

Acute toxicity

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1. Inventory of methods currently available

From the regulatory point of view, the main objective of acute toxicity testing is basically to classify chemicals according to their intrinsic toxicity as required by the EEC directive on classification, packaging, and labelling of dangerous substances (Council Directive 67/548/EEC and subsequent amendments). This requirement aims to protect public health by regulating exposure to potentially dangerous materials.

Classification of chemicals is done on the basis of the medium lethal dose (LD50) value, defined as “the statistically derived single dose of a substance that can be expected to cause death in 50% of the animals in an experimental group”.

The LD50 concept was first introduced in 1927 for establishing the toxic potency of biologically active compounds such as digoxin (1). Since the end of the 1970's the LD50 test has been widely criticised for both scientific and animal welfare reasons (2, 3), and the test procedure has been modified in various ways to reduce the number of animals required, and to reduce the suffering caused to any animal used (4). This modification to the classical LD50 test includes the fixed-dose procedure, OECD TG 420 (5), the acute-toxic-class method, OECD TG 423 (6), and the up-and-down procedure, OECD TG 425 (7). In 2002 the original LD50 test, OECD 401 (8), has been deleted from the OECD guidelines.

TG 420: Fixed Dose procedure

Purpose: the test is of value in minimizing the number of animals required to estimate the acute oral toxicity of a chemical. The method permits estimation of an LD50 with

a confidence interval and the results allow a substance to be ranked and classified according to the Globally Harmonised System (GHS) (9).

Principle: the test consists in dosing groups of animals (single sex, normally females) in a stepwise procedure using the fixed doses of 5, 50, 300, and 2000 mg/kg (exceptionally an additional dose of 5000 mg/kg may be considered). The initial dose level is selected as the dose expected to produce some signs of toxicity without causing severe toxic effects or mortality based on *in vivo/in vitro* data (if no information exists the starting dose will be 300 mg/kg). Further groups of animals may be dosed at higher or lower fixed doses, depending on the presence or absence of signs of toxicity. This procedure continues until the dose causing evident toxicity is identified.

The preferred rodent species is the rat and the test substance is administered in a single dose by gavage (if not possible the dose may be given in smaller fractions over a period not exceeding 24 hours).

Observations: animals are observed daily for a total of 14 days. Observations include changes in skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behaviour pattern. Attention should be given to tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. The principles and criteria summarised in the Humane Endpoints Guidance Document (10) should be taken in consideration. Individual weights of animals have to be determined before and after the substance is administered. All animals should be subjected to gross necropsy and pathological changes should be recorded.

TG 423: Acute Toxic Class Method

Purpose: similar to TG 420.

Principle: the test consists on a stepwise procedure with the use of three animals of a single sex (normally females) per step. Absence or presence of compound-related mortality of the animals dosed at one step will determine the next step (i.e. no further testing is needed; dosing of three additional animals with the same dose; dosing of three additional animals at the next higher or lower dose level). The starting dose level is selected from one of four fixed levels (5, 50, 300, and 2000) and it should be that which is most likely to produce mortality in some of the dosed animals. When there is

no information on a substance to be tested, it is recommended to use the starting dose of 300 mg/kg.

The preferred rodent species is the rat and the test substance is administered in a single dose by gavage (if not possible the dose may be given in smaller fractions over a period not exceeding 24 hours).

Observations: similar to TG 420.

TG 425: Up-and-Down Procedure

Purpose: similar to TG 420.

Principle: the test consists of a single ordered dose progression in which animals of a single sex (normally females) are dosed, one at a time. The first animal receives a dose step below the level of the best estimate of the LD50 (when no information is available to make a preliminary estimate of the LD50, the suggested starting dose is 175 mg/kg). If the animal survives, the dose for the next animal is increased by a factor of 3.2 times the original dose; if it dies, the dose for the next animal is decreased by a similar dose progression.

The preferred rodent species is the rat and the test substance is administered in a single dose by gavage (if not possible the dose may be given in smaller fractions over a period not exceeding 24 hours).

