign compounds may compete with these substrates and undergo active transport. The absorption of particulate matter has also been described.

Absorption is dose dependent. Since most absorption occurs by passive diffusion, in principle, it should be non-saturable. There is nevertheless clearly a limit to the rate of such a process and in addition large doses may be ineffectively distributed and remain at the site of administration as a depot, with consequences for the rate of

absorption. For those cases where active transport is involved, saturation at higher doses will occur, giving rise to altered kinetic behaviour.

3.2.2. Methods

**In vitro methods**

Generally, only specific sub-processes in isolation can be studied using in vitro techniques. Methods exist for assessing the dermal absorption of chemicals using samples of animal or human skin (Bronaugh et al, 1982; Cooper and Berner, 1985). When used with adequate controls, this methodology can give information on the flux of chemicals through the skin. Correlations between in vitro an in vivo dermal absorption has been described. In vitro methods for percutaneous absorption have been reviewed (ECETOC, 1992).

A technique involving inverted intestinal loops may be used as model for studying the potential for absorption from the gut (Acra and Ghishan, 1991).

**In vivo methods**

Several methods can be used, either as an extension of toxicological studies, or as tailored kinetic studies, providing information on absorption.

Where acute toxicity data on a compound are available, a comparison of its toxicity after different dose routes in the same test species can provide information on absorption. Additional experiments using intravenous dosing may provide further insight into the likelihood of absorption.

Information on absorption can be obtained by analysis of body fluids or excreta samples collected, for example in a toxicity study, for the parent compound or its metabolites.

Quantitative information on the overall kinetic behaviour of chemicals, including absorption, can be obtained in specifically designed studies such as a mass balance study. Such studies may include oral, dermal or pulmonary exposure, and can be performed in various species. The chemical used may be radiolabelled (allowing simple quantification), stable isotope labelled or non-labelled. Test guidelines exist for such tests (Section 2).

In mass balance studies, the extent of absorption can be determined by determination of compounds or metabolites in excreta, exhaled air and carcass. In order to obtain information on kinetics of absorption and elimination, plasma samples can be taken at different time points after dosing, to construct a plasma-concentration time curve allowing calculation of kinetic parameters such as the absorption and elimination rate, area under the curve (AUC). The kinetic terms used above, together with others, are defined in the glossary.

As far as practical execution of studies is concerned, care should be taken that in percutaneous absorption studies minimal occlusion occurs. Occlusion has been shown to significantly enhance dermal absorption of chemicals. When conducting inhalation experiments other exposure routes (eg dermal, oral) should be minimised; ideally nose-only exposure should be arranged.

## 3.3. Distribution

### 3.3.1. Principles

Following absorption, compounds are available for distribution throughout the body. The rate of distribution to the tissues of each organ is generally determined by the blood flow through the organ, the ability of the chemical to cross membranes and capillaries, and its affinity for various tissues. Most xenobiotic chemicals cross membranes by passive diffusion, although for some, active transport is involved. Some tissues, such as the central nervous system, are protected from many circulating foreign compounds by a selective membrane barrier.

Special cases of distribution occur with chemicals which bind extensively to plasma protein, thereby reducing the amount of "free" compound available in the plasma for diffusion or filtration. Plasma binding, for instance, affects glomerular filtration of chemicals as only "free" compounds can be filtered into the primary urine. Some chemicals accumulate in organs such as kidney and liver, presumably due to specific intercellular protein binding. Fat can be a storage depot for highly lipophilic compounds. Bone has been shown to be the site of accumulation for some metal ions and chelators.

It should be noted that distribution is a dynamic process involving multiple equilibria between compounds present in organs or tissues and present in plasma. Therefore, a reduction in the plasma level of "free" compound due to excretion may cause redistribution of accumulated compounds in other compartments.

As far as dose dependence of distribution is concerned linear relationships exist in the main for passive diffusion processes. Saturation, giving rise to deviations from linearity, can occur not only for those cases where active transport is involved where saturation limits the extent of transport, but also with compounds which undergo significant plasma protein binding where saturations make more compound "freely" available in the plasma.

### 3.3.2. Methods

**In vitro methods**

In vitro methods are useful in the study of specific elements in the distribution process, for example the determination of plasma protein binding. Studies using plasma, adipose tissue, muscle, liver and lung tissues have been published (Kurz and Fichth, 1983). Experiments using appropriate standards provide useful information on the extent of protein binding of chemicals, eg. equilibrium dialysis, ultrafiltration. Similar methods may be used to determine binding to other tissue proteins.

**In vivo methods**

As a first indication of distribution, where accumulation is expected, organs or tissues collected during toxicity studies or from mass balance studies can be analysed for the presence of parent compound and/or metabolites.

Specially tailored studies can be conducted using radiolabelled compounds to assess the distribution of chemicals. Distribution can be assessed at different time points after dosing either by determination of radioactivity in organs, tissues and carcass, or by whole body autoradiography.

Blood kinetics data can be used to calculate a volume of distribution which can be indicative of unusual distribution behaviour.

## 3.4. Excretion

### 3.4.1. Principles

There are several routes of excretion for most chemicals (especially polar compounds) the major being via the kidneys into urine. Other routes are via the lungs in expired air for volatile materials and for polar metabolites, via the bile into the gastrointestinal tract. Body fluids such as sweat saliva and milk also offer routes for elimination. Material which is not absorbed after oral dosage and compounds secreted in the bile, are eliminated in the faeces [fig.1].

Excretion via the kidneys comprises for the major part passive glomerular filtration, but also active and passive tubular secretion may occur. The kidney is very effective in removing chemicals; 20% of the cardiac output is filtered at the glomeruli, the latter acts as a large pore filter (cut off 60,000 daltons). Once filtered, a chemical may remain in the filtrate and be excreted in urine, or be reabsorbed into the bloodstream via passive diffusion or in some cases by active transport. Foreign compounds can also be excreted from the plasma into the primary urine by

mainly active secretion through the tubules. Proximal tubular cells contain active transport systems for organic anions and cations. Foreign compounds can also act as substrates for this transport system.

Substances that exist predominantly in the gas phase are excreted principally by the lungs. Depending on their vapour pressure, volatile liquids may also be excreted in part via this route.

Excretion of foreign compounds via the bile into the gastrointestinal tract is a route of elimination particularly relevant for large ionised molecules (parent compounds or metabolites) or conjugated compounds. This pathway involves active secretion rather than passive diffusion. Biliary excretion of heavy metal ions has also been reported. Once a compound is excreted in the bile and enters the intestine, it can either be excreted in the faeces, or be absorbed into the bloodstream before or after further metabolism by intestinal enzymes or microorganisms. Such resorption leads to enterohepatic recirculation thereby causing delayed excretion.

Linear dose relationships exist in the main due to the passive diffusion processes. Saturation, giving rise to deviation from linearity, can occur not only in those cases where active transport is involved such as tubular and biliary excretion, but also with glomerular filtration of compounds which undergo significant plasma protein binding.

### 3.4.2. Methods

**In vitro methods**

No methods exist that are specifically designed to measure excretion.

**In vivo methods**

Indications of excretion of compounds may be obtained from toxicity tests. For example recovery from toxic signs may indicate the removal of toxic agent. Qualitative information on the occurrence of urinary

excretion, can be obtained from the analysis of urine samples from toxicity studies for parent compound or metabolites. Faeces samples may also be analysed.

Quantitative information on excretion of compounds comes from mass balance studies; routes and extents of excretion can be obtained by means of measuring amount of compound or metabolites in faeces, urine and exhaled air.

Biliary excretion can be assessed in animals in which the bile duct has been cannulated.

The choice of time intervals in collection of excreta is essential for optimal estimation of excretion rates in mass balance studies.

Blood kinetics studies will provide information on kinetics of excretion.

## 3.5. Metabolism.

### 3.5.1. Principles

Almost every exogenous compound of synthetic or biological origin that is absorbed and distributed within a mammalian system is modified non-enzymatically or by enzyme-catalysed reactions. The sum of these modifications is defined as metabolism. The pathways and the rates of the biotransformation are influenced by a wide variety of factors related to the structure of the compound, the exposure situation and the animal (see section 3.6.). The metabolism of a compound may result in detoxification (the metabolites are less toxic than the parent compound) or in bioactivation (the metabolites are more toxic than the parent compound).

