International Journal of Biology and Life Sciences
ISSN: 2957-9511 | Vol. 6, No. 1, 2024

Research Progress in Acute Oral Toxicity Testing Methods

Meng Li [1], Rui Han [1], Juan Li [2], Wenhui Wu [1], Jianqi Gu [2, *]

[1] School of Stomatology, North China University of Science and Technology, Tangshan, Hebei 063000, China
[2] Department of Stomatology, Hebei general Hospital, Shijiazhuang, Hebei 050000, China

*** Corresponding Author:** Jianqi Gu (Email: Gujq829@163.com)

Abstract: Acute oral toxicity is the first phase of safety toxicological evaluation, with the median lethal dose (LD_{50}) being the most commonly used assessment parameter. This paper aims to summarize and compare conventional methods for determining LD_{50} and alternative approaches, along with their respective advantages and disadvantages, to provide options for further toxicological studies. Alternative tests, which do not require the precise determination of LD_{50} values, minimize animal mortality to the greatest extent and reduce the waste of human and material resources, making them worthy of promotion. Additionally, the development of Quantitative Structure-Activity Relationship (QSAR) models for predicting in vivo acute toxicity and in vitro cell culture toxicity assays are also in progress.

Keywords: Acute Oral Toxicity; Half-lethal Dose; Fixed Dose Method; Alternative Tests.

1. Introduction

The study of pharmacology is essential before drugs are used clinically to assess their toxicity and potential risks. Among them, acute oral toxicity is the first stage of evaluation, based on which subsequent toxicological research is carried out[1]. Therefore, the acute oral toxicity test is the most basic experiment in the detection and assessment of the toxic effects of trial materials, and its results are crucial. The median lethal dose (LD_{50}) [2] [3] is the traditional indicator of acute oral toxicity, which represents the minimum dose of a trial material that causes the death of 50% of the test animals within a specified period. The LD_{50} value may differ depending on the species of test animal, the skill level of the tester, and the testing method, so many experts in pharmacology believe that it is not necessary to require precise values. Therefore, there have been some alternative methods for assessing acute oral toxicity, but the observed indicators are different from those of traditional methods.

2. Traditional Acute Oral Toxicity Test Methods

Traditional methods, such as Horn's method and the Kaber method, require the precise determination of the LD_{50}. Commonly used experimental animals are SPF-grade mice weighing 18-20 g or SD rats weighing 180-220 g, with a weight difference in animals used within the same experiment not exceeding 20% of the batch's average weight. Xu Bingjie et al [4]. also employed zebrafish to determine the lethal concentration 50 (LD_{50}). Zebrafish possess advantages like small size, low cost, ease of rearing and observation, short breeding cycles, and high reproduction frequency. Moreover, zebrafish exhibit high genomic similarity to humans [5] , making them an ideal experimental model.

2.1. Horn's

Horn's[6] is a traditional acute oral toxicity test, using GHS toxicity classification as the evaluation standard. The accurate LD_{50} value can be obtained through a table lookup, and the method is simple and widely applied. The number of animals and the volume of gavage administered vary depending on the solvent and the test substance [7]. Preliminary Experiment: Animals are fasted for 12 hours prior to the start of the experiment but allowed access to water. The test substance is administered via gavage with a volume (determined based on the test substance) of 20 mL/kg.bw. The dose range for the preliminary experiment can be determined from the literature on toxicological studies of similar compounds. This range is used to establish the doses that result in 0% and 100% mortality. If the administered dose exceeds 5000 mg/kg body weight and no deaths occur, or if the LD_{50} is expected to be greater than 5000 mg/kg, a formal test is not required. Instead, only the maximum tolerated dose and repeat experiments are conducted.