Observations: similar to TG 420.

Limitations of TG 420, 423, 425

- Validations against actual data and statistical simulations identified areas where all three methods may have outcomes which result in a more or less stringent classification than that based on the “true” LD50 value (as obtained by the deleted guideline 401) (11). Comparative statistical analysis indicated that all are likely to perform poorly for chemicals with shallow dose-response slopes. For all methods, the study outcome is likely to be influenced by the choice of starting dose level(s), relative to the “true” LD50 value, especially in the case of shallow slopes. Because Guideline 420 uses evident toxicity as an endpoint instead of death, information on toxic effects seen only at dose levels close to a lethal dose will not always be obtained (12).
- Unusually test substances may cause delayed deaths (5 days or more after test substance administration). Substances which cause delayed deaths have an impact on

the practicality of conducting a study to Guideline 425 where the duration of testing will be significantly longer compared with other test methods. However, both in Guideline 420 and 423, the finding of a delayed death may require additional lower dose levels to be used or a study to be repeated.

TG 403: Acute Inhalation Toxicity

Purpose: the test provides information on health hazards likely to arise from short-term exposure by the inhalation route. Data from an acute study (such as the estimation of LC50, median lethal concentration) may serve as a basis for classification and labeling.

Principle: several groups of animals are exposed for a defined period to the test substance in graduated concentrations (at least three), one concentration being used per group.

The preferred species is the rat and at least 10 animals (5 female and 5 male) are used at each concentration level. Animals should be tested with inhalation equipment designed to sustain a dynamic airflow of 12 to 15 air changes per hour, ensure adequate oxygen content of 19% and an evenly distributed exposure atmosphere. Where a chamber is used, its design should minimize crowding of the test animals and maximize their exposure to the test substance.

Observations: animals are observed daily for a total of 14 days. Observations include changes in skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behaviour pattern. Attention should be given to tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. Individual weights of animals have to be determined before and after the substance is administered. All animals should be subjected to gross necropsy and pathological changes should be recorded.

Draft TG 433: Acute Inhalation Toxicity – Fixed Dose Procedure, as an alternative to TG 403

The purpose of the new suggested draft TG 433 (13) is the same as for TG 403. The revised second version of the draft will be submitted for consideration to OECD's Member Countries during the first quarter of 2004. The main difference between TG 403 and the new draft is that a fixed dose procedure is applied in the latter, similar to the strategy used in TG 420. In combination with the use of evident signs of toxicity as

the endpoint rather than death, considerable improvements regarding minimizing animal suffering and reduction of the use of laboratory animals will be achieved.

References

1. Trevan, J.W. (1927). The error of determination of toxicity. Proceedings of the Royal Society (London), Series B 101, 483-514.
2. Zbinden, G. and Flury-Roversi, M. (1981). Significance of the LD50 test for the toxicological evaluation of chemical substances. Archives of Toxicology 47, 77-99.
3. Balls, M. (1991). Why modification of the LD50 test will not be enough. Laboratory Animals 25, 198-206.
4. Balls, M. and Fentem, J.H. (1993). The on-going process to replace the LD50 test. Human Innovations and Alternatives 7, 544-547.
5. OECD (2001). Test Guideline 420. Acute Oral Toxicity – Fixed Dose Procedure.
6. OECD (2001). Test Guideline 423. Acute Oral Toxicity – Acute Toxic Class Method.
7. OECD (2001). Test Guideline 425. Acute Oral Toxicity – Up-and-Down Procedure.
8. OECD (1987). Test Guideline 401. Acute Oral Toxicity (Deleted in 2002).
9. United Nations-Economic Commission for Europe (UN/ECE) (2003). Globally Harmonised System of Classification and Labelling of Chemicals (GHS). UN, New York and Geneva, 2003.
10. OECD (2000). Guidance Document on the Recognition Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation. Environmental Health and Safety Monograph Series on Testing and Assessment No. 19.