Most exogenous compounds, (eg. drugs, plant xenobiotics, environmental pollutants, industrial chemicals) to which mammals are exposed are lipophilic. The evolutionary development of mammalian metabolic systems has provided very efficient mechanisms to remove these compounds from the

body. The mechanisms consist of the introduction or unmasking of polar functions (Phase I reactions) and the subsequent conjugation of these functions with hydrophilic endogenous substrates (Phase II reactions). The resulting polar metabolites are generally eliminated via the kidney or the bile. Whilst usually eliminated, conjugates may be further metabolised leading to re-absorption and enterohepatic circulation.

The organ most important for the biotransformation of xenobiotics is the liver. This organ contains almost all the membrane-bound and cytosolic types of enzymes involved in metabolic processes. In addition to the liver, all other organs especially kidney, lung, the intestine and its microflora show some degree of metabolic activity. The most common reaction types and the enzymes catalysing the reactions are listed in table 1.

These enzymes often exist as a complex pattern of isoenzymes with overlapping substrate specificity and differentially regulated activities thus providing the required broad spectrum for the wide range of compounds metabolised. The metabolism of chemically simple compounds can lead to complicated metabolic profiles [fig.2].

3.5.2. Methods

**In Vitro Methods**

Assays with purified enzymes, organelle preparations, cell systems or intact tissues are used to investigate aspects of metabolism. Detailed information on these topics are available in reviews written by Jakoby (1980), Jakoby et al (1982), Fry (1982), Caldwell and Jakoby (1983), Guengerich (1987), Rauckmann and Padilla (1987), Dolphin et al (1988), Powell et al (1989) and Illing (1989).

Although in vitro methods can not provide exact quantitative data with regard to the in vivo situation, they are very valuable in qualitatively answering questions on the potential metabolic conversion of xenobiotics. Indications of possible detoxification or bioactivation pathways may be

obtained. Selection of one of the experimental systems discussed below is dependent upon the structure of the compound and the availability of appropriate analytical methods.

**Organelle preparations from homogenates:** The biological in vitro systems most commonly used in metabolic studies are fractionated organelle preparations obtained from liver homogenates, eg. the microsomal fraction. The microsomes are isolated by differential centrifugation and consist of fragments of the endoplasmic reticulum. In terms of bioactivation, they contain the most important enzymes metabolising xenobiotics, the membrane-bound cytochrome P-450 dependent monooxygenases. The possible formation of a variety of toxic metabolites can be investigated using this system. In addition, by analysing standard enzyme activities of microsomes isolated from animals from subacute or subchronic studies, enzyme induction phenomena can be elucidated. The soluble fraction of liver homogenates, ie. the cytosol, contains some of the enzymes catalysing the conjugation of xenobiotics to endogenous substrates and is used to evaluate the role of these mostly detoxifying enzymes.

**Cell systems:** Hepatocytes freshly isolated from the mammalian liver are the most commonly employed primary cells mimicking hepatic metabolism. The enzyme activities in these cells are comparable to the activities found in vivo. After isolation of the cells they start to lose these enzyme activities. Co-culture with other rat liver cells may enhance the life time of hepatocytes. Recent results with cryopreserved hepatocytes indicate that it might be possible in the future to use stored cells for in vitro studies. Epithelial kidney cells or Clara cells from the lung are other examples of primary cell cultures with an appreciable level of enzymes metabolising xenobiotics. The constants obtained can serve as first estimate of in vivo constants in pharmacologically based pharmacokinetic modelling (PB-PK) approaches (Section 5.3). The major advantage of primary cell cultures is that the relationships between various types of biotransformation can be analysed under defined conditions. On the

other hand, the disadvantages associated with the loss of tissue organisation may render this method inappropriate for some compounds.

**Other Methods:** Other specialised methods are sometimes used. Thus use of perfused organs is an alternative in vitro method which preserves tissue integrity. Special problems may be solved by these techniques employed for liver, lung, and kidney. Purified enzymes such as cytochrome P-450 dependent monooxygenases or GSH-S-transferases are occasionally used to answer special questions regarding the mechanism of a toxic action.

**In Vivo Methods**

In vivo studies can be performed at very different levels of complexity. The most frequent approach used currently for the identification of metabolites is the sampling and analysis of urine at several time points after a single oral administration. More complex approaches are needed to define the site(s) of each metabolic step and the relative importance of the various organs in the overall process. Analysis of blood, bile, faeces and specific tissues may be required. The role of enterohepatic circulation and of microbial metabolism in various parts of the gastrointestinal tract may have to be specifically studied. Although these latter procedures are commonly used for drugs, they would only be envisaged for industrial chemicals if specific questions have to be answered. The impact of liver enzyme induction on the metabolic pattern can be checked by multiple dosing with cold material followed by one single administration of a radioactive labelled material. The isolation and identification of parent compound and metabolites in biota is greatly facilitated by the use of material labelled with radioactive or stable isotopes. Metabolites may also be traced if the test compound contains specific marker atoms such as Br, F or heavy metals. An overview of the analytical methods used is given in Section 3.1.3.

## 3.6. Factors Influencing Toxicokinetics and Metabolism

### 3.6.1. Introduction

Many factors affect the absorption, distribution, metabolism and elimination of xenobiotics. These have been classified in this report into three groups relating to (i) the exposure (ii) the test animal and (iii) the response of the animal to the test compound. The study of factors affecting metabolism is well-established (Vesell, 1982; Vessel and Penno, 1983; Mucklow, 1988).

### 3.6.2. Factors Deriving from the Nature of the Exposure

**Route of exposure:** The three major routes of exposure to chemicals, oral, dermal and inhalation, may have very different consequences.

When a compound is ingested, several barriers protect the systemic circulation; for example the environment of the stomach (acidity varies with species), the intestinal microflora, the intestinal absorption process, the metabolic activity of the intestinal mucosa and the liver. A chemical may be extensively modified at each of these stages and it may appear in the systemic circulation as a mixture of parent and metabolites. This presystemic removal of a compound which often occurs is known as the first-pass effect (Gibaldi et al, 1970).

Dermal exposure is usually characterised by low and slowly achieved maximum blood concentrations. The stratum corneum and other layers of the skin offer a reasonable barrier to absorption although there are some important exceptions.

The lung is unique in that it receives the entire cardiac output and the blood flow to this organ is about four times that to the liver. Thus a compound which is well absorbed from the lung when inhaled enters the systemic circulation very rapidly and the exposure is akin to an intravenous administration. Biotransformation can occur in the lung and

other parts of the respiratory system (Bond and Dahl, 1989); when this happens a first-pass effect can occur.

**Exposure vehicle:** Vehicles for oral studies (solvents and dispersants for the test substance) must not be chosen purely on the basis of their good solvent properties. Excellent solvents (eg. dimethyl sulphoxide) sometimes exert effects on membranes during absorption, and within cells, thereby distorting one or more of the ADME processes. Dermal and inhalation exposures can be made to mimic real situations; for the former, the substance should be presented as it would be in the workplace and for the latter, the vapour or areosol can be generated with minimal or no carrier.

**Exposure dose:** As the dose of a compound increases, one or more of the processes in an adsorption, distribution, metabolism and excretion study can become saturated. Absorption is likely to be limiting in most cases. Distribution, however, can also be dose-dependent particularly if a compound is transported on a variety of binding sites on lipoproteins or proteins. Saturation of metabolism may occur and often a compound can be metabolised mainly by one route up to saturation, when a second route may come into operation. Toxicokinetics and metabolism will then be completely different at high and low doses.

**Structure of the test chemical:** Of all the variables, the structure of the test compound has the greatest impact in toxicokinetic and metabolic studies. Three general examples will serve to illustrate the dominant effect of structure on metabolism:

- a non-volatile lipophilic hydrocarbon must be metabolised via an oxidative route before clearance;

- a lipophilic carboxylic ester of a primary alcohol will tend to be hydrolysed by esterase action;

- a chemical containing an electrophilic carbon atom, will react with glutathione to form a conjugate; in the absence of glutathione, toxicity may result from reaction with proteins, RNA and DNA.