Formal Experiment: Animals are randomly allocated to groups of approximately 6-10, with body weights distributed in an S-type pattern. They are fasted for 12 hours before dosing but have access to water. The dose range from the preliminary experiment is used, and doses are determined with a ratio of 1:1.2-1.4. The substance is administered via gavage at 20 mL/kg.bw. Depending on the dose, a single administration or 2-3 administrations within 24 hours may be given. Clinical symptoms are monitored continuously for one hour after dosing, then hourly for the next six hours, and daily for 14 consecutive days to record general condition, toxicity symptoms, and mortality. The LD_{50} is calculated based on the number of deaths in each group and the dose series. Animals that do not die are euthanized, autopsied, and their organs visually inspected for tissue lesions. Organ coefficients are calculated, and blood tests may be conducted on individual samples as needed to enhance data reliability.

2.2. Kaber Method

Kaber method [8], proposed by scientist Karber in 1931, has been refined by scholars Finney in 1952 and Sun Ruiyuan in 1963, according to some literature.

The method consists of a preliminary experiment and a formal experiment. In the preliminary experiment, a small number of animals are exposed to a wide range of doses to

determine the lethal dose range, identifying the minimum lethal dose (0% mortality) and the maximum lethal dose (100% mortality). In the formal experiment, the dose range is divided into 5-7 doses based on a geometric progression with a ratio of 1.2-1.5. The drug solution is administered via gavage at a rate of 0.2 mL/10 g body weight, and the drug concentrations are calculated for the average mouse body weight (20 g). Animals are closely monitored for behavior, feeding, secretions, excretions, and signs of intoxication or death over a two-week period.

However, traditional LD_{50} determination methods have significant limitations, mainly in their imprecision and variability. The result is not an exact number but a range, which questions its reliability. Furthermore, due to variations in test animals and experimental conditions, the outcomes often differ significantly, limiting the method's applicability.

Moreover, these methods are costly and inefficient. They require a large number of animals, consuming substantial human and material resources. Critically, the LD_{50} value only reflects the range at which half of the animals die, failing to provide a comprehensive understanding of the acute toxic characteristics of the substance [9].

In the modern era, the demand for efficient and humane practices, in line with the 3R principles (replace, reduce, refine) [10], renders traditional methods outdated. Replacement refers to finding alternatives to animal testing, reduction aims to decrease the number of animals and their suffering, while refinement is about improving experimental methods for better efficiency and accuracy.

Consequently, in Western countries, endpoint methods based on animal mortality have been abolished, and alternative tests have become mandatory. The challenge is to maintain data accuracy while minimizing the number of animals used and their distress. This is an inevitable trend in toxicological assessment and development. Therefore, the pursuit of novel experimental methods and technological advancements is crucial to balance scientific research and animal welfare.

3. Alternative Methods to the Traditional LD50 Approach

3.1. OECD's New Acute Oral Toxicity Test Methods

Alternative methods to traditional acute toxicity testing no longer rely on animal mortality as the endpoint, focusing instead on signs of intoxication in animals. In 2001, the Organization for Economic Co-operation and Development (OECD) revised and published the Fixed Dose Procedure (FDP), Acute Toxicity Classification (ATC), and Up-and-Down Procedure (UDP) as alternatives to conventional acute toxicity tests, marking a significant step towards replacing animal-based oral toxicity studies. To accommodate the needs of drug development, the China National Medical Products Administration (CNMPA) released guidelines for acute toxicology testing in 2014. These guidelines emphasize that most traditional Chinese medicines (TCMs) and natural drugs exhibit relatively mild effects, and that toxicity can be reduced through rational combinations in formulae, resulting in higher safety profiles. As a result, acute toxicity tests for TCMs, natural drugs, and formulae with rational combinations often employ the maximum dose or maximum tolerated dose method for research purposes.

3.1.1. Sequential (Up-and-Down) Procedure

The Sequential, or Up-and-Down Procedure, was adopted by the American Society for Testing and Materials (ASTM) in 1987 and was officially embraced by OECD as an alternative to traditional acute toxicity tests in 2001[11]. Lipnick et al[12]. demonstrated that the UDP requires the least number of animals to yield similar LD_{50} values and consistent toxicity classifications as conventional methods. The procedure involves inputting each dose and animal response into the AOT 425 computer program, which automatically determines the endpoint dose, calculates the LD_{50}, and its 95% confidence interval [13]. The sequential method adheres to the 3R principles, enhancing animal welfare and reducing resource consumption. However, its limitations include the imprecision of estimating the lethal dose with a small number of animals and its applicability only to substances that cause toxicity and death within 48 hours . If delayed death is expected, the method is not suitable and requires further refinement [14].