11. OECD (2001). Guidance Document on Acute Oral Toxicity Testing. Environmental Health and Safety Monograph Series on Testing and Assessment No. 24.
12. Van der Heuvel, M.J., Clark, D.G., Fielder, R.J., Koundakjian, P.P., Olivier, G.J.A., Pelling, D., Tomlinson, N.J., and Walker, A.P. (1990). The international validation of a fixed-dose procedure as an alternative to the classical LD₅₀ test. *Fd. Chem. Toxicol.* 28, 469-482.
13. OECD. Draft Test Guideline on Acute Inhalation Toxicity – Fixed Dose Procedure.

2. Inventory of alternatives methods currently available

a. Test

HepG2 cell/protein content, 24 h.

Short description

The Hepatoma cell line Hep G2 is exposed to the test substances. Cytotoxicity is measured as changes in protein content by use of the method described by Lowry et al 1951 (*J. biol. Chem.* 193, 265).

Purpose

To be used in a test battery, with test *b*, *c* and *d* (see below) or in combination with only test *b*. Animal reduction for oral acute toxicity.

Developer

Described by Dierickx (see references).

References

Dierickx, P. (1989) Cytotoxicity testing of 114 compounds by the determination of the protein content in HepG2 cell cultures, *Toxic. In vitro* 3(3), 189-193.

Clemedson, C., McFarlane-Abdulla, E., Andersson, M., Barile, F.A., Calleja, M.C., Chesné, C., Clothier, R., Cottin, M., Curren, R., Daniel-Szolgay, E., Dierickx, P., Ferro, M., Fiskesjö, G., Garza-Ocanas, L., Gómez-Lechón, M.J., Güllden, M., Isomaa, B., Janus, J., Judge, P., Kahru, A., Kemp, R.B.,

Kerszman, G., Kristen, U., Kunimoto, M., Kärenlampi, S., Lavrijsen, K., Lewan, L., Lilius, H., Ohno, T., Persoone, G., Roguet, R., Romert, L., Sawyer, T., Seibert, H., Shrivastava, R., Stamatii, A., Tanaka, N., Torres Alanis, O., Voss, J-U., Wakuri, S., Walum, E., Wang, X., Zucco, F and Ekwall. B. (1996) MEIC evaluation of acute systemic toxicity: Part I. Methodology of 68 *in vitro* toxicity assays used to test the first 30 reference chemicals. ATLA 24, 251-272.

Known users

Both the cell type and the end point measurement are used by several labs.

Status of validation

Pre-validated in the MEIC programme. The MEIC (Multicenter Evaluation of *in vitro* cytotoxicity Tests) was a large international evaluation study (1989-1999) initiated by Björn Ekwall. The aim of MEIC was to evaluate the relevance of *in vitro* cytotoxicity tests for predicting human acute systemic toxicity. The second goal of the programme was to select the best combination of tests (test battery) for use in acute toxicity testing. The MEIC results demonstrated the relevance of using human cell line tests that determine basal cytotoxicity for estimating human acute toxicity. However, the results also indicated that two types of tests, such as *in vitro* tests relevant for toxicokinetics and *in vitro* test for target organ toxicity, ought to be included in the test battery in order to improve the predictability.

Recommendations

The test was developed to estimate lethal human blood concentrations. Tests need to be combined with data on absorption in order to predict administered doses (versus blood concentrations).

b. Test

HL-60/ATP content, 24 h.

Short description

HL-60 cells (human acute promyelocytic leukaemia) is exposed to the test substance. ATP content is measured by means of a Lucifer-LU plus kit as the bioluminescence generated from the enzymatic luciferin-luciferase reaction (Kangas, L. et al, 1984, Bioluminescence of cellular ATP: a new method for evaluating cytotoxicity agents *in vitro*. Medical Biology 62, 338-343).

Purpose

To be used in a test battery, with test *a*, *c* and *d* (see below) or in combination with only test *a*.

Animal reduction for oral acute toxicity.

Developer

References

Wakuri, S., Izumi, J., Sasaki, K., Tanaka, N., and Ono, H. (1993) Cytotoxicity study of 32 MEIC chemicals by colony formation and ATP assays, *Toxic in vitro* 7(4), 517-521.