Rarely are chemical structures so simple that metabolic routes can be predicted. We can predict the options possible but we cannot predict which of these will operate in a particular species.

3.6.3. Factors Deriving from the Test Animal

**Species Differences:** Species differences in toxicokinetics and metabolism abound (Caldwell, 1980). Differences in rates and in proportions of metabolites formed should be regarded as normal. Absolute differences in metabolic pathways are also encountered quite frequently. Species differences may be due to differences in absorption, distribution, metabolism or elimination (renal or biliary) or to combinations of these. There are various examples of compounds for which species differences in metabolism relate directly with differences in toxicity (eg. methylene chloride, trichloroethylene, formaldehyde and methanol).

**Strain Differences:** Differences between strains of the same species in the disposition of chemicals have been documented. Such differences are largely genetic in origin. They are caused by the genetically controlled expression (or non-expression) of enzymes and can appear as alterations in the composition of an isozyme mixture. They are usually compound-specific and thus prediction based on chemical structure can be made only within narrow limits. Strain differences are effectively avoided in the laboratory by using the same strain in metabolism and toxicity studies.

**Sex Differences:** These have been frequently reported in toxicokinetic and metabolic studies. The most important of these in the current context is the approximately 3-fold difference in the activity of hepatic microsomal mono-oxygenase (cytochrome P-450) in male and female rat (male>female). This is under hormonal control and has been known for a long time (Kamataki et al, 1983). Its significance lies in the dominant

position of the rat in toxicity testing. When a compound is known to be metabolised via mono-oxygenation, sex differences in the rat can be expected in kinetics and are often found also in terminal metabolite ratios.

**Age:** Toxicokinetics and metabolism are a function of age. As neonates, and before sexual maturity, mammals are deficient in several aspects of xenobiotic metabolism. Sexually mature animals (e.g. rat, 8-12 weeks) are always used in routine studies. After maturity, animals develop lowered metabolic competence with age. The effects of age on metabolic competence of experimental species has been well-studied (Rikans, 1989).

**Microflora:** The intestinal microflora are affected by several factors including species, age, disease, drugs, diet and pH (Rowland, 1988). Clearly the toxicological properties of a test compound can be modulated by the action of gut microflora and both detoxication and bioactivation have been observed. Changes can be important because the range of metabolic activities of these microflora rivals that of their hosts (Renwick and George, 1989).

**Other Factors:** Many other host-dependent factors affect the fate of xenobiotic chemicals. Circadian rhythm, exercise, disease and infection have each been shown to affect metabolism and nutritional status is also important. These factors are precisely those which we attempt to eliminate or hold constant in experimental studies.

3.6.4. The Response of the Animal to the Test Compound

**Enzyme Induction:** The induction of the hepatic cytochrome P-450-based mono-oxygenases is the most widely studied phenomenon of this type. One or more of the isozymes may be induced 2-20 fold (or considerably greater) with the consequence that oxidative metabolism is increased in capacity. This may lead to altered toxicity of another xenobiotic depending on whether metabolism effects an increase or a decrease in toxicity of the second compound. Other enzymes (eg. hydrolases and

glutathione transferases) are also induced. Induction has been concisely reviewed recently (Bock et al, 1990).

**Enzyme Inhibition:** Enzyme inhibition (Testa, 1990) can delay metabolically mediated elimination, increasing toxicity (or reducing toxicity of metabolically-activated molecules).

# 4. OVERVIEW OF TOXICOKINETICS AND METABOLISM IN MAN

## 4.1. General

The basis of risk evaluation is that animals serve as models for man and that experimental results can be extrapolated to man. We have seen that toxicokinetics and metabolism can be important determinants of toxicity and that various factors, including species, modulate these phenomena and, therefore, complicate the extrapolation. Confidence in the value of experimental results is enhanced if we can show that the fate of foreign compounds in man is generally similar to that in experimental animals.

Consequently, it is of value to review the existing knowledge available for man. Only in very rare cases would recourse to experimentation in man (ie. in vivo) be sanctioned and, even then, non-invasive procedures and 'normal' (eg. occupational) exposures would be used. Some of these procedures (eg. the measurement of xenobiotic-protein and xenobiotic-DNA adducts) are highly specialised techniques.

Most published information on the fate of xenobiotics in man relates to therapeutic agents. The concentration of free and bound drug in plasma is important information for relating effective dose, toxic dose, bioavailability of various formulations and effects of host-dependent and external factors on therapeutic action. Pharmacokinetic studies of drugs are carried out in man as soon as possible in the development of a new drug. Conversely, for industrial chemicals and for agrochemicals, such studies are rarely carried out. Nevertheless, there are in the literature a number of reports on the fate of industrial chemicals, agrochemicals and food additives in man (for a review based on 795 references, see Miyamoto et al, 1988). The objectives of these studies are usually either to compare the metabolism in man with that in experimental species (as a validation of toxicity studies) or to identify excreted metabolites which can be used as quantitative indicators of occupational exposure (biological monitoring).

The conclusions of Miyamoto et al (1988) are that

- man possesses all of the capabilities for metabolism of xenobiotics found in experimental animals, but
- in contrast to animals, inter-individual differences and a variety of dietary and environmental external factors must be taken into account.

## 4.2. Factors Affecting the Fate of Xenobiotics in Man

### 4.2.1. Life Style

Man varies his diet, consumes drugs and alcohol, takes medicines, interbreeds and undertakes a variety of other activities. The process of maturation (especially during years 0-2) influences the ADME process. These factors can result in a very varied response of man to xenobiotics. Thus interindividual differences in metabolism and response to chemicals are frequently encountered (Vesell and Penno, 1983). The factors leading to such differences are essentially those described in Section 3.6 which can translate into the context of 'life style'.

### 4.2.2. Genetic and Ethnic Differences in Metabolism

These have been reviewed in some detail by Miyamoto et al (1988). The bimodal distribution of N-acetylation between rapid and slow acetylators was the first example of this phenomenon to be described. The discipline of pharmacogenetics derives from this and similar examples (Nebert, 1980). The deficiency in slow acetylators is due to a low level of hepatic N-acetyl transferase. Slow acetylators are particularly sensitive to the effects chemicals which are detoxified via acetylation. The acetylator phenotype is inherited in a simple Mendelian fashion, being a single gene trait, one for rapid acetylators and one for slow acetylators. The defect in the metabolism of isoniazid occurs in approximately half of the world's Caucasian and Negro populations but is rare amongst most Mongoloid races (eg. Eskimos and Japanese) (Kalow, 1982). Genetic influences are also apparent in oxidative pathways.

Differences in oxidation are usually due to the relative amounts of the cytochromes P-450 in the liver; debrisoquine 4-hydroxylation, for example, is catalysed by cytochrome P-450 II D1 which is polymorphic in rat and man (Gonzalez et al, 1989). The evolution of the cytochrome P-450 superfamily is currently under intensive study (Nebert and Gonzalez, 1987). The multiplicity of cytochrome(s) P-450 and their genetic control will afford many more examples of interindividual and ethnic differences in oxidative metabolism in the human population.

## 4.3. Information on Toxicokinetics and Metabolism in Man

### 4.3.1. Available information

Much has been written about species differences in foreign compound metabolism and the selection of species as surrogates for man but the search for the perfect substitute has been abandoned. The fate of xenobiotics is often species-specific but it is also highly compound-specific. There is no single species which, in all cases, behaves like man. Thus available information has been gained from a variety of species and from the fate of analogous compounds which have been studied (usually drugs) in man. A useful generalisation that can be made involves the species difference in the minimum molecular weight requirement for biliary elimination. For organic anions these values are: rat, 325 ± 50; guinea pig, 400 ± 50; rabbit, 475 ± 50; man, ca. 500 (Hirom et al, 1972), monkey, ca. 500 (Calabrese, 1983). Thus, for lipophilic compounds of molecular weight >ca 350 which are eliminated via the bile as metabolite conjugates, urinary metabolite yields in man and rabbit are usual much higher than those in the rat. Another useful generalisation is that chemicals shown to be persistent in 2 or 3 experimental species are likely to be as persistent in man.