Experimental Procedure: The sequential method consists of a limit test and a main test. The limit test is conducted at 2000 mg/kg and 5000 mg/kg dose levels for substances with potentially low toxicity, using a maximum of 5 animals[15].

The main test involves 4-6 different dose levels tailored to the specific drug. One SPF-grade female mouse weighing 18-20 g or one SD female rat weighing 180-220 g is used for each dose. After administration, the animal's survival is observed, and the next animal is selected based on the outcome. If the animal survives, the next receives a higher dose; if it dies, the next receives a lower dose. Through multiple dose adjustments, the survival and death results are obtained, and the LD_{50} is calculated.

Chinese researchers Yu Chun et al [16]. compared Horn's method with the sequential method and found that the sequential method's LD_{50} was comparable to Horn's, with consistent toxicity grading. However, the sequential method reduced the number of animals used by 52.3%, with animal deaths kept below 10, indicating its promising potential for acute oral toxicity testing.

3.1.2. Fixed Dose Procedure (FDP)

In 1987, the European Commission formally revised the guidelines for acute toxicity tests, abolishing the LD_{50} method and adopting the Fixed Dose Procedure as an alternative [17]. OECD then officially adopted FDP in 2001. Currently, FDP is the mainstream substitute for traditional acute toxicity tests internationally. Its key feature is administering a fixed dose to female animals at fixed intervals. FDP reduces resource requirements and aligns better with ethical considerations.

Experimental Procedure: In the preliminary experiment, one animal is tested with fixed doses of 5 mg/kg, 50 mg/kg, and 2000 mg/kg (with an optional 5000 mg/kg if necessary) at specific intervals. The starting dose is typically 300 mg/kg. If severe reactions occur, the animal is euthanized for humane reasons, and the next animal is used for a lower dose until a toxic but non-lethal dose is identified for the formal experiment. If no toxicity is observed at a certain dose and death occurs at the previous dose, an additional dose level is inserted between the two. The formal test usually requires only one dose with five animals (including those from the preliminary test at that dose level).

The FDP classifies the results into four categories [18]: highly toxic, toxic, harmful, and non-toxic, and therefore does not generate data for an LD_{50} curve.

3.1.3. Acute Toxicity Class method (ATC)

The Acute Toxicity Class method (ATC)[19] was introduced in 1990 as an assessment method based on death as the endpoint. It is known for its simplicity and speed. The experiment involves four dose groups (5, 50, 300, and 2000 mg/kg.bw) with a minimum of three animals per group (preferably female). The initial dose is usually 2000 mg/kg.bw, and if no deaths occur, the test concludes. If fatalities are observed, the test is restarted with an initial dose of 300 mg/kg.bw, ensuring at least one surviving animal from the previous round. During the experiment, the dose is adjusted to estimate the LD_{50} range. This method allows for toxicity classification of the test substance according to the GHS toxicity grading system.

3.2. In Vitro Acute Oral Toxicity Alternative Methods

With the rise of the "3R" principles for animals and increased attention to animal welfare, traditional toxicology research systems and evaluation methods based on animal models are being challenged, and alternative non-animal testing methods are gaining prominence. Among these, Quantitative Structure-Activity Relationships (QSAR) and in vitro cell toxicity assays are evolving.