Clemenson, C., McFarlane-Abdulla, E., Andersson, M., Barile, F.A., Calleja, M.C., Chesné, C., Clothier, R., Cottin, M., Curren, R., Daniel-Szolgay, E., Dierickx, P., Ferro, M., Fiskesjö, G., Garza-Ocanas, L., Gómez-Lechón, M.J., Güllden, M., Isomaa, B., Janus, J., Judge, P., Kahru, A., Kemp, R.B., Kerszman, G., Kristen, U., Kunimoto, M., Kärenlampi, S., Lavrijsen, K., Lewan, L., Lilius, H., Ohno, T., Persoone, G., Roguet, R., Romert, L., Sawyer, T., Seibert, H., Shrivastava, R., Stamatii, A., Tanaka, N., Torres Alanis, O., Voss, J-U., Wakuri, S., Walum, E., Wang, X., Zucco, F and Ekwall. B. (1996) MEIC evaluation of acute systemic toxicity: Part I. Methodology of 68 *in vitro* toxicity assays used to test the first 30 reference chemicals. *ATLA* 24, 251-272.

Known users

Shinobu Wakuri, Laboratory of Cell Toxicology, Department of Cell biology, Hatano Institute, Food and Drug Safety Center, 729-5 Ochiai, Hadano, Kanagawa 257, Japan.

Status of validation

Pre-validated in the MEIC programme

Recommendations

The test was developed to estimate lethal human blood concentrations. Tests need to be combined with data on absorption in order to predict administered doses (versus blood concentrations).

c. Test

Chang liver cell/morphology, 24 h (the MIT-24 assay).

Short description

The Chang liver cells are cultured in paraffin-sealed 96-well microtitre plates. Deficient outgrowth of fusiform or spindle-shaped cells is used as a criterion of cyto-inhibition. The cultures are further cultivated for 7 days and used in test *d*.

Purpose

To be used in a test battery, with test *a*, *b*, and *d*.

Animal reduction for oral acute toxicity.

Developer

The MIT-24 assay is described by J. Paul, Cell and Tissue Culture, Churchill-Livingstone, Edinburgh, 1975

References

Ekwall, B. and Sandström, B. 1978, Combined toxicity to HeLa cells of 30 drug pairs, studied by a two-dimensional microtitre method, Toxicol. Lett. 2, 285-292.

Clemedson, C., McFarlane-Abdulla, E., Andersson, M., Barile, F.A., Calleja, M.C., Chesné, C., Clothier, R., Cottin, M., Curren, R., Daniel-Szolgay, E., Dierickx, P., Ferro, M., Fiskesjö, G., Garza-Ocanas, L., Gómez-Lechón, M.J., Güllden, M., Isomaa, B., Janus, J., Judge, P., Kahru, A., Kemp, R.B., Kerszman, G., Kristen, U., Kunimoto, M., Kärenlampi, S., Lavrijsen, K., Lewan, L., Lilius, H., Ohno, T., Persoone, G., Roguet, R., Romert, L., Sawyer, T., Seibert, H., Shrivastava, R., Stamatii, A., Tanaka, N., Torres Alanis, O., Voss, J-U., Wakuri, S., Walum, E., Wang, X., Zucco, F and Ekwall. B. (1996) MEIC evaluation of acute systemic toxicity: Part I. Methodology of 68 *in vitro* toxicity assays used to test the first 30 reference chemicals. ATLA 24, 251-272.

Known users

Dr. Lourdes Garza-Ocanas, Department de Farmacologia y Toxicologia, Facultad de Medicina, Universidad Autonoma de Nuevo Leon, Apartado Postal No. 146, Col. Del Valle, Nuevo Leon, Mexico.

Status of validation

Pre-validated in the MEIC programme.

Recommendations

The test was developed to estimate lethal human blood concentrations. Tests need to be combined with data on absorption in order to predict administered doses (versus blood concentrations).

d. Test

Change cell/pH changes, 168 h.