### 4.3.2. Experimental approaches

The study of non-therapeutic chemicals in human volunteers is severely limited by ethical considerations. The value of kinetic and metabolic studies in man is that part of the interaction between chemical and

subject can be defined in detail at non-toxic doses. This will have already been defined in animals thus affording a soundly-based comparison. A simple dose-excretion study in which a major urinary metabolite is identified and its yield related to the given exposure has proved valuable. The major metabolic route is thus identified and a comparison with animals is enabled. The result also lays the foundation for a method of monitoring exposure.

A way of circumventing in vivo studies has been via the use of isolated tissue preparations (Powis, 1989). This is not an easy alternative. The extrapolation of in vitro results to the in vivo situation has its problems. In addition, the acquisition of suitable human tissue requires the co-operation of the medical profession which may be difficult to obtain for ethical reasons. Liver is the most useful tissue because (i) it is the major organ of biotransformation and (ii) it is the most widely studied animal tissue. Clearly other tissues may be required for specific studies.

In vitro metabolism is studied by incubating test compound with a tissue preparation under appropriate conditions and examining the production of metabolites (including those covalently bound) with time. Various preparations have been used in these studies, eg. isolated perfused organs, tissue explants, slices, isolated cells, homogenates, subcellular fractions and individual enzymes. A recent innovation is the commercial availability of cells into which human cytochrome P-450 has been engineered. Several isozyme types are available. Generally speaking, the more disturbed or fragmented the tissue, the less realistic are the results. The higher the level of cellular organisation used the more difficult it is to obtain the human tissue preparations and this limits the applicability of the approach. However, even given ready access to tissue, the in vivo/in vitro correlation is difficult to maintain in practice for a generalised picture of metabolism. It is very important, therefore, that if they are to be employed, in vitro preparations be validated in laboratory animals with respect to metabolic capacity. This is particularly important when subcellular fractions, which may be lacking in essential cofactors, are used.

# 5. USE OF TOXICOKINETIC AND METABOLIC INFORMATION IN TOXICOLOGY

## 5.1. General

Animal toxicity studies are used to establish a relationship between the dose of a chemical and its toxicological effect(s). In most toxicity studies a range of doses is used to determine a no-effect level and define a dose relationship. The assessment of the dose is based solely on the amount given or accepted for example where administration is by gavage or via diet. The expression of toxicity in any animal study may be influenced by the magnitude of the dose and/or the route of administration as well as the method and frequency of administration. Information about toxicokinetics can be used to evaluate the impact of the above factors and can therefore help to provide a more detailed picture of the hazard of a chemical to experimental animals.

The ultimate goal is to assess the likely hazard of a chemical to man. A safe dose to man is usually assessed from the animal toxicity data by applying safety factors to the no-effect level(s). If data about toxicokinetics can be collected for animals and man these can provide the basis for an improved interspecies extrapolation.

## 5.2. Impact on the Interpretation of Toxicity Studies

### 5.2.1. Dose

The dose to which a target organ is exposed is usually assumed to be linearly related to that administered. This is only reasonable if the metabolism and kinetics of the test chemical are linear over the dose range used in the toxicity study. The processes by which the test chemical is absorbed, distributed, metabolised and excreted may become saturated above a certain dose and the assumption of linearity will be incorrect. Under such circumstances the dose-response data obtained in the toxicity study cannot be simply extrapolated. If absorption is saturated the blood concentration will be constant regardless of the amount of chemical administered above the saturating dose. The amount of

chemical delivered to the target organ(s) will therefore not change and toxicity will either not be apparent or will be the same, irrespective of the dose.

The effect of saturation of metabolism on toxicity will depend on whether toxicity is mediated by the parent chemical or by a metabolite. In the former case toxicity will increase disproportionately with dose whereas in the latter the degree of toxicity may not significantly change with an increase in dose. Studies of dichloromethane in rats provide an example (Green, 1989). In mice this chemical is metabolised by either oxidation or glutathione conjugation. Metabolism by the latter pathway results in the production of reactive intermediates which are believed to be the cause of the formation of liver and lung tumours in this species. In the rat, however, glutathione dependant metabolism is saturated at relatively low substrate concentrations and tumours are not found.

5.2.2. Influence of mode and frequency of administration

The shape of the blood concentration versus time curve may be different following dietary administration than after gavage of an equivalent dose. Rodents consume diet throughout the night and as a result plasma concentrations of the test chemical remain uniform for a long period of time.

Gavage usually produces higher plasma concentrations over a much shorter period. The difference tends to be greatest in chemicals with a short half life of elimination because there is little accumulation following repeated administration. If high plasma concentrations are important for toxicity then dosing of the test chemical by gavage will give lower effect levels compared with dietary administration. Teratology studies with caffeine illustrate this point. Teratogenic effects are observed following administration of a single oral dose of 75mg/kg but not following doses of 200mg/kg in diet or drinking water. Peak blood levels following a single dose are approximately six times those after dietary or drinking water administration and the expression of teratology appears

to be related to the peak blood concentration (Elmazar et al, 1982; Ikeda et al, 1982).

The rate of metabolism or elimination of a test chemical and the frequency of dosing affect the degree of exposure of the test animal. Chemicals which are rapidly eliminated are present in the body for only short periods, if these are administered only once daily the chemical will not accumulate and the animal may not be exposed for a significant proportion of the day. This reduces the likelihood that toxicity will be expressed. Further experimental data for caffeine illustrate this point. Abnormal foetuses were observed following single daily oral doses of 100mg/kg/day to pregnant rats, but no malformations were apparent when the same daily amount was divided into four doses given at equal intervals throughout the day (Smith et al, 1987).

Lipophilic vehicles such as corn oil tend to delay absorption and subsequently can reduce systemic exposure to the test chemical to levels below those at which metabolism is saturated. The no-effect level in studies of carbon tetrachloride in mice was an order of magnitude lower when corn oil was used as vehicle compared with an aqueous emulsion. The corn oil delayed absorption allowing a greater proportion of the dose to be metabolised during the first pass through the liver thus reducing toxicity (Condie et al, 1986).

Chemicals which are slowly eliminated accumulate after repeated daily oral or dietary administration. The time taken to achieve steady state blood and tissue concentrations will depend on the half life of elimination or metabolism of the test chemical. This may be longer than the duration of a short term toxicity test and as a result steady state concentrations may not be attained before the end of the study.

## 5.3. Impact on Hazard Assessment

A relationship between the toxicokinetics and metabolism of a chemical and its toxicity may be established in an experimental animal but in order to use this to predict the hazard to man it can be helpful to have some

toxicokinetic and metabolic information for man. In addition to the possible species differences in distribution, metabolism and excretion which can occur after absorption there are usually significant differences in the exposure of test animals and man. Toxicity studies are often conducted using the oral route of administration whereas human exposure to industrial chemicals is usually by inhalation or by dermal contact. The dose levels used for toxicity studies are also normally significantly greater than the likely human exposure. The frequency of exposure will also often be different. Human exposure may be sporadic whereas animal studies usually involve controlled and more frequent administration of the chemical. As a result steady state concentrations may be achieved in animal studies but not following human exposure. All these factors may influence toxicokinetics and metabolism and therefore need to be taken into consideration when extrapolating to man.

There are limited opportunities to obtain experimental data for man (Section 4.3). Models have therefore been developed to overcome this difficulty, the two principle ones being allometric scaling and PB-PK modelling. These have been reviewed recently by Ings (1990).

The allometric scaling approach uses methods derived from comparative physiology to predict toxicokinetics in man. Small animals have proportionally larger organs and shorter blood circulation times than larger ones. As a result small animals such as those used for toxicity testing will metabolise and/or eliminate a substance more rapidly. Allometry is an empirical technique which examines relationships between size and time and its consequences without necessarily understanding the relationship. Allometry suggests, for example, that in the majority of cases, the extrapolation of a safe dose from animals to man is better done using body surface area than using body weight (the mg/kg values).