3.2.1. QSAR Technology

With the rapid growth of toxicity data and advancements in artificial intelligence, the development of efficient and accurate computational methods for toxicity prediction has become a focal point. ADMET software, developed by Simulations Plus, Inc., is a tool that predicts properties and designs compounds by requiring only the input of a compound's structure. It swiftly and precisely forecasts physicochemical properties, absorption, distribution, metabolism, toxicity, and bioavailability. The advantage of ADMET models and prediction methods is their versatility, reducing reliance on specific test subjects. However, these methods face challenges, primarily the scarcity of reliable toxicity data, which directly impacts prediction accuracy and reliability. Additionally, deciphering the complex relationship between compound substructures and chemical toxicity remains a challenge. Fortunately, the Multi-task Graph Attention (MGA) [20] network framework offers a promising solution by effectively utilizing existing toxicity data and learning both regression and classification tasks, enhancing prediction accuracy and generalization. QSAR models, integrated into ADMET assessments, establish models based on the quantitative relationship between chemical structure and biological activity, enabling the prediction of acute toxicity for test substances and providing a new approach for safety evaluation. However, QSAR models also have issues, such as the need for better adherence to OECD guidelines and improved model performance. Future research should address these challenges to advance toxicity prediction technology.

3.2.2. In Vitro Cell Culture Replacement Experiments

In vitro acute toxicity testing methods involve using cells, tissues, or organs from animals that have been humanely euthanized to conduct tests. These methods aim to evaluate acute toxicity through experimental results. Cell toxicity assays play a crucial role and can be broadly classified into three main aspects: cell viability, metabolic activity, and proliferation rate. By assessing these aspects, in vitro acute toxicity tests provide a comprehensive understanding of a substance's acute toxicity, supporting further medical research and practical applications. Methods that reflect cell proliferation rate include MTT assays [21], EdU labeling, CCK8 assays, thymidine incorporation, and colony formation assays.

While cell toxicity assays offer an efficient means to study the toxic mechanisms of compounds, limitations exist due to the limited variety of cell lines and primary cells, as well as significant differences between in vitro culture conditions and in vivo environments. This limits the in vitro cell assays' ability to accurately reflect the true toxic mechanisms of compounds in biological systems. [22] Therefore, further exploration is necessary to enhance the accuracy and reliability of these methods. Such efforts will contribute to a more comprehensive understanding of the toxic mechanisms of compounds, providing more reliable foundations for future medical research and drug development.

4. Summary and Future Perspectives

While traditional LD_{50} determination methods have demonstrated satisfactory accuracy in experimental data, their reliance on animal mortality as the endpoint has led to significant animal resource consumption and contradicts the humanitarian principles and animal welfare advocated by the 1876 UK Animal Experimentation Act and the 3R principles. Consequently, international organizations have actively pursued and established alternative methods that align with these principles. This review aims to systematically summarize and categorize both traditional LD_{50} methods and in vitro and in vivo replacement experiments. In vivo alternatives rely on animal intoxication symptoms for toxicity grading, thereby approximating the LD_{50} range. These methods not only safeguard animal welfare but also reduce resource expenditure and streamline experimental procedures. Comparative studies have shown comparable accuracy with traditional methods, highlighting their potential for wider adoption. Furthermore, advancements in in vitro cell culture replacement experiments and ADMET predictions have significantly contributed to the progress of acute toxicity testing. These emerging methods and technologies are increasingly playing a crucial role in toxicity assessment, offering more accurate and efficient tools for future medical research and drug development.

Acknowledgments

2024 Government-Funded Excellence Program for Clinical Medicine Talent Cultivation; ZF2024022.

References

[1] Notice of the National Health and Family Planning Commission of the People's Republic of China on Issuing the Guidelines for Management of Chemical Toxicity Assessment [J]. Gazette of the National Health and Family Planning Commission of the People's Republic of China.2015(06):9-12.

[2] Gu B , Li Y P , Yu R Y ,et al. Summary of median lethal dose and its calculation methods[J].China Occupational Medicine, 2009, 72.

[3] Rohit D ,Mahesh S ,Padma D , et al.Evaluation of safety of lipomer doxycycline hydrochloride (lipomer DH).[J].Journal of biomedical nanotechnology,2011.

[4] Xu B J , Zhang L J , Li C Q ,et al.Model validation and evaluation of developmental toxicity of five drugs using

zebrafish embryos[J].Chinese Pharmacological Bulletin, 2016, 32(1):74-79.DOI:10.3969/j.issn.1001-1978.2016.01.016.