Short description

The cultures from test *c* are used. After 168 h the colour of the pH indicator phenol red included in the medium is recorded. Violet colour is judged as total inhibition, while less basic (red) but not normal (orange) colours are considered as partial inhibition.

Purpose

To be used in a test battery, with test *a*, *b* and *c*.

Animal reduction for oral acute toxicity.

Developer

See test *c*.

References

See test *c*.

Known users

See test *c*.

Status of validation

See test *c*.

Recommendations

See test *c*.

e. Test

BALB/c 3T3 Neutral Red Uptake (NRU) Cytotoxicity Assay, 48 h.

Short description

NRU: cell survival/viability assay based on the ability of viable cells to incorporate and bind neutral red (NR), a supravital dye. NR penetrates cell membranes and accumulates in lysosomes. Alterations of the cell surface result in a decreased uptake and binding of NR.

Purpose

Animal reduction for oral acute toxicity.

Developer

Borenfreund E and Puerner J. 1985. Toxicity determination *in vitro* by morphological alterations and neutral red absorption. Toxicol. Lett. 24: 119-124.

References

FRAME. 1992. Balb/c 3T3 NRU cytotoxicity test, INVITOX Protocol n. 56, Nottingham. Liebsch M., and Spielmann H. 1995. Balb/c 3T3 cytotoxicity test. Methods and Molecular Biology, vol. 43, *In vitro* Toxicity testing Protocols. O'Hare S., Atterwill CK., eds. pp. 177-187. Humana Press, Totowa, NJ.

Spielmann H., Liebsch, M., Kalweit S., Moldenhauer F., Wirnsberger, T., Holzhutter HG., Schneider B., Glaser S., Gerner I., Pape W., Kreiling R., Krauser K., Miltenburger HG., Steiling W., Luepke NP., Muller N., Kreuzer H., Murmann P., Spengler J., Bertram-Neis E., Siegemund B., and Wiebel FJ. 1996. Results of a validation study in Germany in two *in vitro* alternatives to the Draize eye irritation test, the HET-CAM test and the 3T3 NRU cytotoxicity test. ATLA. 24: 741-858.

Spielmann H., Genschow E., Liebsch M., Halle W. 1999. Determination of the starting dose for acute oral toxicity (LD50) testing in the up and down procedure (UDP) from cytotoxicity data. ATLA. 27: 957-966.

Known users

Extended use.

Status of validation

Under validation.

Recommendations

f. Test

Normal human keratinocyte Neutral Red Uptake (NRU) Cytotoxicity Assay, 48 h.

Short description

The NRU is a cell survival/viability assay based on the ability of viable cells to incorporate and bind neutral red (NR), a supravital dye. NR penetrates cell

membranes and accumulates in lysosomes. Alterations of the cell surface result in a decreased uptake and binding of NR.

Purpose

Animal reduction for oral acute toxicity.

Developer

Borenfreund E and Puerner J. 1984. A simple quantitative procedure using monolayer cultures for cytotoxicity assays (HTD/NR-90). J. Tissue Culture Meth. 9(1): 7-9.

References

Heimann R., and Rice RH. 1983. Polycyclic aromatic hydrocarbon toxicity and induction of metabolism in cultivated esophageal and epidermal keratinocytes. Cancer Res. 43: 4856.

Spielmann H., Genschow E., Liebsch M., Halle W. 1999. Determination of the starting dose for acute oral toxicity (LD50) testing in the up and down procedure (UDP) from cytotoxicity data. ATLA. 27: 957-966.

Known users

Extended use.

Status of validation

Under validation.

Recommendations

g. Test

In vitro prediction of the maximum tolerated dose (data collected from ECVAM SIS, website: <http://ecvam-sis.jrc.it>).

