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Implementation of toxicokinetics in toxicity studies – Toxicokinetics of 4-methylanisole and its metabolites in juvenile and adult rats

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ABSTRACT

The current risk assessment of compounds is generally based on external exposure and effect relationships. External doses are often not representative for internal exposure concentrations. The aim of this study was to show how the implementation of toxicokinetics in a scheduled toxicity study contributes to improved data interpretation without additional use of animals and to the three goals of the 3R principles for animal testing. Toxicokinetic analyses were implemented in a rat developmental immunotoxicity study with 4-methylanisole without interfering with the outcome of the study and without the use of additional animals. 4-Methylanisole and its metabolites were analysed in plasma of adult rats and in pups at postnatal day 10. 4-Methylanisole has a short half-life in adult animals and the plasma concentrations increased more than proportional with increasing dose. The metabolic profile appeared to be different at low dose as compared to high dose. This information on the dose-proportionality of the internal exposure is crucial for the interpretation of the toxicity data and helps to identify the toxic agent and the appropriate dose metric. The metabolism was similar in adult and juvenile animals. Large inter-individual variability in adult animals, as observed for 4-methylanisole, may hamper dose–response analyses of the results. In addition, 4-metylanisole was excreted via milk, but concentrations in the juvenile animals appeared to be 20- to 100-fold lower than via direct gavage exposure. The toxicokinetic parameters support the data interpretation, among others by providing better insight into internal exposures. Subsequently, it will help to prevent testing of irrelevant exposure scenarios and exposure concentrations. Overall, implementation of kinetics with limited effort provides useful information to support the interpretation of toxicological data and can contribute to reduction and refinement of animal testing.

1. Introduction

Currently, risk assessment of compounds is generally based on external exposure estimates while the actual dose metric of interest for risk assessment should be an appropriate estimate of the internal exposure. The external exposure may not necessarily reflect the internal exposure, *e.g.*, due to limitations in absorption and/or saturation of kinetic processes. Internal exposure over a dose range may therefore be dose proportional, less than dose proportional or more than dose proportional. Information on the internal exposure can be obtained by measuring the kinetics of a compound and, if applicable, of its metabolites.

The importance of toxicokinetic data for chemical safety assessment is increasingly recognised. This may include measurements in blood, plasma, tissues and urine. Saghir et al. (2006) described a method for measuring internal dose (kinetics) of non-pharmaceuticals following exposure through gavage, diet or drinking water and discussed how such measurements provide useful information to improve the study design of subsequent studies and interpretation of results. In addition, Creton et al. (2012) have presented three case studies to display the opportunities for use of toxicokinetics in the selection of dose levels. They showed that information on kinetics can help to prevent unnecessary testing at high dose levels, thereby reducing time, costs and suffering of animals. Further, Adler et al. (2011) and Heringa et al. (2013) provided a general overview of the benefits of

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toxicokinetics for better safety assessment. For example, they described how toxicokinetic data can be easily obtained from *in vitro* and *in vivo* human and animal experiments and showed its usefulness in extrapolation of *in vitro* concentrations to external doses *in vivo* for humans. In general, information on the internal concentrations of a test substance and its kinetics, *e.g.* blood concentrations, will represent actual exposure levels to the parent compound and possible metabolites that will be more closely linked to the toxicity profile than the external exposure concentration or dose of the parent compound. It may also contribute to identification of the toxic agent, *e.g.* parent compound or metabolite(s) that show a dose–response, and thus to understanding the mode of action. Insight in kinetics provides a better understanding of the relationship between external exposure and effects, leading to a better basis for further risk assessment. In addition, toxicokinetic information obtained in an early phase of testing provides a good support for the design of follow-up studies, for example by selecting a more relevant dose range based on internal exposure concentrations. Another important advantage is the contribution of toxicokinetic information to reduction and refinement of the use of laboratory animals, two of the three goals of the 3R principles (Replacement, Reduction, Refinement of animal studies). Toxicokinetic data may indicate that certain dose levels, species or exposure routes are irrelevant for human health risk assessment and thus can be excluded from further testing. Also, internal concentrations may indicate saturation at high dose levels, contributing to prevention of unnecessary (high-dose) testing and thereby animal suffering (refinement). In addition, by implementation of toxicokinetics in toxicity studies, more information can be obtained from the same number of animals (refinement). Thus, toxicokinetic information provides major advantages, including improvement of study design, refinement of animal testing, understanding mode of action, and improving human health risk assessment.