[5] Renata F A ,Guerrieri A K A ,Carlos G , et al.Early Life Stage Assays in Zebrafish.[J].Methods in molecular biology (Clifton, N.J.),2021.

[6] Xiao Jingwei, Cui Tao, Meng Huilin, et al. Comparison of Three Acute Oral Toxicity Testing Methods [J]. Journal of Toxicology.2007(02):135-136.

[7] Ting L I , Shiqi H .Research Progress on Methodsof Acute Oral Toxicity Test[J].Journal of Jilin Institute of Chemical Technology, 2019.

[8] Ling W , Zhi-Ting G , Xiao-Song Z ,et al.Study on Acute Toxicity Test of Anticoccidial Oral Liquid from Dichroa febrifugain Mice[J].China Animal Husbandry & Veterinary Medicine, 2016.

[9] Li Danyu. Acute Oral Toxicity Evaluation of Sichuan Pepper Formula and Comparison of Two Acute Toxicological Experiment Methods [D]. China Academy of Chinese Medical Sciences. 2022. DOI:10.27658/d.cnki.gzzyy.2022.000342.

[10] M K K .ExActa Reduce, Replace, Refine - Animal Experiments.[J].Acta physiologica (Oxford, England),2021.

[11] Rispin, A, Farrar, D, Margosches, E, et al. Alternative methods for the median lethal dose (LD(50)) test: the up-and-down procedure for acute oral toxicity. ILAR J. 2002; 43 (4): 233-43. doi: 10.1093/ilar.43.4.233

[12] Lipnick, RL, Cotruvo, JA, Hill, RN, et al. Comparison of the up-and-down, conventional LD50, and fixed-dose acute toxicity procedures. FOOD CHEM TOXICOL. 1995; 33 (3): 223-31. doi: 10.1016/0278-6915(94)00136-c

[13] Chun Y U , Jun L I , Yan-Li C .Application of Horn's and Dixon-Mood in Acute Oral Toxicity Test of Pesticides[J].Journal of Anhui Agricultural Sciences, 2017.

[14] Zhang Linjuan, Sun Jinxiu. Study on Determination of LD50 by Up-and-Down Dosing Method and Horn’s Method [J]. Journal of Hygienic Toxicology.2003(04):234-236.

[15] Amy R ,David F ,Elizabeth M , et al.Alternative methods for the median lethal dose (LD(50)) test: the up-and-down procedure for acute oral toxicity.[J].ILAR journal,2002.

[16] Chun Y U, Jun L I , Yan-Li C .Application of Horn's and Dixon-Mood in Acute Oral Toxicity Test of Pesticides [J]. Journal of Anhui Agricultural Sciences, 2017.

[17] Nigel, Stallard,Anne,et al.A Statistical Evaluation of the Fixed Dose Procedure[J].Alternatives to Laboratory Animals, 2004, 32(2_suppl):13-21.DOI:10.1177/026119290403202s05.

[18] Hu Qizhi. Introduction to the New OECD Acute Oral Toxicity Testing Method [J]. Journal of Toxicology.2005(04):323-325.

[19] Eva S ,Elke G ,Horst S , et al.Oral acute toxic class method: a successful alternative to the oral LD50 test.[J].Regulatory toxicology and pharmacology : RTP,2005.

[20] Yadav D K , Khan F , Negi A S .Pharmacophore modeling, molecular docking, QSAR, and in silico ADMET studies of gallic acid derivatives for immunomodulatory activity[J]. Journal of Molecular Modeling, 2012, 18(6):2513-2525. DOI: 10.1007/s00894-011-1265-3.

[21] Wentao C , University H .The comparision of MTT and CCK-8 by detecting cytotoxicity of antiviral active components in traditional Chinese medicine[J].Journal of Hubei University (Natural Science), 2017.

[22] Marco H ,Nils H ,Rudolf M , et al.Changing the Way of Entrance: Highly Efficient Transfer of mRNA and siRNA via Fusogenic Nano-Carriers.[J].Journal of biomedical nanotechnology, 2019.