Short description

The minimum *in vitro* drug concentration which induces changes in cell morphology, LDH release or up to 50% cell mortality (CT50) is assumed to correspond to the drug dose *in vivo* which gives rise to initial or mild toxic signs, while the minimum *in vitro* drug concentration which elicits over 90% cell mortality (CT100) is assumed to correspond to the *in vivo* dose which gives rise to marked clinical signs. CT50 and CT100 values (?g/ml) are transformed into mg/kg/day for the *in vivo* threshold. Primary cultures of rat hepatocytes are more sensitive and are used to obtain the *in vivo* values in dogs. MDBK (bovine kidney cells) are less sensitive and are used to obtain the

in vivo values in rats. McCoy (human epithelial cells) serve as *in vitro* control. The following parameters of cell growth and morphology are scored after exposure of the cells (24 h for hepatocytes, 24, 48, 72 h for cell lines) to test compound: surface occupied by growing cells (cell lines only), changes in cell size and shape, presence of cytoplasmic vacuoles, cell detachment, dead and dying cells.

Purpose

Results of cytotoxicity tests in primary cultures of rat hepatocytes, MDBK and McCoy cells can be used to predict the *in vivo* 4-wk maximum tolerated dose in rats and dogs.

Developer

Shrivastava R., John GW., Rispat G., Chevalier A., and Massingham R. 1991. Can the *in vivo* maximum tolerated dose be predicted using *in vitro* techniques? A working hypothesis. ATLA. 19: 393-402.

References

Shrivastava R., Delomenie C., Chevalier A., John G., Ekwall B., Walum E., and Massingham R. 1992. Comparison of *in vivo* acute lethal potency and *in vitro* cytotoxicity of 48 chemicals. Cell. Biol. Toxicol. 8: 157-170.

Known users

Status of validation

In-house development and pre-validated in the MEIC programme.

Recommendations

h. Test

Transepithelial resistance (TER) and paracellular permeability (PCP) in two renal cell lines (LLC-PK1, epithelial proximal tubular cells and MDCK, epithelial distal cells).

Short description

TER measurement and the trans-epithelial transport of uncharged small molecules as FITC-inulin (PCP) are reliable parameters for characterising the intactness of an epithelial barrier. Cells were seeded onto 24 well polycarbonate filter plates and then exposed to test substances. Barrier damage

is measured by using the REMS autosampler for TER assessment and with fluorescence measurement in the base plate for PCP (Duff et al, 2000).

Purpose

Measurements of TER and PCP as generalized predictors of nephrotoxicity.

Developer

Pfaller W., Troppmair E. (2000). Renal transepithelial resistance (TER) and paracellular permeability (PCP) are reliable endpoints to screen for nephrotoxicity. In: Progress in the Reduction Refinement and Replacement of Animal Experimentation. (Ball M., van Zeller A. M., Halder M. eds.) Elsevier Science B. V. Vol. 1, p. 291-304.

References

Duff T., Carter S., Feldman G., McEwan G., Pfaller W., Rhodes P., Ryan M., Hawksworth G. (2002). Transepithelial resistance and inulin permeability as endpoints in *in vitro* nephrotoxicity testing. ATLA, 30 Suppl. 2, 53-59.

Known users

Pfaller W, University of Innsbruck, Austria; Ryan M, Department of Pharmacology, University College Dublin, Ireland; Hawksworth G, University of Aberdeen, Scotland; ECVAM, JRC, Ispra (VA), Italy.

Status of validation

Under prevalidation

Recommendations

The test could be used for cell damage assessment in different epithelia.

i. Test

QSAR's models.

Short description

There are a number of software packages for the prediction of human health effects and related toxicities. These systems allow toxicity to be predicted directly from chemical structure and have been used because of their ease of use and rapid application.

TOPKAT is a statistically based system that consists of a suite of QSAR models. Models are normally derived after the analysis of large data sets of toxicologic information, usually retrieved from the literature. Molecules are characterized by any of a large number of structural, topologic, and

electrotopologic indices. Models are developed using regression analysis for continuous endpoints, and discriminant analysis for categorizing toxicity data.

TOPKAT Model Rat Oral LD50: it comprises 19 QSAR models and the data from which these models are derived: experimental acute median lethal dose (LD50) values of approximately 4,000 chemicals from the open literature. Each quantitative structure –toxicity relationship (QSTR) model assesses oral LD50 for the rat for a specific class of chemicals.