Currently, kinetic parameters are included to a limited extent in the common guidelines for toxicity testing of substances, except in the field of pharmaceuticals (see http://www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/general/general_content_000396.jsp&mid=WC0b01ac058002956e) in which toxicokinetics are commonly included. In addition, toxicokinetics are increasingly incorporated in the field of plant protection products (PPP) (Terry et al., 2014; Saghir et al., 2013 and MacFadden et al., 2012). The inclusion of toxicokinetics in toxicological studies is not routinely performed, even though implementation of only a limited number of measurements (instead of a complete toxicokinetics study according to OECD guideline 417 (OECD, 2010)), can already be of high relevance (Terry et al., 2014; Saghir et al., 2013 and MacFadden et al., 2012). For example, Saghir et al. (2012) have already described a study design for the implementation of toxicokinetics in a safety testing program, demonstrating its usefulness for dose selection and method of oral administration in subsequent studies. Further, efforts have been undertaken to create awareness of the importance of kinetic data to contribute to risk assessment and the 3R principle. In 2011, an expert meeting was held to discuss PBTK modelling and recommendations were made for further use and acceptance of PBTK tools (Bessems et al., 2014). Recently, Geraets et al. (2014) showed the necessity of toxicokinetic information for route-to-route extrapolation and described the impact of its absence on the uncertainty in human health risk assessment. In the last one to two decades, articles and reports have been published to indicate the importance of including TK into toxicity studies (*e.g.* IGHRC 2006). Such discussions have mostly been restricted to theoretical considerations about the value of including kinetics in a toxicity study for chemicals that are not pharmaceuticals. Practical examples are valuable and necessary to show how measurements of kinetic parameters in a toxicity study can contribute to a better interpretation of study results in practice. The current study is a further proof of the usefulness of toxicokinetics.

Our objective was to illustrate the contribution of toxicokinetics to data interpretation by implementing basic toxicokinetic measurements in a scheduled toxicity study, without adding additional animals to the study. The main conditions for implementing toxicokinetics in the scheduled study were: 1) no interference with the outcome of the toxicity study, 2) no use of additional animals and 3) relatively easy to perform with limited additional costs.

The scheduled study selected was a developmental immunotoxicity study with 4-methylanisole (4-MA). 4-MA can be naturally found in Ylang Ylang fragrance oil and is allowed as food flavouring agent according to EU Regulation 872/2012. In addition, it is also used in air care products, cosmetics and toys. In toxicity studies, 4-MA reduced spleen and thymus weights in adults and also caused pup mortality and reduced pup weight, both with a NOAEL of 100 mg/kg bw/day. In Europe, 4-MA received a classification for suspicion of damaging fertility or the unborn child (hazard statement H361, under the Globally Harmonized System for Classification and Labelling of Chemicals). We hypothesized that 4-MA could lead to developmental immunotoxicity. Therefore, a developmental immunotoxicity study was planned according to OECD TG 443 Extended One Generation Reproductive Toxicity Study in which kinetics could be included without the use of additional animals (Tonk et al., 2015). In the selected study, four different cohorts represented different regimens to expose foetuses and pups to 4-MA, providing an excellent opportunity to implement toxicokinetics to study the relationship between external and internal exposure in different exposure scenarios. For this purpose, concentrations of 4-MA and its metabolites in blood were measured to provide insight in the internal exposure of the pups and the individual contribution of multiple exposure routes (*i.e.*, via lactation and via gavage) to the internal exposure. Furthermore, measuring the parent compound and metabolites, in this study performed by GCMS, and relating these measurements to the toxicity enabled the identification of the toxic agent(s). The pups used for kinetic measurements in the study were generated anyway in the study (and were available due to standardisation of litter sizes) and thereby the sampling did not interfere with the toxicity study and noanimals outside those in the study design for toxicity assessment had to be used. To set up the kinetics experiments with the pups, basic information on the biotransformation of 4-MA was needed. Since information from the literature was too limited to set up a time schedule for blood sampling in the pups additional information on kinetics was obtained from male animals in the study that were only used for mating, thereby not interfering with the developmental immunotoxicity study. In this way, the internal concentration of the parent compound and its metabolites over time could be investigated prior to the main pup experiments.

The current study aims to provide an example of how basic toxicokinetic measurements can help in the interpretation of results from toxicity studies and can contribute to refinement of laboratory animal use (3R principle).

2. Materials and methods

2.1. Materials

4-MA was purchased from Sigma–Aldrich (Missouri, USA) (purity of 99%) and dissolved in laboratory-grade corn oil (MP Biomedicals, The Netherlands). Parental (F_0) Wistar rats were obtained from Harlan (The Netherlands). The rats were fed *ad libitum* with a commercial rodent diet (Rat & Mouse No.3 breeding diet, RM3) obtained from SDS Special Diets Services (Witham, England)