PB-PK models divide the body into physiologically realistic compartments and incorporate the major tissues and organs relevant to the absorption, distribution, metabolism, elimination and toxicity of a chemical. The basic unit of construction for a PB-PK model is a series of compartments of uniform concentration connected by the blood circulation. Compartments are arranged

according to the correct anatomical scheme in order to simulate an entire body system. The mathematical formulation of the model consists of a series of mass balance differential equations that account for the handling of the chemical by each compartment. The types of data required for the construction of PB-PK models include tissue and organ blood flow rates and volumes, partition coefficient data describing the distribution of chemical between tissue and blood, and finally metabolic and binding data. Depending on the chemical, other types of data such as transport (eg. diffusion coefficients), permeability and absorption rates, might be required for model construction. This can require the provision of a considerable amount of data. A growing body of information is available for many of the necessary parameters, whilst others will need to be generated by in vivo or in vitro systems. PB-PK models can be constructed with varying degrees of sophistication, the complexity being determined by the type of data simulations required from the model. The models have also to be validated and this may require a large amount of experimental data.

Neither type of model is routinely used to predict metabolic and toxicokinetic data for man, but PB-PK models have found use in recent years in both hazard and risk assessment particularly for volatile chemicals.

## 5.4. Comment

Metabolic and toxicokinetic data can help in hazard evaluation and extrapolation to man. There are experimental, technical and economic reasons why only limited information is generated for most chemicals and in practice acceptable risk assessments are made in the absence of extensive metabolic and toxicokinetic data. Where data are generated for this purpose it is usually after toxicity studies are completed and often when it is not easy to extrapolate from the results of animal toxicity tests to human hazard. For example where a chemical is a carcinogen in one species but not in another and there is doubt about which species is relevant to man. Again the example of methylene chloride serves to illustrate this point (Green, 1989).

# 6. RECOMMENDED APPROACH TO ASSESS THE TOXICOKINETIC AND METABOLIC BEHAVIOUR OF INDUSTRIAL CHEMICALS.

## 6.1. General Considerations

It will be apparent from the previous sections that there are numerous aspects of metabolism and toxicokinetics of a chemical which could be considered in order to satisfy the 7th amendment requirements for notification at Levels 1 and 2. Toxicokinetics and metabolic data for industrial chemicals can be helpful for interpretation of toxicity studies and for the extrapolation of the data to man. An in depth investigation of every substance is clearly impracticable and would be of limited value. It therefore follows that the generation of such data, when necessary, both with regard to the range of factors to be investigated and the depth to which they are studied, should be linked in some way to the development of the toxicity profile of the product.

Industrial chemicals, unlike agrochemicals and drugs, are not designed to have biological activity. Drugs and agrochemicals are intended to enter a biological system (the body, plant, insect, etc) and interfere with normal physiological processes thus producing the desired effect. Moreover, the exposure of man to industrial chemicals is frequently different to the exposure to drugs and pesticides. Industrial chemicals usually enter the biological system incidentally. Sometimes the technical process employed in manufacture may prevent any biological exposure under normal conditions. The considerations which are important in assessing the safety of a drug, for example the margin of safety between a therapeutic and a toxic dose, are therefore not necessarily relevant to an industrial chemical. It is therefore pointless to transfer all of the concepts that have been developed for registration of drugs or agrochemicals into the guidelines for industrial chemicals. Existing registration and notification procedures recognise this difference.

Additionally, drugs and agrochemicals are usually defined chemical compounds for which a kinetic, metabolic or biochemical profile can be elucidated in a

straightforward manner. Industrial chemicals especially technical products are often mixtures. EC legislation (EEC, 1990) defines substances as

> "chemical elements and their compounds in a natural state or obtained by any production process including any additive necessary to preserve the stability of the product, any impurity deriving inevitably from the process used, but excluding any separable solvent".

This includes ill-defined mixtures such as oil additives and catalysts, stabilised products such as pigments, oligomeric materials and chemicals with a high level of impurities. Significant practical problems are associated with designing metabolic and toxicokinetic studies of such "substances". For example, physico-chemical properties may limit the methods of formulation. No single radiolabelled chemical is necessarily representative of a mixture.

The chemical and physical properties of the test chemical may limit the extent to which data can be generated. Such "substances" are currently subjected to the usual array of acute and subchronic toxicity studies with few difficulties but toxicokinetic and metabolic studies present severe problems. Although imaginative approaches to producing data can sometimes be devised, studies with such substances should be approached with caution. In many cases it may be unrealistic to attempt to generate the suggested data.

Recommendations are made in the next section concerning the type of information which should be submitted at each EC notification level. As notification levels are defined by annual and total tonnage, consideration should be given to collect data immediately for higher notification levels in those cases when tonnage is expected to expand rapidly.

## 6.2. Proposed Approaches to meet the 7th Amendment Requirements

### EC NOTIFICATION LEVEL 0

There is no formal requirement for data, nevertheless information predictive of absorption and excretion can be obtained from the data normally reported at notification Level 0.

**EC NOTIFICATION LEVEL 1**

"Basic toxicokinetic information" (sic, EEC, 1990) is *required;* **this is interpreted by ECETOC to mean an assessment of absorption and excretion of the test substance.** This can be obtained from other testing already carried out. Limited additional tests may sometimes be needed.

Information obtained without additional tests:

Physico-chemical parameters (see Section 3.1) such as fat solubility, $\log P_{OW}$, molecular weight, stability in aqueous solutions of different pH, physical state, vapour pressure and particle size are useful in the prediction of toxicokinetic properties.

Systemic toxicity observed after single and repeated exposures indicates absorption whereas specific organ toxicity as demonstrated by macroscopic, microscopic, biochemical or functional changes may suggest specific organ affinity. Differences in toxicity between routes of exposure can highlight differences of absorption.

Recovery from toxic effects during acute toxicity testing can be indicative of the conversion to less toxic derivatives and/or excretion. The absence of increasing toxicity during a repeated dosing experiment may indicate that the toxicant is eliminated from the body before the next dose is administered. Delayed signs of toxicity after single dosing may be the result of delayed absorption or of enterohepatic circulation.

Even the careful observation of the excreta in toxicity studies may provide information about excretion of the parent compound or its metabolites, eg. colour of urine and faeces, pH of urine and consistency of the faeces.

Toxicokinetic and metabolic data on structural analogues may be also used to predict the behaviour of the compound.

These considerations and predictions should be summarised in the form of an appraisal of the substance on the basis of existing knowledge. This appraisal will be used for decision-making about further studies.

Information obtained with specific tests:

If information derived from the above is deemed to be inadequate for evaluation of absorption or excretion it will be necessary to consider additional studies. The design of these studies can be derived from OECD or EC Guidelines (OECD, 1984; EEC, 1988)(see Section 2). At this notification level it is considered that an abbreviated protocol would suffice and this should focus upon absorption and excretion. For example a single oral dose test, but using only one dose level, with typically four rats may be conducted. The animals are placed in metabolism cages and the excreta collected over at least 4 days for analysis.

The method of analysis must be designed to detect the parent compound. In cases where the parent compound would not be expected in the urine, analysis should be directed towards a putative metabolite. Ideally a specific method should be considered, but if one is not available at this stage of testing, then a more general method of analysis may be employed, for example:

- UV absorbance of urine extracts,
- TLC of urine extracts using UV absorbance,
- detection of certain heteroelements such as Br, F and Cl,
- colorimetry of urine as such or after addition of specific reagents (eg. in the case of dyes, salicylates, phenothiazine sulphoxides), or metals when organometal or inorganic compounds are concerned.

With this type of study it is possible to obtain qualitative and in some cases quantitative information.

Alternatively, satellite groups of a 90-day test may be considered whereby the animals are intermittently placed in metabolism cages for 24h to facilitate collection of urine and faeces samples. At termination, blood

samples may also be retained for analysis. This approach may give an indication of absorption and excretion after repeated dosing.

**EC NOTIFICATION LEVEL 2**

"Additional toxicokinetic studies which cover biotransformation, pharmacokinetics" (sic, EEC, 1990) are required. According to the EC guidelines (EEC, 1988) this has been interpreted by ECETOC to mean that quantitative data should be obtained on absorption, excretion and, if appropriate, information on distribution and metabolism. The extent and sequence to which these data are generated should be decided on a case-by-case basis.

If there is sufficient evidence of non-absorption then no further testing is required, otherwise ECETOC proposes that the approach be organised in two stages.