TOPKAT Model for Rat Inhalation Toxicity LC50: it comprises five QSAR models and data from which these models were derived. These multiple regression models were derived from experimental median lethal concentration (LC50) values on more than 643 chemicals after review of the open literature. Reviewed literature data ranged over various time limits; only exposure times in the range of 0.5–14 hr were accepted. Endpoints were modeled as $\log_{10}(1/C) - \log_{10}(\text{hours of exposure})$, where C is the concentration in moles/m³. The chemicals are grouped into five class-specific models: single benzenes, heteroaromatics and multiple benzenes, alicyclics, and acyclics with and without halogens. Each QSTR model assesses acute LC50 to rat of a specific class of chemicals in units of moles per cubic meter per hour.

Purpose

Prediction of toxicity from the chemical structure.

Developer

References

Dearden JC., Barratt MD., Benigni R., Bristol DW., Combes RD., Cronin MTD. (1997). The development and validation of expert systems for predicting toxicity—the report and recommendations of an ECVAM/ECB workshop (ECVAM workshop 24). *ATLA*, 25: 223–252.

Accelrys Inc. 2002. TOPKAT. Cambridge, UK: Accelrys Inc. Available: <http://www.accelrys.com/products/topkat/index.html>.

Cronin MTD., Jaworska JS., Walker JD., Comber MHI., Watts CD., and Worth AP. (2003). Use of QSARs in International Decision-Making Frameworks to Predict Health Effects of Chemical Substances. *Environm. Health Perspectives*, 111 (10): 1391-1401.

Known users

Various european regulatory bodies.

Status of validation

Under research and development.

Recommendations

3. Identified steps or tests with no alternative methods available

There are no validated alternative methods available to account for biokinetic factors. These methods will be needed in combination with alternative methods for toxicity endpoints in order to develop alternative methods schemes for systemic endpoints (including acute oral toxicity).

There are no alternative methods with fully developed protocols for toxicants that may be preferentially toxic to specific cell types/organ systems such as heart and brain. The greatest strides to date have been made with basal cytotoxicity methods. On the other hand, it has been earlier concluded (Report of the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity, October 17-20, 2000) that for acute systemic toxicity it is probably not needed to routinely test for all possible specific organ effects in different *in vitro* models. Instead, tests on energy metabolism and tests assessing the ability of a compound to disrupt epithelial barrier functions may be sufficient.

In addition, there are no *in vitro* methods available to predict acute toxicity by the inhalation route. Some models are currently under development.

4. Summary of the alternative methods currently available and foreseeable time to achieve peer reviewed validation

Look at the following table.

<i>Current endpoints addressed in animal test</i>	<i>Alternative tests available</i>	<i>Endpoints measured</i>	<i>Purpose</i>	<i>Area(s) of application</i>	<i>Validation status</i>	<i>Regulatory acceptance</i>	<i>Comments</i>	<i>Estimated time to have the method validated (ESAC endorsement)*</i>
TG 420 TG 423 TG 425 TG 403	HepG2 cell/protein content	Protein content	Partial replacement (tiered strategy/ test battery)	All soluble chemicals	Prevalidated (MEIC)	–	–	ND ^c
(Changes in skin, eyes, respiratory, circulatory, central nervous system, behaviour pattern, lethargy, sleep, coma, death, necropsy, pathology).	HL60/ATP content	ATP content	Partial replacement (tiered strategy/ test battery)	All soluble chemicals	Prevalidated (MEIC)	–	–	ND ^c
	Chang liver cell/morphology	Morphology	Partial replacement (tiered strategy/ test battery)	All soluble chemicals	Prevalidated (MEIC)	–	–	ND ^c
	Chang cell/pH changes	pH changes	Partial replacement (tiered strategy/ test battery)	All soluble chemicals	Prevalidated (MEIC)	–	–	ND ^c
	BALB/c 3T3 NRU	Cell viability	Reduction ^a , partial replacement (tiered strategy/ test battery)	All soluble chemicals	Under validation (ECVAM, ICCVAM)	–	–	2+ (for the individual test)

Normal human keratinocyte NRU	Cell viability	Reduction ^a , partial replacement (tiered strategy/ test battery)	All soluble chemicals	Under validation (ECVAM, ICCVAM)	-	-	2+ (for the individual test)
<i>In vitro</i> prediction of the MTD	Cell viability	Reduction ^b , partial replacement (tiered strategy/ test battery)	All soluble chemicals	Prevalidated in the MEIC	-	-	ND ^c
TER and PCP in two renal cell lines	Intactness of epithelial barrier	Partial replacement (tiered strategy/ test battery)	All soluble chemicals	Under prevalidation (ECVAM)	-	-	4+ (for the individual test)
QSAR's models	Various	Partial replacement (tiered strategy/ test battery)	All soluble chemicals	R&D	-	-	10+

* This table estimates the time needed to achieve ESAC endorsement for individual alternative tests assuming optimal conditions. It does not indicate the time needed to achieve full replacement of the animal test, nor does it include the time needed to achieve regulatory acceptance. "Optimal conditions" means that all necessary resources, for example technical, human, financial and coordination, are met at all times in the process and that the studies undertaken have successful outcomes.

^a Reduction if the alternative test is considered as single test for the estimation of the starting dose for acute oral toxicity.

^b Reduction since primary cells are used for the *in vitro* prediction of the maximum tolerated dose.

^c ND: not defined.

5. Conclusion

At the moment there are not validated alternative methods able to completely replace the use of animals in the field of acute toxicity. The list of *in vitro* tests presented in this chapter cannot be predictive for acute oral toxicity as single methods, but they become a good alternative if integrated in a tiered approach and/or in a test battery. Such a strategy should include a battery of tests covering general cytotoxicity, metabolism, toxicokinetics, and target organ toxicity.

While good progress has been made on methods for basal cytotoxicity, methods to address metabolism, toxicokinetics and target organ toxicity for potentially sensitive target organs have barely begun. Once methods for individual endpoints are completed and validated, there will be substantial work required to put the individual components into a validated testing battery combined with a predictive model for data extrapolation to the human situation. It should be noted that alternative methods validated to date are for point of contact effects (e.g. skin corrosivity, eye irritancy) and have not needed to address the complexity of toxicokinetics and multiple target tissues which may have differential sensitivities. Validation of an alternative model for acute oral toxicity is significantly more complex than these previous efforts, and shares many of the complexities and challenges of developing alternative models for other systemic endpoints, such as subchronic toxicity and developmental and reproductive toxicity.

Several studies, such as the MEIC study, have shown that cell culture tests give roughly similar results irrespective of the cell type or the growth/viability endpoint measurement used, i.e. almost any cell type and growth/viability endpoint measurement could be used for measurement of basal cytotoxicity. However, the MEIC study also indicated that it is preferably to use human cells for testing.

A proposal for an integrated project (A-cute-tox) has been submitted to the 6FP EC in November 2003. The aim of A-cute-tox is to develop a simple and robust *in vitro* testing strategy for prediction of human acute oral systemic toxicity, which could totally replace the animal acute toxicity tests used today for regulatory purposes. The objectives of the project include: compilation, evaluation and generation of high quality *in vitro* and *in vivo* data for comparative analysis; identifying factors (kinetics, metabolism, and organ toxicity) that influence the correlation between *in vitro* (concentration) and *in vivo* (dose) toxicity; explore innovative tools and cellular

systems to identify new endpoints/strategies to anticipate animal/human toxicity; to design a simple, robust and reliable *in vitro* test strategy amenable for robotic testing, associated with the prediction model for acute toxicity.

The time estimated to achieve complete animal replacement for acute toxicity is strongly dependent on the outcome of this project, which represents the first attempt to create an integrated strategy to be validated with the purpose to predict human systemic toxicity, and it cannot be less than 10 years.