First Stage Tests:

This level of notification requires the conduct of a single oral dose mass-balance study. The study should be designed to meet the requirements of the EC Guideline (EEC, 1988). The animals (typically at least four rats per dose and at two dose levels) are placed in metabolism cages and urine, faeces, carcass and, if appropriate, expired air are retained for analysis. The times at which the samples are collected will largely depend upon the results obtained at Level 1 of notification.

Labelled or non-labelled material can be used. In the latter case, the method of analysis as used at notification Level 1 can be envisaged to determine specific markers of parent compound and or its metabolites. This assay provides quantitative information on absorption, excretion and retention of the chemical and or its metabolites.

Second stage Tests:

The tests to be envisaged at the second stage will be dependent upon a full evaluation of the toxicity profile of the substance and on the toxicokinetic results obtained so far. If non-absorption can be demonstrated at this stage, if no toxicity has been observed in the general testing and if no structure-activity considerations give cause for concern, it may be that no further work need be carried out. If these conditions are not fulfilled, or if there is a specific toxicological problem to address, Stage 2 testing will be necessary. This may involve tissue distribution, blood kinetics or biotransformation.

**Tissue Distribution:** Tissue distribution studies are preferably carried out using radiolabelled material. The distribution can be determined by radiochemical analysis of organs and tissues, or by whole body autoradiography, at various time points.

**Blood Kinetics:** Blood kinetics may be investigated after single oral dosing at appropriate dose levels. The kinetics of the compound can be investigated by analysing the blood for the parent compound and/or its metabolites which may entail very sophisticated methods of analysis. The blood concentrations found are plotted against time and standard toxicokinetic parameters determined such as the elimination half-life (t1/2) and the area under curve (AUC).

**Biotransformation:** The extent of biotransformation is obtained by analysing for parent compound relative to total radioactivity. The excreta can be obtained from the mass-balance study or from animals dosed specifically for the purpose. **ECETOC recommends that no further studies on biotransformation are conducted that do not directly address specific toxicological questions.**

6.3. Comment

Studies on toxicokinetics and metabolism for industrial chemicals and technical products can provide useful supplementary information to existing

toxicity data. It should be kept in mind that these are not routine toxicity studies "ordered by catalogue" and cannot be performed "via a check list" or a standard protocol. Such studies must take into account both the physical and toxicological properties of the test compound in order to provide additional meaningful data to support additional hazard and risk assessments.

As indicated in the EC guidelines, to obtain basic data at notification Level 1 and additional data at Level 2, careful sequential planning and performance of experiments is necessary. It is recommended that an extensive evaluation be conducted, if necessary between manufacturer and authorities, with a view to generating an agreed approach prior to any experimental effort.

# 7. CONCLUSIONS

The central task of this report is to provide interpretation of EC regulatory requirements for new chemicals concerning toxicokinetics and metabolism and to set out a strategy within the framework of the existing and proposed legislation. Due to the complex nature of most new industrial chemicals a critical scientific evaluation should be performed on a case-by-case basis to allow for an individually tailored approach. A thorough consideration of all existing toxicological, physico-chemical and other data is strongly recommended before designing and when evaluating toxicokinetic studies. In practice acceptable hazard assessments of industrial chemicals are made in the absence of extensive metabolic and toxicokinetic data. Extended toxicokinetic experiments and particularly those investigating the metabolism of the chemical should only be considered appropriate if specific toxicological findings need explanation. A stepwise, purpose and problem orientated approach will ensure that studies are designed to produce useful information.

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TABLE 1, Metabolic Reactions of Xenobiotics

PHASE I REACTIONS

| reaction type | enzyme(s) involved |
|---|---|
| Oxidation | |
| aromatic hydroxylation | cytochrome P450 dependent monooxygenases |
| aromatic epoxidation | cytochrome P450 dependent monooxygenases |
| aliphatic hydroxylation | cytochrome P450 dependent monooxygenases |
| olefinic epoxidation | cytochrome P450 dependent monooxygenases |
| O-dealkylation | cytochrome P450 dependent monooxygenases |
| N-dealkylation | cytochrome P450 dependent monooxygenases |
| N-oxidation | cytochrome P450 dependent monooxygenases |
| | flavin dependent monooxgenase |
| dinitrification | cytochrome P450 dependent monooxygenases |
| desamination | cytochrome P450 dependent monooxygenases |
| | FAD dependent monoamine oxidase |
| S-oxidation | cytochrome P450 dependent monooxygenases |
| | flavin dependent monooxygenase |
| alcohol oxidation | NAD dependent alcohol dehydrogenases |
| aldehyde oxidation | NAD dependent aldehyde dehydrogenases |
| Reduction | |
| carbonyl function reduction | alcohol dehydrogenases |
| azo reduction | cytochrome P450 dependent monooxygenases |
| | DT diaphorase |
| N-oxide reduction | cytochrome P450 dependent monooxygenases |
| nitro reduction | cytochrome P450 dependent monooxygenases |
| quinone reduction | cytochrome P450 dependent monooxygenases |
| | DT diaphorase |
| reductive dehalogenation | cytochrome P450 dependent monooxygenases |
| Hydrolysis | |
| ester hydrolysis | esterases |
| amide hydrolysis | amidases |
| epoxide hydrolysis | epoxide hydrolases |
| acetal hydrolysis | glycosidases |

PHASE II REACTIONS

| reaction type | enzyme(s) involved |
|---|---|
| glutathione conjugation | glutathione-S-transferases |
| glucuronic acid conjugation | glucuronyl transferases |
| sulphate conjugation | sulphotransferases |
| acetylation | acetyl transferases |
| amide formation | amino acid N-acyl transferases |

Figure 1: Routes of Absorption, Distribution, and Excretion in the Body (adapted from Klaasen (1986)

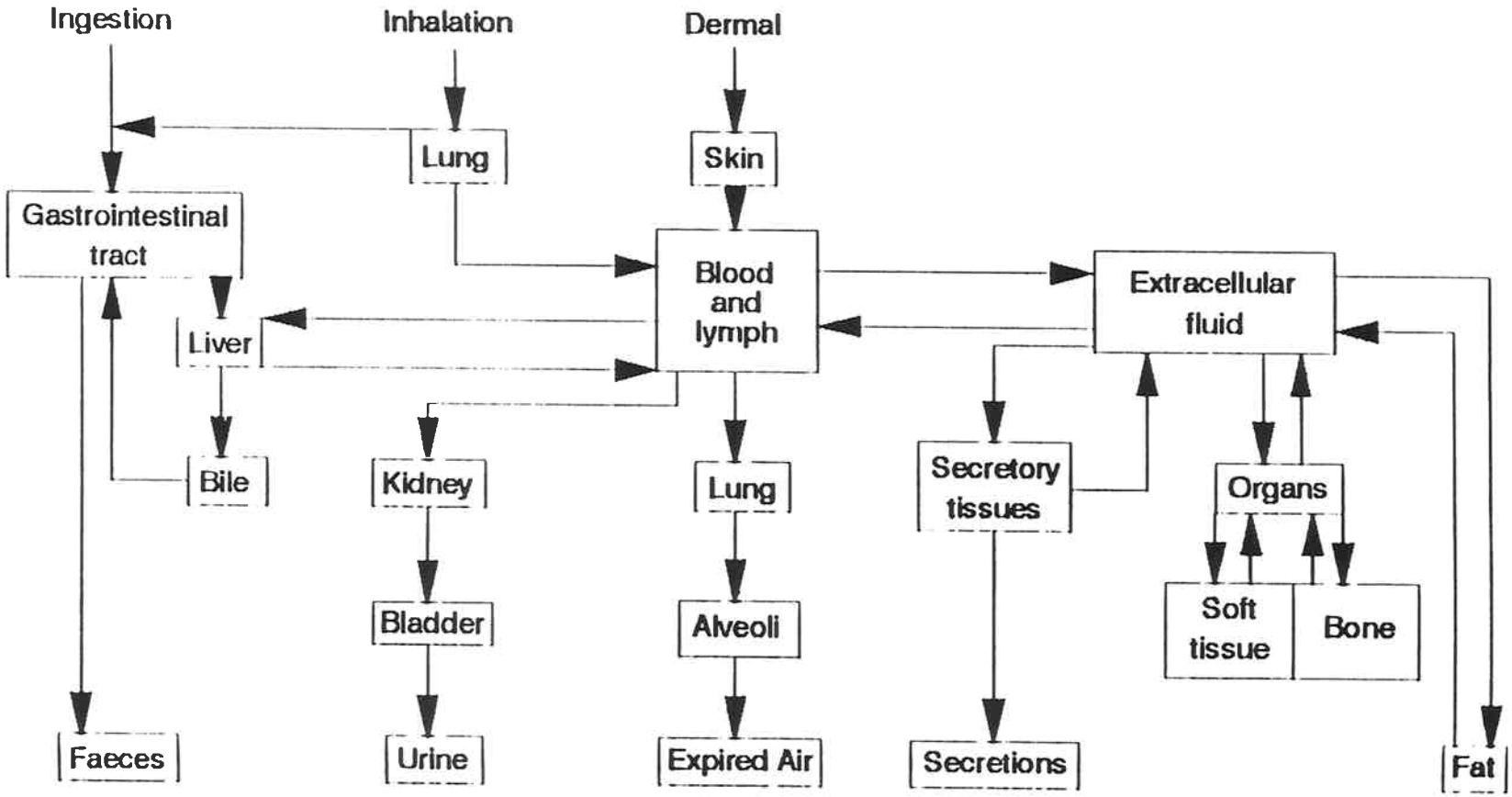
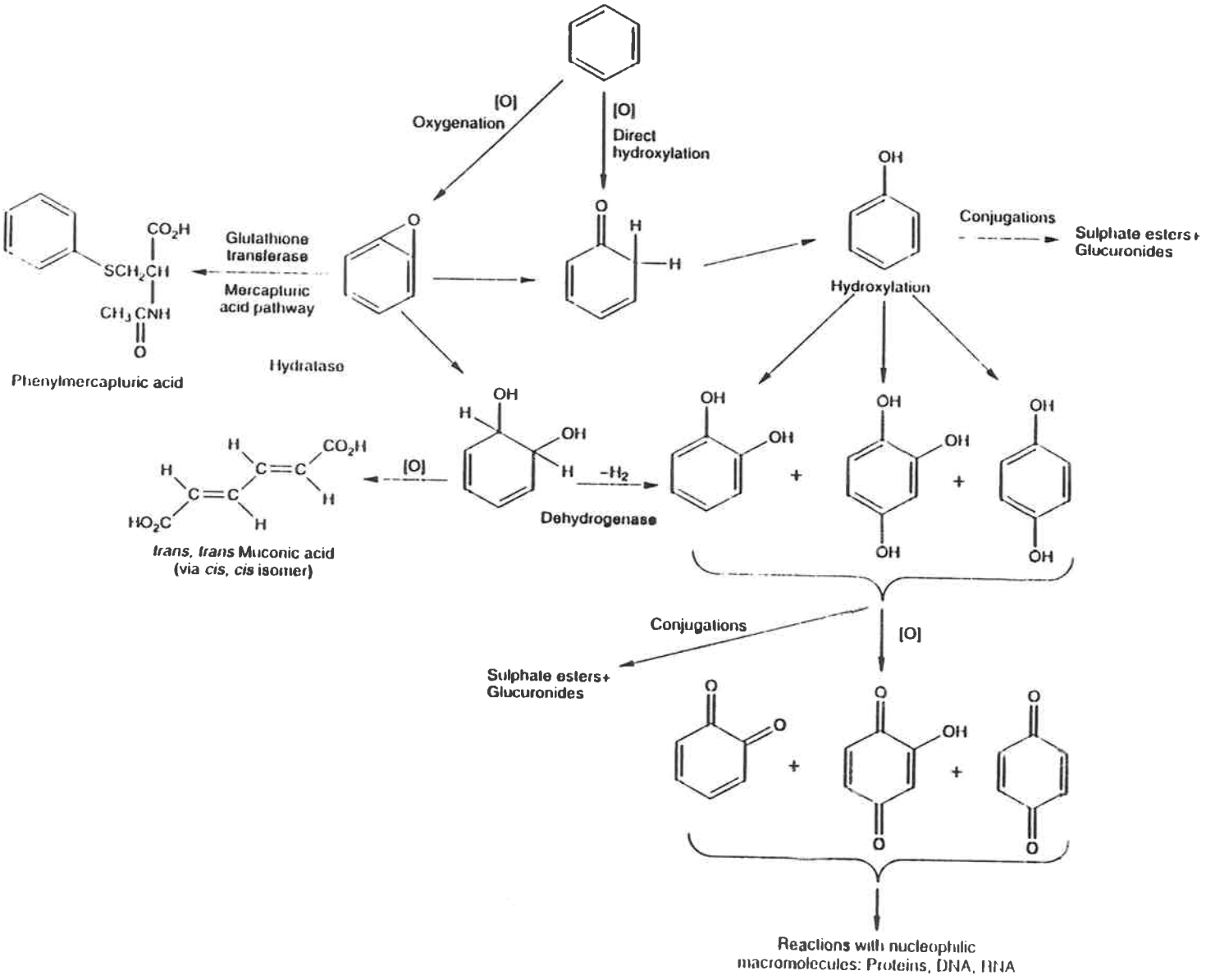


Figure 2: Biotransformation of Benzene (adapted from Snyder, 1987)



GLOSSARY OF TOXICOKINETIC TERMS

First order (linear) kinetics: is process in which the rate of elimination from a compartment is proportional to the amount of chemical present in that compartment.

Zero order (non-linear, saturation) kinetics: is process in which the rate of elimination is not proportional to concentration but is governed by an enzymatic reaction with limited capacity.

Michaelis-Menten kinetics: mathematical description of certain zero-order processes that usually involve saturable enzymatic reactions.

Compartment: is a mathematical entity which does not normally correspond with physiological or anatomical entities. A compartment is a space in which the chemical is rapidly and uniformly distributed.

One compartment model: is a mathematical model whereby it is supposed that the whole organism is one entity in which the chemical is rapidly and uniformly distributed.

Two compartment model: is a mathematical model whereby it is supposed that the organism is composed of a central compartment and a peripheral compartment. The central compartment contains beside plasma the extracellular volume and well perfused organs such as heart, liver and lungs in which the chemical is rapidly and uniformly distributed. The peripheral compartment contains organs and tissues such as muscles and fat in which the distribution of the chemical occurs much slower.

Distribution phase: portion of the plasma concentration versus time curve during which the chemical distributes primarily into the peripheral compartment. Chemicals that follow a one compartment model don't have a distribution phase.

Elimination phase: portion of the plasma concentration versus time curve during which the chemical is eliminated primarily from the central compartment.

Apparent volume of distribution(Vd): hypothetical volume of body fluid that would be necessary if the total amount of chemical were distributed at the same concentration as plasma. This constant has no direct physiological meaning and does not refer to a real volume.

Apparent first order rate elimination constant(ke): represents the fraction of the chemical eliminated per time unit from the central compartment in a one compartment first order model. ke is the sum of individual rate constants: ku

(urinary elimination rate constant), km (metabolic elimination rate constant) and kb (biliary elimination rate constant).

Half-life (t ½): is the time needed to decrease the concentration of the chemical in plasma to one half during the elimination phase and can be calculated from the ke:

$t_{½} = 0.693/ke$ (first order kinetics)

ke is independent of the dose.

Area under the curve (AUC): is the area under the plasma concentration versus time curve and can be calculated from the i.v. dose (D), the volume of distribution (Vd) and the elimination constant (ke):

AUC = D/Vd.ke (first order kinetics, i.v.)

Total body clearance (Cltot): is the measure for the efficiency with which one or more organs eliminate the chemical from the body. Total body clearance is the sum of the clearance of the different elimination processes (urinary excretion, biliary excretion and metabolism) and can be calculated from the volume of distribution (Vd) and the elimination rate constant (ke):

Cltot = Vd.ke (first order kinetics, i.v.)

Renal clearance: is the plasma volume that is cleared from the chemical per time unit by urinary elimination and can be calculated from the volume of distribution (Vd) and the urinary excretion constant (ku):

Clren = Vd.ku (first order kinetics)

When the chemical is completely eliminated via the urinary excretion route than is ku = ke and hence Cltot = Clren.

First-pass effect: process whereby chemicals are metabolised before reaching the systemic circulation.

Protein binding: is the phenomenon that occurs when the chemical combines with plasma (albumin) or tissue protein. It is the unbound drug that is in equilibrium with the biophase.

Enterohepatic circulation: is the process in which the chemical or its metabolites are excreted via the bile into the small intestine and reabsorbed into the systemic circulation after eventual biodegradation in the lumen.

First order absorption rate constant (ka): represents the fraction of chemical absorbed into the central compartment per time unit in a first order kinetics model.

Fraction absorbed (F): the extent to which a chemical is absorbed can be obtained by comparison of the area under the plasma versus time curve after oral and i.v. administration.

Apparent partition coefficient: is the ratio of the concentrations at equilibrium between a lipophilic phase and an aqueous phase. The apparent partition coefficient is not corrected for dissociation or association in either phase.

Xenobiotics: compounds that are foreign to the mammalian organism.

APPENDIX 1.

EC Guidelines on Toxicokinetic Testing for New Chemicals.

# TOXICOKINETICS

## 1. METHOD

### 1.1. Introduction

See General Introduction Part B.

### 1.2. Definitions

See General Introduction Part B.

### 1.3. Reference substances

None.

### 1.4. Principle of the test method

The test substance is administered by an appropriate route. Depending on the purpose of the study, the substance may be administered in single or repeated doses over defined periods to one or several groups of experimental animals. Subsequently, depending on the type of study, the substance and/or metabolites are determined in body fluids, tissues and/or excreta.

Studies may be done with 'unlabelled' or 'labelled' forms of the test substance. Where a label is used it should be positioned in the substance in such a way to provide the most information about the fate of the compound.

### 1.5. Quality criteria

None.

### 1.6. Description of the test method

*Preparations*

Healthy young adult animals are acclimatized to the laboratory conditions for at least five days prior to the test. Before the test, animals are randomized and assigned to the treatment groups. In special situations, very young, pregnant or pre-treated animals may be used.

*Test conditions*

Experimental animals

Toxicokinetic studies may be carried out in one or more appropriate animal species and should take account of the species used or intended to be used in other toxicological studies on the same test substance. Where rodents are used in a test the weight variation should not exceed ± 20 % of the mean weight.

Number and sex

For absorption and excretion studies, there should be four animals in each dose group initially. Sex preference is not mandatory, but under some circumstances both sexes may need to be studied. If there are sex differences in response, then four animals of each sex should be tested. In the case of studies with non-rodents fewer animals may be used.

When tissue distribution is being studied, the initial group size should take into account both the number of animals to be sacrificed at each time point and the number of time points to be examined.

When metabolism is being studied, the group size is related to the needs of the study.

For multiple-dose and multiple-time-point studies, the group size should take into account the number of time points and planned sacrifice(s), but may not be smaller than two animals. The group size should be sufficient to provide an acceptable characterization of uptake, plateau and depletion (as appropriate) of the test substance and/or metabolites.

### Dose levels

In the case of single-dose administration, at least two dose levels should be used. There should be a low dose at which no toxic effects are observed and a high dose at which there might be changes in toxicokinetic parameters or at which toxic effects occur.

In the case of repeated-dose administration the low dose is usually sufficient, but under certain circumstances a high dose may also be necessary.

### Route of administration

Toxicokinetic studies should be performed using the same route and, where appropriate, the same vehicle as that used or intended to be used in the other toxicity studies. The test substance is usually administered orally by gavage or in the diet, applied to the skin, or administered by inhalation for defined periods to groups of experimental animals. Intravenous administration of the test substance may be useful in determining relative absorption by other routes. In addition, useful information may be provided on the pattern of distribution soon after the intravenous administration of a substance.

The possibility of interference of the vehicle with the test substance should be taken into consideration. Attention should be given to differences in absorption between the administration of the test substances by gavage and in the diet and the need for an accurate determination of dose particularly when the test substance is given in the diet.

### Observation period

All the animals should be observed daily and signs of toxicity and other relevant clinical features recorded, including time of onset, degree and duration.

### *Procedure*

After weighing test animals, the test substance is administered by an appropriate route. If considered relevant, animals may be fasted before the test substance is administered.

### Absorption

The rate and extent of absorption of the administered substance can be evaluated using various methods, with and without reference groups (1), for example by:

— determination of the amount of test substance and/or metabolites in excreta, such as urine, bile, faeces, exhaled air and that remaining in the carcase,

— comparison of the biological response (e.g. acute toxicity studies) between test and control and/or reference groups,

— comparison of the amount of renally excreted substance and/or metabolite in test and reference groups,

— determination of the area under the plasma-level/time curve of the test substance and/or metabolites and comparison with data from a reference group.

(1) In this method a reference group is one in which the test substance is administered by another route that ensures complete bioavailability of the dose.

Distribution

Two approaches are available at present, one or both of which may be used for analysis of distribution patterns:

— useful qualitative information is obtained using whole body autoradiographic techniques,

— quantitative information is obtained by sacrificing animals at different times after exposure and determining the concentration and amount of the test substance and/or metabolites in tissues and organs.

Excretion

In excretion studies, urine, faeces and expired air and, in certain circumstances, bile are collected. The amount of test substance and/or metabolites in these excreta should be measured several times after exposure, either until about 95 % of the administered dose has been excreted or for seven days, whichever comes first.

In special cases, the excretion of the test substance in the milk of lactating test animals may need to be considered.

Metabolism

To determine the extent and pattern of metabolism, biological samples should be analysed by suitable techniques. Structures of metabolites should be elucidated and appropriate metabolic pathways proposed where there is a need to answer questions arising from previous toxicological studies. It may be helpful to perform studies *in vitro* to obtain information on metabolic pathways.

Further information on the relationship of metabolism to toxicity may be obtained from biochemical studies, such as the determination of effects on metabolizing enzyme systems, depletion of endogenous non-protein sulphydryl compounds and binding of the substance with macromolecules.

## 2. DATA

According to the type of study performed, data should be summarized in tabular form supported by graphical presentation whenever appropriate. For each test group, mean and statistical variations of measurements in relation to time, dosage, tissues and organs should be shown when appropriate. The extent of absorption and the amount and rates of excretion should be determined by appropriate methods. When metabolism studies are performed, the structure of identified metabolites should be given and possible metabolic pathways presented.

## 3. REPORTING

### 3.1. Test report

According to the type of study performed, the test report shall, if possible, contain the following information:

— species, strain, source, environmental conditions, diet,

— characterization of labelled materials, when used,

— dosage levels and intervals used,

— route(s) of administration and any vehicles used,

— toxic and other effects observed,

— methods for determination of test substance and/or metabolites in biological samples, including expired air,

— tabulation of measurements by sex, dose, regimen, time, tissues and organs,

— presentation of the extent of absorption and excretion with time,

— methods for the characterization and identification of metabolites in biological samples,

— methods for biochemical measurements related to metabolism,

— proposed pathways for metabolism,

— discussion of the results,

— interpretation of the results.

3.2. Evaluation and interpretation

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

## APPENDIX 2

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D-1992-3001-85

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No.7 Recommendations for the Harmonisation of International Guidelines for Toxicity Studies
No.8 Structure-Activity Relationships in Toxicology and Ecotoxicology: An Assessment
No.9 Assessment of Mutagenicity of Industrial and Plant Protection Chemicals
No.10 Identification of Immunotoxic Effects of Chemicals and Assessment of their Relevance to Man
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No.7 Joint Assessment of Commodity Chemicals, Ethylbenzene
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No.9 Joint Assessment of Commodity Chemicals, Chlorodifluoromethane
No.10 Joint Assessment of Commodity Chemicals, Isophorone
No.11 Joint Assessment of Commodity Chemicals, (HFA-132b) 1,2-Dichloro-1,1-Difluoroethane
No.12 Joint Assessment of Commodity Chemicals, (HFA-124) 1-Chloro-1,2,2,2-Tetrafluoroethane
No.13 Joint Assessment of Commodity Chemicals, (HFA-123) 1,1-Dichloro-2,2,2-Trifluoroethane
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No.16 Joint Assessment of Commodity Chemicals, (HCFC-21) Dichlorofluoromethane
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No.21 Joint Assessment of Commodity Chemicals, Tris-(2-butoxyethyl)-phosphate